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Aloysia citrodora Extracts Cultivated in Greece as Antioxidants and Potent Regulators of Food Microbiota

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Featured Application: Recently, plant extracts have attracted scientific and industrial attention as growth stimulators of desired microbes (probiotics), although their antimicrobial properties are well-known. In our study, *Aloysia citrodora* extracts stimulated the growth of a wild-type and a commercial *Lacticaseibacillus rhamnosus* strain, while growth inhibitory activity against common food-spoilage and pathogenic microbes was documented. Thus, they may be considered potential candidates for functional regulation of food microbiota, provided that they are used at the optimum concentration in food systems.

Abstract: Plant extracts contain valuable sources of biologically active molecules and, lately, have attracted scientific and industrial interest as inhibitors of food-borne pathogens and growth stimulators of beneficial microbes. In this vein, the aim of this study was to explore and exploit the effect of *Aloysia citrodora* extracts as potent functional regulators of food microbiota by stimulating the growth of probiotic strains and by suppressing the evolution of common food-spoilage and pathogenic bacteria. Aqueous and ethanolic extracts of *A. citrodora*, rich in polyphenols, were prepared and their phytochemical composition was unveiled by LC-triple quadruple and LC-QToF mass spectrometry. The growth stimulatory activity of a wild-type *Lacticaseibacillus rhamnosus* strain, along with *L. rhamnosus* GG, used as a control, was assessed by monitoring cell growth in the presence of sodium chloride, bile salts, thermal stress, and alcohol. We found that the aqueous extract stimulated the growth of probiotic strains at 0.5 mg/mL. At the same concentration, stimulatory activity was observed for the wild-type *L. rhamnosus* in the presence of bile salts and alcohol and for *L. rhamnosus* GG in the presence of NaCl and under thermal stress. The ethanolic extract of *A. citrodora* exhibited prebiotic activity at 0.25 mg/mL, but did not promote the growth of the strains under the stress conditions tested. In addition, minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) against food-spoilage and pathogenic microbes were determined. The strongest growth inhibitory activity of foodborne pathogens was noted by the *A. citrodora* ethanolic extract, while the aqueous *A. citrodora* extract had no effect against *Escherichia coli*. Importantly, the spoilage and pathogenic microbes tested were more sensitive to the extracts than the probiotic strains, indicating a significant contribution to the functional regulation of food microbiota, provided that they are used at the optimum concentration.

Keywords: growth stimulators; functional regulators; antimicrobials; foodborne pathogens; *Aloysia citrodora*; plant extracts; prebiotics; probiotics

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1. Introduction

An upsurge of interest in functional foods containing prebiotic ingredients and probiotic microorganisms is witnessed today because of the multiple associated effects. Probiotics have been described by the World Health Organization (WHO) as 'live microorganisms which when administered in adequate amounts, confer a health benefit on the host' [1]. Prebiotics refer to dietary non-digestible fibers that have a stimulatory effect on beneficial microbes, such as bifidobacterial and lactobacilli and manipulate the microbiome of the gastrointestinal tract (GI) of the host [2–4].

Lately, polyphenols have already gained scientific and industrial interest as growth stimulators for probiotics. Polyphenols are present in significant amounts in medicinal plants and, along with polyphenols, are plant metabolites that have illustrated a variety of biological actions, making them strong candidates as dietary ingredients. Their activity toward humans is typically mediated through interaction with intestinal microorganisms because of their limited absorbability in the digestive tract. Therefore, they are characterized as a novel class of prebiotics [5]. *In vitro* studies indicated that they may have a dual beneficial effect by simultaneously inhibiting infections and promoting health-promoting bacteria.

Although there is a lack of evidence for the growth stimulatory effect of *A. citrodora* extracts on lactobacilli strains, similar experiments available in the literature demonstrate that the effect of herbal extracts varies and depends usually on the genus, species, and microbial strain, as well as on the extraction method [6]. Certain lactic acid bacteria (LAB) have the ability to degrade certain phenolic compounds found in food, including those with high antioxidant activity [7,8], suggesting increased cell growth of probiotic bacteria in culture media supplemented with plant extracts.

Traditionally, medicinal plants have been consumed by humans for centuries, and plant-based therapies are among the most significant treatment modalities used in traditional medicine of diverse cultures. Plant extracts' wide spectrum of biological activities could be generally linked to their chemical composition [9]. Moreover, as natural alternatives to synthetic food preservatives, plant extracts constitute a potential source of antimicrobial compounds [10].

Aloysia citrodora (common name lemon verbena), a plant from the family of *Verbenaceae*, is one of the well-known medicinal plants with a variety of therapeutic uses [11]. Several studies have shown that *A. citrodora* exhibits therapeutic properties with respect to insomnia, anxiety disorders, asthma, fever, diarrhea, and bloating [12]. The antibacterial, antifungal, anti-inflammatory, and antioxidant properties of verbena infusion have also been noted [9,11,13–15].

Natural antioxidants included in medicinal herbs are responsible for inhibiting reactive oxygen species and lowering the negative effects of oxidative stress. Studies from several countries in the literature revealed the antioxidant activity of extracts from *A. citrodora*. For example, assessment of malondialdehyde (MDA) formation, ferric-reducing ability of plasma (FRAP), and superoxide dismutase activity (SOD) in the plasma samples after oral administration of *A. citrodora* extract in rats demonstrated the potent antioxidant activity of the plant [11].

The aim of the present work was to unveil the phytochemical profile of the plant extracts, the antioxidant activity, and the prebiotic potential of the *A. citrodora* aqueous and ethanolic extracts to promote the growth of *Lacticaseibacillus rhamnosus* strains and suppress the evolution of common food-spoilage and pathogenic bacteria. The procedure followed for the identification of the compounds and the evaluation of the biological activity of the extracts is shown in Figure 1.

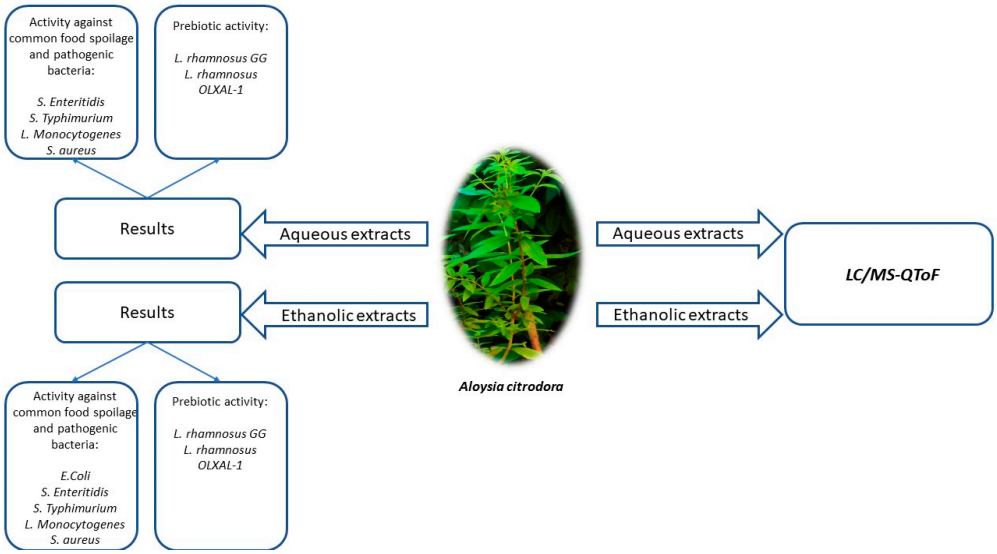


Figure 1. Flowchart of the procedures followed to identify the components of the extracts and their biological activity.

2. Materials and Methods

2.1. Collection of Plant Material

The leaves of *A. citrodora* were collected in March 2022 from the region of Epirus in western Greece. A voucher specimen of the plant was kept in the herbarium "EpirusHerbs" stored at the Department of Chemistry, University of Ioannina, with a voucher code of 0081.

2.2. Preparation of Plant Extracts

The collected leaves of *A. citrodora* were picked and then separated into three batches. The first batch was dried in the shade for two weeks, the second was dried in the oven at 60 °C, and the third underwent lyophilization at −85 °C (Telstar, LyoQuest). The first batch was selected for analysis. Then, the dry material was processed in a commercial blender to create a powder. The resultant powder was then mixed with solvent (10 mL/g of powder) before the resulting slurry was poured into a borosilicate bottle and immersed in an ultrasonic bath at 200 W and 30 °C for one hour. Every 15 min, the bottles were given a vigorous shake. A glass funnel with a sintered glass filter was used to filter the resultant suspension (porosity: 4). The solvent was removed, and the filtrate solution was collected. Then, the solution was frozen and lyophilized for the aqueous extract at 0.01 mbar at 30 °C. For the ethanolic extract, the solvent was removed on a rotary evaporator at 100 mbar and 30 °C (Labtech, EV400-V).

2.3. Analytical Procedures

2.3.1. Chemical Analysis

For the analysis, stock solutions of each extract (1 mg/mL) were prepared; 0.2 mg of dried ethanolic extract were diluted in 1 mL of pure ethanol, and, respectively, 0.2 mg of aqueous extract were diluted in 1 mL of distilled water. Each solution was sonicated for 30 min and filtered through a 0.2 µm RC filter before being analyzed.

To determine the phytochemical content of the plant extracts, we used both a Bruker EVOQ Elite triple quadrupole and a Xevo-G2-XS-QToF. The LC/MS analysis of the extract was performed on a Bruker EVOQ Elite triple quadrupole mass spectrometer in full-scan mode. Spray voltage (−) was set to 4 kV and (+) 3.5 kV, cone gas flow was set to 20 units, heated probe gas flow was set to 50 units, heated probe temperature was 350 °C, cone temperature was 200 °C, and nebulizer gas flow was set at 40 units. The LC-MS/QToF analysis of the extracts was performed on a Xevo-G2-XS-QToF coupled to a Waters UPLC I-Class Solvent Manager (Waters Corp., Milford, MA, USA). The MS conditions were as

follows: scan range was set at m/z 50–1200, and source voltage was 1 kV for negative mode and 0.8 kV for positive mode. The source temperature was 550 °C, the flow of desolvation gas (N2) was set to 1000 L/h, and cone gas flow was set to 20 L/h. For MS/MS, collision energy ramp was set from 25 eV to 45 eV, and the declustering potential was 40 V. The injection volume was 2 µL. For the post-processing and analysis of the acquisition data, the UNIFY software platform was used. For both methods, the eluent flow was set to 0.4 mL/min with H_2O with 0.1% HCOOH on channel A and acetonitrile with 0.1% HCOOH on channel B. The eluent gradient was as follows: from 0 min to 0.07 min 1% B; from 0.07 min to 10 min the eluent composition linearly increased from 1% to 100% B; from 10 min to 12.67 min the composition was maintained at 100% B; from 12.67 min to 12.73 the composition returned to initial conditions of 1% B; from 12.73 min to 15 min the composition was maintained at 1% B, after which point the next sample was injected. The column used in both methods was the Acquity UPLC® HSS T3 1.8 µM.

2.3.2. Determination of Total Phenolic Content

Using the Folin–Ciocalteu reagent and a colorimetric test at 725 nm, the total phenol concentration of the extracts was determined: 480 µL of distilled water, 20 µL of plant extracts (1 mg/mL), and 50 µL of Folin–Ciocalteu reagent were combined; 100 µL of a saturated solution of Na_2CO_3 was added after 3 min, and 350 µL of that solution was then diluted with distilled water. Blank samples were created using 20 µL of ethanol. The absorption was observed at 725 nm after one hour. The same process was used to create the calibration curves, but instead of using plant extract, 20 µL of gallic acid solution was used. Gallic acid's calibration curve required standard solutions with concentrations ranging from 50 to 250 mg/mL. The absorption of the samples was recorded after 1 h using a UV-Vis Spectrometer, DS5, Edinburg Instruments (University of Ioannina, Department of Chemistry, Ioannina, Greece).

2.4. Biological Evaluation

2.4.1. Microbial Strains

The commercial *Lacticaseibacillus rhamnosus* GG and the wild-type *L. rhamnosus* OLXAL-1 isolated from Greek olives [16] were grown in de Man, Rogosa, and Sharpe broth (MRS, Condalab, Spain) at 37 °C for 24 h.

Salmonella enterica subsp. *enterica* ser. Enteritidis FMCC B56 PT4 (kindly provided by Prof. Nychas G. J. E., Agricultural University of Athens, Athens, Greece), *Salmonella enterica* subsp. *enterica* ser. Typhimurium DSMZ 554, *Listeria monocytogenes* NCTC 10527 serotype 4b, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923 were grown in brain–heart infusion (BHI) broth at 37 °C for 24 h.

2.4.2. Growth Stimulatory Activity

The growth stimulatory activity of the plant extracts was assessed as described by Milutinović et al. [5]. Briefly, plant extracts were added to fresh MRS at various concentrations, ranging from 0.25–10 mg (dry matter)/mL. Then, the culture medium was inoculated with an overnight growth culture of *L. rhamnosus* GG or *L. rhamnosus* OLXAL-1 to achieve a final concentration of 5×10^6 cfu/mL and incubated at 37 °C for 24 h. After incubation, samples were 10-fold diluted in one-quarter-strength Ringer's solution, plated on MRS agar medium and incubated at 37 °C for 48 h. MRS broths with no inoculum and inoculated MRS broths with no plant extract served as negative and positive controls, respectively. The effect of plant extracts on cell growth was determined by comparing the number of CFUs in the samples containing the plant extracts to the controls.

2.4.3. Prebiotic Index

The prebiotic index (I_{preb}), defined as the ratio of probiotic growth with the addition of the prebiotic agent to probiotic growth in a control medium, expressed in CFU, was

calculated as described by Palframan et al. [17] and Figueroa-Gonzalez et al. [18], according to the following equation:

$$I_{preb} = \frac{\text{CFU of probiotic cultured with the prebiotic}}{\text{CFU of probiotics on standard medium without the prebiotic}} \quad (1)$$

2.4.4. Osmotic Stress Tolerance Assay

The ability of the lactobacilli strains to tolerate osmotic stress in the presence of plant extracts was tested using various concentrations of NaCl, according to Sampaio et al. [19], with slight modifications. In brief, MRS broth containing various NaCl concentrations (0%, 1%, 2%, 4%, 6%, 8%, and 10% *w*/*v*) was supplemented with plant extract concentrations ranging from 0.25–10 mg (dry matter)/mL) and inoculated with an overnight growth culture of *L. rhamnosus* GG or *L. rhamnosus* OLXAL-1 to achieve a final concentration of 5×10^6 cfu/mL. After incubation at 37 °C for 24 h, samples were 10-fold diluted in one-quarter-strength Ringer's solution and plated on MRS agar medium and incubated at 37 °C for 48 h. Inoculated MRS broth without NaCl served as positive control, and inoculated MRS broths without plant extract served as negative controls.

2.4.5. Bile Salt Tolerance Assay

The bile salt tolerance of the lactobacilli in the presence of the plant extract was determined as described by Nath et al. [20], with slight modifications. In brief, plant extracts were added to MRS broth medium, which contained 0.3% *w*/*v* bile salts at various concentrations, ranging from 0.25–10 mg (dry matter)/mL. Then, the culture medium was inoculated with an overnight growth culture of *L. rhamnosus* GG or *L. rhamnosus* OLXAL-1 to achieve a final concentration of 5×10^6 cfu/mL and incubated at 37 °C for 4 h. Cell growth was monitored at different time intervals (0 h, 2 h, and 4 h), by plate counting in MRS agar. Inoculated MRS broth, with bile salts but no plant extract, served as a control.

2.4.6. Thermal Stress Assay

PBS solutions (pH 7), supplemented with the plant extracts at concentrations of (0.25–10 mg (dry matter)/mL) were inoculated with the lactobacilli cultures and incubated at 52 °C for 35 min. Samples were collected for enumeration at different intervals (0, 10, 20, and 30 min). Culture suspensions without the extracts incubated at 52 °C for 35 min served as controls [21]. All suspensions subjected to thermal stress were maintained in a water bath previously set at 52 °C, and the temperature was continuously monitored.

2.4.7. Alcohol Tolerance

Alcohol tolerance was tested in inoculated MRS broth (10^6 cfu/mL) supplemented with plant extracts at various concentrations (0.25–10 mg (dry matter)/mL) containing various alcohol concentrations (4%, 6%, 8%, 10%, and 12% *v*/*v*). After incubation at 37 °C for 12 h, samples were 10-fold diluted in one-quarter-strength Ringer's solution and plated on MRS agar at 37 °C for 48 h. Inoculated MRS broths without alcohol served as positive controls, and inoculated MRS broths with no plant extract served as negative controls [22].

2.4.8. Antimicrobial Assays

Broth microdilution method was used to determine minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) [23,24]. In brief, bacterial suspensions in MRS or BHI broths supplemented with plant extracts (0.2–400 mg (dry matter)/mL) were incubated at 37 °C for 24 h in a 96-well microplate. After incubation, the optical density was determined at 620 nm (EnSpire Multimode Plate Reader, Perkin Elmer, Waltham, MA, USA), and MIC was defined as the lowest concentration at which no bacterial growth was observed. MRS broths or BHI broths with no inoculum and inoculated MRS broths or BHI broths with no plant extracts were used as controls.

Minimum bactericidal concentration (MBC) was determined, as recently described by Mitropoulou et al. [24].

2.4.9. Antioxidant Activity: DPPH-Radical Scavenging Activity

The evaluation of the DPPH radical-scavenging impact is provided below. Each extract's initial stock concentration (1.0 mg/mL) was diluted to final concentrations of 500, 250, 100, 50, and 10 µg/mL in methanol; 2.5 mL of sample solutions at various concentrations were combined with 1 mL of a 0.3 mM DPPH• methanolic solution, and the mixture was left to react at room temperature. The following equation was used to translate the absorbance (Ab) at 517 nm into the percentage antioxidant activity after 30 min:

$$\text{Scavenging capacity \%} = 100 - [(\text{Ab of sample} - \text{Ab of blank}) \times 100/\text{Ab of control}] \quad (2)$$

DPPH solution plus methanol was used as the control, while methanol (1 mL) and extract compound solution (2.5 mL) were utilized as the blank. The absorption of samples was recorded after 30 min of incubation, using a UV-Vis Spectrometer, DS5, Edinburg Instruments, (University of Ioannina, Department of Chemistry, Ioannina, Greece).

2.5. Statistical Analysis

All experiments were performed at least in triplicate and the mean values ± SD are presented. The results were analyzed for statistical significance with analysis of variance (ANOVA). Tukey test and independent *t*-test were used to determine significant differences. [Coefficients, ANOVA tables, and significance ($p < 0.05$) were computed using IBM SPSS Statistics for Windows, v.24.0 (IBM Corp., Armonk, NY, USA)].

3. Results and Discussion

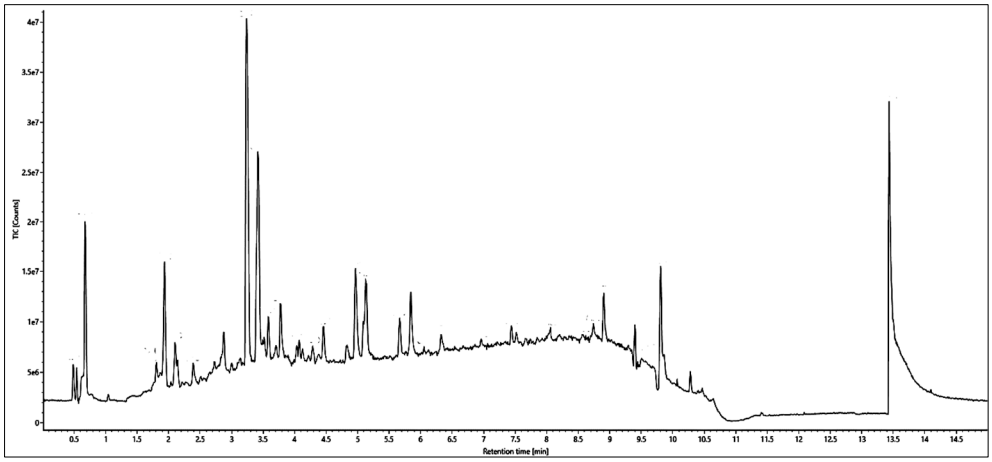
3.1. Plant Extracts Preparation

A. citrodora aqueous and ethanolic extracts from plant samples originating from the Epirus region in northwest Greece were produced. Several techniques were taken into consideration to provide the optimum plant preparation and extraction conditions. Three techniques for drying plants were examined, starting with newly harvested plant material. The first approach involved natural drying in a temperature-controlled room at 30 °C; the second involved drying material in an oven at 60 °C; and the third involved lyophilization to remove water content. The finely ground material was sonicated with solvent for three days at room temperature, and the resulting extract was evaluated under these extraction conditions. We identified that for the specific plant material, the sonication procedure, followed by lyophilization, provided the optimal results for the specific medicinal plant. We found that lyophilization followed by sonication delivered, for the specific medicinal plants, the optimal phytochemical content as screened by 1D NMR.

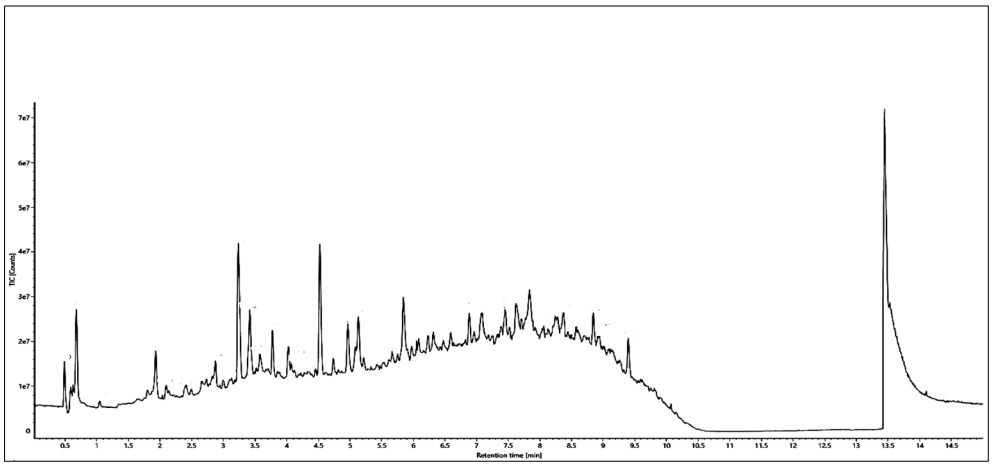
3.2. Phytochemical Analysis

We utilized LC-MS to analyze the plant extracts. For the elucidation of the discovered ions, triple quadruple mass spectrometry employed internal libraries. Analysis using the LC-QToF was used to supplement compound characterization. Data from both techniques were cross-referenced to produce the final report. We decided to remove substances with minimal response factors for the sake of simplicity. It is interesting that the resulting, distinctive pieces ($M^+ - [C_6O_6H_5]$) were employed as validation for the anticipated molecules in the case of hexosides, such as glucosides, glucoronides, etc. Additionally, because analysis was performed in both the ethanolic and aqueous extracts, we were able to rule out false positives that might have occurred because of the makeup of any component.

In Figure 2a,b, we illustrate the LC-MS chromatographs (negative and positive mode) for *A. citrodora* ethanolic extract, while in Figure 2c,d, we illustrate the LC-MS chromatograph (negative and positive mode, respectively) for *A. citrodora* aqueous extract. The compounds identified in the *A. citrodora* extracts can be seen in Tables 1–4 in negative and positive ESI modes.



(a)



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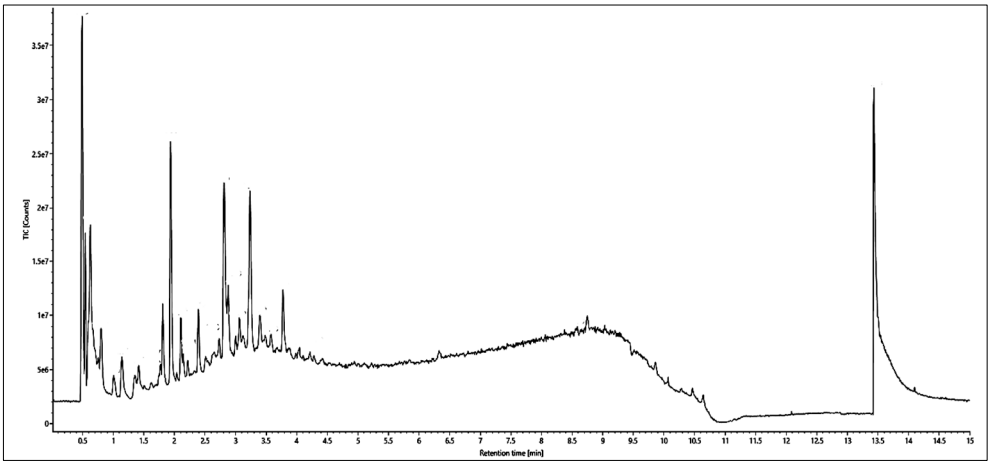
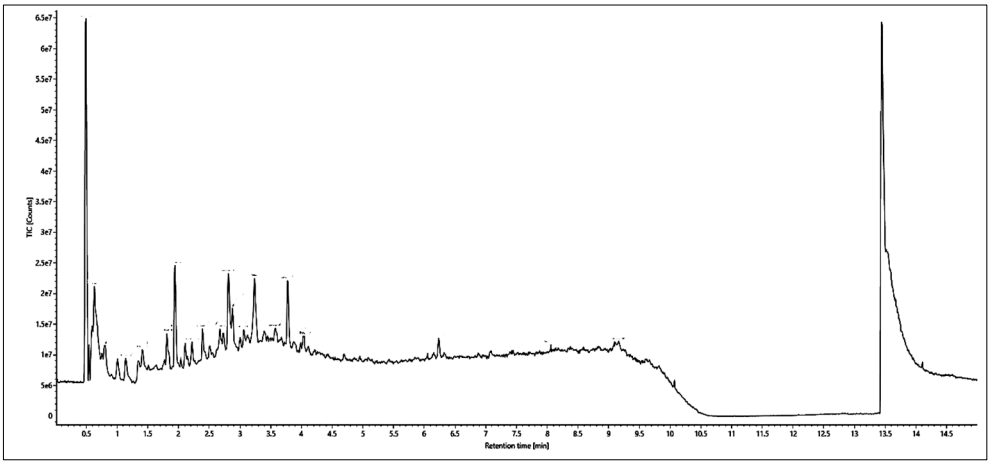


(c)

Figure 2. *Cont*.



(**d**)

Figure 2. LC/MS chromatographs of the *Aloysia citrodora* ethanolic (**a**) negative mode and (**b**) positive mode and of the aqueous extracts (**c**) negative and (**d**) positive modes.

Table 1. Identified compounds in *A. citrodora* ethanolic extract- (ESI negative mode).

***Aloysia citrodora* EtOH Extract—Negative**				
Row No.	**Component Name**	**Observed *m*/*z***	**Mass Error (mDa)**	**Response**
1	Acteoside	623.1986	0.4	2,292,685
2	Isoacteoside	623.1982	0	1,882,823
3	Cirsiliol	329.0668	0.1	963,036
4	Hispidulin	299.0562	0.1	823,986
5	Eupatorin	343.0822	−0.1	591,793
6	Pectolinarigenin	313.072	0.2	387,648
7	Isorhamnetin	315.051	0	299,038
8	Geniposidic-Acid	373.1138	−0.2	295,613
9	Leucosceptoside A	637.2136	−0.2	208,446
10	Apigenin	269.0456	0.1	132,449
11	Naringenin	271.0611	−0.1	123,043
12	Luteolin	285.0403	−0.2	35,290
13	Isorhamnetin 3-O-glucoside	477.1037	−0.1	27,382
14	Homoplantaginin_Tectoridin	461.1086	−0.4	23,435
15	4-Hydroxybenzoic acid 4-O-glucoside	299.0768	−0.4	13,422

Table 2. Identified compounds in *A. citrodora* ethanolic extract- (ESI positive mode).

***Aloysia citrodora* EtOH Extract—Positive**				
Row No.	**Component Name**	**Observed *m*/*z***	**Mass Error (mDa)**	**Response**
1	Acteoside	647.1942	−0.5	797,019
2	Isoacteoside	647.1949	0.3	498,939
3	Cirsiliol	331.0807	−0.6	486,000
4	Eupatorin	345.0961	−0.7	434,876
5	Hispidulin	301.0708	0.2	354,196
6	Geniposidic-Acid	397.11	−0.6	287,194
7	Dimethylmatairesinol	409.1614	−0.7	165,605
8	Pectolinarigenin	315.0855	−0.8	86,037
9	Isorhamnetin	317.0647	−0.9	73,872
10	Pinoresinol-4-O-Beta-Monoglycoside	543.1831	−0.5	58,992
11	Leucosceptoside A	661.2101	−0.2	55,225
12	Kaempferol	286.0465	−0.7	30,280
13	Apigenin	271.0594	−0.7	16,561
14	Naringenin	273.0749	−0.8	14,227

Table 3. Identified compounds in *A. citrodora* aqueous extract- (ESI negative mode).

Aloysia citrodora H_2O Extract—Negative				
Row No.	**Component Name**	**Observed *m/z***	**Mass Error (mDa)**	**Response**
1	Luteolin 7-O-diglucuronide	637.104	−0.6	1,157,953
2	Acteoside	623.198	−0.2	971,531
3	Geniposidic-Acid	373.1143	0.3	399,595
4	Isoacteoside	623.1985	0.4	269,646
5	Malic acid	133.0143	0.1	223,063
6	Apigenin 7-O-diglucuronide	621.1094	−0.3	202,647
7	4-Hydroxybenzoic acid 4-O-glucoside	299.0772	0	54,020
8	Eupatorin	343.0819	−0.5	33,223
9	Leucosceptoside A	637.2129	−0.9	23,761
10	Vicenin-2	593.151	−0.2	22,973
11	Cirsiliol	329.0665	−0.2	14,545
12	Pectolinarigenin	313.0716	−0.2	13,039
13	Protocatechuic acid 4-O-glucoside	315.0723	0.1	12,191
14	Eriocitrin	595.1663	−0.6	11,704
15	Coumaric acid (p-)	163.0399	−0.2	8432

Table 4. Identified compounds in *A. citrodora* aqueous extract- (ESI positive mode).

Aloysia citrodora H_2O Extract—Positive				
Row No.	**Component Name**	**Observed *m/z***	**Mass Error (mDa)**	**Response**
1	Luteolin 7-O-diglucuronide	639.1197	0.5	655,236
2	Geniposidic-Acid	397.1095	−1	380,211
3	Acteoside	647.1952	0.6	236,018
4	Apigenin 7-O-diglucuronide	623.1246	0.3	134,959
5	Dimethylmatairesinol	409.1614	−0.8	124,934
6	Pinoresinol-4-O-Beta-Monoglycoside	543.1842	0.5	41,603
7	Isoacteoside	647.1966	2	37,067
8	4-Hydroxybenzoic acid 4-O-glucoside	323.0729	−0.8	34,696
9	Eupatorin	345.0958	−1.1	17,851
10	Leucosceptoside A	661.2112	0.9	6345
11	Coumaric acid-4′-O-glucoside	349.0882	−1.2	4471
12	Herniarin	177.0538	−0.8	3055
13	Cirsiliol	331.0797	−1.5	2977
14	Apigenin-7-O-glucuronide	447.0926	0.4	2594
15	Vicenin-2	617.1463	−1.4	2533

During our LC-MS-based analysis, several categories of phytochemicals have been identified. Various flavonoids have been determined in the ethanolic and aqueous plant extracts. Caffeoyl phenylpropanoid, known as acteoside or verbascoside, is extensively found in the plant kingdom, particularly in the *Aloysia* species [13]. Based on our analysis, both ethanolic and aqueous extracts are rich in acteoside. The second-most abundant compounds in the ethanolic extract were isoacteoside, eupatorin, pectolinarigenin, isorhamnetin, leucosceptoside A, cirsiliol, apigenin, narigenin, hispidulin, and kaempferol. In the aqueous extract, luteolin 7-O-diglucuronide was a major component, as were apigenin 7-O-diglucuronide, eupatorin, vicenin-2, eriocitrin, and geniposidic acid.

The majority of the identified compounds from the plant extracts have been reported to have significant biological activities. For example, verbascoside is a potent antioxidant agent. Thiobarbituric acid-reactive substances (TBARS) and trolox equivalent antioxidant

capacity (TEAC) assays revealed a protective effect on the ocular tissue and fluids against oxidative stress following oral administration of verbascoside to hares (maximum dose of 3 mg/day) [11]. Yan Liu et al. [25] reported that verbascoside alleviated experimental colitis in mice by regulating the gut microbiota. The outcomes demonstrated that in colitic mice, acteoside reduces intestinal inflammation, oxidative stress, and nuclear factor-B (NF-B) activation. Additionally, acteoside partially reversed the dysbiosis of the gut microbiome in colitic mice, which was evident in the altered gut microbiome structure and the enrichment of helpful bacteria (*Akkermansia muciniphila* and *Bacteroides thetaiotaomicron*) [25]. A hydroxycinnamic acid that has been identified in the extracts was isoacteoside, a compound that exhibited antioxidant activity by scavenging intracellular ROS, improving the effects of antioxidant enzymes and promoting cell survival. It also prevented H_2O_2-induced apoptosis [26]. Moreover, Gao et al. [27] demonstrated the anti-inflammatory activity of isoacteoside *in vitro* and *in vivo*. The underlying process might occur through modifications of the NF-kB and MAPK pathways [27]. A derivative of acteoside, leucosceptoside A, also has a significant antioxidant activity, as indicated in the international literature [28].

Another compound, cirsiliol, a flavone with various biological activities, has been identified in the plant extracts. Hyung-Jin Lim et al. [29], investigated the anti-inflammatory effects of cirsiliol on IL-6-induced activity. The results showed that cirsiliol controls JAK2 phosphorylation to inhibit IL-6-induced cellular signaling and, therefore, can be used as a treatment for inflammatory illnesses linked to IL-6 [29].

During our analysis, an interesting bioactive flavonoid has been revealed: eupatorin, which has potent anti-inflammatory activities [30]. Moreover, eupatorin exhibits antioxidant activity against various oxidative modifications of endothelium and LDL [30]. In the ethanolic extract, isorhamnetin, an O-methylated flavonol from the class of flavonoids and a metabolite of quercetin, has been identified. Isorhamnetin has been reported to have various pharmacological activities, including anti-inflammation, antioxidation, and apoptosis modulation actions. In addition, it is a potent antioxidant and antiviral agent [31]. Li et al. [32] revealed the ability of isorhamnetin to prevent type 2 diabetes in rats by regulating their gut microbiome.

Geniposidic acid, which is classified as an iridoid glucoside, has been identified in our analysis. Wang et al. [33] have demonstrated that geniposidic acid regulates autophagy and oxidative stress. In addition, this compound has antiviral activity against white spot syndrome virus replication in red swamp crayfish [34].

Apigenin, a natural product belonging to the flavone class, is also present in both studied extracts. Apigenin has numerous health benefits, including antitumor activity, protection of biological organs (heart, brain, liver, and lung), lipid-lowering action, hypotension, hypoglycemia, antioxidation, anti-inflammation, anti-osteoporosis, and immune regulation, which is useful for understanding the health benefits of apigenin-rich foods. Moreover, Fu et al. (2022) have indicated that apigenin modifies the gut microbiota to treat ulcerative colitis [35]. Their study demonstrated that apigenin potentially improves the symptoms of ulcerative colitis (UC) by enhancing the intestine's biological, mechanical, and immunological barriers in UC mice [35]. Another study showed that some gut bacteria can regulate the human body when apigenin or its glycosides are absorbed by breaking them down into smaller metabolites [36]. Additionally, apigenin itself is used as a potential functional food for the prevention and treatment of several associated ailments, although considerable research needs to be done to pinpoint its precise mechanisms. Future research is still needed to determine the safety of apigenin because a high dose may cause hepatotoxicity [37].

Naringenin is a flavonoid belonging to the flavanones. Several biological activities have been ascribed to this phytochemical, among them antioxidant, antitumor, antiviral, antibacterial, anti-inflammatory, anti-adipogenic, and cardioprotective effects [38]. Lin et al. [39] evaluated the inhibitory effects of naringenin on ovarian cancer, and the results showed that naringenin inhibits the expression of the PI3K pathway, improves gut microbiota, and suppresses epithelial ovarian cancer.

Luteolin, a common phytochemical, was found in *A. citrodora*'s extracts. Preclinical research has established a wide range of pharmacological properties for this flavone, including antioxidant, anti-inflammatory, and antibacterial properties. Li et al. [40] found that in UC rats, luteolin therapy may reduce colonic inflammation and injury. In the same study, results showed that after luteolin therapy, the diversity of the gut microbiota in UC rats changed. Luteolin could improve UC by controlling the structure and composition of the gut microbiota [40]. Another antimicrobial phytochemical is homoplantaginin. Li et al. [41] demonstrated the antimicrobial activity of this compound, which has been isolated from the leaves of *Scrophularia ningpoensis*.

Tectoridin, an isoflavone, is the 7-glucoside of tectorigenin, a phytochemical with multiple biological activities, which was found during the analysis. Niu X. et al. [42], exhibited the anti-platelet, anti-angiogenic, and anti-proliferative activities of tectoridin.

The chemical structures of the main identified compounds are shown in Figure 3.

3.3. Determination of Total Phenolic Content

The ethanol extract of *A. citrodora* revealed the highest gallic acid equivalent amount with a value of 69.10 ± 0.02 mg gallic acid/g dry extract and for the aqueous extract 60.70 ± 0.02 mg gallic acid/g dry extract.

3.4. Growth Stimulatory Activity

The Greek endemic plant extracts of *A. citrodora* were tested for their potential growth stimulatory effects on two lactobacilli strains. The results indicated significant ($p < 0.05$) growth stimulatory of *L. rhamnosus* GG and *L. rhamnosus* OLXAL-1 strains at 0.5 mg (dry matter)/mL of the aqueous extract (Figure 4a). Furthermore, *A. citrodora* ethanolic extract stimulated the growth of both lactobacilli only at 0.25 mg (dry matter)/mL ($p < 0.05$), while no stimulatory activity was recorded at the other concentrations tested ($p > 0.05$, Figure 4b).

In addition, the prebiotic index ranged above 1 for both *L. rhamnosus* strains incubated with *A. citrodora* aqueous extract at all concentrations with a significantly increased value at 0.5 (dry matter)/mL ($p < 0.05$, Figure 5a). For the ethanolic extract, prebiotic index values ranged at lower levels, but a significantly increased prebiotic index was recorded at 0.25 mg (dry matter)/mL ($p < 0.05$, Figure 5b). A prebiotic index exceeding the value of 1 suggests a positive stimulatory effect on probiotic growth, while values below 1 indicate low effectiveness.

The results of the osmotic stress using NaCl at various concentrations demonstrated that *A. citrodora* aqueous extract significantly promoted the cell growth of *L. rhamnosus* GG at 0.5 mg (dry matter)/mL in NaCl-supplemented MRS (1–4% w/v, $p < 0.05$), while no significant growth was observed at 6–10% w/v NaCl ($p > 0.05$) (Figure 6a,b). On the contrary, *A. citrodora* aqueous extract reported no substantial contribution to *L. rhamnosus* OLXAL-1 growth in the presence of NaCl (1–10% w/v, $p > 0.05$).

Bile salt tolerance assay showed an increased survival rate of *L. rhamnosus* OLXAL-1 incubated with *A. citrodora* aqueous extract [0.5 mg (dry matter)/mL, $p < 0.05$] after 4 h (Figure 7b). Interestingly, no effect was observed for *L. rhamnosus* GG incubated with the *A. citrodora* aqueous extract ($p > 0.05$) (Figure 7a).

The results of the stimulatory activity of the aqueous *A. citrodora* extract [0.25–0.5 mg (dry matter)/mL] on the lactobacilli strains regarding their performance under thermal stress at 52 °C revealed that the plant extract had a positive effect on *L. rhamnosus* GG cell growth ($p > 0.05$) even after 30 min of exposure. On the other hand, no growth stimulatory effect was reported for *L. rhamnosus* OLXAL-1 at 52 °C, since the final cell counts ranged at similar levels between the examined cases and the control ($p > 0.05$) (Figure 8a,b). The above mentioned result might suggest that *L. rhamnosus* OLXAL-1 possibly possesses thermally resistant properties.

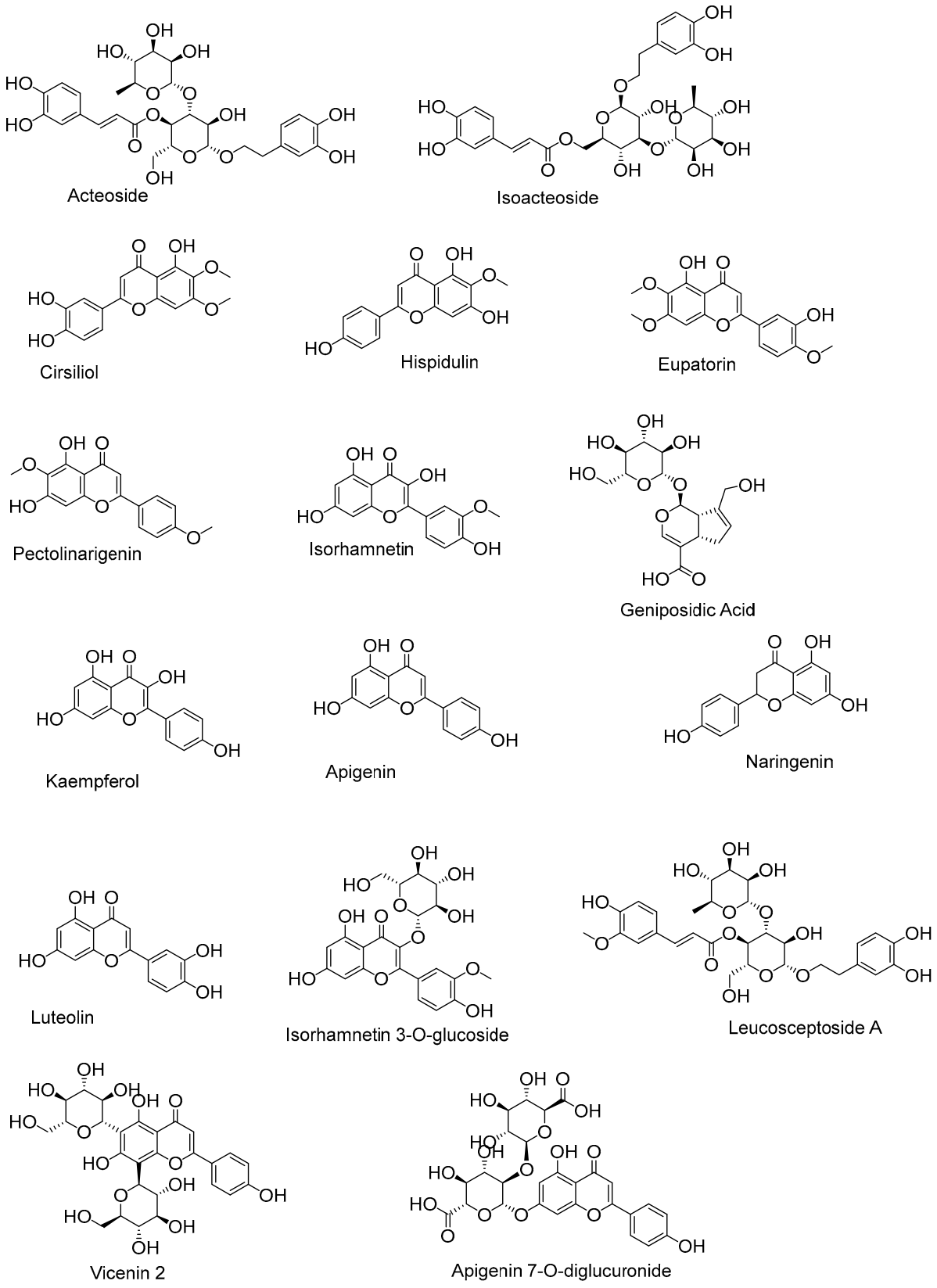
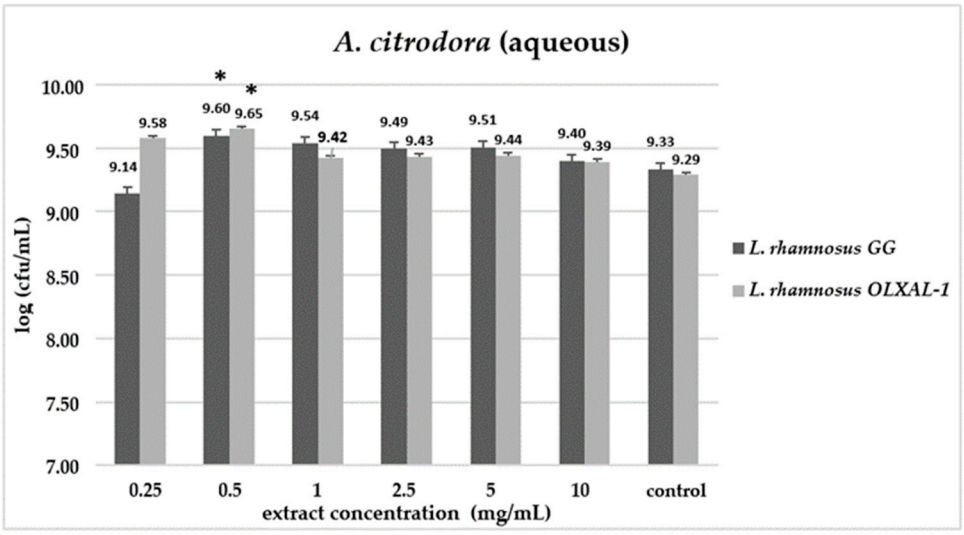


Figure 3. Chemical structures of the main identified phytochemicals.



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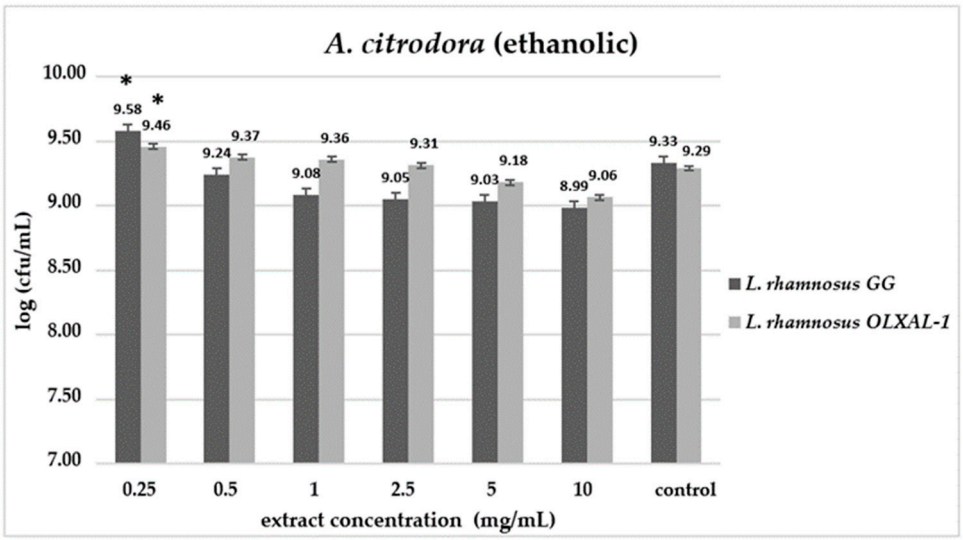
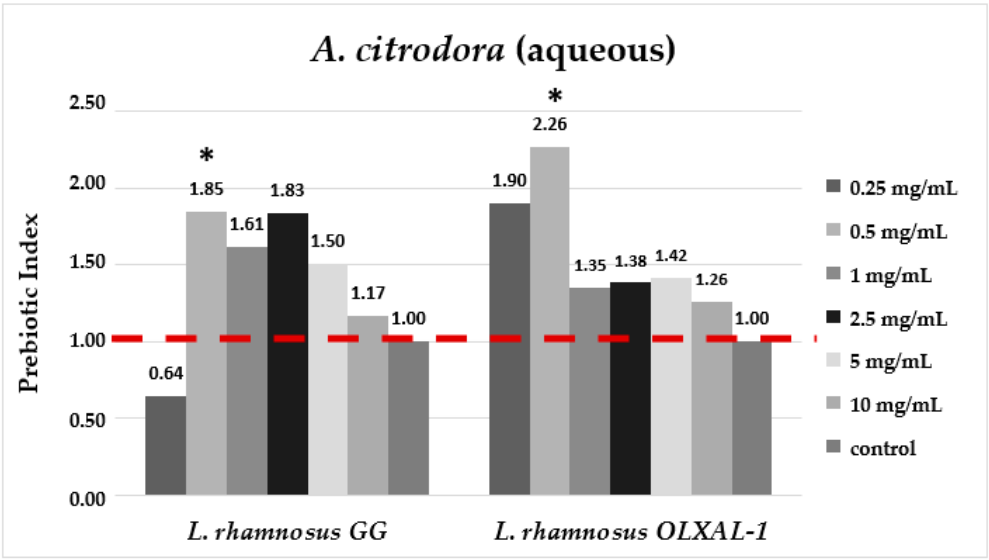


(**b**)

Figure 4. Effect of (**a**) aqueous and (**b**)_ethanolic *A. citrodora* extracts [mg (dry matter)/mL] as growth stimulators of *Lacticaseibacillus rhamnosus* GG and *Lacticaseibacillus rhamnosus* OLXAL-1. Significant difference ($p < 0.05$) compared to the control (MRS broth) is indicated with *.

When subjected to alcohol stress, cell growth of *L. rhamnosus* OLXAL-1 was positively affected by *A. citrodora* aqueous extract at 0.5 and (dry matter)/mL ($p < 0.05$) compared to the controls, even at the highest alcohol concentration, 12% v/v (Figure 9b). A positive effect was also documented on *L. rhamnosus* GG ($p > 0.05$), only at 4% and 6% alcohol, where the addition of 0.5 (dry matter)/mL *A. citrodora* aqueous extract significantly promoted cell growth ($p < 0.05$, Figure 9a). No stimulatory activity was reported for *L. rhamnosus* GG in other alcohol concentrations.



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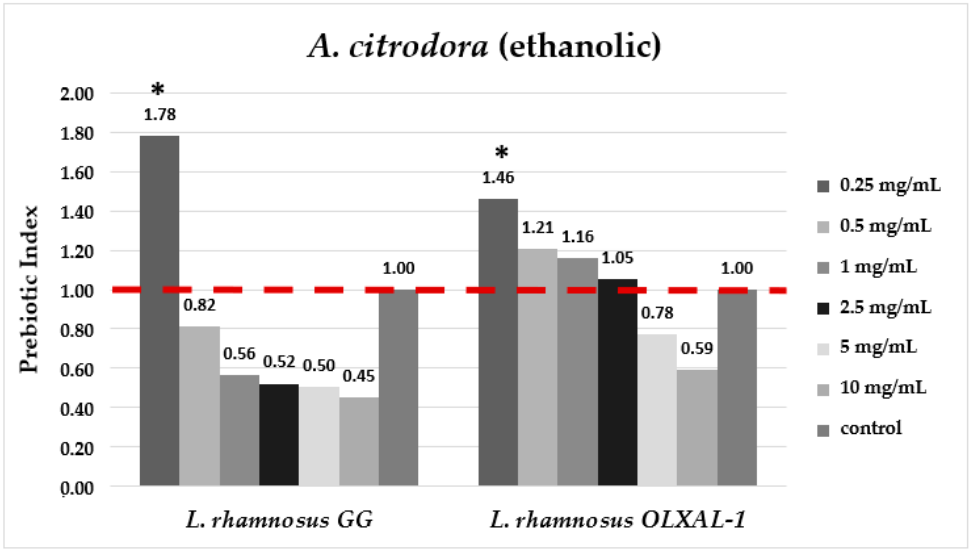
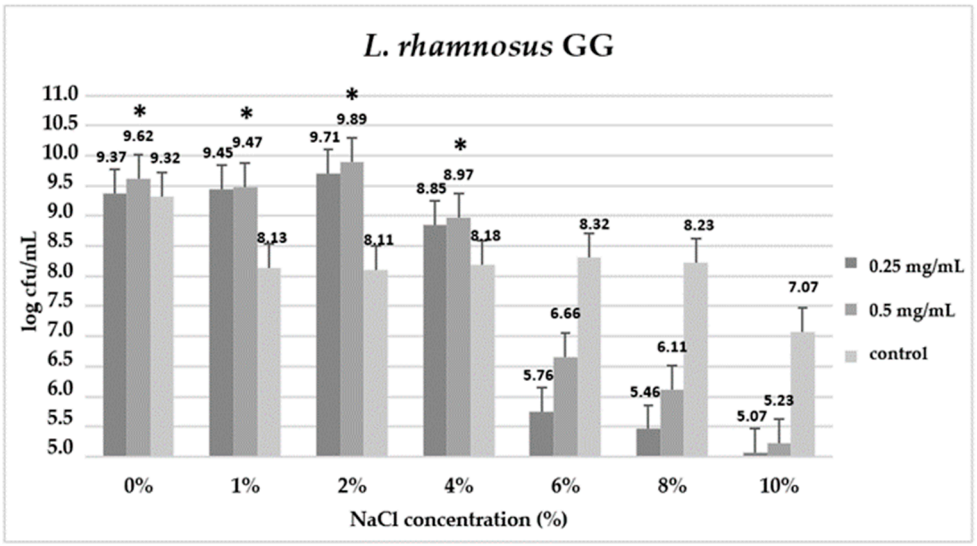


(**b**)

Figure 5. Prebiotic index values of (**a**) aqueous and (**b**) ethanolic *A. citrodora* extracts based on *Lacticaseibacillus rhamnosus* GG and *Lacticaseibacillus rhamnosus* OLXAL-1. Significant difference ($p < 0.05$) compared to the control (MRS broth) is indicated with *.

Similar results regarding the stimulatory effect of plant extracts were observed in peonidin-based anthocyanins extracted from purple sweet potato (*Ipomoea batatas* (L.) Lam.) [43], as well as in culinary spice extracts on various beneficial microbes, including *L. rhamnosus* strains [44]. Polysaccharides and phenolic compounds can increase the proportion of probiotic bacteria in the gut, regulate the intestinal microenvironment, and stimulate the innate immune system to fight infections by promoting macrophages and lymphocytes within the intestinal mucosa [2,45,46].



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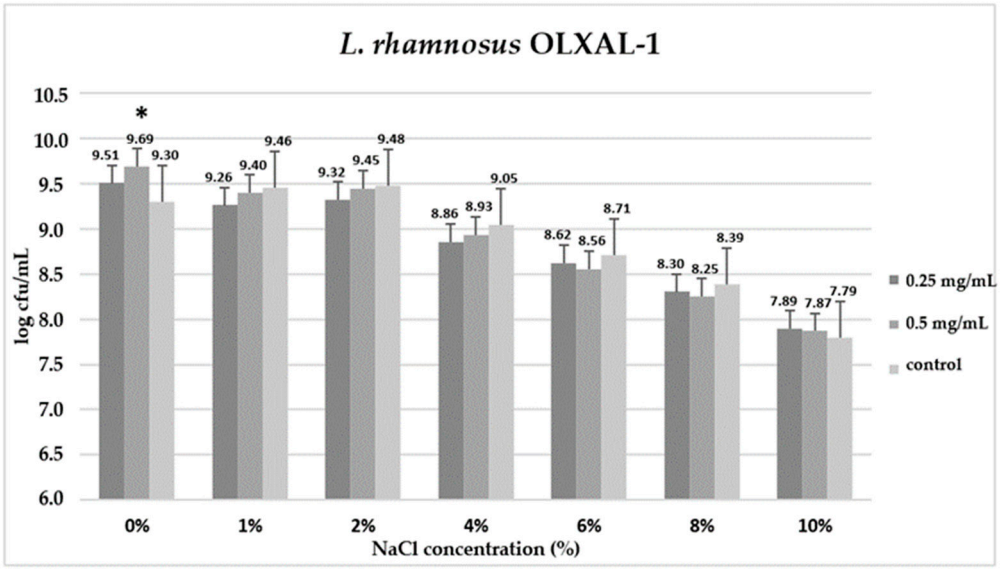

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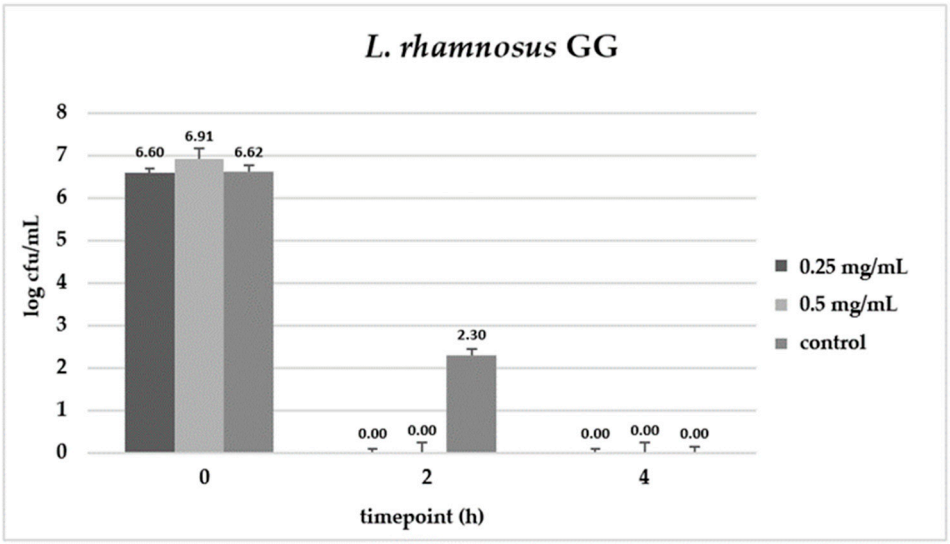
Figure 6. Effect of aqueous *A. citrodora* extract (mg (dry matter)/mL) as growth stimulator of (**a**) *Lacticaseibacillus rhamnosus* GG and (**b**) *Lacticaseibacillus rhamnosus* OLXAL-1 in MRS supplemented with different NaCl concentrations. Significant difference ($p < 0.05$) compared to the control (MRS broth, 0% NaCl) is indicated with *.

Recent studies have demonstrated the beneficial effects of polyphenols in human and animal microbiota [47], as well as in the survival of various lactobacilli strains, such as *L. johnsonii* and *L. rhamnosus* GG [48]. Dietary fibers, plants, fruits, and secondary plant metabolites are the most common and richest sources of polyphenols (PP) and polysaccharides (PS) that serve as substrates for the probiotic strains. Hence, they effectively support bacterial growth. However, the level of this probiotic cell growth is dependent on the various components each plant extract possesses. Simply structured components, such as galactose, are more likely to be consumed as substrates by probiotic strains, while more complex compounds, such as pectin, are selectively processed by a small number of bacterial strains [8]. This finding is indicative of the various responses that bacteria have to different prebiotic substrates as a result of differential molecule and compound

utilization. A recent study investigated the capacity of different ginseng berry extract components to support probiotic bacterial growth. The results revealed that galactose was the most-consumed substrate by *L. plantarum*, *L. reuteri*, *B. longum* subsp. *Infantis*, and *B. longum*, while pectin was utilized mostly by lactobacilli strains [8].



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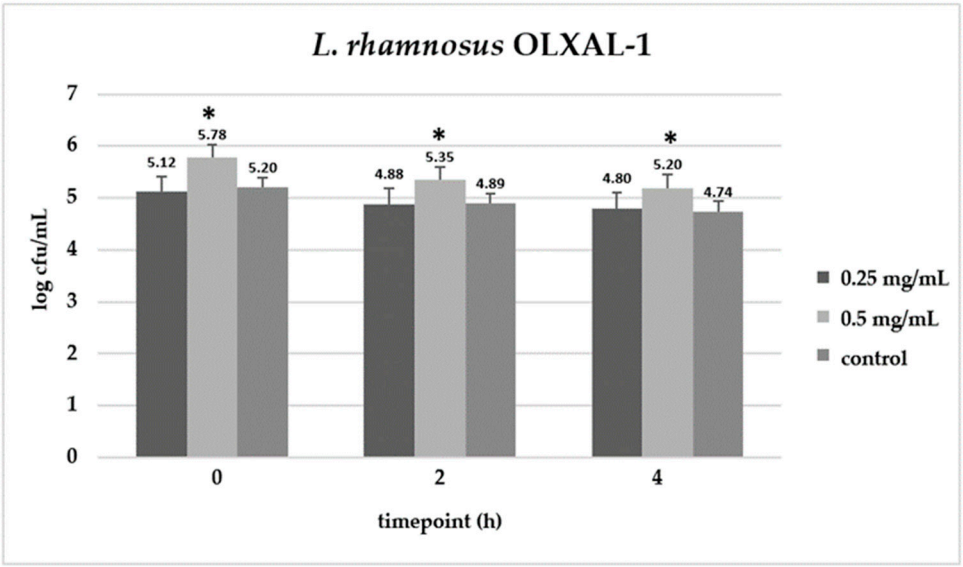

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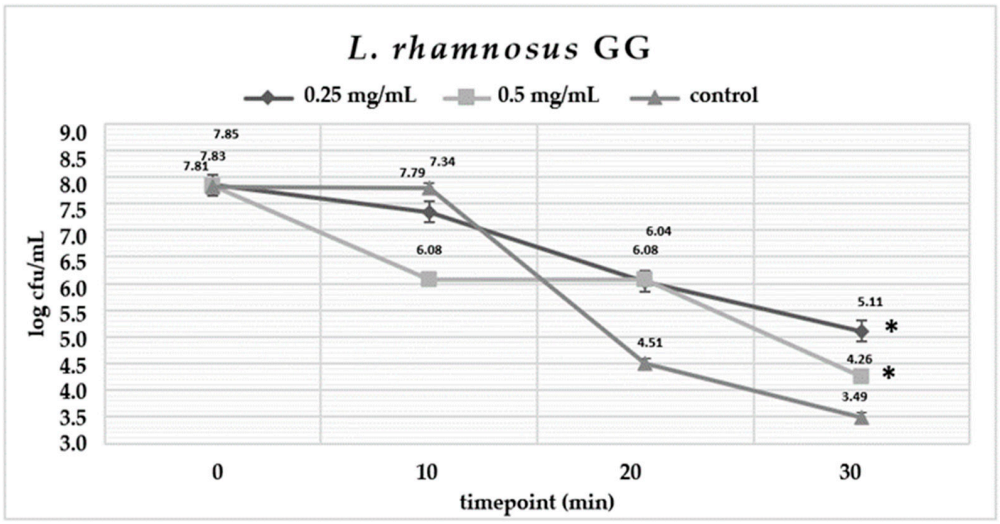
Figure 7. Effect of aqueous *A. citrodora* extract (mg (dry matter)/mL) as growth stimulator of (**a**) *Lacticaseibacillus rhamnosus* GG and (**b**) *Lacticaseibacillus rhamnosus* OLXAL-1 in MRS enriched with bile salts (0.3% *w*/*v*). Significant difference ($p < 0.05$) compared to the control (MRS broth) is indicated with *.



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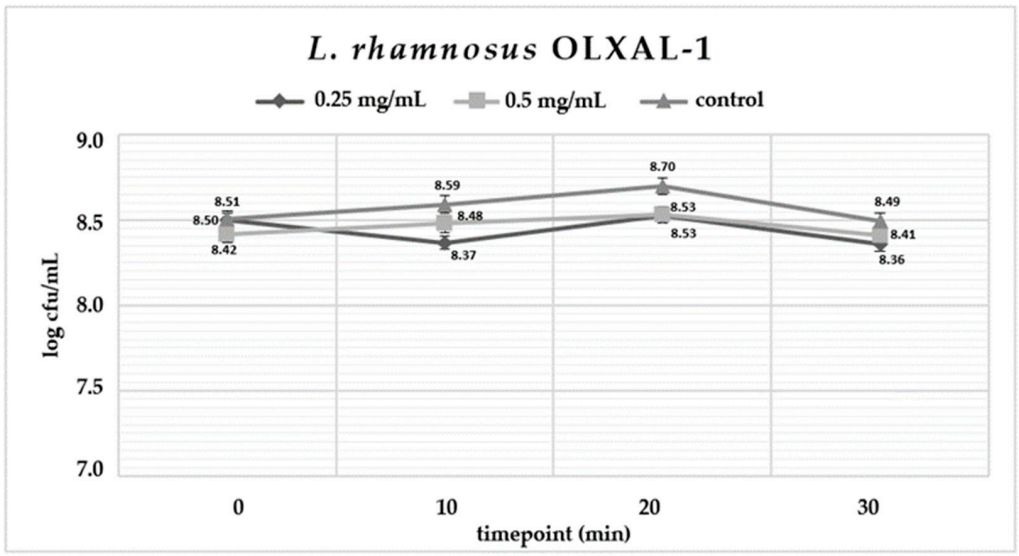
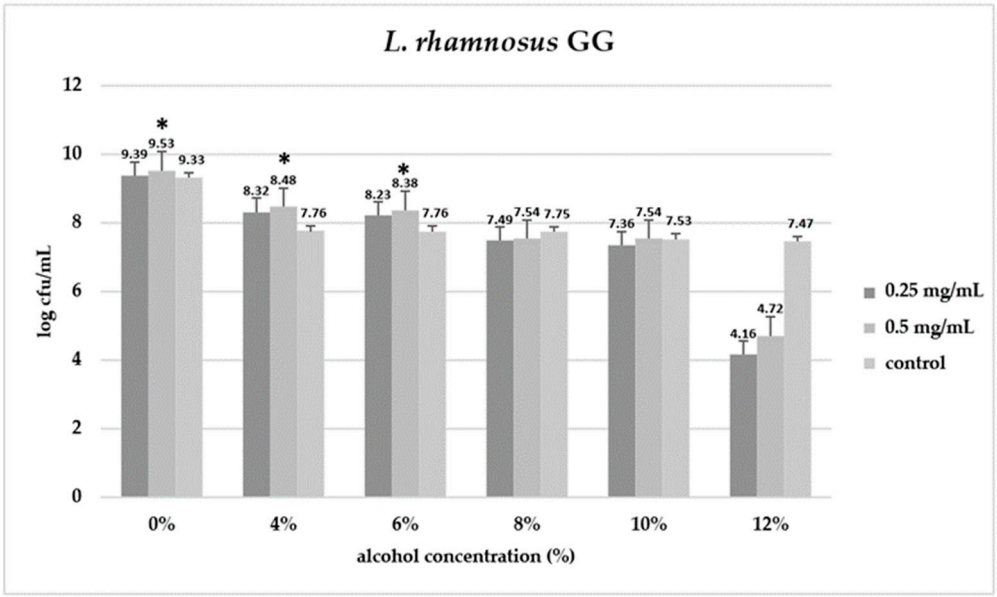


(**b**)

Figure 8. Effect of aqueous *A. citrodora* extract (mg (dry matter)/mL) as growth stimulator of (**a**) *Lacticaseibacillus rhamnosus* GG, and (**b**) *Lacticaseibacillus rhamnosus* OLXAL-1 under thermal stress (52 °C). Significant difference ($p < 0.05$) compared to the control (52 °C, without plant extract) is indicated with *.

A. citrodora extracts had an overall beneficial and growth stimulatory effect on lactobacilli strains (*L. rhamnosus* GG, *L. rhamnosus* OLXAL-1). In addition, the aqueous extract of *A. citrodora* seems to be a valuable substrate, increasing the survival rate of the lactobacilli strain in various assays (osmotic, acid, thermal, and alcohol stress), indicating that it could be used as a prebiotic agent or even as a food preservative. Yet, not many studies exist evaluating plant extracts as prebiotic agents and their protective, supportive effects on probiotic strains. However, further research is required to validate their activity in food systems and provide more insights on their mode of action.



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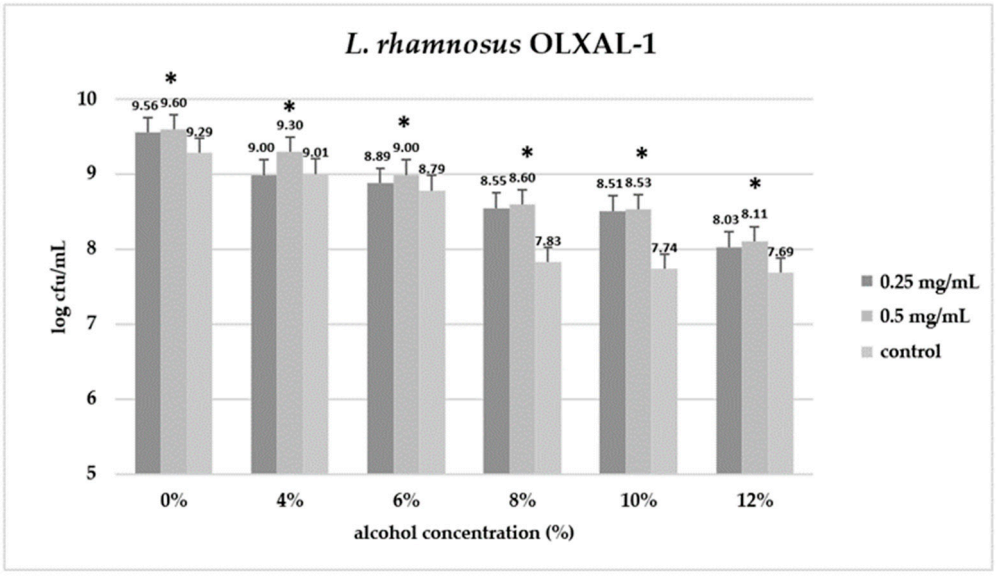

(**b**)

Figure 9. Effect of aqueous *A. citrodora* extract (mg (dry matter)/mL) as growth stimulator of (**a**) *Lacticaseibacillus rhamnosus* GG and (**b**) *Lacticaseibacillus rhamnosus* OLXAL-1 at different alcohol concentrations. Significant difference ($p < 0.05$) compared to the control (MRS broth, 0% alcohol concentration) is indicated with *.

3.5. Antimicrobial Activity against Spoilage and Foodborne Pathogenic Bacteria

A. citrodora is one of the well-known medicinal plants with therapeutic properties [11]. In our study, the antimicrobial activity of *A. citrodora* aqueous and ethanolic extracts against five common foodborne and spoilage pathogenic bacteria was evaluated, and the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) are shown in Table 5. In order touse plant extracts in the food industry, it is very critical to determine precisely the MIC and MBC values for each extract and regulate the optimum amount of the antimicrobial agent to secure microbial safety. Gram-positive bacteria were more sensitive than Gram-negative bacteria. The strongest antimicrobial activity was demonstrated by the ethanolic extract, whereas the aqueous extract had no effect on *E.*

coli. However, MIC and MBC values were significantly ($p < 0.05$) higher compared to gentamycin, which was used as a positive control [24].

Table 5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (mg/(dry matter)/mL) of *A. citrodora* aqueous and ethanolic extracts against common food-spoilage and pathogenic bacteria.

Microbial Species	*A. citrodora* (Aqueous)		*A. citrodora* (Ethanolic)	
	MIC	MBC	MIC	MBC
S. Enteritidis	12.5	50	6.25	25
S. Typhimurium	12.5	50	6.25	25
L. monocytogenes	6.25	25	1.56	6.25
E. coli	NA	NA	12.5	50
S. aureus	6.25	25	3.12	12.5

NA: no activity.

A. citrodora ethanolic extract was rich in acteoside compared to the aqueous extract.

Acteoside is a glycoside, which, according to de Miera et al. [49], has the ability to interact with ionized phosphocholine or phosphoglyceride phosphate groups in phospholipid model membranes, an interaction that probably enhances the antimicrobial properties of acteoside [49]. Similar to our results, Mothana et al. [50] showed that *S. aureus* was sensitive (MIC 4 mg/mL) to the methanolic extract of *A. citrodora*, while *E. coli* was resistant, and Elgueta et al. [51] determined the MIC of the hydroethanolic extract at 9 ± 1.5 mg/mL against *S.* Enteritidis [51].

Importantly, the spoilage and pathogenic microbes tested were more sensitive to the extracts than the *L. rhamnosus* strains, as MIC and MBC values were significantly ($p < 0.05$) lower as shown in Table 6, in agreement with the literature [52,53].

Table 6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (mg/(dry matter)/mL) of *A. citrodora* aqueous and ethanolic extracts against *Lacticaseibacillus rhamnosus* GG and wild-type *Lacticaseibacillus rhamnosus* OLXAL-1.

Microbial Species	*A. citrodora* (Aqueous)		*A. citrodora* (Ethanolic)	
	MIC	MBC	MIC	MBC
L. rhamnosus GG	50	200	25	100
L. rhamnosus OLXAL-1	100	400	50	200

3.6. Antioxidant Activity: DPPH-Radical Scavenging Activity

The DPPH-radical scavenging assay is a common *in vitro* test to determine the ability of plant extracts to scavenge free radicals. In the current study, this colorimetric method was used to evaluate the antioxidant activity of the extracts from *A. citrodora* leaves harvested in Greece. The results showed that the IC_{50} for the aqueous extract was 25.51 μg/mL and for the ethanolic extract the IC_{50} was 18.76 μg/mL. The aqueous extract of lemon verbena had lower antiradical activity than the ethanolic extract. The most effective scavengers of the DPPH radical were discovered to be ethanolic, followed by aqueous extracts. The quantity and identity of phenols and flavonoids in the ethanolic extract could be attributed to this result [54].

4. Conclusions

A. citrodora is a medicinal plant, known especially for its antioxidant activity. Herein, we first identified the phytochemical profile of the generated ethanolic and aqueous extracts using LCMS/MS and UPLC/MS Q-TOF. The richly identified phytochemical profile led us to further evaluate the potential health benefits of these plant extracts. Interestingly, we

determined that both the aqueous and ethanolic extracts stimulated the growth of the probiotic strains, wild-type *L. rhamnosus* and *L. rhamnosus* GG. Furthermore, we reported growth inhibitory activity against several foodborne pathogens for both extracts. Importantly, we found that the spoilage and pathogenic microbes evaluated were more sensitive to the extracts than the probiotic strains. This further strengthens the potential of the studied *A. citrodora* extracts to adopt a beneficial role in the functional regulation of food microbiota, a relatively understudied area.

The results of the present study demonstrated for the first time that *Aloysia citrodora* extracts may stimulate the growth of probiotic strains even in a variety of tolerance assays, highlighting their potential as prebiotics in the food industry. However, further research is required to validate their activity in food systems and provide more insights on their mode of action.

Author Contributions: Conceptualization, Y.K. and A.G.T.; data curation, V.K.G., V.A.A., G.M., V.K., I.K., C.K.F., E.P.V., Y.K. and A.G.T.; formal analysis, V.K.G., V.A.A., G.M., V.K. and E.P.V.; funding acquisition, Y.K. and A.G.T.; investigation, V.K.G., V.A.A., G.M., I.K. and C.K.F.; methodology, V.K.G., V.A.A., G.M. and C.K.F.; project administration, Y.K. and A.G.T.; resources, V.K., Y.K. and A.G.T.; supervision, Y.K. and A.G.T.; validation, V.K.G., V.A.A., G.M., V.K., I.K., C.K.F., Y.K. and A.G.T.; visualization, Y.K. and A.G.T.; writing—original draft, V.K.G., V.A.A., G.M., V.K., I.K. and C.K.F.; writing—review and editing, Y.K. and A.G.T. All authors have read and agreed to the published version of the manuscript.

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