RESEARCH ARTICLE

Hasan U. Celebioglu[1]*
Emre Evin[2]
Sena Davran Bulut[1]
Buse N. Derebasi[1]
Orhan Adali[2]

Exploring the Impact of Hesperidin on Probiotic Properties of *Lactobacillus acidophilus* LA-5 and Its Synbiotic Interaction with Colon Cancer Cells

Authors' addresses:
[1]Department of Biotechnology, Faculty of Science, Bartin University, Bartin, Turkiye
[2] Department of Biological Sciences, Faculty of Arts and Sciences, Middle East Technical University, Ankara, Turkiye

Correspondence:
Hasan U. Celebioglu
Department of Biotechnology, Faculty of Science, Bartin University, Bartin, Turkiye
Phone:
e-mail: ufukcelebioglu@gmail.com

Article info:
Received: 15 November 2023
Accepted: 11 January 2024

ABSTRACT

Probiotics are live microorganisms that provide health benefits to the host when taken in adequate amounts. Hesperidin is a phenolic compound found in banana fruit, lemon fruit, and lemon peel. It exhibits anti-inflammatory, anti-oxidative, and anti-cancer activities. Although these compounds are present in the gastrointestinal tract (GIT), their synbiotic interaction on the GIT is not well understood. This study aims to investigate the effects of hesperidin on the probiotic properties of *Lactobacillus acidophilus* LA-5 (LA-5) and the impact of their synbiotic interaction on the cancer cell line HT-29. Results showed that hesperidin did not negatively affect the auto-aggregation, adhesion, and antioxidant capacity of LA-5. LA-5 grown with hesperidin exhibited greater resistance to pepsin and bile salt compared to LA-5 alone. Furthermore, *in vitro* cancer studies indicated that bacteria grown in the presence of hesperidin may increase caspase-3 activity in HT-29 cells and thus induce apoptosis in this way. This study suggests that hesperidin may enhance and contribute to the probiotic properties of LA-5. Consequently, LA-5 grown with hesperidin may exert more beneficial effects on the host.

Key words: Hesperidin, Probiotic Bacteria, *Lactobacillus acidophilus* LA-5

Introduction

Probiotics are defined as live microorganisms that, when consumed in sufficient quantities, confer health benefits to the host, according to the Food and Agriculture Organization and the World Health Organization (FAO/WHO) (Hill et al., 2014). Probiotic bacteria contribute to the gut microbiota with their advantageous effects, including the improvement of the immune system, normalization of the mucosal barrier, reduction of oxidative stress, and prevention of various diseases (Celebioglu et al., 2021; Onur et al., 2022).

Commonly known probiotic genera include *Lactobacillus* and *Bifidobacterium*. *Lactobacillus acidophilus*, a member of lactic acid bacteria (LAB), is well-documented and fully characterized. *L. acidophilus* exhibits several health benefits, such as inhibiting the growth of pathogens by producing lactic, propionic, and acetic acids, which lower the pH (Muyyarikkandy & Amalaradjou, 2017). Additionally, *L. acidophilus* is associated with the improvement of symptoms related to intestinal diseases. Despite extensive studies on the protective properties of probiotics against cancer, particularly colon cancer, molecular explanations for these protective properties have not been elucidated yet (Śliżewska et al., 2021).

Plant phenolic compounds are secondary metabolites commonly found in foods and beverages, providing bitterness, astringency, color, flavor, odor, and oxidative stability (Pandey & Rizvi, 2009). Daily intake of phenolic compounds typically ranges from less than 100 mg to over 2 g. Over 95% of dietary phenolic compounds reach the colon and undergo metabolism by the gut microbiota (Clifford, 2004). While phenolic compounds are generally known to have positive effects on human health, their efficacy depends on bioavailability and hydrolysis by intestinal enzymes or microbiota before absorption (Marín et al., 2015). Hesperidin (Fig. 1), found in banana fruit, lemon fruit, and lemon peel, is a phenolic compound known for its anti-inflammatory, anti-oxidative, and anti-cancer activities (Xiong et al., 2019). Studies have shown that hesperidin has been used to prevent/treat various diseases, including cardiovascular diseases, diabetes, Alzheimer's disease, and colon cancer (Wilmsen et al., 2005); Man et al., 2019).

Research indicates that phenolic compounds and probiotic bacteria interact when coexisting in food products, dietary supplements, or the digestive tract (Duda-Chodak et al., 2008). This interaction has led to increased attention in studies exploring the effects of the interplay between phenolic compounds and probiotics on human health. The

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20241043

objective of this study was to investigate the effects of hesperidin on the probiotic properties (auto-aggregation, adhesion, antioxidant capacity, tolerance to pepsin, and bile salt) of *L. acidophilus* LA-5 and the effects of their synbiotic interaction on the human colon cancer cell line HT-29.

Materials and Methods

Growth Kinetics of Lactobacillus acidophilus LA-5

Lactobacillus acidophilus LA-5 was a kind gift of Chr. Hansen, Turkey and grown in semisynthetic medium for lactic acid bacteria (LABSEM) without shaking at 37ºC (Celebioglu et al., 2018). The bacterial cultures were divided into groups, and hesperidin was used in different concentrations (0-200 µg/mL), one of which as control without hesperidin. The growth of probiotic bacteria was observed every four hour by measuring their absorbance at wavelength of 600 nm in microplates.

Pepsin and Bile Salt Resistance Assay of Lactobacillus acidophilus LA-5 Grown with Hesperidin

Pepsin and bile salt tolerances were determined in 96-well microplates by the Thiazolyl Blue Tetrazolium Bromide (MTT) assay. Control and treated groups were collected after growth by centrifugation, washed with PBS. For the analysis of pepsin resistance, groups were incubated for 3 hours at 37 ℃ in PBS containing 3 mg/mL pepsin. For the determination bile salt tolerance, groups were incubated for 3 hours at 37 ℃ in PBS containing 2 mg/mL bile salts. Then, samples were incubated in to 96-well microplates with 1 mg/mL MTT for 2 hours. The optical densities of the samples in the plates were measured in the micro-plate reader at 570 nm. Bacterial viability was calculated as percentage of absorbance measured for treated groups relative to absorbance of the control group.

Effects of Hesperidin on Auto-aggregation Property of Lactobacillus acidophilus LA-5

Lactobacillus acidophilus LA-5, which was kind gift of Chr. Hansen, Turkey, were grown in medium for semisynthetic lactic acid bacteria (LABSEM) including different concentrations (0–200 µg/mL) of hesperidin without shaking at 37°C (Celebioglu et al., 2018). Bacterial cells were collected in the late logarithmic phase (3200×g, 15 min), washed with phosphate-buffered saline (PBS) and re-suspended in PBS to OD600 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 s (1 h, RT). After incubation, 100 µL from the upper portion of the suspensions 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 using the following formula:

$$\%Auto - aggregation = (1 - \frac{A_t}{A_0}) \times 100$$

where A_t is the absorbance measured after incubation (every hour for 3 h), and A_0 is the absorbance measured before incubation (Kos et al., 2003).

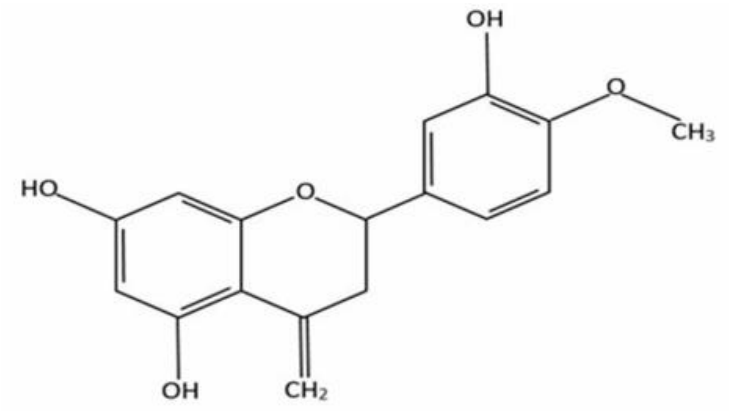

Figure 1. *Chemical structure of hesperidin (Musa et al., 2019)*

Adhesion Property of Lactobacillus acidophilus LA-5 Grown with Hesperidin

Adhesion was assayed in 96-well microplates according to the technique described previously (Leccese Terraf et al., 2014). Briefly, microplates were covered with 200 µL of 1 mg/mL mucin in PBS and incubated at 4°C overnight, followed by washing with PBS.

For the adhesion assay, LA-5 grown in LABSEM with and without hesperidin was collected in the late logarithmic phase and centrifuged at 3200×g at 15 min. After centrifugation, pellets were washed with PBS and re-suspended in PBS to OD600 1.5.

200 µL of LA-5 suspension were added to each well and plates were incubated for 2 h at 37°C. After removing non-adhered cells by washes with 200 µL of PBS, adhered cells were detected by staining with crystal violet. Crystal violet was added to each well and incubated for 30 min. Then, crystal violet was removed and 200 µL of iodine solution was added to each well. After 2-3 min, the plate was washed with PBS twice and the stain was released with 30% acetic acid (200 µL per well). Absorbance was measured at 570 nm in a plate reader (Leccese Terraf et al., 2014).

Preparation of Cell Free Supernatants (CFS) of Lactobacillus acidophilus LA-5

After growth of probiotic bacteria, cultures were centrifuged (3200xg, 15 min) and the supernatant was

removed and filtered (0.22 µm syringe-filters). The cell-free supernatants (CFS) were stored at -80ºC until use.

Antioxidant Effects of Lactobacillus acidophilus LA-5 Grown with Hesperidin

Antioxidant effects of LA-5 grown in the presence of hesperidin were examined using the free radical 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging assay. ABTS scavenging was assayed in 96-well plate according to the technique described by Re et al. with some modifications (Re et al., 1998). ABTS was dissolved in water to a 7 mM concentration. ABTS stock solution was mixed with 2.45 mM potassium persulfate (final concentration) and mixture was kept in the dark at room temperature for 12–16 h before use. The ABTS solution was diluted with ethanol to an absorbance 0.7 at 734 nm. CFS (100 µL) and ABTS (100 µL) were added to a 96-well plate, incubated (6 min, RT, in the dark) and the absorbance measured spectrophotometrically at 734 nm. Decreased absorbance, so the remaining amount of ABTS was determined as the amount of free radical scavenging, i.e. antioxidant activity. Relative antioxidant activities were calculated as percentage of reduction in the absorbance to the absorbance of initial solution.

In vitro Cytotoxicity Assay of Lactobacillus acidophilus LA-5 and Hesperidin for HT-29 Cells

Cell viabilities of HT-29 cells, and thus the cytotoxic effects of LA-5 grown in the presence of hesperidin (100 µg/mL) were determined by the Alamar Blue cell viability assay (Dinh et al., 2023). Human colon cancer cell line HT-29 was grown in MEM-Eagle medium.

CFSs and LABSEM medium were diluted with MEM-Eagle medium as 1/5, 1/10, and 1/50 (v/v). CFS of probiotic bacteria grown in LABSEM without hesperidin were used as probiotic control. Hesperidin (100 µg/mL) in LABSEM (without bacteria) was used to determine the effects of only hesperidin on the cells. HT-29 cells were plated into 96-well plates ($15x10^3$ cells/well). Then, cells were treated with the diluted LABSEM medium and CFSs of LA-5 grown either with hesperidin (hesperidin treated group) or without hesperidin (CFS group) for 24 h at 37°C. Following the treatments, the Alamar Blue cell viability assay (Invitrogen™, DAL1100) was performed according to the manufacturer guidelines and absorbances were measured at 570 and 600 nm using Multiskan™ FC Microplate Photometer. Cell viability was calculated as the percentage of Alamar Blue reduction according to the given formula (Invitrogen™, DAL1100). HT-29 cells were treated with LABSEM medium, which was diluted to 1/5 with MEM-Eagle medium, was accepted as 100 % cell viability.

Cancer Cell Invasion Assay

Cancer cells able to metastasize and invade possess the capability to pass through the extracellular matrix (ECM). Therefore, Boyden Chamber method coated with a thin ECM was used to determine the ability of cancer invasion after treatment of HT-29 cells with hesperidin and CFS. In this method, serum-free medium (100 µL) was added into the Boyden chamber with a cell count of $1x10^5$. These chambers were placed in wells filled with medium containing 150 µL of serum. After 24 hours at 37°C with 5% CO_2 incubation, the chamber was removed and washed with PBS and placed back in the well. The well was filled with 150 µL of separating buffer solution in order to separate the invasive cells from the membrane, which had migrated to the outer surface of the membrane at the bottom of the chamber and adhered to the membrane. After 30 minutes of incubation at 37°C, 50 µL of lysis buffer/dye mixture was added to the well and 150 µL of the mixture was taken from these wells and transferred to a new 96-well plate. Measurements were made with a 480/520 nm filter set using the SpectraMax ID3 fluorescent plate reader spectroscopy device.

Determination of Apoptosis by Caspase-3 Activity

The colorimetric Caspase-3 Activity Assay Kit (Abcam, UK) was used for the determination of caspase-3 activity. Cells were grown by treatment with hesperidin and CFS, and precipitated by centrifugation. Then, the cell pellet was re-suspended with 50 µL of lysis buffer and incubated for 10 minutes. It was centrifuged at 10,000 g for 1 minute and the supernatant was transferred to another tube to be used in the activity determination. 50 µL of cell lysate was transferred to wells of 96-well plates. 50 µL of reaction mixture and 5 µL of substrate solution were added to these wells. After incubation at 37°C for 90 minutes, absorbance was measured at 405 nm using the MultiSkan FC ELISA Plate Reader (ThermoFisher Scientific, US). Caspase-3 enzyme activity was calculated using the standard curve created with the standards given in the kit.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8 package program. One-way ANOVA was used to determine the differences between the groups and Tukey's test was used for multiple comparisons. Quantitative data were expressed as mean with standard deviation (mean ± SD) and $p < 0.05$ was considered as statistically significant.

Results

Effects of Hesperidin on Growth Kinetic of Lactobacillus acidophilus LA-5

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20241043

Effects of different concentrations of hesperidin (0-200 µg/mL) were investigated on growth kinetics of *Lactobacillus acidophilus* LA-5 (Fig. 2). None of the hesperidin concentrations showed any inhibitory effects on LA-5.

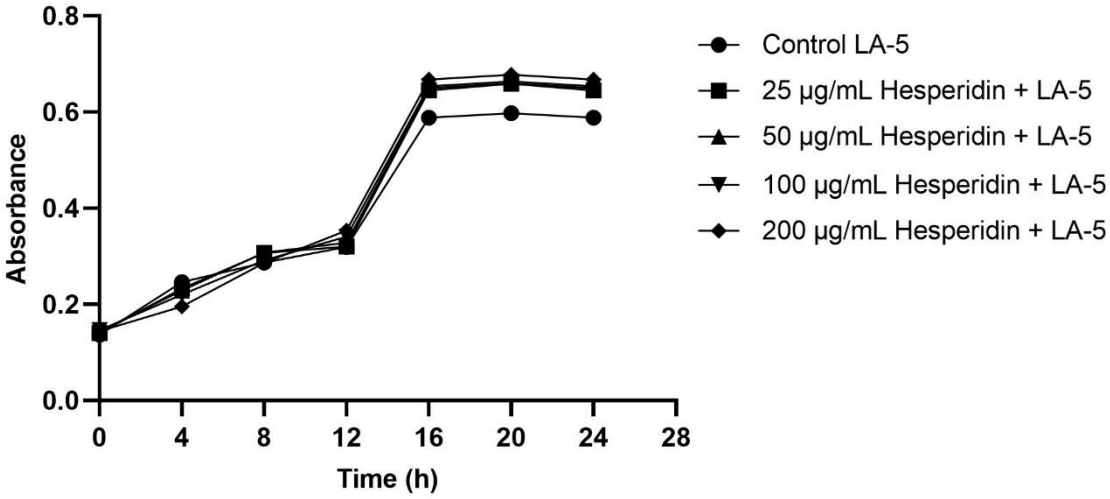

Figure 2. *Growth kinetics of Lactobacillus acidophilus LA-5 grown in the presence of hesperidin.*

Effects of Hesperidin on Pepsin and Bile Salt Resistance of Lactobacillus acidophilus LA-5

Pepsin resistance of *Lactobacillus acidophilus* LA-5 grown with hesperidin was investigated. Figure 3A shows effect of hesperidin on the pepsin tolerance of *Lactobacillus acidophilus* LA-5. Pepsin tolerance of LA-5 was around 30%. When LA-5 grown with 25, 50, 100, and 200 µg/mL hesperidin, ability of pepsin resistance was statistically ($p<0.05$) increased approximately 24%, 56%, 20%, and 14%, respectively.

Figure 3B indicates the effect of hesperidin on the

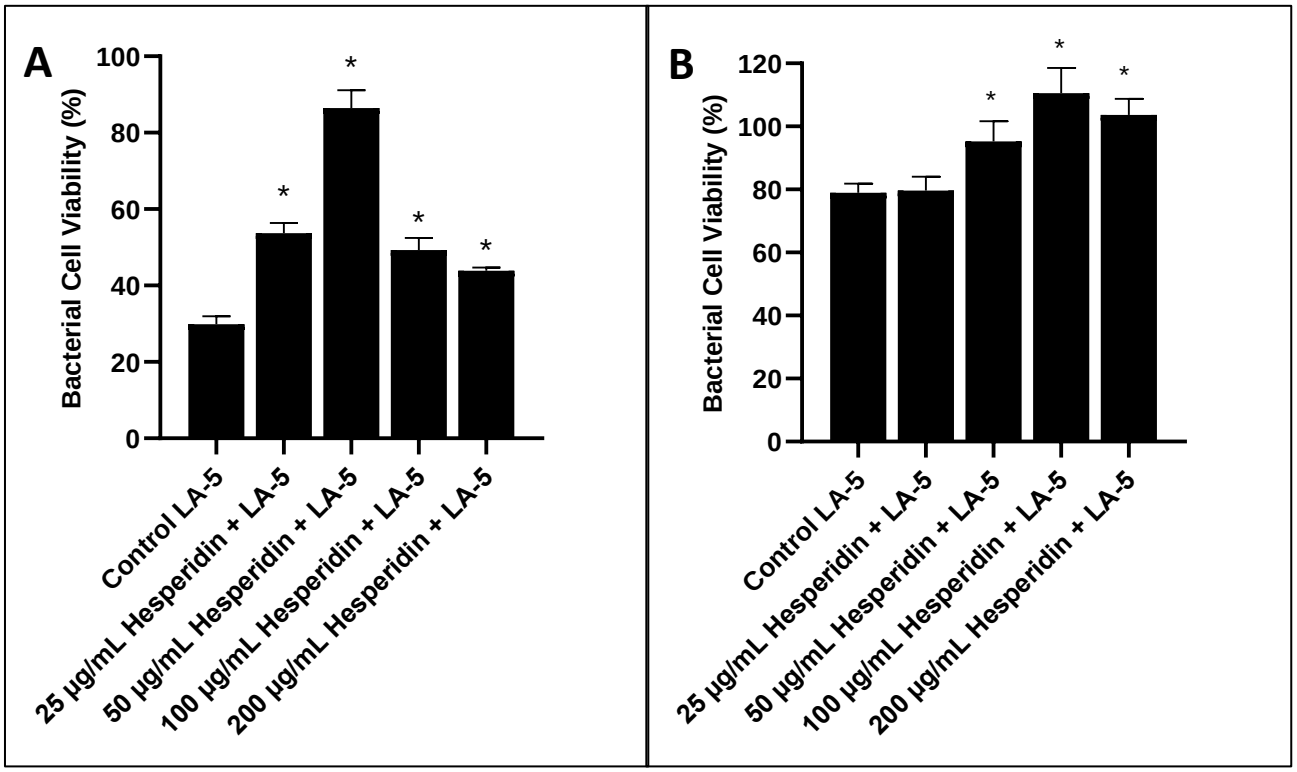

Figure 3. *(A.) Pepsin (B.) Bile Salt resistances of Lactobacillus acidophilus LA-5 grown in the presence of hesperidin. * indicates statistically significant results compared to control ($p<0.05$).*

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20241043

resistance of bile salt of *Lactobacillus acidophilus* LA-5. LA-5 and LA-5 grown with 25 µg/mL hesperidin has around 80% tolerance to bile salt while LA-5 grown with 50, 100 and 200 µg/mL hesperidin showed 95%, 110%, and 103% bile salt tolerance, respectively. 50, 100 and 200 µg/mL hesperidin increased to potential of bile salt resistance of LA-5 statistically ($p<0.05$). Thus, 50, 100 and 200 µg/mL hesperidin contributed the bile salt tolerance property of LA-5.

Effects of Hesperidin on Auto-aggregation Ability of Lactobacillus acidophilus LA-5

In this study, the effects of hesperidin on the auto-aggregation of *L. acidophilus* LA-5 bacteria were investigated. Different concentrations of hesperidin did not show any statistically significant ($p<0.05$) effect on auto-aggregation of LA-5 (Fig. 4A).

Effects of Hesperidin on Antioxidant Property of Lactobacillus acidophilus LA-5

In this study, the effect of hesperidin on antioxidant property of *Lactobacillus acidophilus* LA-5 was investigated. Figure 5 indicated that LA-5 had 58% antioxidant capacity against ABTS radical group while 25, 50, 100, and 200 µg/mL hesperidin showed 21%, 25%, 14%, and 64% antioxidant potential, respectively. However, there is no difference statistically significant ($p<0.05$) between LA-5 and LA-5 grown with 25, 50, 100, 200 µg/mL hesperidin. Hesperidin did not affect the antioxidant property of LA-5, positively.

Effects of Lactobacillus acidophilus LA-5 Grown with Hesperidin on the Human Colon Cancer Line HT-29 Cell Line

Cancer cell viability was determined by the Alamar Blue

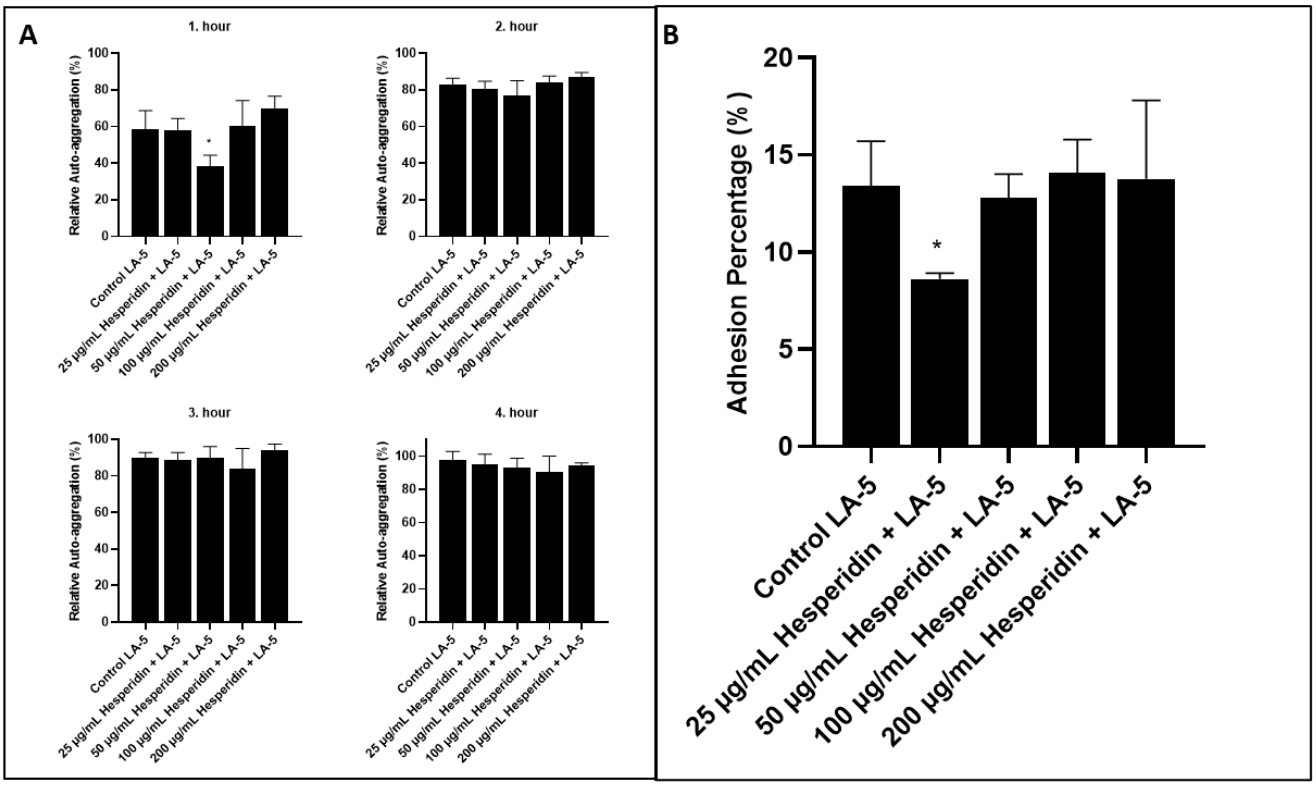

Figure 4. *(A.) Auto-aggregation and (B.) Adhesion of Lactobacillus acidophilus LA-5 grown in the presence of hesperidin. * indicates statistically significant results compared to control ($p<0.05$).*

Effects of Hesperidin on Adhesion Property of *Lactobacillus acidophilus* LA-5

Figure 4B showed the effects of hesperidin on the adhesion ability of *Lactobacillus acidophilus* LA-5. LA-5 has around 12% adhesion potential to mucin. While 25 µg/mL of hesperidin negatively affected the adhesion capability of LA-5, LA-5 grown with 50, 100, 200 µg/mL hesperidin had same percentage adhesion property of LA-5 statistically ($p<0.05$).

method. The CFS of LA-5 grown in the presence of hesperidin (100 µg/mL) was diluted with MEM-Eagle medium at 1/5, 1/10 and 1/50 ratios and given to HT-29 cell line. According to the results of the study, the 1/5 and 1/10 dilutions of the LABSEM (probiotic bacteria free) and CFS of LA-5 (hesperidin free) decreased cell viability compared to the control (no treatment) group significantly ($p<0.05$) (Fig. 6A). CFS of LA-5 grown in the presence of hesperidin decreased cell viability compared to the control in the same

diluted conditions, but no difference was found between the LABSEM, CFS of LA-5 and CFS of LA-5 grown in the presence of hesperidin.

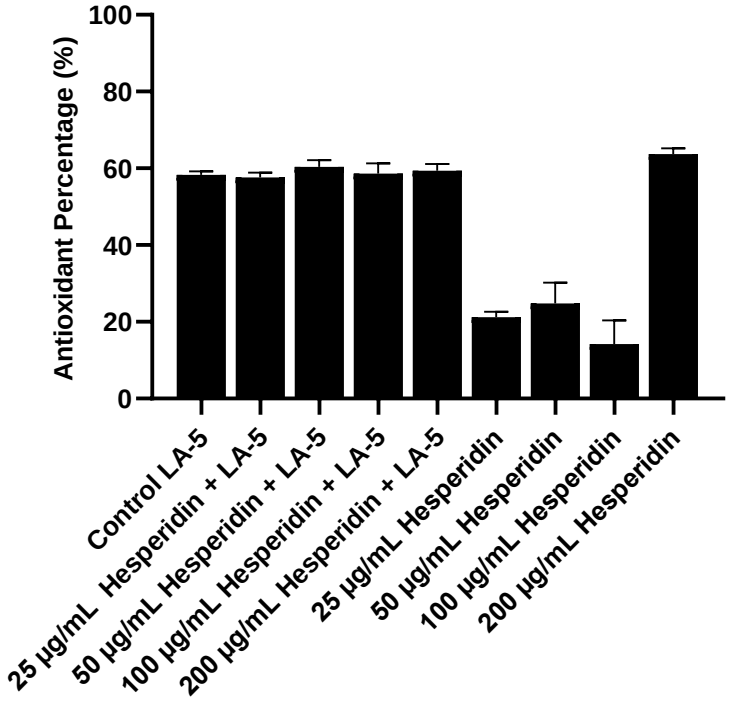

Figure 5. *Effects of Hesperidin on Antioxidant Property of Lactobacillus acidophilus LA-5. * indicates statistically significant results compared to control (p<0.05).*

Cancer invasion was studied with the Boyden Chamber Technique. According to the results, none of the CFSs had a statistically significant change on cancer invasion (Fig. 6B).

Caspase activity, which is a marker of apoptosis in cancer cells, is one of the routine studies performed as well as cell viability experiments. In this respect, the effects of CFSs on caspase activity in HT-29 cells were also investigated in this study. First, a standard curve was created for caspase activity (Supplementary Figure S1), and then the effects of CFSs on caspase activity were calculated using this curve (Table 1).

As seen in Table 1, 1/5 dilution of LA-5 grown in the presence of hesperidin statistically significantly increased the caspase activity compared to the control group (p=0.0089). Caspases are important mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease that catalyzes the specific degradation of many key cellular proteins (Porter & Jänicke, 1999).

There are two major intracellular pathways to induce apoptosis; one begins with ligation of cell surface death receptors, the other involves the release of mitochondrial cytochrome c. Some anticancer drugs kill susceptible cells, especially by inducing the expression of death receptor ligands, while others induce apoptosis by inducing cytochrome c release from mitochondria. Caspase-3 has important functions in these pathways. In this regard, the fact that anti-cancer molecules increase caspase-3 activity means that apoptosis can occur (Kaufmann & Earnshaw, 2000). CFS of LA-5 grown in the presence of hesperidin may have increased caspase-3 activity in HT-29 cells and thus induced apoptosis in this way.

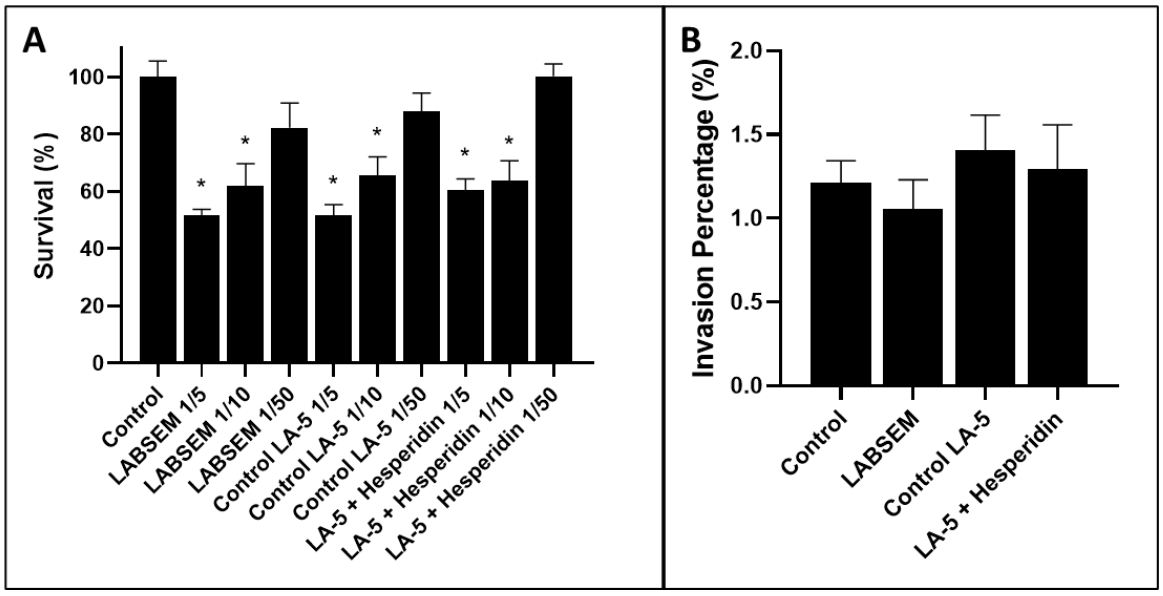

Figure 6. *Effects of L. acidophilus bacterial CFS growth in the presence of hesperidin on the (A.) viability and (B.) invasion of HT-29 cells. CFSs were diluted 1/5, 1/10 and 1/50 with cell medium. * indicates statistically significant results compared to control (p<0.05).*

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20241043

Table 1. *Average caspase enzymatic activity (mM/g protein), standard deviation, and p values for different treated groups.*

	Average Activity (mM/g prt)	Standard Deviation	p Values
Control	442.8	6.1	
Medium Control 1/5	952.2	97.8	0.1196
EtOH Control 1/5	895.5	64.1	0.0872
Hesperidin 1/5	802.0	1.0	0.0089
Medium Control 1/10	761.8	46.5	0.0273
EtOH Control 1/10	792.1	36.6	0.0601
Hesperidin 1/10	637.9	36.2	0.1089
Medium Control 1/50	429.7	63.8	0.8714
EtOH Control 1/50	363.6	12.3	0.0553
Hesperidin 1/50	430.4	2.5	0.2610

The p-values represent the statistical significance of differences between each treated group and the control group. A p-value less than 0.05 is considered statistically significant.

Discussion

In this study, we investigated the impact of hesperidin on the probiotic properties (growth kinetics, resistance to pepsin, and bile salt, auto-aggregation, adhesion, antioxidant activity,) of *L. acidophilus* LA-5, as well as the effects of their synbiotic interaction on the human colon cancer cell line HT-29.

Hesperidin is phenolic compound that has antibacterial activity against Gram-positive and Gram-negative bacteria (Kırcı et al., 2023). In the present study, all the hesperidin concentrations did not affect as inhibitor of the growth kinetics of LA-5 which is Gram-positive bacterium. Hesperidin may be selective against LA-5.

Probiotic bacteria's ability to tolerate the presence of pepsin and bile salt is crucial for their survival and colonization in the gastrointestinal tract (GIT) to exert their probiotic properties (Farid et al., 2021). Our findings revealed that all 25, 50, 100, and 200 µg/mL hesperidin contributes to the pepsin resistance of LA-5. Especially, 50 µg/mL hesperidin increased the pepsin tolerance of LA-5 than 25, 100, and 200 µg/mL concentrations of hesperidin. While 25 µg/mL hesperidin did not affect the bile salt resistance potential of LA-5, 50, 100, and 200 µg/mL hesperidin increased the 15%, 30%, 23% bile salt resistance of LA-5, respectively. This suggests that hesperidin, especially 50, 100, and 200 µg/mL of hesperidin, may contribute LA-5 to survive and colonize in harsh environmental conditions. Thus, when orally administered, the number of surviving LA-5 reaching the intestinal mucosa may be higher.

Auto-aggregation is a crucial property facilitating the formation of multicellular clusters of the same bacterial strains, an essential process for probiotics to bind to the intestinal mucosa and act as a barrier to protect epithelial cells (Trunk et al., 2018; Etzold et al., 2014; Pedersen & Tannock, 1989). The auto-aggregation of bacterial strains can be altered under different conditions such as stress, change in temperature or presence/absence of oxygen depending on the bacterial strain (Trunk et al., 2018; McLean et al., 2008). García-Cayuela et al. found that *L. acidophilus* LA-5 has 5.76% and 15.23% auto-aggregation ability at 2 and 6 h, respectively (García-Cayuela et al., 2014). Our investigation into the auto-aggregation ability of *L. acidophilus* LA-5, with and without hesperidin, revealed no statistically significant difference. This suggests that hesperidin does not negatively affect the auto-aggregation ability of LA-5, indicating its potential to bind to the intestinal mucosa and protect epithelial cells even when exposed to hesperidin.

Adhesion to the mucus layer is crucial for probiotic colonization in the intestines, contributing to reduced pathogenic colonization and better regulation of the immune system (van Tassell & Miller, 2011). Our study on the mucosal adhesion ability of LA-5, with and without hesperidin, demonstrated no significant difference in percentage adhesion ability. This implies that hesperidin does not impact the adhesion potential of LA-5, suggesting that adhesion potential may be linked to the transient colonization of intestinal cells, in line with the auto-aggregation ability of probiotic bacteria (Collado et al., 2007; Servin & Coconnier, 2003; Buck et al., 2005).

Hesperidin is phenolic compound that has strong antioxidant capacity. Choi et al. (2022) indicated that 100, 250, and 500 µM hesperidin had 10%, 20% and 40% ABTS scavenging activity, respectively (Choi et al., 2022). Also, probiotic bacteria have potential to remove reactive oxygen species (ROS) due to their strong antioxidant property. Chan et al. (2018) showed that lipophilic and hydrophilic fraction of *Lactobacillus acidophilus* had roughly 40 and 25 µmol Trolox/mL ABTS scavenging activity, respectively (Chan et al., 2018). Our study demonstrated that LA-5, both with and without hesperidin, exhibited significant (around 60%) ABTS scavenging activity. This indicates that hesperidin does not compromise the antioxidant potential of LA-5.

Furthermore, effect of hesperidin on the cytotoxic effect of LA-5 was investigated. It has been reported in previous studies that different strains of *L. acidophilus* exert cytotoxic effects on HT-29 cells (Zhang et al., 2020). Although the cytotoxic effect is dependent on the bacterial strain and the cancer cell type used, it is generally known that probiotic bacteria have a cytotoxic effect against cancer cells (Celebioglu, 2020; Celebioglu et al., 2020). According to our

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20241043

results, hesperidin and LA-5 did not have significant effects on HT-29 cancer cell line. This may be related to the bacterial strain and cancer cell line used because previous studies have demonstrated the anti-cancer properties of LA-5 in different cell lines.

In conclusion, our results indicate that hesperidin may enhance the potential of LA-5 to survive and colonize in the harsh conditions and thus, when LA-5 taken orally, it may contribute to its potential in enhancing the beneficial effects of probiotics on human health by increasing the number of LA-5 reaching the intestines. The results may support further exploration of the synbiotic interaction between phenolic compounds and probiotics, especially effects on caspase, for potential applications in promoting gastrointestinal health and preventing diseases.

Acknowledgments

The probiotic strain was obtained from Chr. Hansen, Turkey. This study was supported by Bartin University (Project No. 2019-FEN-B-007).

References

Buck BL, Altermann E., Svingerud T., & Klaenhammer TR. 2005. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Applied and Environmental Microbiology, 71(12):8344–8351. https://doi.org/10.1128/AEM.71.12.8344-8351.2005

Celebioglu HU. 2020. Probiotic bacteria grown with chestnut honey enhance *in vitro* cytotoxicity on breast and colon cancer cells. Archives of Biological Sciences, 72(3):329–338 https://doi.org/10.2298/ABS200114027C

Celebioglu HU, Delsoglio M, Brix S, Pessione E, & Svensson B. 2018. Plant Polyphenols Stimulate Adhesion to Intestinal Mucosa and Induce Proteome Changes in the Probiotic *Lactobacillus acidophilus* NCFM. Molecular Nutrition and Food Research, 62(4): https://doi.org/10.1002/mnfr.201700638

Celebioglu HU, Ejby M, Majumder A, Køber C, Goh YJ, Thorsen K, Schmidt B, O'Flaherty S, Abou Hachem M, Lahtinen SJ, Jacobsen S, Klaenhammer TR, Brix S, Mølhave K, & Svensson B. 2016. Differential proteome and cellular adhesion analyses of the probiotic bacterium *Lactobacillus acidophilus* NCFM grown on raffinose - an emerging prebiotic. Proteomics, 16(9): https://doi.org/10.1002/pmic.201500212

Celebioglu HU, Celebi B, Erden Y, Evin E, & Adali O. 2020. Cytotoxic Effects of Probiotic Bacteria *Lactobacillus acidophilus* and *Lactobacillus* rhamnosus Grown in the Presence of Oleuropein on Human Prostate Cancer Cells. Celal Bayar University Journal of Science, 16(1):55–59. https://doi.org/10.18466/cbayarfbe.633237

Celebioglu HU, Erden Y, & Ozel HB. 2021. *In vitro* cytotoxic effects of *lactobacilli* grown with lime honey on human breast and colon cancer cells. Food Bioscience, 41(March):101020. https://doi.org/10.1016/j.fbio.2021.101020

Chan CL, Gan R-you, Shah NP, & Corke H. 2018. Enhancing antioxidant capacity of *Lactobacillus acidophilus*-fermented milk fortified with pomegranate peel extracts. Food Bioscience, 26(May):185–192. https://doi.org/10.1016/j.fbio.2018.10.016

Choi SS, Lee SH, & Lee KA. 2022. A Comparative Study of Hesperetin, Hesperidin and Hesperidin Glucoside: Antioxidant, Anti-Inflammatory, and Antibacterial Activities. In Vitro Antioxidants, 11(8): https://doi.org/10.3390/antiox11081618

Clifford MN. 2004. Diet-derived phenols in plasma and tissues and their implications for health. Planta Medica, 70(12):1103–1114. https://doi.org/10.1055/s-2004-835835

Collado MC, Meriluoto J, & Salminen S. 2007. Adhesion and aggregation properties of probiotic and pathogen strains. European Food Research and Technology, 226(5):1065–1073. https://doi.org/10.1007/s00217-007-0632-x

Dinh MN, Hitomi M, Al-Turaihi ZA, & Scott JG. 2023. Alamar Blue assay optimization to minimize drug interference and inter-assay viability. BioRxiv : The Preprint Server for Biology. https://doi.org/10.1101/2023.03.16.532999

Duda-Chodak A, Tarko T, & Statek M. 2008. The Effect of Antioxidants on *Lactobacillus casei* Cultures. Acta SciPol. Technol Aliment, 7(4):39–51.

Etzold S, Kober OI, Mackenzie DA, Tailford LE, Gunning AP, Walshaw J, Hemmings AM, & Juge N. 2014. Structural basis for adaptation of lactobacilli to gastrointestinal mucus. Environmental Microbiology, 16(3):888–903. https://doi.org/10.1111/1462-2920.12377

Farid W, Masud T, Sohail A, Ahmad N, Naqvi SMS, Khan S, Ali A, Khalifa SA, Hussain A, Ali S, Saghir M, Siddeeg A, & Manzoor MF. 2021. Gastrointestinal transit tolerance, cell surface hydrophobicity, and functional attributes of *Lactobacillus acidophilus* strains isolated from Indigenous Dahi. Food Science and Nutrition, 9(9):5092–5102. https://doi.org/10.1002/fsn3.2468

García-Cayuela T, Korany AM, Bustos I, Gómez de Cadiñanos Luz P, Requena T, Peláez C, & Martínez-Cuesta MC. 2014. Adhesion abilities of dairy *Lactobacillus plantarum* strains showing an aggregation phenotype. Food Research International, 57:44–50. https://doi.org/10.1016/j.foodres.2014.01.010

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, & Sanders ME. 2014. Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology, 11(8):506–514. https://doi.org/10.1038/nrgastro.2014.66

Kaufmann SH, & Earnshaw WC. 2000. Induction of apoptosis by cancer chemotherapy. Experimental Cell Research, 256(1):42–49. https://doi.org/10.1006/excr.2000.4838

Kırcı D, Demirci F, & Demirci B. 2023. Microbial Transformation of Hesperidin and Biological Evaluation. ACS Omega, 8(45):42610–42621. https://doi.org/10.1021/acsomega.3c05334

Kos B, Šušković J, Vuković S, Sĭmpraga M, Frece J, & Matošić S. 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. Journal of Applied Microbiology, 94(6):981–987. https://doi.org/10.1046/j.1365-2672.2003.01915.x

Leccese Terraf MC, Mendoza LM, Juárez Tomás MS, Silva C, & Nader-Macías MEF. 2014. Phenotypic surface properties (aggregation, adhesion and biofilm formation) and presence of related genes in beneficial vaginal lactobacilli. Journal of Applied Microbiology, 117(6):1761–1772. https://doi.org/10.1111/jam.12642

Man MQ, Yang B, & Elias PM. 2019. Benefits of Hesperidin for Cutaneous Functions. Evidence-Based Complementary and Alternative Medicine, 2019. https://doi.org/10.1155/2019/2676307

Marín L, Miguélez EM, Villar CJ, & Lombó, F. 2015. Bioavailability of dietary polyphenols and gut microbiota

metabolism: Antimicrobial properties. BioMed Research International, 2015. https://doi.org/10.1155/2015/905215

McLean JS, Pinchuk GE, Geydebrekht OV, Bilskis CL, Zakrajsek BA, Hill EA, Saffarini DA, Romine MF, Gorby YA, Fredrickson JK, & Beliaev AS. 2008. Oxygen-dependent autoaggregation in *Shewanella oneidensis* MR-1. Environmental Microbiology, 10(7):1861–1876. https://doi.org/10.1111/j.1462-2920.2008.01608.x

Muyyarikkandy MS, & Amalaradjou MA. 2017. *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus* and *Lactobacillus paracasei* attenuate *Salmonella enteritidis*, *Salmonella heidelberg* and *Salmonella typhimurium* colonization and virulence gene expression *in vitro*. International Journal of Molecular Sciences, 18(11): https://doi.org/10.3390/ijms18112381

Onur E, Gökmen GG, Nalbantsoy A, & Kışla D. 2022. Investigation of the supportive therapy potential of propolis extract and *Lactobacillus acidophilus* LA-5 milk combination against breast cancer in mice. Cytokine, 149(September 2021): https://doi.org/10.1016/j.cyto.2021.155743

Pandey KB, & Rizvi SI. 2009. Plant polyphenols as dietary antioxidants in human. Oxidative Medicine and Cellular Longevity, 2(5):270–278.

Pedersen K, & Tannock GW. 1989. Colonization of the porcine gastrointestinal tract by lactobacilli. Applied and Environmental Microbiology, 55(2):279–283. https://doi.org/10.1128/aem.55.2.279-283.1989

Porter AG, & Jänicke RU. 1999. Emerging roles of caspase-3 in apoptosis. Cell Death and Differentiation, 6(2):99–104. https://doi.org/10.1038/sj.cdd.4400476

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, & Rice-Evans C. 1998. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. EMPA Activities, 26(1998):

Servin AL, & Coconnier MH. 2003. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Bailliere's Best Practice and Research in Clinical Gastroenterology, 17(5), 741–754. https://doi.org/10.1016/S1521-6918(03)00052-0

Śliżewska K, Markowiak-Kopeć P, & Śliżewska W. 2021. The role of probiotics in cancer prevention. Cancers, 13(1):1–22. https://doi.org/10.3390/cancers13010020

Trunk T, S Khalil H, & C Leo J. 2018. Bacterial autoaggregation. AIMS Microbiology, 4(1):140–164. https://doi.org/10.3934/microbiol.2018.1.140

van Tassell ML, & Miller MJ. 2011. *Lactobacillus* adhesion to mucus. Nutrients, 3(5):613–636. https://doi.org/10.3390/nu3050613

Wilmsen PK, Spada DS, & Salvador M. 2005. Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. Journal of Agricultural and Food Chemistry, 53(12):4757–4761. https://doi.org/10.1021/jf0502000

Xiong H, Wang J, Ran Q, Lou G, Peng C, Gan Q, Hu J, Sun J, Yao R, & Huang Q. 2019. Hesperidin: A therapeutic agent for obesity. Drug Design, Development and Therapy, 13:3855–3866. https://doi.org/10.2147/DDDT.S227499

Zhang T, Pan D, Yang Y, Jiang X, Zhang J, Zeng X, Wu Z, Sun Y, & Guo Y. 2020. Effect of *Lactobacillus acidophilus* CICC 6074 S-Layer Protein on Colon Cancer HT-29 Cell Proliferation and Apoptosis. Journal of Agricultural and Food Chemistry, 68(9):2639–2647. https://doi.org/10.1021/acs.jafc.9b06909