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Research Article

Effects of Loganic Acid isolated from *Vinca soneri* on Surface Hydrophobicity and Auto-Aggregation of Probiotic Bacteria, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*

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Abstract

Loganic acid is an ridoid glycoside found in various plants, having anti-inflammatory activities. In the present study, loganic acid was isolated from *Vinca soneri,* used in folk medicine due to laxative, diuretic and antipyretic effects. Probiotics, a group of functional foods, are microorganisms that have positive effects on human health when taken into the body in the required amounts. The most studied and known probiotics are *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*. These bacteria are used as “starters” in various foods or for different formulations, as well as food supplements. The aim of this study was to investigate the *in vitro* effects of loganic acid on *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG. Surface hydrophobicity and auto-aggregation are important functions for a probiotic bacterium and the results indicated that loganic acid can modulate the probiotic functions, thus there is a potential for beneficial health benefits of combinations of loganic acid and probiotic strains.

Keywords: Auto-aggregation, *Lactobacillus*, Loganic acid, Surface Hydrophobicity, *Vinca soneri*

Vinca soneri'den İzole Edilen Loganic Asidin Probiyotik Bakteriler *Lactobacillus acidophilus* ve *Lactobacillus rhamnosus*'un Yüzey Hidrofobisitesi ve Oto-Agregasyonu Üzerine Etkileri

Öz

Loganic asit, çeşitli bitkilerde bulunan ve anti-enflamatuar aktivitelere sahip bir iridoid glikozittir. Bu çalışmada loganik asit, laksatif, idrar söktürücü ve antipiretik etkiler nedeniyle halk hekimliğinde kullanılan *Vinca soneri* bitkisinden izole edilmiştir. Bir grup fonksiyonel gıda olan probiyotikler, vücuda yeteri miktarlarda alındığında insan sağlığını olumlu yönde etkileyen mikroorganizmalardır. En çok çalışılan ve bilinen probiyotikler *Lactobacillus acidophilus* ve *Lactobacillus rhamnosus* bakterileri olmakla beraber bu bakteriler, çeşitli gıdalarda veya farklı formülasyonlarda, ayrıca besin takviyelerinde “starter” olarak kullanılır. Bu çalışmanın amacı, loganik asidin *Lactobacillus acidophilus* LA-5 ve *Lactobacillus rhamnosus* GG üzerindeki *in vitro* etkilerini araştırmaktır. Yüzey hidrofobisitesi ve oto-agregasyon, bir probiyotik bakteri için önemli fonksiyonlardır ve bu çalışmanın sonuçları, loganik asidin, probiyotik fonksiyonları modüle edebileceğini, dolayısıyla, loganik asit ve probiyotik suşların kombinasyonlarının daha iyi bir potansiyele sahip olabileceğini göstermiştir.

Anahtar Kelimeler: Laktobasil, Loganik asit, Oto-agregasyon, *Vinca soneri,* Yüzey hidrofobisitesi

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1. Introduction

Vinca genus belongs to *Apocynaceae* family and widespread in Europa, Asia and Africa. *Vinca major*, *Vinca minor*, and *Vinca herbacea* are grown in Turkey. In addition, two endemic species were recorded to Turkey Flora; *Vinca soneri* (Koyuncu et al. 2012) and *Vinca ispartensis* (Koyuncu et al. 2015). *Vinca* species are used in folk medicine due to laxative, diuretic and antipyretic effects (Baytop, 1999). Although some other members of *Vinca* species have some compounds that have pharmaceutically importance, far too little attention has been paid to investigation of chemical content of *Vinca soneri* (Sezer et al. 2018; Bahadori 2012). Loganic acid is a cyclopentapyran that is 1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylic acid substituted at positions 1, 6 and 7 by beta-D-glucosyloxy, hydroxy and methyl groups, respectively (Figure 1). It is a plant secondary metabolite and the anti-inflammatory and protective effects of loganic acid have been known for a while (Del Carmen Recio et al. 1994; Wei et al. 2013). However, other biological activities of loganic acid are not known.

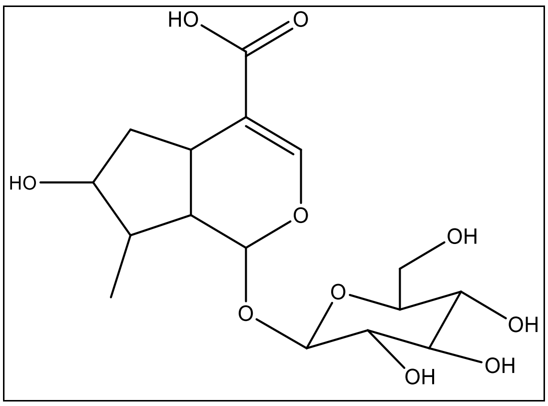

Figure 1. Chemical structure of loganic acid.

Probiotics, a group of functional foods, are microorganisms that have positive effects on human health when taken into the body in the required amounts (Hill et al. 2014). The best known probiotics are *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*. These bacteria are used in formulating various foods and as supplements for the protection of human health (Puupponen-Pimia et al. 2002). The ability of probiotic bacteria to adhere to intestinal epithelial surfaces is of great importance for colonization in the intestinal region, preventing the attachment of pathogenic microorganisms, modulation of the immune system, improving the structure of damaged mucosa, in order to provide more and longer probiotic effect (Mills et al. 2011).

One of the most commonly studied bacteria is *Lactobacillus rhamnosus*, which is added to dietary supplements and various foods such as dairy products. It is a bacterial species that expresses the lactase enzyme, producing lactic acid. *L. rhamnosus* has been shown to prevent diabetes and to be useful in the treatment of gastrointestinal problems such as irritable bowel syndrome (Spiller 2008; Yadav et al. 2013). Another very well-known probiotics is *Lactobacillus acidophilus*, whose probiotic activities are very well documented *in vitro* and *in vivo* including attenuation of lactose intolerance, exclusion of pathogens, immunomodulation and reduction of cholesterol level (Lebeer et al. 2008; Sanders & Klaenhammer 2001).

The aim of this study was to investigate the *in vitro* effects of loganic acid on *Lactobacillus acidophilus* LA-5 (LA-5) and *Lactobacillus rhamnosus* GG (GG). For this purpose, loganic acid was added to bacterial cultures and its effects on bacterial growth kinetics, surface hydrophobicity (Microbial Adhesion to the Solvent - MATS Test) and aggregation of bacteria (Auto-Aggregation Test) were investigated.

2. Material and Method

2.1. Plant Extraction and Loganic Acid Isolation

100 g of aerial parts of *Vinca soneri* were cut into small pieces and macerated two times with 500 mL of methanol overnight. Methanolic extract (30 g) was fractionated over sephadex LH-20 using methanol as mobile phase. Five main fractions were obtained according to TLC analysis. The 2nd main fraction (5 g) was applied to C18 packed column and eluted with mixture of water:methanol from 100:0 to 50:50. The eluted fractions with high percentages of water (3-9 frcs) were combined and purified using sephadex LH-20 with 50:50 water:methanol to give loganic acid (530 mg).

2.2. Growth of probiotic bacteria in the presence of loganic acid and bacterial growth kinetics

Lactobacillus acidophilus LA-5 and *Lactobacillus rhamnosus* GG, which are kind gifts of Chr. Hansen, Turkey, were grown in Man, Rogosa and Sharpe (MRS) medium without shaking (37ºC) (Celebioglu et al. 2018). The bacteria were divided into groups and treated with loganic acid. Loganic acid was not added to the control group (MRS only), and 5 µg/mL, 10 µg/mL, and 20 µg/mL of loganic acid were added to the treated groups in MRS medium. Bacterial optical density was determined by densitometry. Reading the densitometry every four hour, the effects of loganic acid on bacterial growth were investigated.

2.3. Microbial Adhesion to Solvents (MATS)

Bacterial surface hydrophobicity was measured by the method of microbial adhesion to solvents (MATS) (Kos et al. 2003), with some modifications (Celebioglu et al. 2016). The bacteria (control and treated groups) were harvested in the stationary phase (3200xg, 15 min), washed with PBS (Phosphate-saline buffer), and suspended in 0.1 M KNO_3 (pH 6.2) to have OD_{600} of 0.5. One mL of xylene (nonpolar solvent) was added to 3 mL of bacterial suspension and incubated at RT for 10 min. The two-phase system was vortexed for 2 min, the aqueous phase was separated and incubated for another 20 min at RT. Absorbance was measured at 600 nm and the bacterial adhesion to the solvent was calculated using the formula

$$\%\,Adhesion = \left(1 - \frac{A1}{A0}\right) \times 100$$

where, A1 is the absorbance measured after the incubation and A0 is the absorbance measured before the incubation (Kos et al. 2003).

2.4. Probiotic Auto-Aggregation

Bacterial cells were collected in the early stationary phase (3200xg, 15 min), washed with PBS and re-suspended in PBS to OD_{600} 0.5 (Celebioglu et al. 2016). Auto-aggregation was determined by adding 4 mL of bacterial suspensions to the test tubes after vortex for 10 sec. for one hour-incubation at room temperature. After incubation, 100 µL of suspension was taken, added to the tube containing 900 µL of PBS, and the absorbance was measured at 600 nm. The percentage of auto-aggregation was calculated with the formula

$$\% \, Autoaggregation = \left(1 - \frac{At}{A0}\right) \times 100$$

where At is the absorbance measured after incubation and A0 is the absorbance measured at 0^{th} hour (Kos et al. 2003).

2.5. Statistical Analysis

Each assays were replicated at least three times. The results were compared using Student's *t*-test and $p<0.05$ was considered as statistically significant.

3. Research Findings

3.1. NMR Results

^{1}H NMR (400 MHz, MeOD) δ_H 7.31 (s, 1H, H3), 5.10 (d, J=5.1, 1H, H1), 4.49 (d, J=7.6, 1H, H1'), 3.89 (m, 1H, H7), 3.67 (dd, J=10.9, 5.5, 1H, H6'a), 3.44 (m, 1H, H6'b), 3.15 (m, 1H, H3'), 3.24 (m, 1H, H5'), 3.05 (m, 1H, H4'), 2.98 (m, 1H, H2'), 2.92 (m, 1H, H5), 2.09 (ddd, J=13.5, 7.70, 4.5 1H, H6a), 1.81 (m, 1H, H9), 1.72 (m, 1H, H8), 1.45 (ddd, J= 13.5, 7.7, 4.5, 1H, H6b), 0.99 (d, J=6.9 3H, H10). ^{13}C NMR (101 MHz, MeOD) δ_C 168.5 (C11), 150.6 (C3), 113.1 (C4), 99.0 (C1), 96.5 (C1'), 77.7 (C5'), 77.3 (C3'), 73.6 (C2'), 72.6 (C7), 70.6 (C4'), 61.6 (C6'), 45.2 (C9), 42.2 (C6), 41.0 (C8), 31.4 (C5), 14.0 (C10).

NMR data are fully agreement with Dei et al. (2011). Occurrence of loganic acid in *Vinca major* and *Vinca minor* was reported previously (Bianco et, al. 1984; Şöhretoğlu et. al. 2013).

3.2. Bacterial Growth Kinetics

Figure 2 shows the bacterial growth of *Lactobacillus acidophilus* LA-5 in the presence of different concentrations of loganic acid. Even though 5 and 10 µg/mL of loganic acid seem to have impact on the growth of this bacterium, 20 µg/mL of loganic acid had no inhibitory effect on the growth, thus no significant anti-bacterial effect of loganic acid against *Lactobacillus acidophilus* LA-5 was observed.

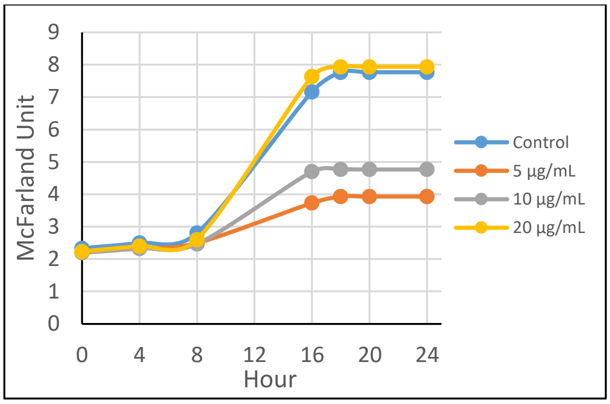

Figure 2. Bacterial growth kinetics of *Lactobacillus acidophilus* LA-5 grown in the presence of different concentrations of loganic acid.

Figure 3 shows that bacterial growth of *Lactobacillus rhamnosus* GG was not effected by loganic acid.

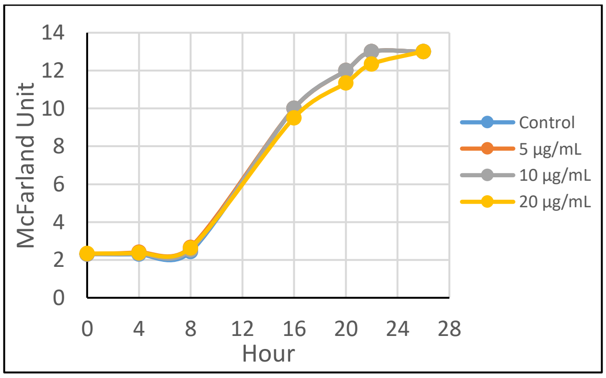

Figure 3. Bacterial growth kinetics of *Lactobacillus rhamnosus* GG grown in the

presence of different concentrations of loganic acid.

difference when compared to control group is statistically significant (p<0.05).

3.3. Bacterial Surface Hydrophobicity

Bacterial surface hydrophobicity plays a role for adhesion of especially probiotic or pathogenic bacteria to the mucosal surfaces (Krasowska & Sigler 2014). Increase in surface hydrophobicity of probiotic bacteria could prevent the pathogenic bacteria to adhere to the mucosa; thus, indicating that it could be an important factor for maintenance of the intestinal health.

Figure 4 shows the surface hydrophobicity of *Lactobacillus acidophilus* when treated with different concentrations of loganic acid. MATS test showed that at concentrations of 25 and 50 µg/mL, loganic acid significantly reduced the surface hydrophobicity of LA-5 but increased at 100 µg/mL.

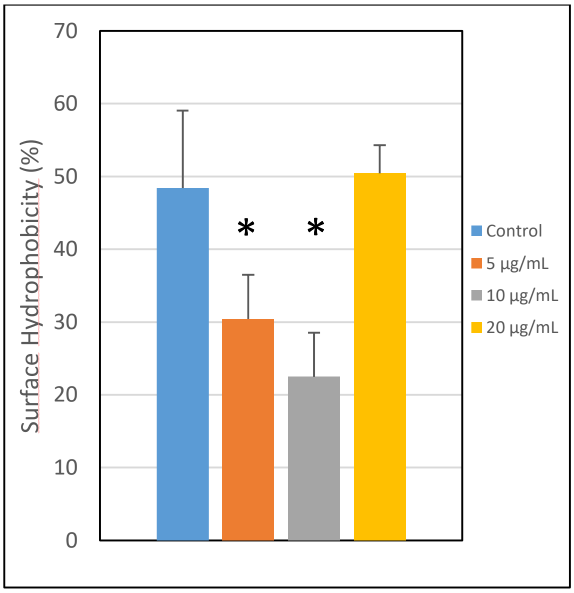

Figure 4. Surface Hydrophobicity of *Lactobacilus acidophilus* LA-5 grown in the presence of different concentrations of loganic acid. Asterisks (*) show that the difference when compared to control group is statistically significant (p<0.05).

Figure 5 shows the surface hydrophobicity of *Lactobacillus rhamnosus* GG and at only 50 µg/mL concentration, loganic acid decreased the surface hydrophobicity (p <0.05) when compared to the control group.

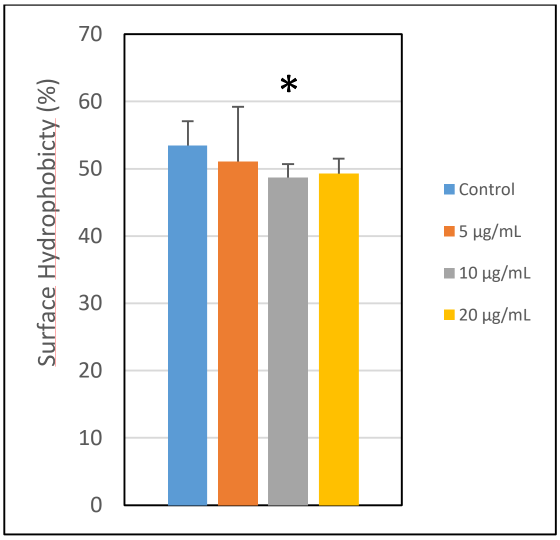

Figure 5. Surface Hydrophobicity of *Lactobacilus rhamnosus* GG grown in the presence of different concentrations of loganic acid. Asterisks (*) show that the difference when compared to control group is statistically significant (p<0.05).

In a similar study, different probiotic *lactobacillus* strains (*L. plantarum* 49, *L. plantarum* 53, *L. paracasei* 106, *L. paracasei* 108, *L. fermentum* 263, *L. fermentum* 296) were used (dos Santos et al. 2019). The effects of quercetin and resveratrol on auto-aggregation and surface hydrophobicity properties of these six *lactobacillus* strains were investigated. Quercetin increased the cell surface hydrophobicity of *L. fermentum* 296 at all concentrations, and resveratrol at ½

MIC, the minimum concentration of the compound that inhibits all bacteria, increased the hydrophobicity of *L. paracasei* 106 and *L. paracasei* 108 of. However, quercetin's ¼ MIC and all resveratrol concentrations decreased the hydrophobicity of *L. plantarum* 49. Cell surface hydrophobicities of *L. plantarum* 53 and *L. paracasei* 108 were not affected at any concentrations. The results indicated that resveratrol and quercetin were found to be useful on *L. fermentum* 263 (dos Santos et al. 2019).

3.4. Bacterial Auto-Aggregation

Bacterial auto-aggregation is the aggregation of bacteria by adhering each other. High auto-aggregation properties of probiotic bacteria are important for their binding to the intestinal mucosa (Kos et al. 2003). Higher auto-aggregation may lead to greater adhesion.

Figure 6 shows the auto-aggregation of *Lactobacillus acidophilus* LA-5 when grown on loganic acid. Only one concentration (5 µg/mL) had slight decrease on the auto-aggregation (p<0.05).

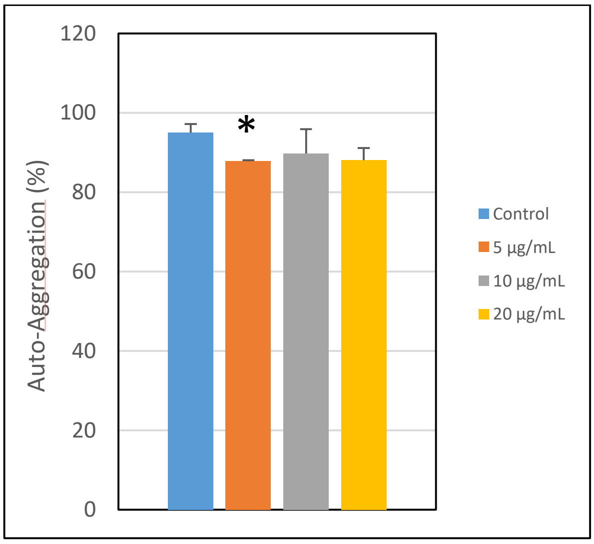

Figure 6. Auto-Aggregation of *Lactobacilus acidophilus* LA-5 grown in the presence of different concentrations of loganic acid. Asterisks (*) show that the difference when compared to control group is statistically significant (p<0.05).

Figure 7 shows the auto-aggregation of *Lactobacillus rhamnosus* GG grown in the presence of loganic acid. The concentrations of 5 and 20 µg/mL decreased the auto-aggregation significantly (p<0.05), while 10 µg/mL had no effect.

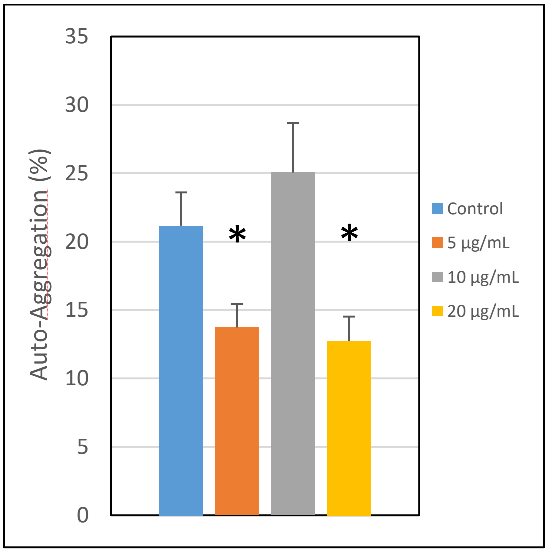

Figure 7. Auto-Aggregation of *Lactobacilus rhamnosus* GG grown in the presence of different concentrations of loganic acid. Asterisks (*) show that the difference when compared to control group is statistically significant (p<0.05).

According to study done by dos Santos et al., different concentrations had different effects on tested strains of *Lactobacillus* (dos Santos et al. 2019). This is also confirmative with the results obtained in the present study, meaning that different concentrations could have different effects. Aggregation is also important for biofilm formation, thus helping the bacteria to bind to the mucous layer of the intestines (de Souza et al. 2019).

Even though *in vitro* assessments of physiological properties of probiotic bacteria and their modulation by dietary components are indicative for probiotic functionality, *in vivo* studies are required for the confirmation and further analyses (Bustos et al. 2012; dos Santos et al. 2019). This is because *in vivo* conditions are much more complex than the *in vitro* conditions due to the presence of number and types of microorganisms, dietary factors, cell-to-cell communication, peristatic flow, and individual host response (Bustos et al. 2012; Lebeer et al. 2008).

4. Conclusions

Recently, the combinations of different kinds of functional foods, defined as food or food ingredients that have positive health effects, are very popular research subject. Here, we present the *in vitro* effects of loganic acid on two *lactobacillus* strains. Even though the *in vivo* studies are required to investigate the interplay between loganic acid and the probiotic bacteria in detail, as well as more molecular studies for clarifying the mechanisms how loganic acid can affect the probiotic functionality, the preliminary results suggest that there is a potential for beneficial health benefits of combinations of loganic acid and probiotic strains.

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