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In vitro, modulation of the dominant intestinal microbiota in type 2 diabetics by controlling antimicrobial activity with the methanolic extract of *Pistacia lentiscus* L.

Bachir Bourroubey[1,2*], Nadia Chelli[1], Aicha Tir Touil[1] and Boumediene Meddah[1]

[1]Laboratory of Bioconversion, Microbiological Engineering and Health Safety, Faculty of Natural and Life Sciences, University Mustapha Stambouli of Mascara, Sidi S'aid, 29000, Mascara, Algeria. [2]Ain Tadles Hospital, EPH Belatrech Ladjel, Mostaganem, 27000, Algeria. *Author for correspondence. E-mail: bachir.bourroubey@univ-mascara.dz

ABSTRACT. The intestinal microbiota has become known as the 'second brain' a complementary organ for most metabolic and defensive reactions. The study aims to investigate the potential of the methanolic extract of *Pistacia lentiscus* leaves to modulate the dominant flora in type 2 diabetic patients compared to healthy subjects, by controlling growth kinetics. Comparative study between the dominant flora of type two diabitics (DT2) and helthy subjects (HS) stools. Using fully sterilized experimental space and materials, the germs were isolated through an antimicrobial study, and their microbial growth kinetics, were analyzed both with and without phytotherapeutic treatment *in vitro*. This was carried out as part of a study into the effect of methanolic extract from the leaves of the *Pistacia lentiscus* L. plant harvested in north-west Algeria. The study found a decrease in the quantity of lactobacilli and streptococci in DT2 and an inverse relationship between enterobacteria and streptococci in all microbiotas. On the molecular side. The methanolic extract of *P lentiscus* leaves gave a super antimicrobial effect for *E coli* and *Clostridium sp* at concentrations below their Minimum Inhibitory Concentration (2 mg mL^{-1}), compared with the other gram positives studied, *lactobacillus* and *Streptococcus* (4 mg mL^{-1}). This beneficial action confirms the high antimicrobial effect of the methanolic extract of *P lentiscus* leaves with lower concentration for the quantitative restauration of intestinal microbiota, the intermediary between insoluno-resistance and metabolism. *Pistacia lentiscus* has the potential to modulate the dominant flora in type 2 diabetics, through its regulatory antimicrobial power.

Keywords: MIC; antimicrobial activity; intestinal microbiota; *Pistacia lentiscus L*; type two diabete.

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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 body's overall health (Yatsunenko 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 negatively impact the body's health (Holdeman, Good, & Moore, 1976).

The intestinal microbiota consists 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 & Quévrain, 2016). With a weight of more than 1.5 kg and a microbial diversity of approximately 10^{14} 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). Quantitative and qualitative imbalance in the intestinal microbiota is often a major and direct cause of metabolic diseases and their complications, such as type 2 diabetes (Everard & Cani, 2013).

Type two diabetes 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 (International Diabetes Federation [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-Wisnewsky, Lefevre, & Bindels, 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 & 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 realistic average (Mehenni, 2016). This plant, which belongs to the Anacardiaceae family (Al-Saghir & Porter, 2012), called 'Darw', a tree of about 3 meters (Rodríguez-Pérez et al., 2013), is widespread in the north of Africa, especially in Algeria and Marocco (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, Cakir, Zengin, & Duru, 2003; Benhammou & Bekkara, 2009; Maxia et al., 2011; Ait-Idir & Bouyoucef, 2017; Charid et al., 2020; Pachi et al., 2020).

This study aimed to identify qualitatively and quantitatively some microbial groups isolated from the faecal 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). Afterwards, all selected microbial strains were tested with antimicrobial 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).

Materials and methods

Study population and clinical examination

This study was a single-anonymized, randomized, placebo-controlled clinical trial. It was conducted on two groups of males aged between 25 and 45 years in April 2021 at the Ain Tadles Hospital (Mostaganem, Algeria). The survey involved a structured questionnaire (Henry et al., 2019) followed by an examination by a diabetologist. Type 2 diabetes was identified according to the World Health Organization (WHO, 2016) criteria, and healthy subjects without metabolic diseases were also included. Volunteers who had taken antibiotics within three months before the study were excluded. A simplified questionnaire was administered to collect information on the participant's health status and their dietary and pharmacological regimen followed in their daily life. Informed consent was obtained from all participants.

Fresh faecal samples (n = 32) were collected from each subject (diabetic and healthy) in sterile copro-parasitology boxes. Immediately after the sample was taken from the volunteer, it was transported in boxes with a temperature not exceeding 15° C to the hospital's internal microbiology laboratory for the first isolation operations, in order to avoid any quantitative increase or decrease.

Microbiological analysis

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, 48, and up to 72 hours according to the investigated strains, and with the addition of 5% CO_2 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 diabetic and healthy subjects.

In vitro antimicrobial activities of *Pistacia lentiscus*

Pistacia lentiscus (Figure 1) 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 leaves of the *Pistacia lentiscus* plant were harvested during the last 15 days of April 2022 in the Yennaro region, located in Masra, Mostaganem, Algeria, characterized by moderate humidity and temperature.

The methanolic extraction of *Pistacia lentiscus* leaves was carried out using the maceration technique, after drying under amber and grinding. 90% pure methanol was used (volume/weight). The final extract was recovered by rotavapeuration. The phytochemical characteristics of the extract have already been published (Bourroubey, Chelli, Touil, & Meddah, 2023).

Figure 1. *Pistacia lentiscus* L. leaves.

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^{-1}) into well 2 to obtain a negative control and into well 2 to obtain the first and highest concentration for the test. Transfer 50 µL of the mixture from well to well, 3 to 12, and add 50 µL of inoculum calibrated at 10^6 CFU mL^{-1}. Incubate at 37°C and perform a 0 and 24 hours 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/CMI ratio is greater than or less than or equal to 4 or bacteriostatic when this ratio is greater than 4 (Nikaido, 2009). The growth kinetics of each species was carried out by reading 0, 2, 6, 18 and 24 hours at 600 nm, with 2 mg mL^{-1} of the extract (the MIC value decreases the most) compared to other concentrations. Log (CFU mL^{-1}) = (ODt − ODnct)/(ODt0 − ODnct0) where OD: optical density at 600 nm, t: incubation time (2, 6, 18 and 24 hours), t0: before incubation and nc: negative control. Except for the first value of less than 10^6 CFU mL^{-1} for each concentration and each strain, the conventional method was used for enumeration on glosis, and it is first of all to determine the MBC and growth carefully.

Statistical study

The microbial variation between type 2 diabetics and healthy individuals, 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 study

The partial study of the fecal flora identified six microbial groups, and qualitative and quantitative differences were observed between carriers of type 2 diabetics (DT2) and healthy subjects (HS)

Microbial Qualitative assessment of isolates

The total number of strains isolated showed a very similar diversity between DT2 and HS, based on the microbial groups studied. The total average of isolated microorganisms was 15.7 ± 3.2 in DT2 patients, slightly lower than HS (16.2 ± 1.14) (Figure 2).

We assessed (49.45 and 43.31%) of Gram-positive and Gram-negative bacteria in diabetic patients 2, comparatively to healthy subjects with (45.06 and 48.76%) (Figure 3).

The qualitative variation of the intestinal flora is influenced by various factors, including natural, environmental, nutritional (Wu et al., 2020; Duru 2022), and genetic factors (Qin et al., 2012; Al-Muhanna et al., 2021), which lead to modifications in the intestinal complex. Previous studies have reported similar results, indicating that the abundance and diversity of gut microbiota are altered in individuals with type 2 diabetes (Larsen et al., 2010; Qin et al., 2012). The variations in the intestinal flora could lead to metabolic disorders and inflammation, which are common in diabetic patients (Wu et al., 2020).

DT2 patients achieve diversity in firmicutes compared to healthy patients who achieve greater diversity in beneficial microorganisms, confirmed by previous studies (Aljahdali, 2022).

Statistically, no significant effect was observed when using qualitative variation in isolated strains and the faecal flora of the two groups as comparison factors, except for lactobacilli; a significant difference was identified between the two types of samples, as the diversity in DT2 decreased to two-thirds (3.3 ± 1.3), in contrast to HS (2.2 ± 0.7). On the other hand, there is a significant difference between the microbial families, the diversity increasing at the level of Enterobacteria and decreasing at the level of Clostridia and Yeasts ($p < 0.05$) (Figure 2).

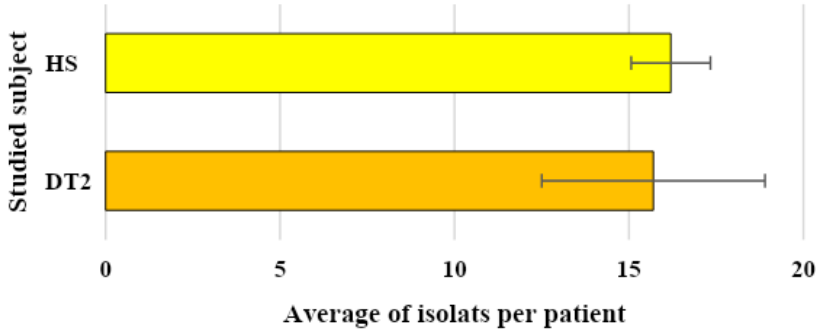

Figure 2. Average number of various isolates for each fecal flora.

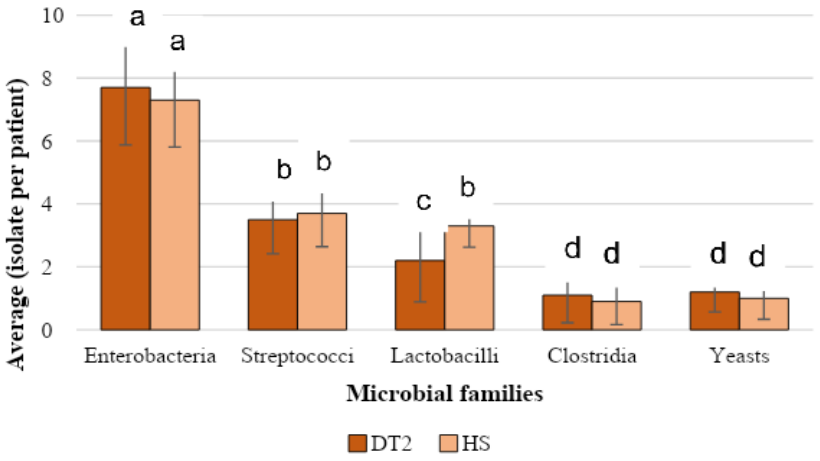

Figure 3. Average of the qualitative diversity of the isolates between the two faecal flora studied (DT2 v HS). (a, b, c and d: Graduations with significant difference).

Quantitative assessment

Overall, the microbial groups in each faecal flora studied showed a clear predominance of Enterobacteria and Streptococci with (173.53 ± 3.56) * 10^6 CFU g^{-1} and (74.64 ± 2.02) * 10^6 CFU g^{-1} in diabetic subjects. Streptococci (102.66 ± 5.23) * 10^6 CFU g^{-1} was more critical in healthy volunteers than Enterobacteriaceae (87.97 ± 2.53) * 10^6 CFU g^{-1}.

Regarding the beneficial flora (lactobacilli), a low proportion was recorded (20.52 ± 1.46) * 10^2 CFU g^{-1}) in DT2 intestinal microbiota compared to the control group (HS) with (86.50 ± 3.33) * 10^2 CFU g^{-1} ($p < 0.05$).

The levels of *Clostridium* and yeasts [(59.70 ± 1.33) * 10^2 CFU g^{-1} and (95.09 ± 3.38) * 10^2 CFU g^{-1}], respectively, were found to be higher in type 2 diabetics (DT2) than in healthy subjects (HS) [(34.60 ± 3.35) * 10^2 CFU g^{-1} and (44.55 ± 2.72) * 10^2 CFU g^{-1}].

In contrast, the levels of *Salmonella* and *Shigella* were found to be higher in type 2 diabetics (DT2) (79.52 ± 4.12) * 10^2 CFU g^{-1} than in healthy controls (68.48 ± 0.46) * 10^2 CFU g^{-1}. Regarding the statistical analysis, a significant difference was observed between Enterobacteria, Streptococci, and the remaining microbial groups. In contrast, no significant difference was found between Lactobacilli, Clostridium, and yeasts in the faecal flora of the two ($p < 0.05$) (Figure 4).

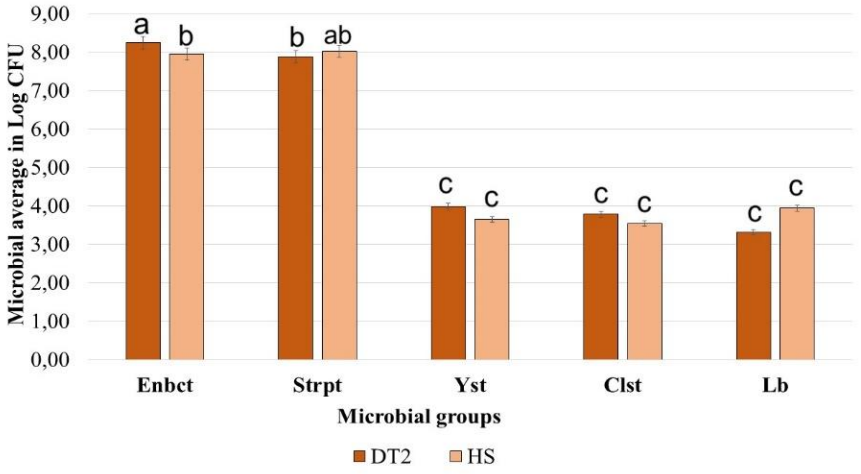

Figure 4. Microbial quantitative assessment in Log CFU comparison in studied groups (DT2 vs HS). (a, ab, b and c: Graduations with significant difference).

The findings of quantitative assessment follow previous studies that differentiate between the two types of gut microbiota (Khan, Zakir, Khanam, Shakil, & Khan, 2010). Extensive evidence has shown that diabetic patients exhibit changes in their intestinal microbiota, with a decrease in the proportion of functional bacteria and an increase in various opportunistic pathogens and some Gram-negative endotoxin-producing bacteria (Zhang & Zhang, 2013; Aljahdali, 2022). These changes can affect intestinal permeability and contribute to insulin resistance (Everard & Cani, 2013). *Lactobacillus* is a beneficial bacterium that resides in the digestive tract in small amounts and varies by species (Delroisse et al., 2008). Moreover, several studies have confirmed that certain probiotic strains can modulate blood glucose homeostasis (Panwar, Jain, Bhargaya, Akthtar, & Yun, 2012). The results of *Clostridium* and yeasts are consistent with previous research, as many species of these microorganisms have the potential to negatively affect metabolic pathways by producing biologically active metabolites, which can lead to various intestinal infections. Furthermore, these microorganisms often coexist with individuals with metabolic disorders such as type 2 diabetes (Duru, 2022). In contrast, the levels of *Salmonella* and *Shigella* were found to be higher in type 2 diabetics. This may be attributed to opportunistic and harmful strains in the intestinal microbiota of individuals with metabolic disorders (Khan et al., 2014).

Biological activity of *Pistacia lentiscus* on the dominant flora

After a comparative study of the most biofilm-producing strains in HS and DT2 patients, two main molecular factors, the first is the production of EPS**,** and the second is hydrophobicity**,** the microbiological activity of the methanolic extract of *Pistacia lentiscus* leaves was studied on certain strains of the dominant flora of the intestinal microbiota (Table 1).

Table 1. Identified strains that demonstrated significant superiority in terms of hydrophobicity and exopolysaccharide productivity between the two types of microbiota. (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*).

	Microorganismes					
	E coli	*Sr ala*	*Lb brv*	*Lb pln*	*Lb slv*	*Cls*
EPS	DT2*	HS*			HS*	HS*
Hydrophobicity	DT2*		HS*	HS*		HS*

Determination of MIC and MBC

Table 2 presents the values of MIC (Minimum Inhibitory Concentration), MBC (Minimum Bacteriocidal Concentration), and MIC/MBC ratio. The study determined that the methanolic extract of *Pistacia lentiscus* had a MIC value of 2 mg mL^{-1} for *Escherichia coli* and *Clostridium* and 4 mg mL^{-1} for *Streptococcus alactolyticus* and all the strains of *Lactobacillus* studied, except *Lactobacillus salvarius* in HS. The extract has a bacteriostatic effect for *Clostridium* and *Streptococcus*, and a bactericidal one for the other isolated strains.

Table 2. MIC and MBC values of the methanolic extract of *P. lentiscus* leaves in the bacteria studied. (MIC: Minimum Inhibitory Concentration, MBC: Minimum Bacteriocidal Concentration).

Bacteria	Group	MIC (mg mL^{-1})	MBC (mg mL^{-1})
Escherichia coli	DT2	2	8
	HS	2	8
Clostridium	DT2	2	16
	HS	2	16
Streptococcus alactolyticus	DT2	4	32
	HS	4	32
Lactobacillus brevis	DT2	4	8
	HS	4	8
Lactobacillus plantarum	DT2	4	8
	HS	4	16
Lactobacillus salivarius	DT2	4	16
	HS	8	16

Growth kinetics

Figure 5 illustrates the kinetics of bacterial growth at various concentrations of plant extract against different bacterial strains. At the minimum inhibitory concentration MIC values (2 mg mL^{-1}), there was little difference in bacterial growth between the strains with or without the extract, except for a qualitative increase in the presence of the extract from *Lactobacillus plantarum*, *Lactobacillus salivarius*, and *Streptococcus alactolyticus* during the first 2 and 4 hours of incubation. After 24 hours, a significant decrease in bacterial growth was observed for all strains in the presence of the extract, with some strains exhibiting CFU mL^{-1} values higher than the initial 10^6 (e.g., *Streptococcus* and some *Lactobacillus* strains) and others exhibiting values below the initial 10^6 (e.g., *Escherichia coli* and *Clostridium* strains), isolated from the faecal flora of two different groups (DT2 and HS). The results suggest that *P. lentiscus* extract may be effective in inhibiting the growth of certain bacterial strains, but further research is needed to determine its potential use as an antimicrobial agent.

Bacterial growth kinetics vary depending on the bacterial strain, its resistance to biologically active substances resulting from genetic changes (Nikaido, 2009; Guimarães et al., 2019), and the mode of action in the microbial cell. *Pistacia lentiscus* has an antimicrobial effect on different strains. According to Azzouzi and Brahimi in 2019 (Azzouzi & 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 acts 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 and 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. A significant effect exists between dominant and subdominant bacteria in T2D compared to HS in 2 and 4 mg mL^{-1} concentrations.

The methanolic extract of *Pistacia 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 *Pistacia 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, Morin, & Monsan, 1986; Cerning, 1990; Osman, El-Shouny, Talat, & El-Zahaby, 2012).

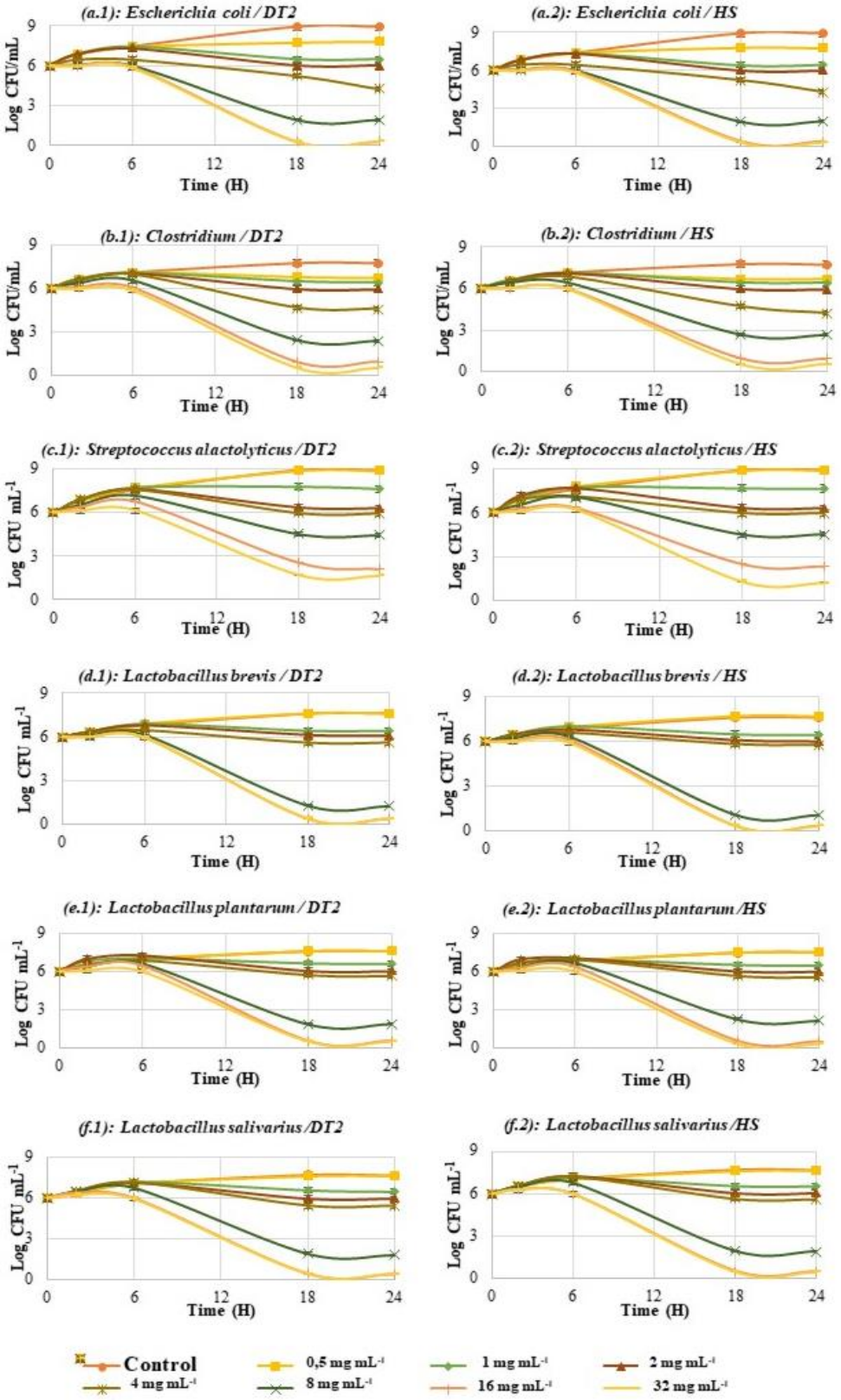

Figure 5. Growth kinetics of selected strains of dominant faecal flora in healthy subjects and type 2 diabetic patients at different concentrations of methanolic extract of *Pistacia lentiscus*.

The growth kinetics of the selected strains were graphically represented in these curves, with higher antimicrobial activity recorded after 18 hours incubation, in *E coli* and *Clostridium sp* compared to *Lactobacillus* and *Streptococcus* with the lower concentrations (0.5, 1 and 2 mg mL^{-1}),

A MIC value of 2 mg mL^{-1} was determined for *E coli* and *Clostridium* compared with those of other Gram-positive bacteria (4 mg mL^{-1}). This beneficial action confirms the high antimicrobial effect of the methanolic extract of *P lentiscus* leaves with lower concentration for the quantitative restauration of intestinal microbiota, the intermediary between insoluno-resistance and metabolism.

Abbreviations

EPS, Exopolysaccharides microbiens; DT2, Type 2 diabetic, Type two diabete; HS, Healthy subjects; MIC, Minimum Inhibitory Concentration; MBC, Minimum Bacteriocidal Concentration; CFU, Colony Forming Unit; *P. lentiscus*, *Pistacia lentiscus* L; EMB, Eosin Methylene Blue; MRS, deMan, Rogosa, and Sharpe; BEA, Bile Esculin Azide; SDA, Sabouraud Dextrose Agar.

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. A minimum inhibitory concentration (MIC) value of 2 mg mL^{-1} was determined for *E coli* and *Clostridium* compared with those of other Gram-positive bacteria (4 mg mL^{-1}). This beneficial action confirms the high antimicrobial effect of the methanolic extract of *Pistacia lentiscus* leaves with lower concentration for the quantitative restauration of intestinal microbiota, the intermediary between insoluno-resistance and metabolism.

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