

***In vitro*, REGULATION OF EXOPOLYSACCHARIDE PRODUCTION BY *Pistacia lentiscus* L. TO MODULATE INTESTINAL MICROBIOTA IN TYPE 2 DIABETICS**

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ABSTRACT

With the development of research, the intestinal microbiota has become known as the "second brain" a complementary organ for most metabolic, defensive reactions and an important intermediate in metabolic reactions. The study aims to investigate the potential of the methanolic extract of *Pistacia lentiscus* L. leaves to modulate the dominant flora in type 2 diabetic patients (DT2) compared to healthy subjects (Hs). The researchers conducted a comparative study between the dominant flora of stools of DT2 and Hs, analyzing the microbiological, molecular, and biological activity of the extract. Microbiologically, the study found a decrease in the quantity of lactobacilli and streptococci in DT2, and an inverse relationship between enterobacteria and streptococci in all microbiota. On the molecular side, the production of exopolysaccharides (EPS) increased in beneficial Gram-positive bacteria and in Hs compared to DT2, but not in Gram-negative bacteria. A significant increase in hydrophobicity was recorded in *Escherichia coli* for DT2 and *Clostridium* sp. for Hs. The study also found that the methanolic extract of *Pistacia lentiscus* L. controls the production of EPS and hydrophobicity, with a minimum inhibitory concentration (MIC) on *Escherichia coli* and *Clostridium* of 2mg/mL. The extract allowed the growth of beneficial bacteria such as *Streptococcus* and *Lactobacillus*, which continued to produce EPS with variable and significant rates that helped them fix and establish a biofilm. The study suggests that *P. lentiscus* L. has the potential to *in vitro* modulate the dominant flora in DT2 to Hs, through an intermediate action on microbial EPS.

Keywords: anti-adhesion, anti-biofilm, Exopolysaccharides, intestinal microbiota, *Pistacia lentiscus* L., type two diabete

INTRODUCTION

Recent extensive studies on the lower part of the digestive tract have highlighted the significance of the intestinal microbiota as the "second brain" of the human body due to its multiple functions and its control over the overall health of the body (Yatsunen et al., 2012 ; Doré et al., 2017). The safety of the intestinal microbiota is vital to ensure the safety of the body's vital functions, and any defects in it can have negative impacts on the body's health (Holdeman et al., 1976).

The intestinal microbiota consisted of microorganisms (Savage, 1977) such as bacteria, microscopic fungi, yeasts, microalgae, and parasites, which reside in the digestive tract as an accessory organ of the body's systems and are critical to the interconnection of many vital functions (Landman and Quévrain, 2016). With a weight of more than 1.5 kg and a microbial diversity of approximately 1014 germs in more than 160 species, it can coexist in complex interrelationships with each other, the internal wall, and the lumen of the digestive tract for the continuity of the life of each species (Doré et al., 2017).

Type two diabete is a metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Li et al., 2020). The International Diabetes Federation estimates that there will be 537 million adults (20-79 years old) with diabetes in 2021 (IDF, 2021), more than 90% of whom will have type 2 diabetes (Xu et al., 2021). According to statistical projections, this number will rise to 643 million by 2030 (IDF, 2021). The intestinal microbiota composition is associated with many characteristics of digestive pathologies, mainly type 2 diabetes (Knauf, 2022; Aron-Wisniewsky et al., 2022). There is growing evidence that the gut microbiota contributes to the low-grade inflammation that characterizes these metabolic disturbances through mechanisms associated with intestinal barrier dysfunction controlling intestinal permeability and metabolic endotoxin (Everard and Cani, 2013). Traditional medicine employed alternative treatments mainly based on natural plant extracts, with *Pistacia lentiscus* being used as an antidiabetic to restore blood sugar levels to their natural average (Mehenni et al., 2016). This plant, which belongs to the Anacardiaceae family (AL-Saghir and Porter, 2012), called "Darw", a tree of about 3 meters (Rodríguez-Perez et al., 2013), is widespread in the north of Africa, especially in Algeria and Morocco (Belfadel, 2009; Brahmi et al., 2020), has been used by traditional medicine to treat many symptoms. Recent studies have confirmed that it is an antioxidant, anti-inflammatory,

antimicrobial, anti-ulcer, anti-cancer and antitoxin in general (Kordali et al., 2003; Benhammou et Atik Bekkara, 2009; Maxia et al., 2011; Ait-Idir and Bouyoucef, 2017; Charid, 2020; Pachi et al., 2020).

The objective of this study was to identify qualitatively and quantitatively some microbial groups isolated from the fecal material of two groups of people: the first group consisted of subjects with type 2 diabetes (DT2), and the second group consisted of healthy subjects (Hs). The study aimed to distinguish some groups of intestinal microbiota qualitatively in these two categories of patients (DT2 and Hs). Afterward, all selected microbial strains were tested for the production of biofilm, exopolysaccharides, hydrophobicity, and the biological activity of the methanolic extract of the leaves of the selected medicinal plant *Pistacia lentiscus* L., collected in the northwest of Algeria (city of Mostaganem): antimicrobial activity, Exopolysaccharids production (EPS), antiadhesion and antibiofilm powder.

Abbreviations: EPS: Exopolysaccharides microbiens, DT2: Type 2 diabetic, Hs: Healthy subjects; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bacteriocidal Concentration; CFU: Colony Forming Unit, *P. lentiscus*: *Pistacia lentiscus* L.

MATERIALS AND METHODS

Study population and clinical examination

This study was an *in vitro* characterization. It was conducted on two groups of males aged between 25 and 45 years in April 2021 at Ain Tadles Hospital (Mostaganem, Algeria). The survey included a structured questionnaire (ENTRED, 2019) followed by an examination by a diabetes specialist. DT2 was defined according to the World Health Organization criteria (WHO, 2016), and diabetics without metabolic diseases were included. Exclusion criteria were anyone older than 45 years or younger than 25 years, female, with acute or chronic infectious diseases and/or intestinal diseases and/or health issues that may affect the intestinal flora (diarrhea, constipation, gastroenteritis). Volunteers who had taken antibiotics within three months prior to the study, whether healthy or diabetic, were excluded. A simple questionnaire was administered to collect information about the participants' health status and their diet and medication regimen. Informed consent was obtained from all participants.

Fresh fecal samples (n=40) were collected from each patient (DT2 and Hs) in sterile boxes and transported to the laboratory for microbiological analyses.

Microbiological analyses

Faecal samples were analyzed for aerobic, aero-anaerobic, and anaerobic bacterial and fungal groups using non-selective and selective media (Blood agar, Chapman medium, EMB medium, Nutritive agar medium, MRS, BEA medium, Sabouraud Dextrose Agar SDA). The media were incubated at 37 °C for 24 h, 48 h, and up to 72 h according to the investigated strains, and with the addition of 5% CO₂ in the atmosphere for lactobacilli and streptococci. The strains isolated from these standard culture procedures were identified using commercial kits (API Staph, API 20 E, and API 50CH, Biomerieux, Marcy étoile, France). The tests were conducted twice for each sample, and the mean Colony Forming Unit (CFU) count was determined. After logarithmic transformation, the number of microbial colonies was presented as mean and SD. The frequency of carriage for each identified strain was calculated in percentage, and a comparison was made between the two groups of DT2 and Hs.

Detection of biofilm production power

12 microbial strains shared between diabetics and healthy controls were isolated from feces. After studying the quality of biofilm production for all isolates, the most biofilm-producing strain was determined using the Stepanovic Congo Red Agar supplemented with 5% sucrose (Freeman et al., 1989; Mahamat et al., 2013; Jung-Su et al., 2016).

Quantification of biofilm production

To quantify biofilm production for each isolated and identified strain, a modified version of Stepanovic's method (Stepanović et al., 2007) was used. A 2% hyper-glucose selective agar and broth were prepared and inoculated with each strain for 24h for all bacteria and yeast expected *Lactobacillus* (48 hours). A 1/100 dilution was then prepared in triplicate, and 200 µL of each microbial suspension was inoculated into microplate wells along with a negative control. After incubation at 37°C, the microbial suspension was aspirated and washed three times with 200 µL of PBS (Buffer Solution). Biofilm attachment was then ensured by incubating with 200 µL of ethanol for 15 minutes, followed by drying at room temperature. Fixed biofilm was stained with 200 µL of crystal violet (2%) for 5 minutes, washed with distilled water, and then solubilized with ethanol (95%). The optical density was read at 570 nm using an ELISA plate spectrophotometer (Moreira et al., 2017; Lei et al., 2020). The percentage of biofilm formation (%Bf) was calculated using the following equation (Richard et al., 2018): % Bf = ((ODs - ODC) / ODs) x 100, where ODs is the optical density of the strain at 570 nm, and ODC is the optical density of the blank control at 570 nm. % Bf provides a quantitative measure of biofilm production and can be used to compare the biofilm-forming abilities of different microbial strains.

Determination of the most biologically effective strains

In order to limit the study to one strain of each genus among the high biofilm producers, the tests were carried out in three examinations, the most productive of the exopolysaccharides, then the hydrophobicity.

Quantification of EPS

Extraction and purification of EPS

Preparation of the cultures was carried out by spreading on selective agar hyper sucrose (2%) in 20 to 30 Petri dishes (depending on the macroscopic appearance of the microbial mucus). After incubation for six days at 37°C, we collected all the colonial mucus with a scraper and placed them in physiological water (0.9% NaCl), then put the solution at 80°C for 5 minutes. In order to precipitate the particles by molar mass, we centrifuged (5000 rpm for 10 min) and then ultrafiltered the supernatant (3 times at <10°C). First dissolved in ethanol (3 volumes) at -20°C and then centrifuged (10,000 rpm for 20 min at 4°C). Then, we repeated the technique of dissolution and centrifugation of the pellet under the same conditions. Final dissolution was done with physiological water (NaOH 0.9%), and finally, we recovered the particles in powder form after lyophilisation (Domenico et al., 1989; Al-Muhanna et al., 2021).

Dosage of exopolysaccharides (EPS)

Quantification of the Exopolysaccharides (EPS) was carried out by the technique of Dubois et al. (1956), where 1ml of 5% phenol (5g phenol in 100mL of distilled and iced water) was poured. 1 mL of sample (1mg/mL) was added and homogenized by vortexing. Then 5 mL of concentrated sulfuric acid was poured gently over the prey in the tube. After incubation for 5 minutes at 100°C, reactions were ensured by storing the assembly in the dark for 30 minutes. The absorbance reading was taken at 490 nm. Calibration law was plotted using half-reduction

concentrations in µg/mL of glucose (100, 50, 25, 12.5, and 6.25) by the same technique (Domenico et al., 1989).

Membrane Hydrophobicity test

A fundamental laboratory protocol was followed to evaluate the hydrophobic membrane (H%) of the tested isolates: After microbial culture for 18 hours at 37°C in sterile selective broth, centrifugation was performed (6000 rpm at 4°C) for 10 minutes. Then, the supernatant was discarded and washed twice by adding PBS with centrifugation under the same previous conditions. After recovery of the microbial strains, 3 mL of 0.1 M KNO₃ solution was added to each tube. 200 µL of each suspension was poured into the microplate wells to measure initial absorbance (ODi) at 620 nm. Then, 1 mL of xylene was added to each suspension in the tubes to form two phases (aqueous/organic). After vortex homogenization for 2 minutes and incubation at 37°C for 10 minutes, 200 µL of the aqueous phase was collected to the microplate wells to measure the final absorbance (ODf) at 620 nm (González Arias et al., 2013). %H = ((ODi - ODf) / ODi) x100. Microorganisms isolated with hydrophobicity greater than 40% were classified as hydrophobic surface cells.

In vitro antimicrobial activities of methanolic extract of *P. lentiscus* L.

P. lentiscus was identified by a botanist in the department of Biology at Mascara University. A referenced specimen, AN00001, was introduced in our university's WAMAP-base of the Laboratory of Bioconversion, Microbiological Engineering and Health Safety (LBGMSS). The methanolic extract of *Pistacia lentiscus* leaves has been characterized in another study published by our laboratory (Bourroubey et al., 2023).

Determination of MIC and MBC and growth kinetics

Based on the precise method using sterile 96-well microplates, in the wells of columns 1 to 12, introduce aseptically, using a micropipette, 50 µL of selective broth. Introduce 50 µL of the calibrated bacterial suspension into well 1 to obtain a positive control, 50 µL of the methanolic extract of *P. lentiscus* diluted in DMSO (32 mg/mL) into well 2 to obtain a negative control and into well 2 to obtain the first and highest concentration for the test. Transfer 50µL of the mixture from well to well, 3 to 12, and add 50µL of inoculum calibrated at 10⁶ CFU/mL. Incubate at 37°C and perform a 0 H and 24 H reading at 600 nm using a spectrophotometer (CGOLDENWALL 722N is a visible ultraviolet light scanner with wavelengths between 320 and 1020 nm with 2 nm resolution, connected to a computer). The Log CFU versus time curve was plotted to determine each microorganism's MIC (minimum inhibitory concentration). MBC is the lowest extract concentration that lyses microbes (less than 0.001% survivors), they were tested by conventional counting on agar from wells that gave an optical density lower than the initial density. An extract is said to be bactericidal when the MBC/MIC ratio is less than or equal to 4 or bacteriostatic when this ratio is greater than 4 (Nikaido, 2009).

Quantification of exopolysaccharides (EPS) before and after treatment with the extracts

To immobilize the bacteria, the bacterial suspensions were exposed to ultrasound waves at 52 kHz for 10 minutes. The extract was added at a concentration of 2 mg/mL and 4 mg/mL (lower MICs) and incubated at 37°C for 24 hours. The extraction of EPS was carried out using the technique described by (Ricciardi and Clementi, 2000; Ricciardi et al., 2022). After 24 hours, the bacteria were recovered by centrifugation at 5000g/15 min following boiling at 80°C for 15 min. The EPS was then allowed to precipitate, and the supernatant was filtered at 4°C before adding three volumes of ethanol. The mixture was then centrifuged at 10,000g/20 min at 4°C, and the resulting pellet was redissolved in sterile physiological water. EPS quantification was performed using the total sugar assay technique described by Dubois et al., (1956).

Anti-adhesion activity

Initially, the culture was centrifuged (3000 rpm at 4°C for 10 minutes) after being incubated for 18 hours at 37°C in BHI broth. The cell pellet was recovered in PBS to prepare a suspension with an optical density adjusted to 1 at 600 nm. Then, 1.5 mL of the 1/100 dilution of this suspension with BHI sucrose broth (0.25%) was added to each tube. On the other hand, dilutions (2000, 1500, 1000, 500, 250 and 125 µg/mL) of the methanolic extract of *P. lentiscus* L. leaves, harvested from the Mesra region of Mostaganem (Algeria), were prepared with PBS. Next, 1.5 mL of the dilution was added to the tubes in triplicate. After incubation at 37°C for 24 hours, the suspensions were discarded, and 3 mL of sodium hydroxide was added to each well to remove non-adherent and weakly adherent microbial cells. The mixture was aspirated, and 3 mL of PBS was added. The reading was taken at 600 nm, and the positive control was carried out by adding 1.5 mL of PBS instead of the extract (Khan et al., 2011; Rohazila et al., 2014). The anti-adhesion activity was calculated using the formula: % Anti-adhesion = ((ODc - ODt)/ODc) x100,

where ODC is the positive control optical density at 600 nm, and ODT is the optical density of the treated well at 600 nm (Awadelkareem et al., 2022).

Anti-biofilm activity

The microbial suspension was prepared from an 18-hour culture at 37°C in sterile BHI broth (OD = 1 at 600 nm), and a 1/100 dilution was made. Next, 100 µL of the diluted suspension was inoculated into the microplate wells with 100 µL of different concentrations (2000, 1500, 1000, 500, 250, and 125 µg/mL) of the methanolic extract of *P. lentiscus* L., harvested from the Mesra region of Mostaganem (Algeria). After incubation at 37°C for 24 hours, the mixture in the wells was aspirated, and the wells were washed twice with 200 µL of sterile distilled water. After drying, the remaining biofilm was stained with 200 µL of crystal violet (0.2%) for 15 minutes. The wells were washed twice with distilled water, and the dye was solubilized with 200 µL of ethanol (95%). The absorbance reading was taken at 600 nm. Suspension with PBS and medium only were used as negative control and blank control, respectively (Rohazila et al., 2014). The anti-biofilm activity was calculated using the formula: % Anti-Bf = ((ODcn – ODT)/ODcn) x100, where ODcn is the negative control optical density at 600 nm, and ODT is the optical density of the treated well with the extract at 600 nm (Swetha et al., 2019).

Statistical study

The microbial variation between DT2 and Hs, as well as biofilm production, hydrophobicity, anti-adhesion activity, and anti-biofilm activity, were analyzed for their statistical significance based on triple repetition of all experiments. An analysis of variance (ANOVA) test and post hoc test (where available in IBM SPSS statistics version 25) were used, and all data were presented as means ± standard deviation (SD). A *p*-value of 0.05 was set as the threshold for determining statistical significance.

RESULTS AND DISCUSSION

Microbial qualitative production biofilm

To determine the microorganisms with the potential to produce biofilm, all strains isolated on Congo red agar were cultured, and those producing completely black colonies were identified. These results are summarized in Table 1.

Table 1 Comparison of Biofilm-Producing Isolates and EPS Among Intestinal Microbiota of DT2 and Hs.

Microbial group	Common isolates between diabetics and healthy subjects
Enterobacteria	<i>Escherichia coli</i> , <i>Salmonella</i> sp, <i>Klebsilla aerogenes</i> , <i>Klebsilla pneumoniae</i>

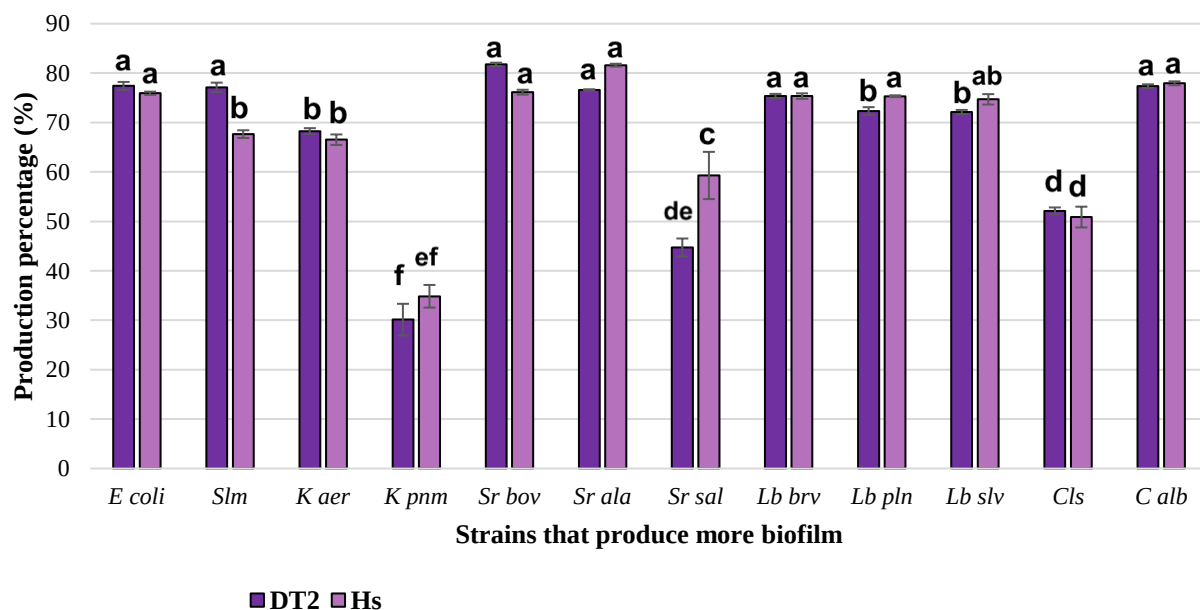


Figure 1 Comparison of the most biofilm-producing strains for each isolated species in DT2 and Hs. a, ab, b, c, d, de, ef and f: Graduations with significant difference (*p* < 0.05). *E coli* : *Escherichia coli*, *Slm* : *Salmonella*, *K aer* : *Klebsilla aeruginas*, *K pnm* : *Klebsilla pneumoniae*, *Sr bov* : *Streptococcus bovis*, *Sr ala* : *Streptococcus alactolyticus*, *Sr sal* : *Streptococcus salvarius*, *Lb brv* : *Lactobacillus brevis*, *Lb pln* : *Lactobacillus plantarum*, *Lb slv* : *Lactobacillus salvarius*, *Cls* : *Clostridium*, *C alb* : *Candida albicans*.

Based on previous research and hypotheses, multiple factors play a role in the microorganism's ability to produce biofilm. These factors include the composition of the outer layer and its cohesion with other members of the same species and the

Streptococci	<i>Streptococcus bovis</i> , <i>Streptococcus salvarius</i> , <i>Streptococcus alactolyticus</i>
Lactobacilli	<i>Lactobacillus brevis</i> , <i>Lactobacillus salvarius</i> , <i>Lactobacillus plantarum</i>
Clostridium	<i>Clostridium</i> sp
Yeast	<i>Candida albicans</i>

Multiple research studies have provided evidence of various microbial species in the intestinal lumen having the capability to produce biofilms, albeit in differing ratios. The binding ability of each strain to each other and the surface is determined by the glycoprotein nature produced. Therefore, biogeographical mapping of the microbiota's structural elements is essential to comprehensively comprehend the functional dynamics of this intricate community (Hanne et al., 2019; Hussain et al., 2020; Arias and Brito, 2021).

Interactions between microorganisms and secondary metabolites have been identified as a contributing factor to the varying biofilm production capabilities of different microbial strains and intestinal microbiota (Hanne et al., 2019). Such metabolites can act as inhibitors or growth stimuli for different species, thereby affecting biofilm production. Additionally, the genetic factors of microorganisms can vary between strains of the same species, leading to differences in their biofilm production capabilities (Pratt and Kolter, 1999; McCarty et al., 2014). Furthermore, the nature of biotic and abiotic nutrients can also impact biofilm production. Such nutrients may contain toxic molecular elements or inhibit microbial growth and catalysis under certain conditions (Mah and O'Toole, 2001; Satpathy et al., 2016).

Biofilm-producing strain

The most biofilm-producing strain of each common microbial isolate in the fecal material of both groups (DT2 and Hs) was selected (Figure 1)

The study aimed to investigate the biofilm production ability of all isolated strains from fecal material of both DT2 and Hs. The most biofilm-producing strain for each common microbial isolate in the fecal material of both groups was selected (Figure 1). *Streptococcus bovis* strains had the highest biofilm production, with 82.22% in Hs and 81.77% in DT2, followed by *Streptococcus alactolyticus* and *Streptococcus salvarius* (76.61% and 44.75% respectively) in DT2 patients compared to Hs (59.69% and 81.61%, respectively). *Escherichia coli*, *Salmonella*, and *Klebsilla aerogenes* had the highest biofilm production among the Facultative aero-anaero Enterobacteria in DT2 patients (77.43%, 15.77%, and 68.27%, respectively), while Hs had (75.93%, 67.67%, and 66.56%, respectively). High biofilm production was recorded for *Lactobacillus brevis* and *Candida albicans*, with no significant difference between the two types of fecal microbiota. *Lactobacillus plantarum* and *Lactobacillus salvarius* revealed low biofilm production (72.33% and 72.12% respectively) in DT2 patients compared to Hs (74.72% and 75.33%, respectively). The capacity for biofilm production in *Clostridium* and *Klebsilla pneumoniae* was determined at much lower rates in all volunteers.

host, which various molecules and the level of hydrophobicity can influence. Additionally, external environmental factors can either positively or negatively impact this productivity (Satpathy et al., 2016).

A significant difference in the potential for biofilm production was observed in five strains (*Salmonella*, *Klebsiella pneumoniae*, *Streptococcus salvarius*, *Lactobacillus plantarum*, and *Lactobacillus salvarius*) between the two microbiota studied. Only *Salmonella* exhibited a higher biofilm production potential than four strains isolated in DT2. *Streptococcus alactolyticus*, *Lactobacillus brevis*, and *Candida albicans* were identified as the most biofilm-producing strains, with no significant difference in their potential for biofilm production observed between them.

Exopolysaccharides amount

The exopolysaccharide amounts produced by the studied strains are represented in Figure 2. Our findings show that all microorganisms produce these molecules but with significant differences. *Candida albicans* and Gram-positive bacteria (*Lactobacillus*, *Streptococcus*, and *Clostridium*) were more productive than Gram-negative ones (*Escherichia coli*, *Klebsiella*, and *Salmonella*). Furthermore, the dominant flora of the intestinal microbiota of DT2 was found to produce EPS at reduced levels in certain Gram-positive strains (*Lactobacillus salvarius*, *Streptococcus alactolyticus*, and *Clostridium*), but at high levels in certain Gram-negative strains (*Escherichia coli*, *Klebsiella aerogenes*, and *Salmonella*), compared to their counterparts in Hs.

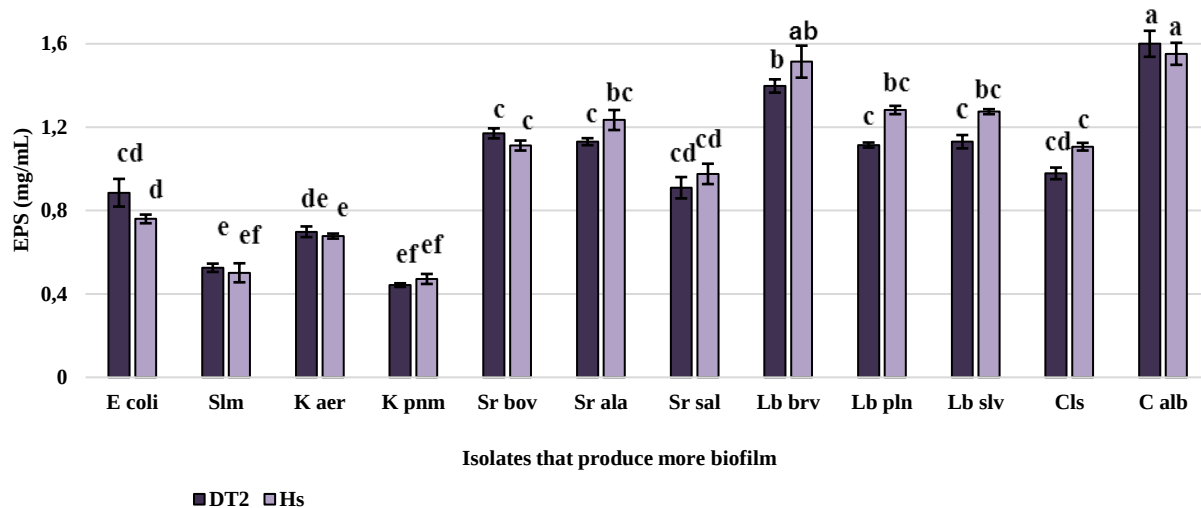


Figure 2 Exopolysaccharide levels in biofilm-producing isolates at 490 nm (DT2vs Hs). a, ab, b, bc, c, cd, d, de, e and ef: Graduations with significant difference ($p < 0.05$). *E. coli* : *Escherichia coli*, *S. lmn* : *Salmonella*, *K. aer* : *Klebsiella aeruginas*, *K. pnm* : *Streptococcus bovis*, *S. ala* : *Streptococcus alactolyticus*, *S. sal* : *Streptococcus salvarius*, *L. brv* : *Lactobacillus brevis*, *L. pln* : *Lactobacillus plantarum*, *L. slv* : *Lactobacillus salvarius*, *Cls* : *Clostridium*, *C. alb* : *Candida albicans*.

Our study supports previous research that has demonstrated the presence of polysaccharides and other polymers in the outer layer of microorganisms. The density of these substances may differ based on various factors, including the host, outer layer thickness, capsule presence or absence, enzymatic activity, and production of secondary metabolites. These findings are consistent with studies conducted by Paul *et al.* (1986); Read and Costerton (1987) and Cerning (1990). It is worth noting that microbial EPS concentration and composition can change significantly under stress conditions, as Konnova *et al.* (2001) reported. The type of microorganism and the culture media's composition can directly impact EPS production, as Osman *et al.* (2012) described. For instance, Zisu and Shah (2003) found that a *Streptococcus* strain produced up to 406 mg/L EPS in an MRS-based medium. In contrast, Vijayendra *et al.* (2008) observed that fermentation of a non-stringing strain of *Leuconostoc sp.* in modified MRS broth for 72 hours at

22°C resulted in the production of 13.8 g/L EPS. The quantity of EPS produced by *Lactobacillus sp.* may vary considerably, ranging from 80 mg/L (Khalil *et al.*, 2018) to 1700 mg/L (Van Der Meulen *et al.*, 2007).

Statistical analysis revealed a significant difference between the two types of intestinal microbiota (DT2 and Hs) regarding EPS production. It was lower in DT2 patients, particularly in the predominant Gram-positive bacteria. *Candida albicans* was identified as the most productive, while Gram-negative bacteria were found to be the least productive group. When comparing the same strain between the two microbiota, a significant difference in EPS production was observed for all strains of *Lactobacillus*, *Streptococcus alactolyticus*, and *Clostridium*, with higher productivity in Hs. However, for *Escherichia coli*, *Klebsiella aeruginas*, and *Salmonella*, the productivity was more elevated in DT2.

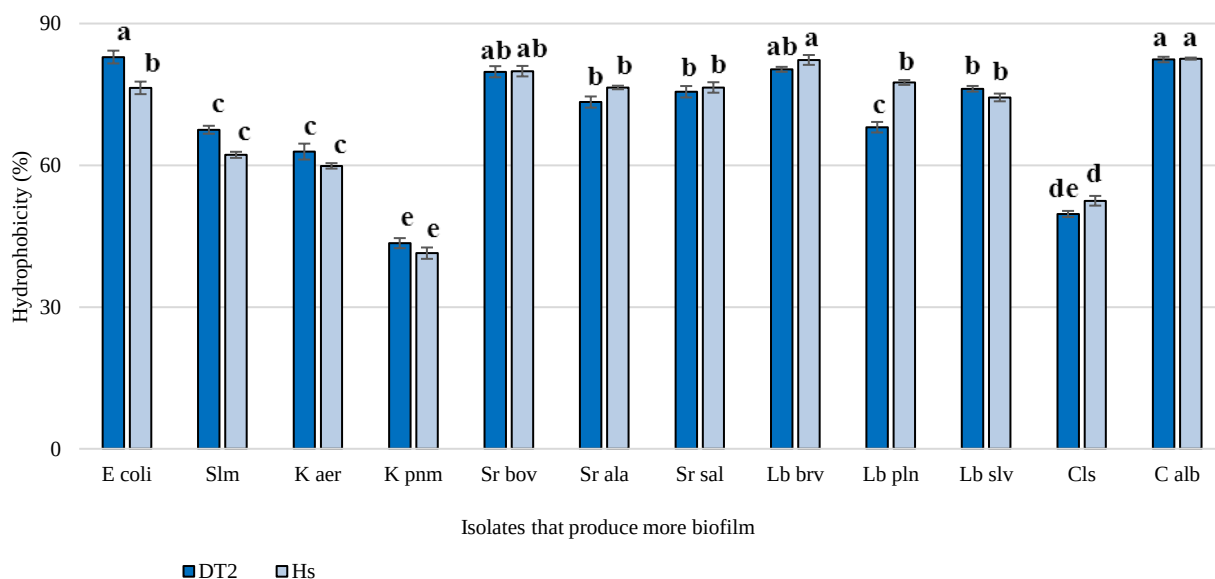


Figure 3 Membrane hydrophobicity of the most biofilm-producing isolates at 600 nm (DT2vs Hs). a, ab, b, c, d, de and e: Graduations with significant difference ($p < 0.05$). *E. coli* : *Escherichia coli*, *S. lmn* : *Salmonella*, *K. aer* : *Klebsiella aeruginas*, *K. pnm* : *Streptococcus bovis*, *S. ala* : *Streptococcus alactolyticus*, *S. sal* : *Streptococcus salvarius*, *L. brv* : *Lactobacillus brevis*, *L. pln* : *Lactobacillus plantarum*, *L. slv* : *Lactobacillus salvarius*, *Cls* : *Clostridium*, *C. alb* : *Candida albicans*.

Surface Hydrophobicity

A difference was found in microbial surface hydrophobicity between the two faecal microbiota and between strains within each flora. DT2 exhibited higher hydrophobicity levels in Gram-negative bacteria and *Lactobacillus salivarius*, compared to Hs. Conversely, all Gram-positive strains showed a relative increase in hydrophobicity in Hs compared to DT2, with the exception of *Lactobacillus salivarius*. Notably, *Candida albicans* exhibited very high hydrophobicity levels, exceeding 82%. Results are shown in the figure 3. Cell hydrophobicity can be measured by the water contact angle, as demonstrated in previous studies (Mozes and Rouxhet, 1987). Hydrophobicity has been shown to play a critical role in biofilm formation (Wijesundara et al., 2022), as the microbial surface contains large molecules such as EPS, glycoproteins, and proteins that are difficult to hydrate efficiently in aqueous media (Gonzalez et al., 1988; Kiely and Olson, 2000; Vadillo-Rodríguez et al., 2005).

Statistically, *Candida albicans*, *Escherichia coli*, *Lactobacillus* and *Streptococcus* with very high hydrophobicity were determined compared to the rest of the strains. However, a significant direct effect was recorded on *Escherichia coli*, *Streptococcus bovis* and *Lactobacillus plantarum*. Hs was distinguished by higher hydrophobicity for the latter species and DT2 by the remaining two.

In vitro, iological activity of *P. lentiscus* on the dominant flora

After a comparative study of the most biofilm-producing strains in Hs and DT2, two main molecular factors, the first is the production of EPS, and the second is hydrophobicity, the biological activity of the methanolic extract of *P. lentiscus* L. leaves (Paul et al., 1986) was studied on certain strains of the dominant flora of the intestinal microbiota (Tab 2).

Table 2 Identified strains that demonstrated significant superiority in terms of hydrophobicity and EPS productivity between the two types of microbiota.

Microorganismes	<i>E coli</i>	<i>Sr ala</i>	<i>Lb brv</i>	<i>Lb pln</i>	<i>Lb slv</i>	<i>Cls</i>
EPS	DT2*	Hs*			Hs*	Hs*
Hydrophobicity	DT2*		Hs*	Hs*		Hs*

DT2*: A significant superiority in type tow diabetics. Hs*: A significant superiority in healthy subjects. *E coli* : *Escherichia coli*, *Sr ala* : *Streptococcus alactolyticus*, *Lb brv* : *Lactobacillus brevis*, *Lb pln* : *Lactobacillus plantarum*, *Lb slv* : *Lactobacillus salivarius*, *Cls* : *Clostridium*.

Determination of MIC and MBC

Table 3 presents the values of MIC (Minimum Inhibitory Concentration), MBC (Minimum Bacteriocidal Concentration), and MIC/MBC ratio. The study determined that the methanolic extract of *P. lentiscus* L. had a MIC value of 2 mg/mL for *Escherichia coli* and *Clostridium* and 4 mg/mL for *Streptococcus alactolyticus* and all the strains of *Lactobacillus* studied, except *Lactobacillus salivarius* in Hs. The extract has a bacteriostatic effect for *Clostridium* and *Streptococcus*, and a bactericidal one for the other isolated strains.

Table 3 MIC and MBC values of the methanolic extract of *P. lentiscus* leaves in the bacteria studied.

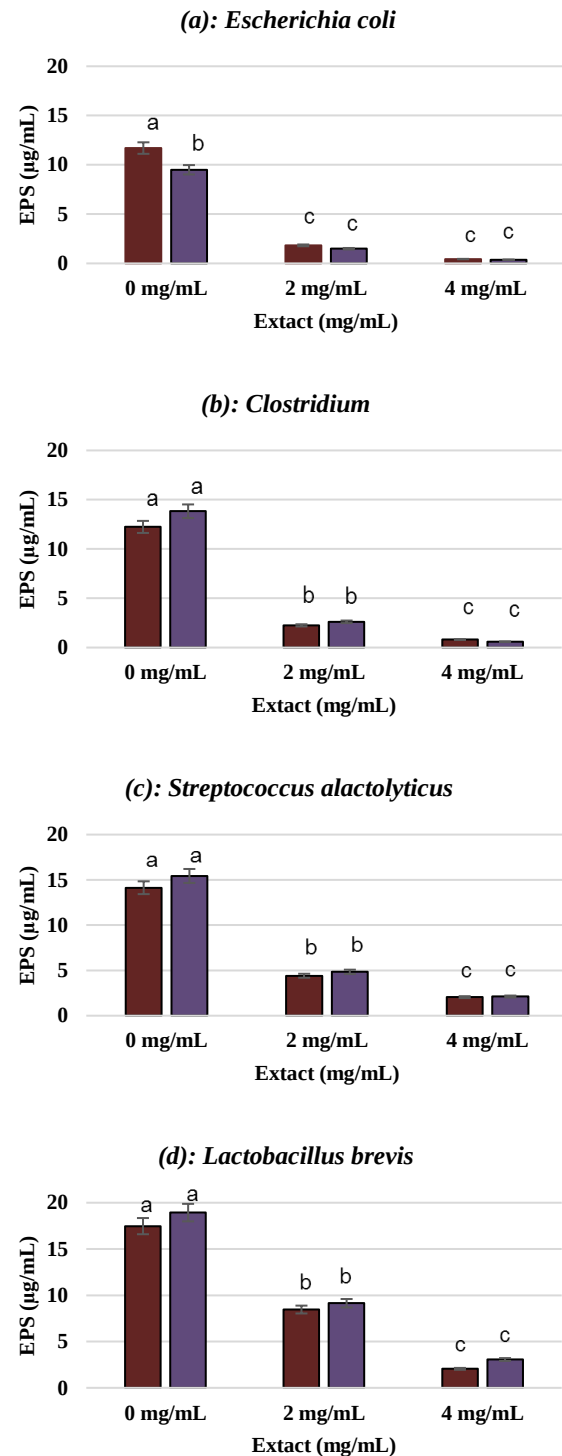
Bacteria	Group	MIC (mg/mL)	MBC (mg/mL)	
<i>Escherichia coli</i>	DT2	2	8	bactericidal
	HS	2	8	bactericidal
<i>Clostridium</i>	DT2	2	16	bacteriostatic
	HS	2	16	bacteriostatic
<i>Streptococcus alactolyticus</i>	DT2	4	32	bacteriostatic
	HS	4	32	bacteriostatic
<i>Lactobacillus brevis</i>	DT2	4	8	bactericidal
	HS	4	8	bactericidal
<i>Lactobacillus plantarum</i>	DT2	4	8	bactericidal
	HS	4	16	bactericidal
<i>Lactobacillus salivarius</i>	DT2	4	16	bactericidal
	HS	8	16	bactericidal

Bacterial growth kinetics vary depending on the bacterial strain and its resistance to biologically active substances resulting from genetic changes (Nikaido, 2009; Azzouzi and Brahimi, 2019), as well as the mode of action in the microbial cell. *P. lentiscus* L. has an antimicrobial effect on different strains. According to Azzouzi and Brahimi (2019), the difference in the cell wall structure between Gram-positive and Gram-negative bacteria and the composition of the plant extract contributes to its antimicrobial activity. Which act as a growth factor in certain strains, as in the case of *Streptococcus* and *Lactobacillus* studied in this research, and sometimes as an antimicrobial agent as has been recorded in *Escherichia coli* and *Clostridium*. Azzouzi et Brahimi (2019) cited the difference in the cell

wall structure between Gram-positive and Gram-negative bacteria; moreover, the plant extract's composition is attributed to the antimicrobial activity. There is a significant effect between dominant and subdominant bacteria in T2D compared to Hs in 2 and 4 mg /mL concentrations.

Estimation of the yield of EPS before and after treatment with the *P. lentiscus* extract

Figure 4 illustrates the effect of various concentrations of the methanolic extract of *P. lentiscus* on EPS production by the investigated bacteria. A significant reduction in EPS amount was observed at 2 mg/mL of extract. *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Streptococcus alactolyticus* had a higher ratio of EPS at the concentration of 2 mg/mL of *P. lentiscus* extract (8.46, 5.21, and 4.41 µg/mL for DT2 and 9.14, 6.37, and 4.84 µg/mL for Hs). In contrast, *Lactobacillus salivarius* and *Clostridium* had a lower ratio (2.97 and 2.25 µg/mL for DT2 and 3.83 and 2.61 µg/mL for Hs). *Escherichia coli* had very low percentages (0.47 and 0.37 µg/mL) in both microbiotas.



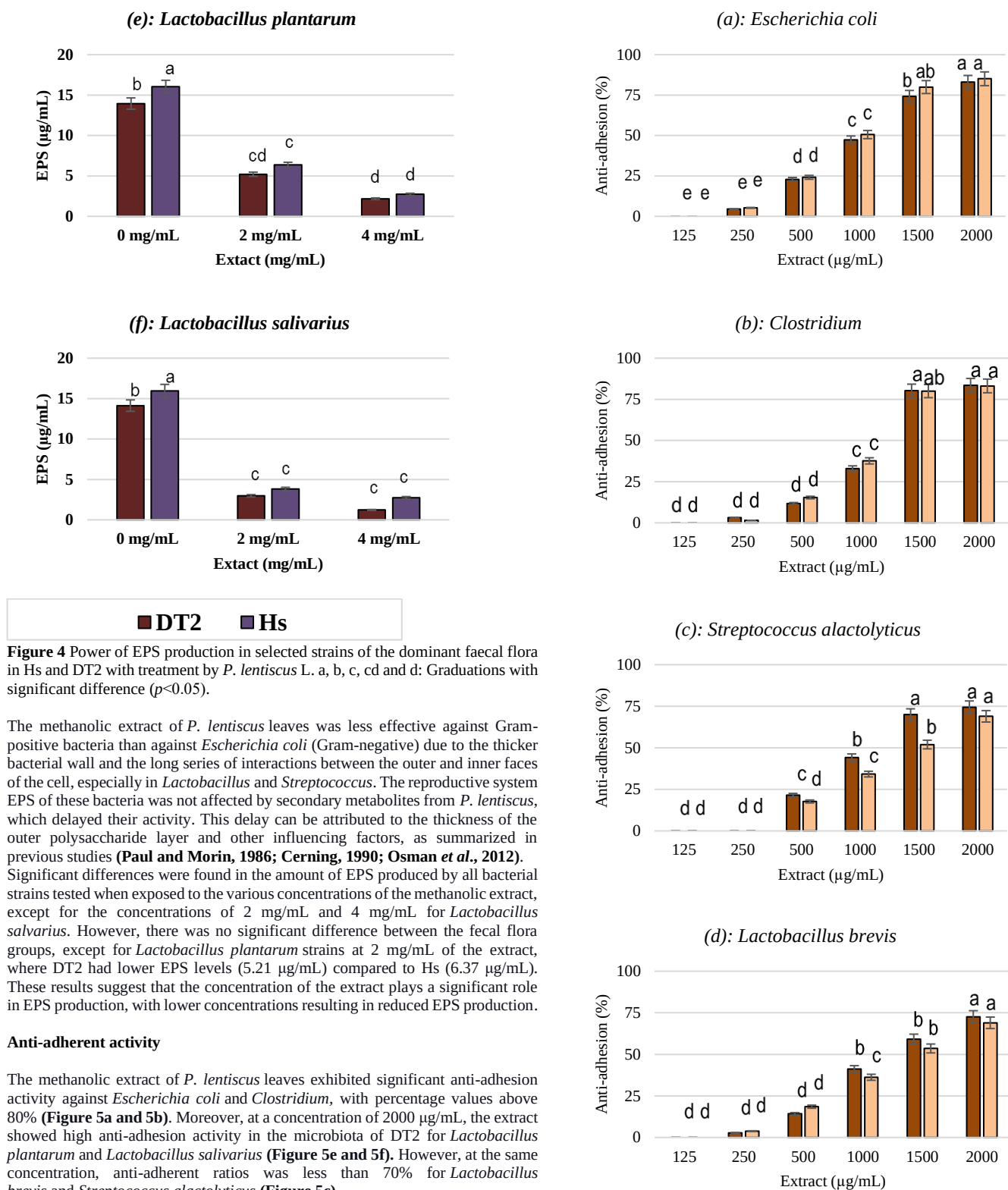


Figure 4 Power of EPS production in selected strains of the dominant faecal flora in Hs and DT2 with treatment by *P. lentiscus* L. a, b, c, cd and d: Graduations with significant difference ($p < 0.05$).

The methanolic extract of *P. lentiscus* leaves was less effective against Gram-positive bacteria than against *Escherichia coli* (Gram-negative) due to the thicker bacterial wall and the long series of interactions between the outer and inner faces of the cell, especially in *Lactobacillus* and *Streptococcus*. The reproductive system EPS of these bacteria was not affected by secondary metabolites from *P. lentiscus*, which delayed their activity. This delay can be attributed to the thickness of the outer polysaccharide layer and other influencing factors, as summarized in previous studies (Paul and Morin, 1986; Cerning, 1990; Osman et al., 2012). Significant differences were found in the amount of EPS produced by all bacterial strains tested when exposed to the various concentrations of the methanolic extract, except for the concentrations of 2 mg/mL and 4 mg/mL for *Lactobacillus salivarius*. However, there was no significant difference between the fecal flora groups, except for *Lactobacillus plantarum* strains at 2 mg/mL of the extract, where DT2 had lower EPS levels (5.21 µg/mL) compared to Hs (6.37 µg/mL). These results suggest that the concentration of the extract plays a significant role in EPS production, with lower concentrations resulting in reduced EPS production.

Anti-adherent activity

The methanolic extract of *P. lentiscus* leaves exhibited significant anti-adhesion activity against *Escherichia coli* and *Clostridium*, with percentage values above 80% (Figure 5a and 5b). Moreover, at a concentration of 2000 µg/mL, the extract showed high anti-adhesion activity in the microbiota of DT2 for *Lactobacillus plantarum* and *Lactobacillus salivarius* (Figure 5e and 5f). However, at the same concentration, anti-adherent ratios was less than 70% for *Lactobacillus brevis* and *Streptococcus alactolyticus* (Figure 5c).

The dominant flora of DT2 patients showed less adhesion in most concentrations of *P. lentiscus* methanolic extract, except for *Escherichia coli* and *Clostridium*, which exhibited higher resistance, as observed in *Escherichia coli* (Figure 5a) and homogeneous resistance between the two strains of *Clostridium* (DT2 and Hs) (Figure 5b).

Anti-adhesion activity was recorded in very low percentages, below 6% at most for low concentrations of the methanolic extract of *P. lentiscus* leaves below 250 µg/mL, and no resistance was recorded in the two strains of *Streptococcus alactolyticus* and *Lactobacillus salivarius* in HS (Figure 6c and 6f).

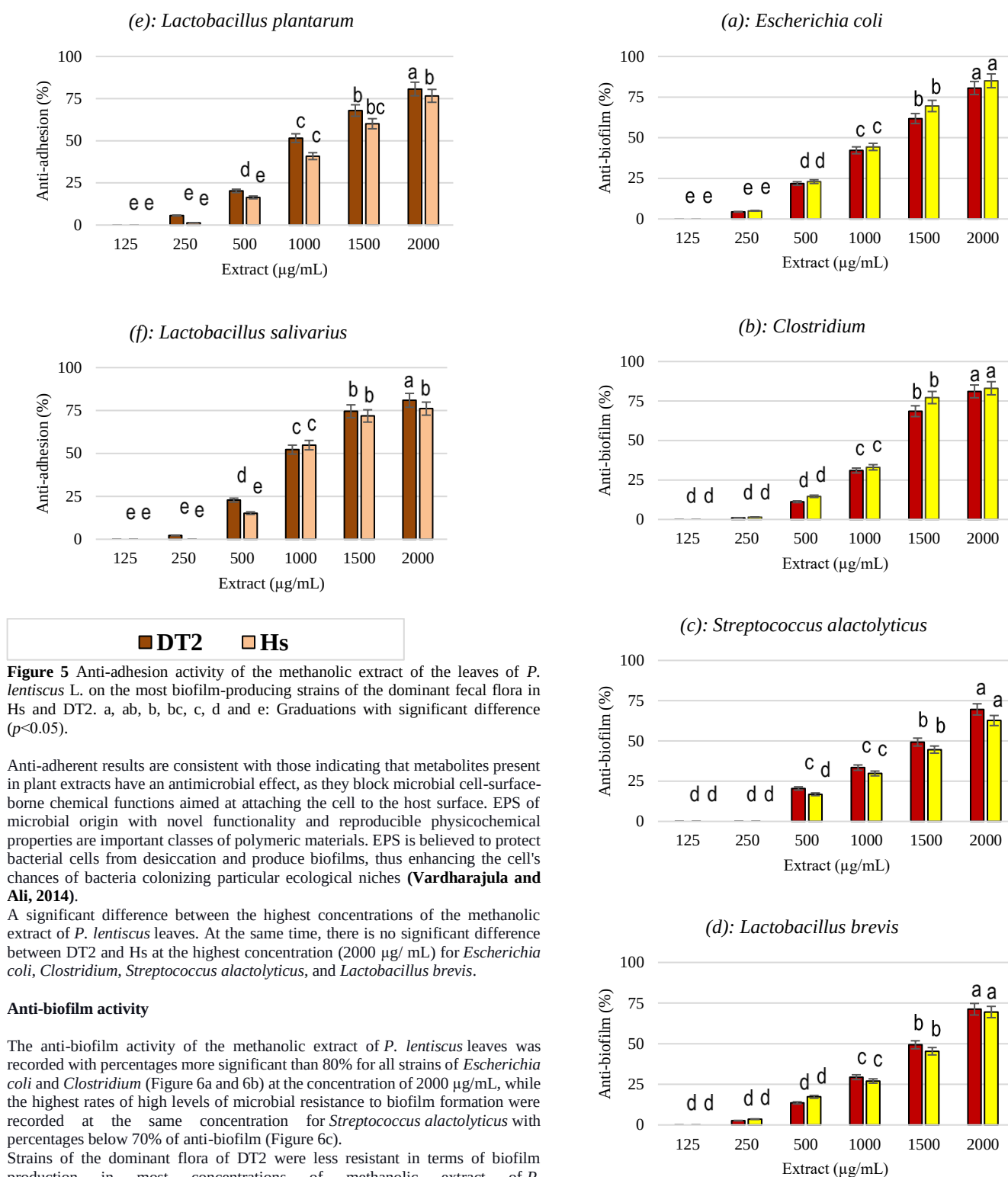


Figure 5 Anti-adhesion activity of the methanolic extract of the leaves of *P. lentiscus* L. on the most biofilm-producing strains of the dominant fecal flora in Hs and DT2. a, ab, b, bc, c, d and e: Graduations with significant difference ($p < 0.05$).

Anti-adherent results are consistent with those indicating that metabolites present in plant extracts have an antimicrobial effect, as they block microbial cell-surface-borne chemical functions aimed at attaching the cell to the host surface. EPS of microbial origin with novel functionality and reproducible physicochemical properties are important classes of polymeric materials. EPS is believed to protect bacterial cells from desiccation and produce biofilms, thus enhancing the cell's chances of bacteria colonizing particular ecological niches (Vardharajula and Ali, 2014).

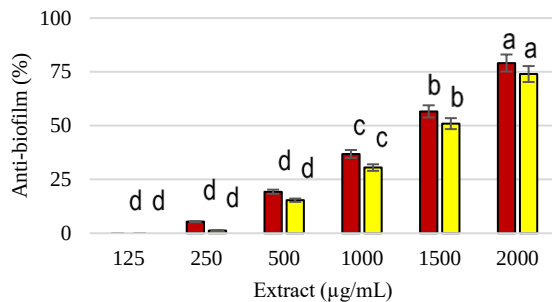
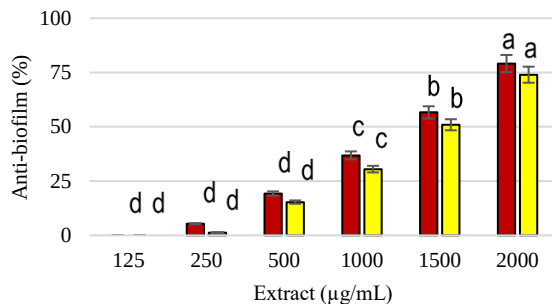
A significant difference between the highest concentrations of the methanolic extract of *P. lentiscus* leaves. At the same time, there is no significant difference between DT2 and Hs at the highest concentration (2000 µg/mL) for *Escherichia coli*, *Clostridium*, *Streptococcus alactolyticus*, and *Lactobacillus brevis*.

Anti-biofilm activity

The anti-biofilm activity of the methanolic extract of *P. lentiscus* leaves was recorded with percentages more significant than 80% for all strains of *Escherichia coli* and *Clostridium* (Figure 6a and 6b) at the concentration of 2000 µg/mL, while the highest rates of high levels of microbial resistance to biofilm formation were recorded at the same concentration for *Streptococcus alactolyticus* with percentages below 70% of anti-biofilm (Figure 6c).

Strains of the dominant flora of DT2 were less resistant in terms of biofilm production in most concentrations of methanolic extract of *P. lentiscus* leaves (Figure 6c, 6d, 6e and 6f), except for *Escherichia coli* and *Clostridium*, where these strains recorded higher opposition, especially for high concentrations of *P. lentiscus* leaf extract, and homogeneous activity was registered between the two strains of *Lactobacillus salivarius* (Figure 6f) for concentrations equal to or greater than 1000 µg/mL.

Low concentrations of the methanolic extract of *P. lentiscus* leaves below 250 µg/mL recorded anti-biofilm activity in very low percentages, below 5.5% at most. No resistance was recorded in the two strains of *Streptococcus alactolyticus* and *Lactobacillus salivarius* in Hs (Figure 6c and 6f).

(e): *Lactobacillus plantarum*(e): *Lactobacillus plantarum*

■ DT2 ■ Hs

Figure 6 Anti-biofilm activity of the methanolic extract of the leaves of *P. lentiscus* L. on the most biofilm-producing strains of the dominant fecal flora in Hs and DT2. a, b, c, d and e: Graduations with significant difference ($p < 0.05$).

Anti-biofilm results consistently show that plant extracts have an anti-biofilm effect. They prevent microbial agglomerations by inhibiting interactions between chemical functions carried on the surface of microbial cells that change their nature from one strain to another. During biofilm formation, EPS plays a crucial role in building up biofilm aggregate, an important quality of these large molecules responsible for maintaining biofilm integrity (Powell et al., 2018). According to Harimawan and Ting, a significant reduction in EPS production was recorded upon treatment with plant extracts suggesting their role in disrupting biofilm dynamics (Harimawan et al., 2016).

The strains with high percentages of the EPS have a high performance to resist the methanolic extract of leaves of *P. lentiscus* where it is anti-adhesion and anti-biofilm in other strains. There is a direct relationship between the level of EPS produced and hydrophobicity in some microorganisms of the dominant intestinal flora (*Escherichia coli* and *Clostridium* sp).

A significant difference between the highest concentrations. At the same time, there is no significant difference between DT2 and Hs bacteria, except for the concentration of 500 µg/mL in *Streptococcus alactolyticus*, which also did not cause a difference compared to the concentration of 1000 µg/mL in DT2.

On this basis, we consider that the proportion of EPS produced by the intestinal microbiota has an immediate positive effect on the motility of the outer wall of bacteria in adhesion and aggregation when there is an exogenous biochemical performance of the methanolic extract of *P. lentiscus* L. leaves. Since the range of concentration of the extract between 500 and 1000 µg/mL achieves anti-adhesion and anti-biofilm biological activity at 50% federal rates, this range of concentrations achieves the restoration and modulation of the intestinal microbiota of carriers of metabolic diseases to its healthy nature. The effect of secondary metabolites of the methanolic extract of *P. lentiscus* leaves showed more significant biological activity against Enterobacteria than Streptococci, with low percentages of this activity against benign bacteria, thus restoring the intestinal microbiota in DT2 to normal. Where there are previous studies that confirmed that the secondary metabolites of plant extracts play an important role in restoring the gut microbiota (Yong Z, Heping, 2013; Zhao et al., 2018; Bonfili et al., 2021; Lin et al., 2021; Leilei et al., 2022). Citing the health category, concentrations of less than 1000 µg/ml of the methanolic extract of *P. lentiscus* are values that preserve the healthy nature of the intestinal microbiota, which is the direct manifestation of metabolic diseases. Overall, we conclude that the methanolic extract of *P. lentiscus* leaves for concentrations greater than 500 µg/mL and less than 1000 µg/mL is a factor for restoring and modulating the intestinal microbiota of DT2 patients towards the healthy nature found in Hs.

CONCLUSION

The intestinal microbiota differs from person to person, allowing it to influence the nature of the secondary metabolites that control human health. This is the reason for our study to find a solution to normalize it naturally in patients with type two diabetes, who suffer from an increase in enterobacteria and a decrease in streptococci and lactobacillus. Exopolysaccharides with a greater percentage of hydrophobicity control the adhesion and formation of biofilm as well as the resistance of the intestinal microbiota to external agents of biological activity. Gram-positive bacteria are characterized by producing lower than normal levels of exopolysaccharides, and Gram-negative bacteria produce higher than normal percentages of people with type two diabetes. On the other hand, the biological activity of the methanolic extract of *Pistacia lentiscus* leaves is very important in modulating the dominant intestinal flora in patients with type two diabetes since it is a powerful anti-adhesion and anti-biofilm, especially against undesirable Gram-negative strains and *Clostridium*. However, it is less effective with probiotic strains, in which it acts as a growth factor. In perspective, our laboratory is working on the molecular part to determine the direct reactions between the *Pistacia lentiscus* metabolites and the microbial EPS to formulate an active galenic form.

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