

Insight into effects of Axillarin and Verbascoside isolated from *Tanacetum alyssifolium* and *Plantago euphratica* on probiotic properties of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*

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Abstract

Phenolic compounds are found in the seeds, flowers, leaves, stems, branches, and fruits of plants. They have an important place in human nutrition as many fruits and vegetables have phenolic compounds. Recently, many phenolic compounds have been used as functional foods and research of new phenolic compounds that are candidate to become functional foods is ongoing. Probiotics which are beneficial microorganisms, are living microbes that benefit the host when taken in sufficient amounts and they have an important role in human gastrointestinal microbiota. The present study aims to investigate effects of Axillarin and Verbascoside, two phenolic compounds isolated from *Tanacetum alyssifolium* and *Plantago euphratica*, respectively, on two very common probiotic bacteria *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*. For this, growth kinetics, auto-aggregation, and microbial adhesion to solvents, which are some critical properties of probiotics, were investigated. Axillarin and verbascoside showed significant improvement on growths of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG depending on the dose. Auto-aggregation properties were enhanced by these phenolic compounds, even though surface hydrophobicities were decreased. In conclusion, the present study indicates that the vital conditions of the probiotics could be modulated by Axillarin and/or Verbascoside, consequently their adhesion and colonization capabilities could be altered.

Introduction

Phenolic compounds are one of the highly studied topics for proving nature-based treatments. There are approximately 8000 phenolics present in plants, of which 4000 are elucidated (Bravo, 2009). Phenolic compounds are found in the seed flowers, leaves, stems, branches and fruits of plants. These compounds originated from pentose phosphate, shikimate, and phenylpropanoid pathways in plant metabolisms and are secondary metabolites in plants (Arceusz et al., 2013; Balasundram et al., 2006; Harborne et al., 1992; Silva et al., 2016). Phenolic compounds are effective in the growth, development, and the protection of plants against pathogenic organisms, giving taste and color

properties to vegetables and fruits (Alasavar et al., 2001). These compounds, also called as polyphenols because of their structure, are found in many herbal foods and beverages, therefore they are present in significant amounts in human nutrition (Balasundram et al., 2006; Isavar et al., 2001). Polyphenols are thought to have many positive effects, including anti-allergic, anti-viral, antioxidant, anti-mutagenic, anti-carcinogenic, anti-inflammatory, anti-microbial, and anti-thrombotic activities (Balasundram et al., 2006; Moure et al., 2001). The health benefits of phenolic compounds are mostly related to their antioxidant and anti-inflammatory properties. Although phenolic compounds do not have a direct nutritional value, only 5-10% are absorbed in the small intestine and 90-95% pass into the large intestine

(Crozier et al., 2010). The compounds that interact directly with intestinal microflora may have positive or negative effects on these microorganisms (Cueva et al., 2017; Liano et al., 2016; Pereira-Caro et al., 2015). Probiotics are living microbes that benefit the host in sufficient amounts of practice and they have an important role in human nutrition and health. Such behavior of probiotics makes them functional foods that interact directly with gastrointestinal microflora (Arjmandi 2014; FAO, WHO 2006; Reuter 2001; Saarela et al., 2000). The most well-known probiotics belong to *Lactobacillus* genus. It is possible to find numerous studies regarding to modern-day *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*. *Lactobacilli* are involved in human nutrition through their presence in milk and dairy products. Daily consumption of various types of food may alter the effects of probiotic bacteria on the intestinal system (Billoo et al., 2006; Kim et al., 2006; Lee & Salminen, 1995; Salminen et al., 1998).

The reported antibacterial and antioxidant properties of phenolic compounds and how these compounds affect probiotic microorganisms that benefit their hosts, are among the topics that are still being investigated and discussed among the community today. Verbascoside, which was isolated from the *Tanacetum alyssifolium* for this research, is phenylethanoid glycoside, belonging to the phenylpropanoid family. The other phenolic compound is Axillarin which is an o-methylated flavanol belonging to the Flavonoid group isolated from the *Plantago euphratica*. Previous studies on the phenolic compounds of Verbascoside and Axillarin (Figure 1) have been studied on their antioxidant and anti-inflammatory effects (Georgiev et al. 2012; Hu et al. 2017). Therefore, the present study aimed to elucidate the effects of these polyphenol compounds on probiotics microorganisms, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*.

Materials and Methods

Isolation of Axillarin

20 g of methanolic extract of *Tanacetum alyssifolium* was firstly fractionated over sephadex LH-20 using methanol as a mobile phase. According to the thin-layer chromatography (TLC) basis, Axillarin containing fractions (1.2 g) were combined and separated over C18 packed column. Water and methanol mixture were used as follows: 100:0, 80:20, 70:30 and 60:40 (v:v) each 0.5 L. Axillarin was obtained from fractions eluted with 60:40 water: methanol (v:v) system. The solvents were evaporated to dryness to give 875 mg of Axillarin.

Isolation of Verbascoside

Ten grams (10 g) of methanolic extract of *Plantago euphratica* was dissolved in hot water, then kept at +4°C overnight and non-soluble parts were removed by filtration. The water extract was lyophilized to give 4 g of pale-yellow solid. One gram (1 g) portion of extract was dissolved in 20 mL deionized water and repeatedly injected to HPLC ten times using 2 mL sample loop. The HPLC system used was a Shimadzu Prep-HPLC instrument with recycling mode equipped with LC20AR pump, SPD-20A UV-Vis detector and FRC-10A fraction collector. The mobile phase was a mixture of deionized water/acetonitrile (ACN); the UV wavelengths were 235 and 280 nm. The preparative column (250×20 mm, 10 μm) used was C18 (EMR Chrometsil). The flow rate was 8 mL/min. Verbascoside was purified from crude extract using a gradient elution from 90:10 to 50:50 (water: ACN) with 8 mL/min flow rate. The main peaks were collected according to the absorbance at 235 and 230 nm. Each collected fraction was purified using recycling mode with isocratic elution with 85:15 (Water: ACN), with 8 mL/min flow rate. The recycling process was continued until a clear separation was observed.

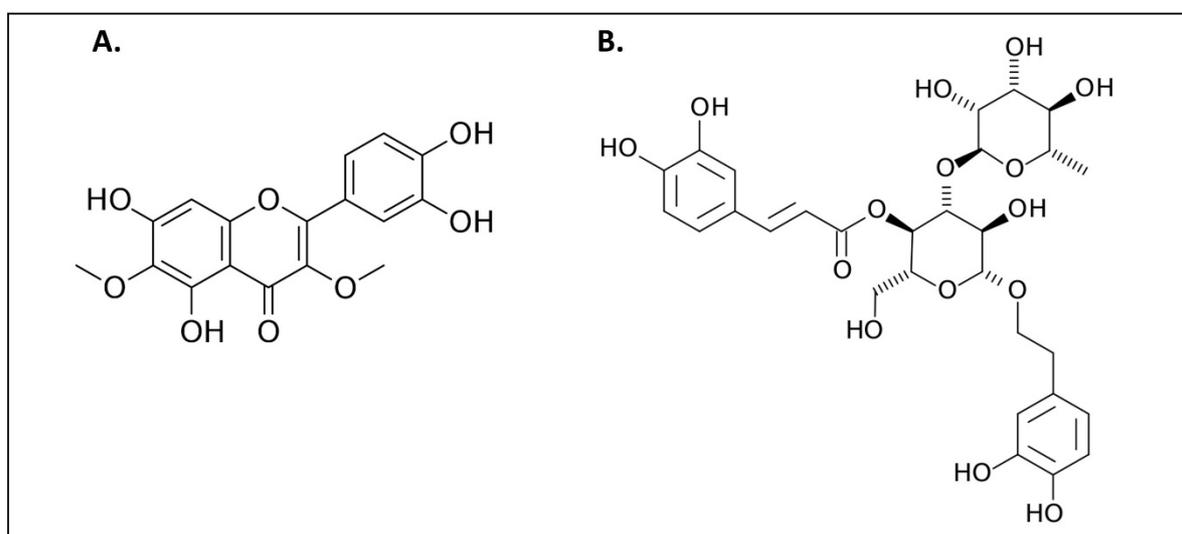


Figure 1. Chemical structures of (A) Axillarin and (B) Verbascoside.

^1H and ^{13}C NMR spectra were recorded on 400 MHz and 600 MHz, respectively (Bruker). Chemical shifts were in ppm from Me_4Si , generated from MeOD.

Growth of probiotic bacteria in the presence of Axillarin and Verbascoside, and their bacterial growth kinetics

Lactobacillus acidophilus LA-5 and *Lactobacillus rhamnosus* GG, kindly provided from Chr. Hansen, Turkey, were grown in Man, Rogosa and Sharpe (MRS) medium without shaking, at 37 °C (Celebioglu et al., 2018). The bacteria were treated separately with Axillarin and Verbascoside, not in mixture and Axillarin and Verbascoside were not added to the control groups (MRS only). Two different experiments were prepared for Axillarin and Verbascoside under the same conditions, and 5 µg/mL, 10 µg/mL and 12.5 µg/mL Axillarin and Verbascoside were added to bacterial growth media for each treatment groups. Bacterial optical density measurements were determined every four-hours with McFarland tube densitometer (Buch-Holm).

Probiotic auto-aggregation

Bacterial cells were incubated for 16 hours by treatment with Axillarin and Verbascoside, harvested in stationary phase (3200 g, 15 min), washed with Phosphate-saline buffer (PBS) and re-suspended in PBS to OD₆₀₀ 0.5 (Kos et al., 2003). The percentage of auto-aggregation is calculated according to equation 1;

$$\text{Auto-Aggregation\%} = \left(1 - \frac{A_t}{A_0}\right) \times 100$$

where A_t is the absorbance measured after incubation and A_0 is the absorbance measured at 0th hour (Kos et al., 2003).

Microbial adhesion to solvent (MATS)

Microbial adhesions of probiotic bacteria to solvents were measured by using the method described previously in the study of Kos et al. (2003). After growing, harvesting, and washing the bacterial cells as described above, they were suspended with 0.1 M KNO_3 (pH 6.2) to have OD₆₀₀ of 0.5. One mL of Xylene (non-polar solvent) was added to 3 mL of bacterial suspension and incubated at room temperature (RT) for 10 min then the two-phase system was vortexed for 2 min, the aqueous phase was separated and incubated again for 20 min at RT. Absorbance was measured at 600 nm and bacterial adhesion solvent was calculated according to equation 2;

$$\text{Adhesion \%} = 1 - \left(\frac{A_1}{A_0}\right) \times 100$$

where, A_1 is the absorbance measured after the incubation and A_0 is the absorbance measured before the incubation (Kos et al., 2003).

Statistical analysis

Each experiment was designed as three biological replicates, and measurements were repeated three times. Results of measurements were expressed as mean ± standard deviation and One-way ANOVA was used to evaluate the results using GraphPad Prism version 8.0. Differences were considered as significant for $p < 0.05$.

Results and Discussion

NMR assay of Axillarin

^1H NMR (600 MHz, MeOD) δ_{H} 7.61 (s, 1H), 7.52 (d, $J = 8.0$ Hz, 1H), 6.89 (d, $J = 8.0$ Hz, 1H), 6.48 (s, 1H), 4.84 (brs, 4H, -OH), 3.87 (s, 3H, -OCH₃), 3.78 (s, 3H, -OCH₃). ^{13}C NMR (150 MHz, MeOD) δ_{C} 178.8 (C-4), 157.4 (C-7), 156.7 (C-2), 152.3 (C-5), 152.2 (C-9), 148.5 (C-4'), 145.0 (C-3'), 137.8 (C-3), 131.2 (C-6), 121.5 (C-1'), 120.9 (C-6'), 115.1 (C-5'), 115.0 (C-2'), 104.9 (C-10), 93.6 (C-8), 59.5 (C-6-OCH₃), 59.1 (C-3-OCH₃).

NMR assay of Verbascoside

^1H NMR (400 MHz, MeOD) δ_{H} 7.62 (d, $J = 15.87$, 1H, H β '), 7.08 (d, $J = 2.12$, 1H, H2'), 6.98 (dd, $J = 8.21$, 2.12, 1H, H6'), 6.80 (d, $J = 8.09$, 1H, H5'), 6.72 (d, $J = 2.09$, 1H, H2), 6.70 (d, $J = 7.91$, 1H, H5), 6.59 (dd, $J = 8.00$, 2.14, 1H, H6), 6.30 (d, $J = 15.88$, 1H, H α'), 5.21 (d, $J = 1.83$, 1H, H1'''), 4.94 (m, 1H, H4''), 4.40 (d, $J = 7.86$, 1H, H1''), 4.07 (dt, $J = 9.71$, 7.32, 1H, H $\alpha\alpha$), 3.94 (m, 1H, H2'''), 3.84 (t, $J = 9.20$, 1H, H3''), 3.74 (m, 1H, H $\alpha\beta$), 3.65 (m, 1H, H6''a), 3.61 (m, 1H, H3'''), 3.58 (m, 1H, H5'''), 3.56 (m, 1H, H5''), 3.54 (m, 1H, H6''b), 3.41 (t, $J = 8.48$, 1H, H2''), 3.32 (m, 1H, H4'''), 2.81 (dt, $J = 7.35$, 2.45, 2H, H β), 1.11 (d, $J = 6.21$, 3H, H6'''). ^{13}C NMR (101 MHz, MeOD) δ_{C} 166.93 (C=O), 148.38 (C4'), 146.63 (C β '), 145.43 (C3'), 144.74 (C3), 143.28 (C4), 130.15 (C1), 126.32 (C1'), 121.83 (C6'), 119.91 (C6), 115.76 (C2), 115.17 (C5'), 114.96 (C5), 113.91 (C2'), 113.37 (C α'), 102.83 (C1''), 101.63 (C1'''), 80.27 (C3''), 74.83 (C2''), 74.65 (C5'').

NMR assignments were fully agreed with literature for Verbascoside and Axillarin (Akdemir et al., 2004). Verbascoside is a common phenyl ethanoid for *Plantago* species and was previously isolated from *P. lagopus* (Harput et al., 2012), *P. pellardii* (Gálvez et al., 2005), and *P. cynops* (Debrauwer et al., 1989).

Bacterial growth kinetics

In this study, Axillarin and Verbascoside were used in concentrations of 5, 10 and 12.5 µg/mL. The groups treated with Axillarin and Verbascoside showed significant improvement on *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG depending on the dose of phenolic compounds, when compared with control groups (Figure 2). Previous studies have examined the antimicrobial effect of Axillarin and found that it inhibited the development of pathogenic bacteria (Avila et al., 1999). However, in the present study, Verbascoside have positively influenced growth of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*.

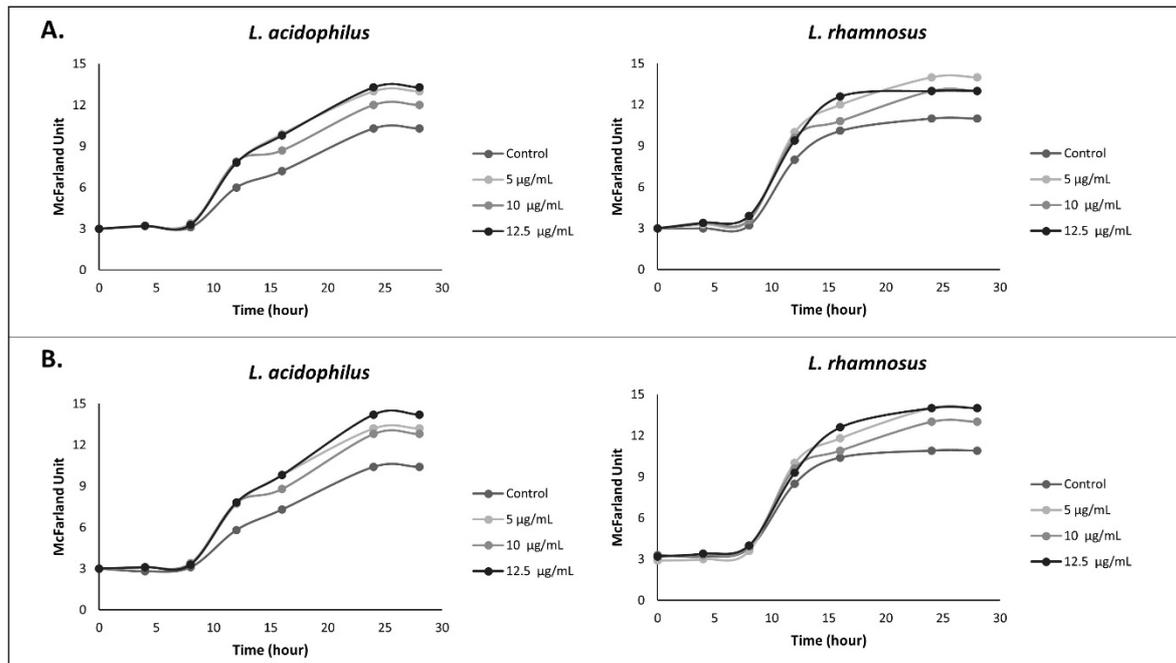


Figure 2. Growth kinetics of probiotic bacteria. (A) Growth curve of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG when grown in the presence of Axillarin. (B) Growth curve of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG when grown in the presence of Verbascoside.

Anti-inflammatory effect studies have also been reported for Axillarin, but on *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG have shown an effect that promoted development (Beg et al., 2011). The fact that these compounds have no antimicrobial effects on *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* suggests that they have the potential to have selectively positive effects on beneficial bacteria in the intestinal microflora.

Bacterial auto-aggregation

Even though there are many protective mechanisms present in the gastrointestinal mucosa, humans are occasionally exposed to enteric pathogens.

Enteric pathogens cause infection by colonizing on microflora present in the gastrointestinal tract (Sekirov et al., 2010). Therefore, adhesion to intestinal epithelial cells is a prerequisite for colonizing probiotic strains in the gastrointestinal tract, preventing their immediate elimination (Alander et al., 1997; Freter 1992; Pedersen and Tannock 1989). Consequently, auto-aggregation is an important condition for the adhesion and survival of probiotic strains to the intestinal epithelium (Boris et al., 1997; Del Re et al., 1998). The concentration of 5 µg/mL of Axillarin significantly decreased the auto-aggregation of *Lactobacillus acidophilus* at 1st hour, then the next hours of the auto-aggregation, it significantly ($p < 0.05$) increased the auto-aggregation, when compared to

Table 1. Auto-aggregation percentages of probiotics grown in the presence of Axillarin. The results are given as mean with standard deviations. Asterisks (*) indicate the differences are statistically significant ($p < 0.05$) when compared to respective control groups, according to One-Way ANOVA.

	1 st Hour	2 nd Hour	3 rd Hour	4 th Hour	5 th Hour
<i>L. acidophilus</i>					
Control	59.2 ± 0.6	21.8 ± 0.7	59.7 ± 13.0	70.4 ± 17.6	83.8 ± 9.5
5 µg/mL	20.5 ± 1.4 *	42.7 ± 1.3 *	70.4 ± 4.5 *	79.1 ± 1.5 *	81.6 ± 0.7
10 µg/mL	21.8 ± 0.3 *	65.0 ± 0.2 *	70.1 ± 0.3 *	72.1 ± 0.5	84.1 ± 7.0
12.5 µg/mL	29.6 ± 0.2 *	32.9 ± 0.1 *	64.2 ± 3.8	79.8 ± 2.0 *	87.4 ± 6.1 *
<i>L. rhamnosus</i>					
Control	84.5 ± 2.6	N.D.	N.D.	N.D.	N.D.
5 µg/mL	84.3 ± 2.4	N.D.	N.D.	N.D.	N.D.
10 µg/mL	88.7 ± 5.2 *	N.D.	N.D.	N.D.	N.D.
12.5 µg/mL	89.1 ± 3.0 *	N.D.	N.D.	N.D.	N.D.

N.D.: Not Determined

control (Table 1). The second concentration is 10 µg/mL and showed a significant decrease at 1st hour, but at 2nd and 3rd hour, it increased the auto-aggregation, compared to control group. The last concentration is 12.5 µg/mL and showed a significant decrease in the first two hours but increase in auto-aggregation in the last hours. This could mean Axillarin has a positive effect on auto-aggregation of *Lactobacillus acidophilus*. On the other hand, the auto-aggregation of *Lactobacillus rhamnosus* was significantly increased by 10 µg/mL and 12.5 µg/mL of Axillarin in the first hour as compared to control group. In the next hours of the assay, the auto-aggregation was reached to the maximum, which means the comparison and measurement were not possible (stated in the table as ND). The results of auto-aggregation indicate that these polyphenols can positively affect the adhesion of the probiotic bacteria as auto-aggregation which is very important for probiotic microorganisms to adhesion and live in the gastrointestinal tract (GIT) (Boris et al., 1997; Del Re et al., 1998).

Auto-aggregation of Verbascoside showed a significant decrease ($p < 0.05$) in auto-aggregation of *L. acidophilus* at the concentration of 5 µg/mL in the 1st and 2nd hours as compared with control groups; however, increase in the 5th hour (Table 2). The concentration of 10 µg/mL showed an important decrease in auto-aggregation in the 1st and 2nd, but significantly increased in the 3rd and 5th hours. The concentration of 12.5 µg/mL significantly decreased auto-aggregation in the 2nd and there are significant increases at 3rd, 4th, and 5th hours when compared with control group. On the other hand, auto-aggregation of *Lactobacillus rhamnosus* was significantly increased by Verbascoside with every concentration. In the next hours of measurements, the auto-aggregations were not able to be obtained, which means there were no bacteria found on the surface of suspension because

they were aggregating completely down to the tubes (Table 2).

Auto-aggregation of probiotics grown in the presence of phenolic compounds was mostly lower than the control group at 1st hour. This could be explained by that bacteria started interacting each other at the beginning and after one hour, these interactions could not be fully achieved. Thus, in time, bacteria could interact fully, and the effects of phenolic compounds could be observed at the later hours. Increased auto-aggregation, at later hours, can indicate these beneficial bacteria are positively affected by Axillarin and Verbascoside, thus they have potential to adhere more in the GIT and show their probiotic activities to the host (Boris et al., 1997; Del Re et al., 1998).

Microbial adhesion to solvents (MATS)

The surface properties of *Lactobacilli* contribute to their interactions with the host and gastrointestinal microbiota. Thus, this can affect their residence in GIT by allowing *Lactobacilli* adhering to intestinal tissue, as well as affecting interactions with their own species and other bacteria (Kleerebezem et al., 2010; Lebeer et al., 2008). The adhesion behavior of microbial cells has been shown to depend on the van der Waals interactions and balance of electrostatic on the hydrophobic character of the surfaces (Boonaert & Rouxhet 2000). Hydrophobicity plays an important role for the contact between a bacterial cell and mucous or epithelial cell (Schillinger et al., 2005). In this assay, Xylene (non-polar solvent) was used to assess the hydrophobic and hydrophilic characteristics of bacterial surface (Bellon-Fontaine et al., 1996; Kos et al., 2003). As a result of the assays conducted, surface hydrophobicity of both bacteria was significantly ($p < 0.05$) decreased by presence of Axillarin and Verbascoside at every concentration, as compared to control (Figure 3).

Table 2. Auto-aggregation percentages of probiotics grown in the presence of Verbascoside. The results are given as mean with standard deviations. Asterisks (*) indicate the differences are statistically significant ($p < 0.05$) when compared to respective control groups, according to One-Way ANOVA applied for each column.

	1 st Hour	2 nd Hour	3 rd Hour	4 th Hour	5 th Hour
<i>L. acidophilus</i>					
Control	31.4 ± 12.2	47.5 ± 13.2	47.4 ± 1.1	69.7 ± 7.9	64.5 ± 3.6
5 µg/mL	23.1 ± 7.9 *	17.1 ± 4.8 *	49.3 ± 1.9	63.2 ± 5.3	71.5 ± 0.5 *
10 µg/mL	28.0 ± 2.3 *	11.6 ± 1.3 *	61.8 ± 0.2 *	68.2 ± 0.7	77.0 ± 10.1 *
12.5 µg/mL	30.4 ± 0.9	34.6 ± 6.9 *	65.0 ± 14.6 *	72.9 ± 0.1 *	62.7 ± 0.6
<i>L. rhamnosus</i>					
Control	65.8 ± 2.1	N.D.	N.D.	N.D.	N.D.
5 µg/mL	80.5 ± 13.4 *	N.D.	N.D.	N.D.	N.D.
10 µg/mL	84.6 ± 13.0 *	N.D.	N.D.	N.D.	N.D.
12.5 µg/mL	81.0 ± 0.6 *	N.D.	N.D.	N.D.	N.D.

N.D.: Not Determined

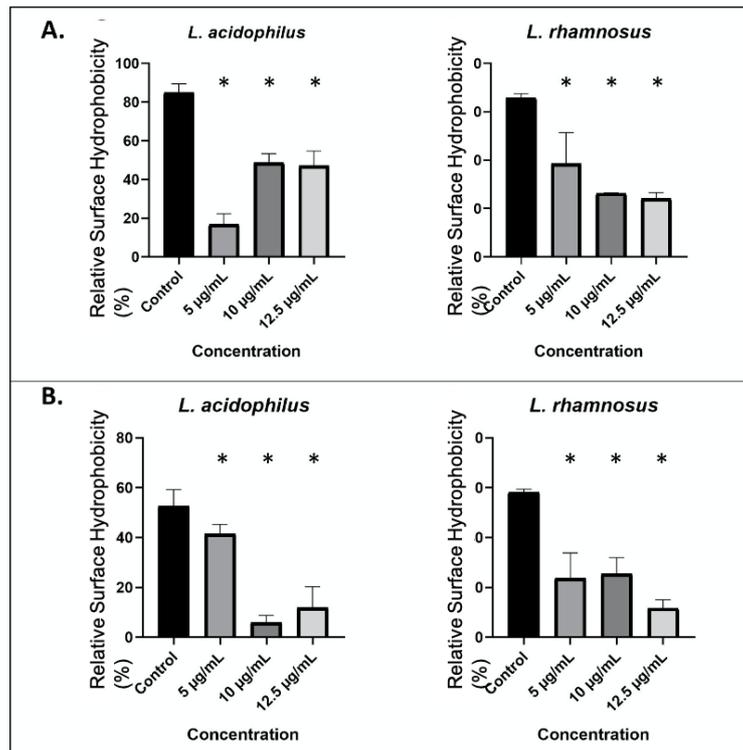


Figure 3. Surface hydrophobicity (A) of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG when grown in the presence of Axillarin and (B) of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG when grown in the presence of Verbascoside. Asterisks (*) indicate the differences are statistically significant ($p < 0.05$) when compared to respective control groups, according to One-Way ANOVA.

Lactobacillus may well serve as model systems for the study of structure-property functions of the bacterial cell envelope (Schär-Zammaretti & Ubbink 2003). Physicochemical properties of the cell surface, such as hydrophobicity, can affect the automatic aggregation and adhesion of bacteria to different surfaces. Cell adhesion is a complex process involving contact between bacterial cell membrane and interacting surfaces (Del Re et al., 1998; Perez et al., 1998; Wadström et al., 1987). On this topic, reported investigations were on the structure and forces of interaction and composition related to bacterial adhesion to intestinal epithelial cells (Del Re et al., 1998; Perez et al., 1998) and mucus (Collado et al., 2005). However, in addition to surface properties of the bacteria, the surface proteins that many *lactobacilli* possess, specifically S-layer proteins binding to the cell wall in a non-covalent manner, play significant roles in adhesion (Celebioglu & Svensson, 2017). The biological functions of the S-layer include protection, determination of cell shape, molecular and ion capture to surfaces where adhesion takes place. It is thought that the structure responsible for the attachment of a bacterial cell to the intestinal epithelium is the S-layer (Meng et al., 2017; Wasko et al., 2014). Therefore, not only hydrophobicity and aggregation are sufficient for good adhesion ability, but more importantly, surface proteins of bacteria play a key role on bacterial adhesion (Schär-Zammaretti & Ubbink 2003; Sengupta et al., 2013).

Conclusion

In the present study, growth kinetics, auto-aggregation, and microbial adhesion to solvents assays were tested using Axillarin and Verbascoside polyphenols on probiotics, which are beneficial microorganisms to the host (*Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG). Probiotic bacteria tested here, treated with Axillarin and Verbascoside polyphenols, showed significant alterations in auto-aggregation and hydrophobicity experiments. The results from the auto-aggregation and hydrophobicity (MATS) experiments indicate that the vital conditions of the probiotics could be modulated by Axillarin and/or Verbascoside, consequently alter their adhesion and colonization capabilities. Furthermore, future studies could investigate how polyphenols affect the surface proteins of probiotic bacteria, which are of great importance for bacterial adhesion.

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