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Research Article

Insight into Effects of Ipolamiide isolated from *Plantago euphratica* on Probiotic Properties of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*

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Abstract

Iridoid glycosides are 2-cyclopentanoid derivatives of terpene origin naturally occuring in the leaves, fruits, seeds, bark, roots of various plants. They are medically important because used in the treatment of many diseases while normally protect plants from biotic and abiotic attacks. They have anti-microbial, anti-tumor, anti-cardiac and anti-inflammatory effects. Ipolamiide is one of the iridoid glycosides and naturally present in many plants. Ipolamiide is very little known compound in terms of the biological activities.

The beneficial microorganisms in the body and the plant secondary metabolites can interact in the human gastrointestinal tract. Probiotics are live microorganisms that have many health benefits by improving microbial balance of the intestines. Among the most known and most studied probiotics are *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*.

The aim of the present study is to investigate the effects of ipolamiide on probiotic bacteria *Lactobacillus rhamnosus* GG (GG) and *Lactobacillus acidophilus* LA-5 (LA-5). For this purpose, ipolamiide was added to the growth of probiotics in different concentrations and its effects on bacterial growth kinetics, bacterial surface hydrophobicity (Microbial Adhesion to Solvents - MATS Test) and bacterial aggregation (Auto-Aggregation Test) were investigated.

The results showed that ipolamiide did not show any important change in surface hydrophobicity of probiotic bacteria. Dose-dependent increases in auto-aggregation properties of the LA-5 and GG were observed. However, further detailed studies are required to give insight into other possible biological activities of ipolamiide.

Keywords: Aggregation, Iridoid Glycosides, MATS Test, Probiotics

Plantago Euphratica Bitkisinden İzole Edilen İpolamiidin *Lactobacillus acidophilus* ve *Lactobacillus rhamnosus* Bakterilerinin Probiyotik Özellikleri Üzerine Etkileri

Özet

İridoid glikozitler, doğal olarak yapraklarda, meyvelerde, tohumlarda, ağaç kabuğunda, bitki köklerinde bulunan terpenin 2-siklopentanoid türevleridir. Tıbbi olarak önemlidir, çünkü normalde bitkileri biyotik ve abiyotik ataklardan korurken birçok hastalığın tedavisinde kullanılır. Anti-mikrobiyal, anti-tümör, anti-kardiyak ve anti-enflamatuar etkilere sahiptirler. Ipolamiid, iridoid glikozitlerden biridir ve birçok bitkide doğal olarak bulunur. Ipolamiid biyolojik aktiviteleri açısından çok az bilinen bir bileşiktir.

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Vücuttaki faydalı mikroorganizmalar ve bitki kökenli bileşikler, insan gastrointestinal kanalında etkileşime girebilir. Probiyotikler, bağırsakların mikrobiyal dengesini geliştirerek birçok sağlık yararına sahip olan canlı mikroorganizmalardır. En bilinen ve en çok çalışılan probiyotikler arasında *Lactobacillus acidophilus* ve *Lactobacillus rhamnosus* bulunur.

Bu çalışmanın amacı, ipolamiidin probiyotik bakteriler *Lactobacillus rhamnosus* GG (GG) ve *Lactobacillus acidophilus* LA-5 (LA-5) üzerindeki etkilerini araştırmaktır. Bu amaçla probiyotiklerin büyümesine farklı konsantrasyonlarda ipolamiid eklenmiş ve bakteriyel büyüme kinetiği, bakteriyel yüzey hidrofobisitesi (Solventlere Mikrobiyel Yapışma - MATS Testi) ve bakteriyel agregasyona (Oto-Agregasyon Testi) etkileri incelenmiştir.

Sonuçlar, ipolamidin probiyotik bakterilerin yüzey hidrofobisitesinde önemli bir değişikliğe sebep olmadığını göstermiştir. LA-5 ve GG'nin oto-agregasyon özelliklerinde doza bağlı artışlar gözlenmiştir. Bununla birlikte, ipolamiidin diğer olası biyolojik aktiviteleri hakkında bilgi edinmek için daha ayrıntılı çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Agregasyon, İpolamiid, İridoid glikozitler, MATS Testi, Probiyotik

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1. Introduction

Plantago species are represented by about 275 species that grown all over the world. The most common use of *Plantago* species as herbal remedy is in the treatment of skin disorders and wound healing. In addition, several studies were reported previously analgesic (Das et al., 1984), anti-inflammatory (Barua et al., 2011), antiviral (Chiang et al., 2002), antioxidant (Gálvez et al., 2005) and anticancer (Moon & Zee, 1999) activity of various member of *Plantago* species. According to the literature; *Plantago* species include many secondary metabolites such as phenyl ethanoids (Nishibe et al., 1993; Murai et al., 1995), flavonoids (Jurišić Grubešić et al., 2013), iridoids (Handjieva et al., 1991; Jensen et al., 1996; Darrow & Bowers, 1997; Fuchs & Bowers, 2004), terpenoids (Venditti et al., 2015) and steroids (Zacchigna et al., 2009; Najib et al., 2012). *Plantago euphratica* is one of the endemic species of Turkey. To the best of our knowledge, no previous studies have been reported the biological activity and phytochemical content of *Plantago euphratica.*

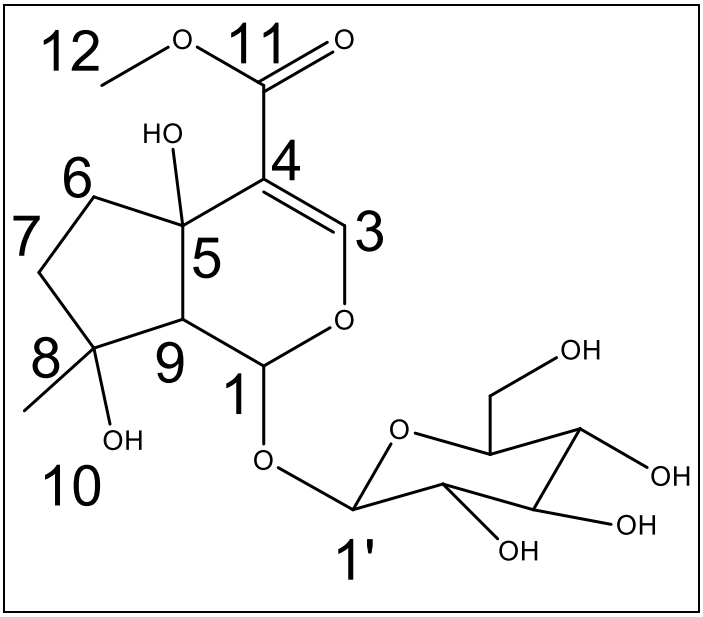

Figure 1. Chemical structure of ipolamiide

Probiotics are defined as living microorganisms that have a beneficial effects on the host by improving the microbial balance of the intestinal system (Hill et al., 2014). Among these, *Bifidobacterium* and *Lactobacillus* species are widely used. Lactic acid bacteria are among the group of microorganisms that possess effects on human health and constitute the most important group of probiotic microorganisms (Uymaz, 2010). Foods containing probiotic microorganisms constitute an important part of the food market. Increasing consumer awareness and better understanding of the importance of diet for a healthy life result in increasing demand for probiotic foods (Kleerebezem & Vaughan, 2009). *Lactobacillus plantarum, L. rhamnosus, L. paracasei, L. acidophilus* and *L. salivarius* are commonly found in the mucosa from the mouth to the rectum (Alp & Ertürkmen, 2017). In order to use a microorganism in food as probiotic; it should be preferably of human origin, able to survive in the gastrointestinal tract, not be pathogen, not have antibiotic resistance, produce antimicrobial compounds, stimulate the immune system, be resistant to stomach acid and bile salt (Collins & Gibson, 1999; Dunne et al., 2001; Erem, Küçükçetin, & Certel, 2013; Kechagia et al., 2013; Uymaz, 2010). Probiotic microorganisms compete with pathogenic

microorganisms for adherence to the intestinal wall and the consumption of nutrients contained in the lumen, produce substances with antimicrobial properties and regulate the immune functions associated with the intestinal mucosa to demonstrate these beneficial effects (Alp & Ertürkmen, 2017).

The beneficial microorganisms in the body and the compounds of plant origin interact in the human digestive tract. The aim of this study was to investigate the *in vitro* interaction of ipolamiide and probiotic. For this purpose, *L. acidophilus* LA-5 and *L. rhamnosus* GG bacteria were grown in the presence of different concentrations of ipolamiide and the effects of ipolamiide on bacterial growth kinetics, surface hydrophobicity and autoaggregation were investigated.

2. Material and Method

2.1. Plant Collection and Ipolamiide Isolation

Plantago euphratica was collected from Erzincan (Kemah-İliç Road 32th km, jipsy rock) at June 2018. The plant sample was dried under room conditions without direct exposure sunlight. 100 g of well-grounded aerial parts of *Plantago euphratica* were extracted with 2X600 mL of methanol for 12 h. After removing solvent, a dark green slurry extract (9.8 g) was obtained. In order to remove chlorophylls, extract was dissolved in hot water then cooled to rt., non-soluble parts were filtrated, and the filtrate lyophilized overnight to give 4 g of pale-yellow solid. 1 g of extract dissolved in 20 mL of water and repeatedly injected to semi prep-HPLC ten times with 2 mL sample loop 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 nm. The collected fractions between 16-18 minutes were purified using recycling mode with isocratic elution with 85:15 (Water: ACN), with 8 mL/min flow rate. After 10th cycle, a clear separation was observed then peaks were collected. The solvents were evaporated to give ipolamiide (128 mg) with high purity.

2.2. Growth of probiotic bacteria in the presence of ipolamiide 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, at 37ºC (Celebioglu, Delsoglio, Brix, Pessione, & Svensson, 2018). The bacteria were divided into groups and treated with ipolamiide. Ipolamiide was not added to the control group (MRS only), and 5 µg/mL, 10 µg/mL, and 20 µg/mL ipolamiide was added to the treated groups in MRS medium. Bacterial optical density was determined by densitometry. Reading the densitometry every four hour, the effects of ipolamiide 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). 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 stationary phase (3200xg, 15 min), washed with PBS and re-suspended in PBS to OD_{600} 0.5 (Kos et al., 2003). 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 0th hour (Kos et al., 2003).

2.5. Statistical Analysis

Every experiments were performed with at least three replicates and the results were expressed as mean ± Standard deviation and compared using Students's *t*-test. $p<0.05$ was considered as statistically significant.

3. Results and Discussion

3.1. NMR Study

^{1}H NMR (400 MHz, MeOD)) δ_H 7.47 (s.1H, H3), 5.83 (brs, 1H, H1), 4.61 (d, J=7.84, 1H, H1'), 3.92 (dd, J=11.9, 2.3, 1H, H6'a), 3.75 (s, 3H, H12), 3.68 (dd, J=11.9, 5.9, 1H, H6'b), 3.40 (m, 1H, H3'), 3.34 (m, 1H, H5'), 3.30 (m, 1H, H4'), 3.21 (dd, J=9.2, 7.8, 1H, H2'), 2.50 (brs, 1H H9), 2.27 (m, 1H, H6a), 2.09 (m, 1H, H6b), 1.96 (m, 1H, H7a), 1.59 (m, 1H, H7b), 1.17 (s, 3H, H10). ^{13}C NMR (101 MHz, MeOD) δ_C 166.7 (C11), 151.3 (C3), 113.8 (C4), 98.2 (C1'), 92.9 (C1), 77.6 (C8), 76.9 (C5'), 76.0 (C3'), 73.0 (C2'), 70.4 (C5), 70.3 (C4'), 61.45 (C6'), 60.3 (C9), 50.4 (C12), 39.0 (C7), 37.5 (C6), 22.0 (C10).

The NMR spectra correspond to the ipolamiide. Thus, this confirms that the isolation of ipolamiide was successful.

3.2. Bacterial Growth Kinetics

In this study, 0, 5, 10, and 20 µg/mL concentrations of ipolamiide were used. Ipolamiide showed a statistically significant decrease in *Lactobacillus acidophilus* LA-5 at 5 and 10 µg/ml, whereas 20 µg/ml was not observed. When *Lactobacillus rhamnosus* GG was examined, no significant change was observed (Fig. 2). This means ipolamiide did not Show very effective against the probiotic bacteria, and even higher concentrations could have positive effects on the growth. It is important that such compounds have no effect on the growth of beneficial microorganisms, while their anti-bacterial activities against pathogenic bacteria are present. Thus, this compound can selectively affect on beneficial bacteria.

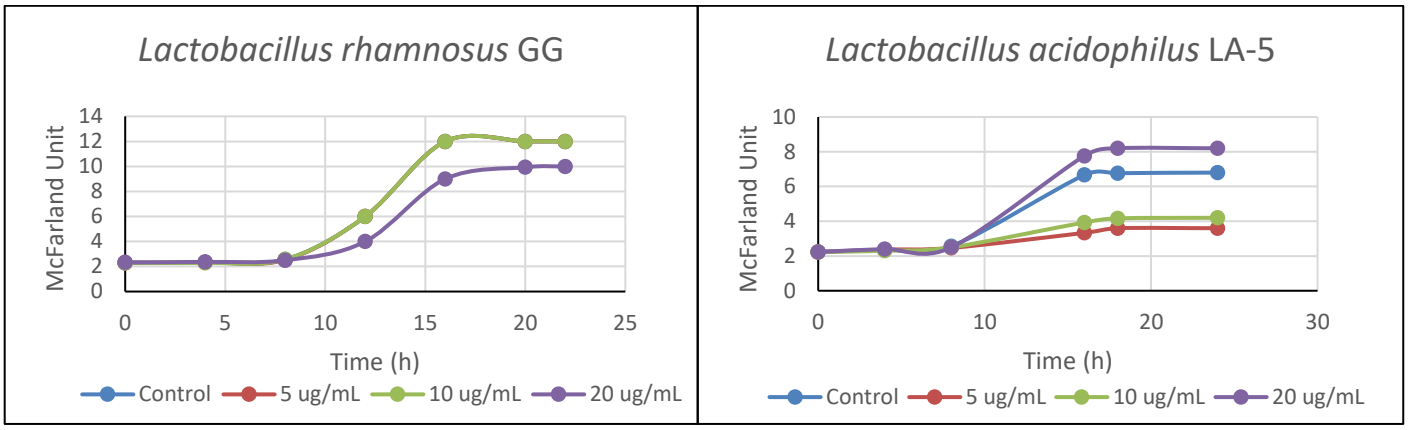

Figure 2. Effects of different concentrations of ipolamiide on growth kinetics of probiotic bacteria.

3.3. Bacterial Surface Hydrophobicity

Bacterial surface hydrophobicity plays an important role in the adhesion of bacteria to the mucosa in the intestines (Liu et al., 2004). Therefore, the more surface hydrophobicity increases, the more likely the beneficial bacteria will bind to the mucosa in the gastraointestinal tract. Only at 10 µg/mL concentration of ipolamiide applied on probiotic bacteria, the surface hydrophobicity of *Lactobacillus rhamnosus* GG bacteria was significantly ($p < 0.05$) decreased, while no other concentrations showed any alteration on either *Lactobacillus acidophilus* LA-5 or *Lactobacillus rhamnosus* GG surface hydrophobicity (Fig. 3). This means that no changes observed on the hydrophobicity could not alter the adhesion of the probiotic bacteria to the host components, such as mucus layer and epithelial cells.

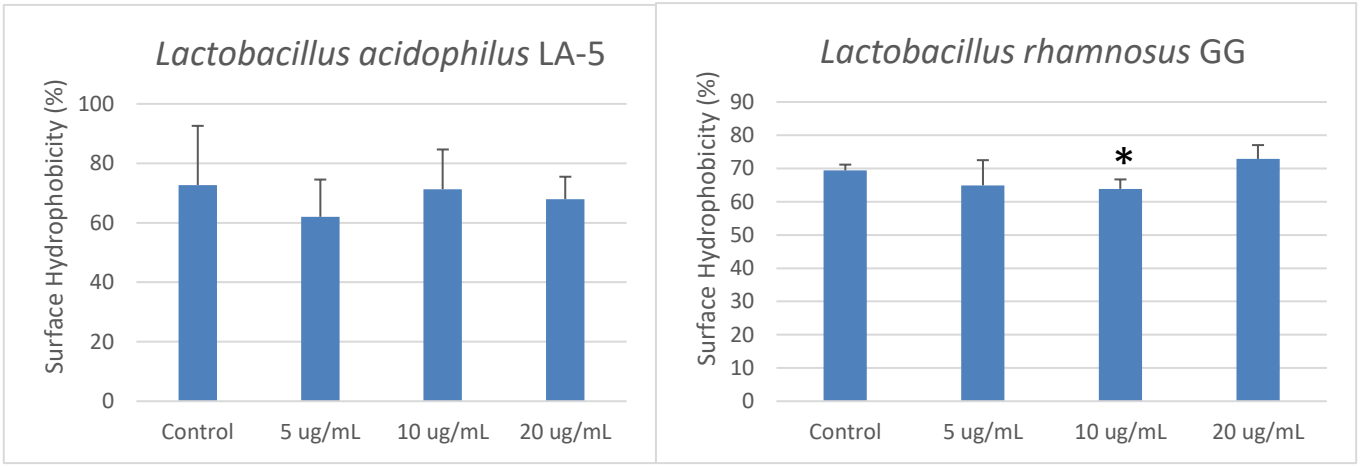

Figure 3. Effects of different concentrations of ipolamiide on probiotic bacterial surface hydrophobicity. . Asterisks (*) indicate $p<0.05$, when compared to control.

3.4. Bacterial Auto-Aggregation

Bacterial aggregation is also of great importance for attachment of probiotics to the intestinal mucosa (Etzold et al., 2014). High aggregation is an indication of better adhesion. When the auto-aggregation of the bacteria was examined (Fig. 4), significant increases (10 and 20 µg/mL) were observed in *Lactobacillus acidophilus* LA-5, as well as in the auto-aggregation of *Lactobacillus rhamnosus* GG bacteria ($p <0.05$). however, a significant decrease was observed in the auto-aggregation of *Lactobacillus rhamnosus* GG when the concentration of 5 µg/mL was used.

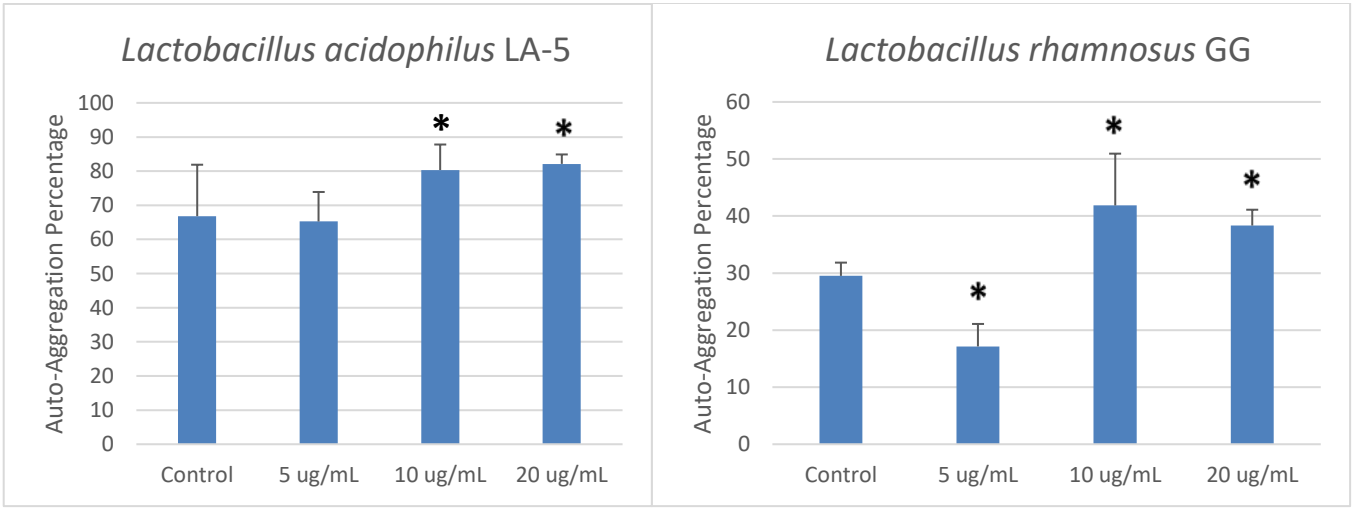

Figure 4. Effects of different concentrations of ipolamiide on probiotic auto-aggregation. Asterisks (*) indicate $p<0.05$, when compared to control.

4. Conclusions and Recommendations

Probiotics and plant-derived compounds are great canditates for the functional foods, defined as food or food ingredients that have posivitive health benefits on human. Thus, interplay between probiotics and plant compounds should be studied in terms of more bioactive compounds could be produced by the metabolism of the beneficial microorganisms. Furthermore, such compounds could affect the probiotic activities of the microorganisms. Thus, the present study was conducted as preliminary screening of this interaction between ipolamiide and very well known probiotic bacteria *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*.

Further studies are required in order to investigate how these bacteria can metabolize the ipolamiide and when it is metabolized, whether its biological activity is increased.

4. Acknowledge

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