

Investigation of Possibilities of Using *Nerium oleander* L. Extract as Prebiotic for *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*

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Abstract

The use of microorganisms that support human health and stimulate the immune system is gaining more importance. It is thought that probiotic bacterial strains, representing a significant portion of these microorganisms, may be treated in different disease groups. For instance, prevention of gastrointestinal diseases, ulcerative colitis, diarrhea, necrotizing enterocolitis, inflammatory bowel diseases, constipation, urogenital system health, rotavirus diarrhea as well as colon cancer can be prevented using probiotics. The probiotic bacterial strains, have basic needs such as bacterial auto-aggregation and cell surface hydrophobicity. Due to the phytochemicals contained in the *Nerium oleander* L., this plant has been used as medicinal plants. Such plants, when ingested, contact with the human gastrointestinal microbiome, so beneficial microorganisms can be affected. Thus, present study aimed to investigate the effects of *Nerium oleander* L. extract on two of the most studied and very well-known probiotic bacteria, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*.

Nerium oleander L. was extracted using ethanol. Probiotics were grown in the presence of this extract. Surface hydrophobicity of probiotics grown in the presence of plant extract was assessed by Microbial Adhesion to Solvents (MATS) Assay, and auto-aggregation was performed.

Auto-aggregation and surface hydrophobicity properties of these bacteria can be modulated by this plant, due to its phytochemicals. We have demonstrated that *Nerium oleander* plant extract could have a positive effect on the specific properties of probiotic bacteria, such as auto-aggregation and hydrophobicity. Auto-aggregation, as well as surface hydrophobicity were increased or decreased dependent on the dose as the plant extract

The combinations of this plant and a probiotic strain could be next generation functional food, defined as food ingredient that has positive health benefits to the human.

Keywords: auto-aggregation, *Nerium oleander* L., prebiotics, probiotics, synbiotics, surface hydrophobicity

Lactobacillus acidophilus ve *Lactobacillus rhamnosus* için *Nerium oleander* L. Ekstraktının Prebiyotik Olarak Kullanım Olanaklarının Araştırılması

Öz

İnsan sağlığını destekleyen ve bağışıklık sistemini uyaran mikroorganizmaların kullanımı gün geçtikçe önem kazanmaktadır. Bu mikroorganizmaların önemli bir bölümünü temsil eden probiyotik bakteri suşlarının farklı hastalık gruplarında tedavi edilebileceği düşünülmektedir. Örneğin, gastrointestinal hastalıkların önlenmesi, ülseratif kolit, ishal, nekrotizan enterokolit, inflamatuvar bağırsak hastalıkları, kabızlık, ürogenital sistem sağlığı, rotavirüs ishal ve kolon kanseri probiyotikler kullanılarak önenebilirliği çalışılmaktadır. Probiyotik bakteri suşları, bakteriyel oto-agregasyon ve hücre yüzeyi hidrofobitesi gibi temel faktörlere sahiptir. *Nerium oleander* L.'de bulunan fitokimyasallar nedeniyle, bu bitki tıbbi bitki olarak kullanılmaktadır. Bu tür bitkiler, insan gastrointestinal mikrobiyomuyla temas eder, bu nedenle yararlı mikroorganizmalar etkilenir. Bu nedenle, bu çalışmada *Nerium oleander* L. ekstresinin en çok çalışılan ve çok iyi bilinen probiyotik bakteriler, *Lactobacillus acidophilus* ve *Lactobacillus rhamnosus* üzerindeki etkileri araştırılmıştır. *Nerium oleander* L. etanol kullanılarak ekstrakte edilmiştir. Probiyotikler bu ekstraktın varlığında büyütülmüştür. Bitki ekstraktı varlığında geliştirilen probiyotiklerin yüzey hidrofobitesi ve oto-agregasyon testleri yapılmıştır. *Nerium oleander* L. bitki özütünün probiyotik bakterilerin oto-agregasyon ve hidrofobiklik

gibi belirli özellikleri üzerinde olumlu bir etkiye sahip olabileceği gösterilmiştir.

Bu bakterilerin oto-agregasyonu ve yüzey hidrofobisite özellikleri, fitokimyasalları nedeniyle bu bitki tarafından düzenlenebilir bulunmuştur. Bakterilerin oto-agregasyon ve yüzey hidrofobisitesi, bitki özütünün dozuna bağlı olarak artmış veya azalmıştır.

Daha detaylı çalışmalarla birlikte, bu bitkinin ve bir probiyotik türün kombinasyonlarının, insan sağlığına yararlı olan gıda bileşeni olarak tanımlanan yeni nesil fonksiyonel gıda formülasyonlarını oluşturabilir.

Anahtar Kelimeler: *Nerium oleander* L., Oto-agregasyon, prebiyotik, probiyotik, sinbiyotik, yüzey hidrofobisitesi

1.

Introduction

The presence of bacteria harmful to human body and causing diseases has been known for a long time. However, today's scientific studies show that some living microorganisms can be used in the treatment and prevention of diseases (Fotiadis et al. 2008; Huang, Wang, and Hu 2016; Anandharaj, Sivasankari, and Parveen Rani 2014; Douglas and Sanders 2008; Ishikawa et al. 2011; Soa, Wana, and El-Nezami 2017; Kumar et al. 2010).

Prebiotics are defined as “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal (GI) microflora that confer benefits upon host wellbeing and health” (Gibson et al. 2004; Gibson et al. 2010). On the other hand, probiotic was defined as “a living microbial nutritional supplement that improves bowel balance and positively affects the host”. Recently, it is now known as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” by FAO/WHO (Hill et al. 2014). Both group are member of functional foods, defined as the food or food ingredient that has positive health benefits to the human.

Lactic acid bacteria species such as *Lactobacillus*, *Bifidobacterium*, *Enterobacterium*, which are in accordance

with the definition, are frequently used in probiotic foods produced for human consumption because they live in native intestinal flora (Gill and Prasad 2008). These microorganisms, which do not show pathogenic and toxic properties, do not lose their viability in the designed product and the benefit increases as people take part in the intestinal flora after consumption (Kleerebezem and Vaughan 2009). The probiotic product contemplated may comprise one or more of these microorganisms. These lactic acid bacteria strains are likely to be found in infant formula, probiotic supplemented milk and various pharmaceutical preparations (Sanders 2009).

The basic requirement for probiotic bacteria is to survive in the gastrointestinal tract (GIT) (González-Rodríguez et al. 2013). Mucus, the first contact in the intestine, is known as an important site for bacterial adhesion and colonization. The fact that bacteria have the ability to aggregate forms a biological barrier by adhering to the surface and to each other, which is an important criterion in terms of probiotics since it will attach to the cells and colonize in GIT (Collado, Meriluoto, and Salminen 2007). The bacterial cell surface is the component that mediate the adhesion of bacteria to

intestinal epithelial cells (Servin and Coconnier 2003). The necessary mechanisms for the adhesion activities of probiotic bacteria differed among the species and it was thought that the adhesion of *Lactobacilli* to human intestinal cells is due to the mechanism consisting of different combinations of proteins and carbohydrates present on the bacterial surface (Buck et al. 2005). Adhesion of probiotics to the intestinal mucosa is important as it reduces the colonization of pathogens, regulates the immune system, and provides healing of the damaged mucosa (Van Tassell and Miller 2011). *Lactobacillus* colonization may also be important for the prevention of gastrointestinal diseases (Siciliano and Mazzeo 2012). For example, a reduction in the amount of mucosal *lactobacillus* is thought to cause ulcerative colitis, diarrhea, necrotizing enterocolitis, inflammatory bowel diseases, constipation, urogenital system defects, rotavirus diarrhea and colon cancer (Drisko, Jeanne A. Giles, Cheryl K. Bischoff 2003).

Easily grown in the Mediterranean countries and Turkey, *Nerium oleander* L. is known to be used as folk treatment against parasitic, bacterial infections, heart abnormalities, ringworm, skin diseases, warts, malignancies and vomiting (Kiran and Prasad 2014; Gayathri et al. 2011; Rashan et al. 2011). In the phytochemical analysis of oleander plant, the presence of various phytochemicals such as cholesterol, proteins, amino acids, alkaloids, flavonoids, tannins, tanonins, glycosides, terpenoids and flobatinins has been identified (Kiran and Prasad 2014). Furthermore, presence of *Nerium oleander* L. when ingested as folk medicine in GIT can alter the balance of microbiota. Thus, the aim of the present study is to investigate effects of *Nerium oleander* L. extract on very well-

known probiotic bacteria *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*. To achieve this purpose, auto-aggregation and surface hydrophobicity properties of these probiotic bacteria were investigated after exposed to ethanol extract of *Nerium oleander* L.

2. Material and Method

2.1. Ethanol extract of *Nerium oleander* L.

Green leaf parts of *Nerium oleander* L., were washed with tap water, then left to dry in a 60°C oven overnight. The dried leaves were removed from the oven and pulverized in mortar. The powdered green leaf fractions were treated with 70% ethanol (1:10 (g/ml)) for 6 hours. The solvent was then removed using a rotary evaporator. The stock solution obtained was concentrated with dH₂O and stored at + 4 ° C for experimental use. Different concentrations used in experimental studies were prepared from the stock extract prepared.

2.2. Growth of probiotic bacteria

Lactobacillus acidophilus LA-5 and *Lactobacillus rhamnosus* GG, which are kindly gifted by Chr. Hansen, Turkey, were grown in Man, Rogosa and Sharpe (MRS) medium at 37°C without shaking (Celebioglu et al. 2016; Celebioglu et al. 2018; Celebioglu et al. 2017). Bacteria were divided into groups and treated with ethanol extracts of *Nerium oleander* L. (25, 50, and 75 µg/mL). The control group was not treated with the extract.

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 KNO₃ (pH 6.2) (OD₆₀₀ 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 (1)

$$(1-A1/A0) \times 100 \quad (1)$$

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

2.4. Bacterial Auto-Aggregation

Bacterial cells were collected in the stationary phase (3200xg, 15 min), washed with PBS and re-suspended in PBS to OD₆₀₀ 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 5 hour-incubation at room temperature. Every hour, 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 (2)

$$(1-A1/A0) \times 100 \quad (2)$$

where A1 is the absorbance measured at every hour (1st, 2nd, 3rd, 4th, or 5th hour) and A0 is the absorbance measured at 0th hour (Kos et al. 2003).

2.5. Statistical Analysis

Each experiments were conducted as at least three biological replicates and three technical replicates. Non-parametric One-Way Analysis of Variance (ANOVA) was used to evaluate the significance in differences between treated and control groups (pair-wise comparison) using IBM SPSS Statistics Version 25. The significant differences were accepted when p<0.05.

3. Research Findings

Recently, the main focus on functional foods, defined as the food or food ingredient that has positive health benefits to the human, is the combining two different groups of functional foods, called synbiotics (i.e. combination of prebiotics and probiotics) (Guiné and Silva 2016). It is suggested that *Nerium oleander* L. could have beneficial effects, and used as traditional folk medicine, but when ingested and reached to GIT, it contacts with GIT microbiome, consisting of mainly beneficial microorganisms. Thus, the investigation of effects of this plant on especially beneficial microorganisms in the GIT is of considerable importance.

Figure 1 shows that ethanol extract of *N. oleander* L. has no negative effect on probiotic growths.

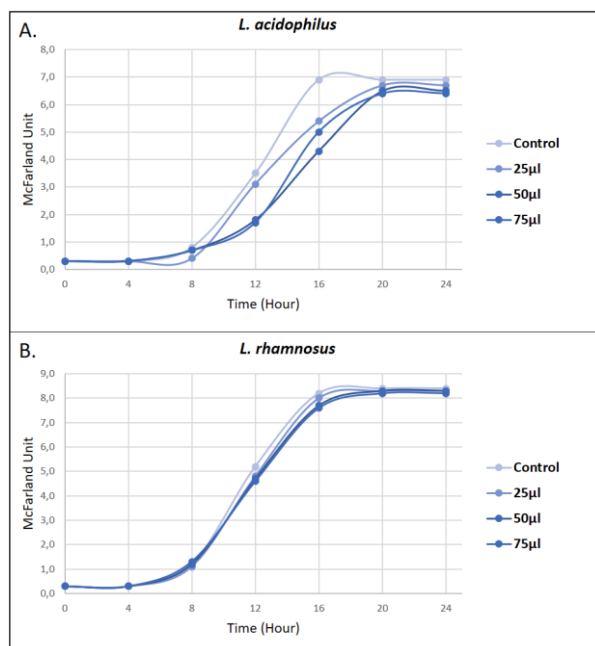


Figure 1. Growth kinetics of (A) *Lactobacillus acidophilus* LA-5 and (B) *Lactobacillus rhamnosus* GG when grown in the presence of different concentrations of *Nerium oleander* L. ethanol extract.

3.1. Auto-aggregation of Probiotics

Adhesion of beneficial microorganisms to GIT has paramount importance as it can lead to good colonization of the bacteria (Laparra and Sanz 2009). It is a complex phenomenon that consists multiple processes in the microorganisms, where non-specific and specific mechanisms play roles (Del Re et al. 2000). One of the non-specific mechanisms involved in adhesion of bacteria to the host is cellular aggregation between microorganisms of the same strain, called auto-aggregation (Kos et al. 2003).

Figure 2 and 3 show the auto-aggregation properties of two probiotic *Lactobacillus* strains affected by *Nerium oleander* L. extract. Auto-aggregation of the bacteria was observed for 5 hours, the absorbances were measured every hour (1st, 2nd, 3rd, 4th, and 5th hour).

Regarding *L. acidophilus* LA-5, statistical analyses showed that 75 µg/mL of *Nerium oleander* L. extract significantly ($p < 0.05$) decreased the auto-aggregation at 1st, 2nd, and 3rd hours (Figure 2). Furthermore, at these times, there are also significant changes between 25 and 75 µg/mL of *Nerium oleander* L. extract treatment groups, which indicates the decrease in auto-aggregation of *L. acidophilus* LA-5 could be dose-dependent. At the fourth hour, 50 µg/mL of plant extract significantly decreased the auto-aggregation, when compared to control group, while at the fifth hour, there was no significant alteration in auto-aggregation observed.

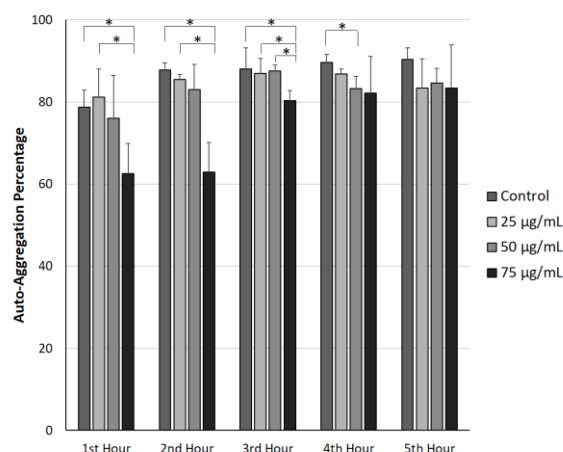


Figure 2. Auto-aggregation of *Lactobacillus acidophilus* LA-5 when grown in the presence of different concentrations of *Nerium oleander* L. ethanol extract. Asterisks (*) indicate that the difference between the groups is statistically significant ($p < 0.05$) according to ANOVA.

Regarding *Lactobacillus rhamnosus* GG, at 1st to 3rd hours, 50 µg/mL of *Nerium oleander* L. extract significantly increased the auto-aggregation, while at fourth hour, 25 and 50 µg/mL of extract increased the auto-aggregation (Figure 3). Even though at first and second hours, 25 µg/mL of extract seems

to increase the auto-aggregation, this is not statistically significant ($p > 0.05$) according to pair-wise comparison in ANOVA. This is most probably due to the lack of number of subjects (n). At the fifth hour, no significant changes were observed in any concentration.

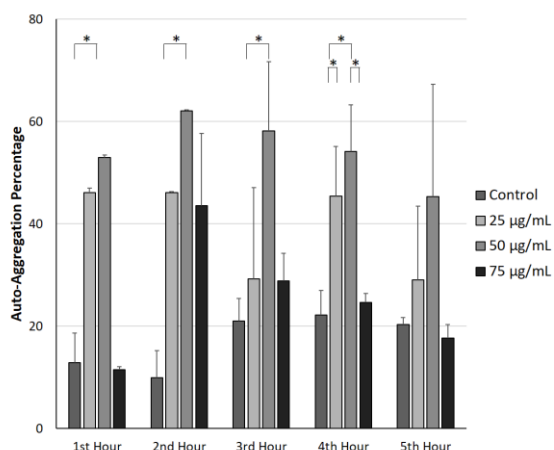


Figure 3. Auto-aggregation of *Lactobacillus rhamnosus* GG when grown in the presence of different concentrations of *Nerium oleander* L. ethanol extract. Asterisks (*) indicate that the difference between the groups is statistically significant ($p < 0.05$) according to ANOVA.

Lactic acid bacteria that have good aggregation abilities could be adhere to mucous layer of GI, than those having poor aggregation (Li et al. 2015). For instance, it has been shown that aggregating *Lactobacillus crispatus* had good adhesion to colonic cells, Caco-2, than its non-aggregating mutant (Ocaña and Nader-Macias 2002).

In the present study, it was observed that *Lactobacillus rhamnosus* GG had higher auto-aggregation ability in the presence of *Nerium oleander* L. ethanol extract than *Lactobacillus acidophilus* LA-5.

3.2. Bacterial surface hydrophobicity

Another non-specific mechanism promoting the adhesion is the surface hydrophobicity of the microorganisms (Van Loosdrecht et al. 1987a). The role of hydrophobic interactions in bacterial adhesion has been known due to physicochemical properties of the cell wall (or cell surface) can control important bacterial behavior (Van Loosdrecht et al. 1987b). Surface hydrophobicity may be different between the bacterial strains, but could be important for possible correlation between physicochemical properties of the bacteria and their ability to adhere to the intestinal mucus (Liu et al. 2004). Thus, in the present study, these two properties (auto-aggregation and surface hydrophobicity) were examined for two different probiotic strains when grown in the presence of *Nerium oleander* L. ethanol extract.

Figure 4 and 5 show the bacterial surface hydrophobicity of two *Lactobacillus* strains grown on different concentrations of *Nerium oleander* L. ethanol extract in terms of adhesion to hydrophobic solvent, xylene. According to these results, a significant ($p < 0.05$) increase was observed in *Lactobacillus acidophilus* LA-5 at 25 µg/mL concentration of the plant extract, compared to the control group, but no significant change was observed at 50-75 µg/mL concentrations (Figure 4).

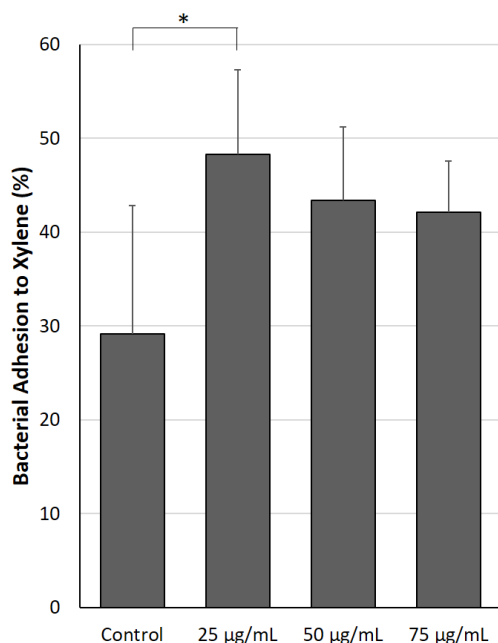


Figure 4. Bacterial surface hydrophobicity for *Lactobacillus acidophilus* LA-5 when grown in the presence of different concentrations of *Nerium oleander* L. ethanol extract. Asterisks (*) indicate that the difference between the groups is statistically significant ($p < 0.05$) according to ANOVA.

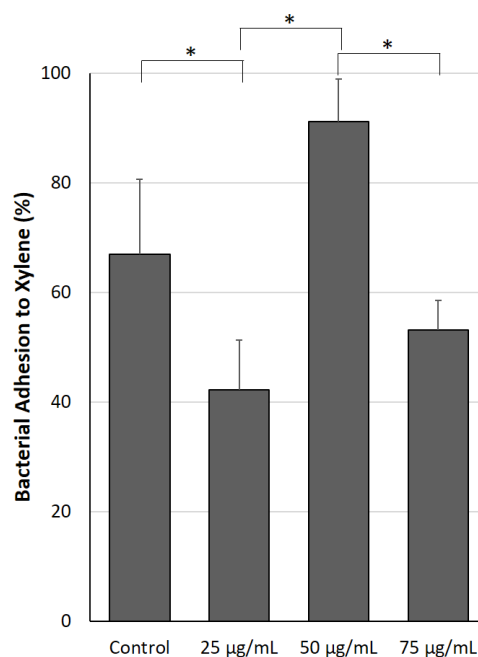


Figure 5. Bacterial surface hydrophobicity for *Lactobacillus rhamnosus* GG when grown in the presence of different concentrations of *Nerium oleander* L. ethanol extract. Asterisks (*) indicate that the difference between the groups is statistically significant ($p < 0.05$) according to ANOVA.

On the other hand, 25 µg/mL of the plant extract significantly ($p < 0.05$) decreased the surface hydrophobicity of *Lactobacillus rhamnosus* GG, while 50 µg/mL of the extract significantly ($p < 0.05$) increased the surface hydrophobicity, and 75 µg/mL did not change it (Figure 5). However, according to ANOVA, there is also significant alterations between the treated groups.

It was found that some concentrations of plant extract decreased the auto-aggregation *Lactobacillus acidophilus* LA-5. Decreased auto-aggregation, even though the rate is not so high, could lead to decreased adhesion of the bacteria. However, as the adhesion is multi-step process, other factors can be involved and when looking at the hydrophobicity results, especially 25 µg/mL concentration of the plant extract increased the surface hydrophobicity of this bacterium. Hydrophobic interactions between the surface of bacterium and mucus layer in the GIT can still lead to good correlation in the adhesive properties.

On the other hand, the auto-aggregation ability of *Lactobacillus rhamnosus* GG was

increased by some concentrations of ethanol extract of *Nerium oleander* L. Auto-aggregation capability of *Lactobacillus rhamnosus* GG was increased with 50 µg/mL *Nerium oleander* L extract. This can indicate that these bacteria can aggregate and so residing in the GIT can be improved. This is also positively correlated with surface hydrophobicity; especially 50 µg/mL of plant extract significantly increased both auto-aggregation and surface hydrophobicity of this bacterium.

Previous studies also showed that there could be a correlation between auto-aggregation and surface hydrophobicity. In the research done by Nikolic et al., auto-aggregating strains of *lactobacilli* (*Lactobacillus casei* group, including *Lactobacillus paracasei*) had high hydrophobic nature of the cell surface (Nikolic et al. 2010). In another study, there has been shown a significant correlation between auto-aggregation and hydrophobicity of 15 *Lactobacillus plantarum* strains (Nikolic et al. 2010).

Adhesion ability is important for probiotic bacteria to colonize the gastrointestinal tract and can provide a healthy benefit to the host. According to the study by Ehrmann et al., a correlation was observed between adhesion ability and hydrophobicity in some *lactobacilli* (Ehrmann et al. 2002). However, this correlation is most probably strain-specific, thus higher auto-aggregation does not mean every time that the strain has also higher adhesion. Non-specific and specific ligand-receptor mechanisms can be involved in adhesion because it is a complex process (García-Cayuela et al. 2014; Sánchez, Urdaci, and Margolles 2010; Greene and Klaenhammer 1994) Since the different elements present in the dynamic environment

of the gastrointestinal tract are likely to change bacterial adhesion, it is difficult to extrapolate the *in vitro* results of bacterial adhesion to *in vivo* conditions (Lebeer, Vanderleyden, and De Keersmaecker 2008). Thus, *in vitro* experiments are required to resolve adhesion mechanisms, as well as to investigate the effects of dietary constituents on probiotic adhesion (Li et al. 2015).

4. Conclusions

In conclusion, the present study showed that different *Nerium oleander* concentrations exhibited different effects on probiotic properties of two different bacterial strains, such as auto-aggregation and surface hydrophobicity. We have demonstrated that *Nerium oleander* plant extract could have a positive effect on the specific properties of probiotic bacteria, such as auto-aggregation and hydrophobicity. Thus, the findings of our study are of great importance and we think that they will guide the future studies.

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