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Probiotic bacteria grown with chestnut honey enhance *in vitro* cytotoxicity on breast and colon cancer cells

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Abstract: Chestnut honey has been used as ethnomedicine. Probiotics are defined as live microorganisms that can provide a health benefit, impeding the development of several health conditions and diseases, including cancer. This study aims to investigate the effects of chestnut honey on probiotic bacteria and the *in vitro* cytotoxic effects of the combination of probiotics and chestnut honey on cancer cells. First, the effects of chestnut honey on the growth of bacteria were examined, followed by its effects on the probiotic properties of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG. Once the bacteria had grown on chestnut honey, the *in vitro* cytotoxic effects on breast and colon cancer cell lines, MCF-7 and Caco-2, respectively, and a non-cancerous breast epithelial cell line, MCF-10A, were investigated. Chestnut honey positively affected the probiotic bacteria by increasing the growth and modulating probiotic properties such as autoaggregation and surface hydrophobicity. Furthermore, probiotics grown on chestnut honey had more cytotoxic effects on the cancer cell lines than probiotics or honey alone. The present study showed that new combinations of honey and probiotics have the potential to formulate new nutraceuticals.

Keywords: auto-aggregation; chestnut honey; cytotoxicity; *Lactobacillus*; MCF-7 cell line; Caco-2 cell line; surface hydrophobicity

INTRODUCTION

From ancient times, bee products, especially honey, have been one of the main dietary components for humans. As synthetic drugs and chemicals have adverse effects on human health, natural products containing bioactive compounds, one of which is honey, have gained paramount importance [1]. Apitherapy is known as protection and strengthening of the immune system, prevention or treatment of illnesses and promotion of healing processes through therapeutic techniques by using bee products such as honey, pollen, propolis, royal jelly, bee venom, wax and apilarnil [2].

Honey contains mainly carbohydrates (95-98% of the dry weight) and a very small portion is secondary metabolites (2-4%) [1]. The major carbohydrates in honey include glucose and fructose, but it also contains small amounts of oligosaccharides [3]. However, the main potential of the therapeutic effects of honey lies in their bioactive compounds, especially phenolic compounds such as phenolic acids, flavonoids, procyanidins and anthocyanins [4-6]. The total phenolic contents of honey vary according to floral source, geographical position and climatic conditions, from 16 mg gallic acid equivalents per 100 g (mg GAE/100 g) for acacia honey to 120 mg GAE/100 g for oak honey [1]. Turkey is one of the richest regions in terms of honey varieties and production, thanks to its geography, climate, seasonal production and pollen varieties that are very suitable for honey [1].

Castanea sativa Mill., also known as sweet chestnut, is a multipurpose species intensively cultivated as monoculture in the Mediterranean and Central Europe [7]. Chestnut forests are widely spread and they are an important landscape ecosystem on the coast of the Black Sea of Turkey, and also in France, Italy, Greece and Spain [7]. Thus, chestnut-based honeys are one of the main bee products in this region [8]. Chestnut honey has been used in ethnomedicine, especially for respiratory diseases like asthma, as well as for cancer

[4]. Generally, it is unifloral honey, meaning the source is from one plant species only, and is darker than other types of honey [8].

Probiotics are defined by the FAO/WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [9]. To be classified as a probiotic, microorganisms should survive in the gastrointestinal tract (GIT) [10]. The main genera identified as probiotics are lactobacilli and bifidobacteria. Lactobacilli, which are best characterized as probiotics, are *Lactobacillus acidophilus* and *L. rhamnosus*. They are available as a dietary supplement and are added to a variety of foods, such as dairy products, and can survive in acidic conditions and tolerate bile in the human GIT. Probiotics have numerous potential applications and uses in the digestive system as well as in other health issues [11]. They are known to produce antimicrobial agents such as hydrogen peroxide (H_2O_2), diacetyl and bacteriocin as well as organic acids, which inhibit pathogenic organisms [12]. Recent studies have shown that probiotic bacteria have the potential to prevent several adverse health conditions and diseases, including lactose intolerance, antibiotic- and age-related diarrhea, as well as cancer [12-14].

The cancer prevention ability of lactobacilli has been gaining increasing attention, due to the difficulty of successful treatment of most cancers [15]. The most frequent malignancy in women worldwide is breast cancer, whose heterogeneous nature makes it a global problem [16]. Lactobacilli have been determined to exhibit cytotoxic effects on several cancer types [17]. However, because probiotics combined with other dietary constituents, called synbiotics, can exert more health benefits [18], the present study aimed at investigating the effects of chestnut honey on the probiotic bacteria *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG, and at evaluating the *in vitro* cytotoxic effects of the combination of probiotics and chestnut honey on breast and colon cancer cell lines, MCF-7 and Caco-2, respectively.

MATERIALS AND METHODS

Collection of chestnut honey

Chestnut honey was collected in Bartın Province, Kumluca Town, Kirsin Village, western Black Sea region of Turkey, with an altitude of 625-730 m. In this region, the dominant pollen is 85% *Castanea sativa* Mill. (Sweet chestnut). The samples were stored at room temperature. The honey was dissolved as 10% (w/v), either in ultra-pure water or as a 1:1 (v/v) ethanol:water solution.

Effects of chestnut honey on growth of bacteria

The broth microdilution assay was used to investigate the antimicrobial activities of the chestnut honey [19]. *Escherichia coli* (Gram negative) and *Staphylococcus aureus* (Gram positive) bacterial cultures from frozen stocks were inoculated into nutrient broth (NB), while *Lactobacillus acidophilus* LA-5 and *L. rhamnosus* GG were inoculated into Man, Rogosa and Sharpe (MRS) medium broth for 24 h at 37°C. Subculturing was performed and new cultures were incubated at 37°C until 0.5 McFarland Unit was reached. Twenty µL of bacterial cultures were added in microtiter plate wells and the volumes were brought to 200 µL with medium containing different concentrations of the honey ranging from 0-10 mg/mL. Negative controls were prepared using medium without bacteria. Absorbances of microtiter plates at 600 nm were read using an ELISA reader before (0^{th} h) and after (24^{th} h) incubation at 37°C.

Growth of probiotics in the presence of chestnut honey

L. acidophilus LA-5 and *L. rhamnosus* GG, which were kind gifts of Chr. Hansen, Turkey, were grown in MRS medium without shaking (37°C) [20]. The bacteria were divided into groups, and chestnut honey was used as a carbon source (1% w/v). Glucose was used as control carbon source (MRS only).

Effects of chestnut honey on probiotic properties

Determination of auto-aggregation

Bacterial cells were collected in the late logarithmic phase (3200 *x* g, 15 min), washed with phosphate-buffered saline (PBS) and resuspended in PBS to OD_{600} 0.5 [21]. Autoaggregation was determined by adding 4 mL of the bacterial suspension to the test tubes after vortexing for 10 s (1 h, RT). After incubation, 100 µL from the upper portion of the suspension was taken, added to the

tube containing 900 µL of PBS, and the absorbance was measured at 600 nm. The percentage of autoaggregation was calculated using the following formula:

$$\% \text{ Autoaggregation} = (1\text{-At/A0}) \times 100,$$

where At is the absorbance measured after incubation and A0 is the absorbance measured before incubation [22].

Determination of bacterial surface hydrophobicity

Surface hydrophobicity was measured by the method of microbial adhesion to solvents (MATS) [22]. Once the bacteria were harvested in the late logarithmic phase, they were washed with PBS and suspended in 0.1 M KNO_3 (pH 6.2) to OD_{600} of 0.5. One mL of xylene (nonpolar solvent) was added to 3 mL of the bacterial suspension, followed by incubation at room temperature for 10 min. The two-phase system was vortexed for 2 min, the aqueous phase was separated and incubated for another 20 min at room temperature. Absorbance was measured at 600 nm and the bacterial adhesion to the solvent was calculated using the formula:

$$\% \text{ Adhesion} = (1\text{-A1/A0}) \times 100,$$

where A1 is the absorbance measured after the incubation and A0 is the absorbance measured before the incubation [22].

Preparation of cell-free supernatants of probiotic bacteria

After growth of probiotic bacteria, the cultures were centrifuged (3200 *x* g, 15 min) and the supernatant was taken and filtered using 0.22-µm pore-sized syringe-filters. The cell-free supernatants were stored at -80°C until use.

In vitro cytotoxicity assay of probiotics and chestnut honey for MCF-7, MCF-10A and Caco-2 cells

Cell viabilities of MCF-7 and Caco-2 cells, and thus the cytotoxic effects of probiotics grown on chestnut honey, were determined by the thiazolyl blue tetrazolium bromide (MTT) assay [23]. The human breast cancer cell line MCF-7 (ATCC, USA) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.01 mg/mL human recombinant insulin, 1% penicillin-streptomycin solution and 1% non-essential amino acid solution. The medium of the cells was changed twice a week, and the cells were incubated at 37°C with 5% CO_2 at all steps. The non-cancerous human breast epithelial cell line MCF-10A (ATCC, USA) was grown in DMEM/F12 medium supplemented with 10% FBS, 20 ng/mL epithelial growth factor (EGF), 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL human recombinant insulin and 1% penicillin-streptomycin solution.

Cell-free supernatants were diluted with respective medium as 1/2, 1/5 or 1/10 dilutions. One percent (w/v) chestnut honey in MRS medium (without growing bacteria) was used to determine the effects of only the honey on the cells. Cell-free supernatants of probiotic bacteria grown in MRS with glucose as carbon source were used as the probiotic control (LA-5 Ctrl or GG Ctrl). Then, cancer cells were seeded in 96-well plates at a density of $15x10^3$ cells per well, and were treated with cell-free supernatant of bacteria for 24 h. Following the treatments, the cells were incubated in 0.5 mg/mL MTT solution. The optical densities of the cells in the plates were read in a microplate reader at a wavelength of 570 nm [23]. Cell viability was calculated as the percentage of absorbance measured for treated groups to the absorbance of the control group.

Statistical analysis

Statistical analyses were performed using the 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 the mean with standard deviation (mean±SD) and $p<0.05$ was considered significant.

RESULTS

Effects of chestnut honey on bacterial growth

Different concentrations (100-10000 µg/mL) of chestnut honey dissolved in either ultra-pure water or as an ethanol:water mix (1:1) were used to investigate its effects on the growth of different bacteria, including

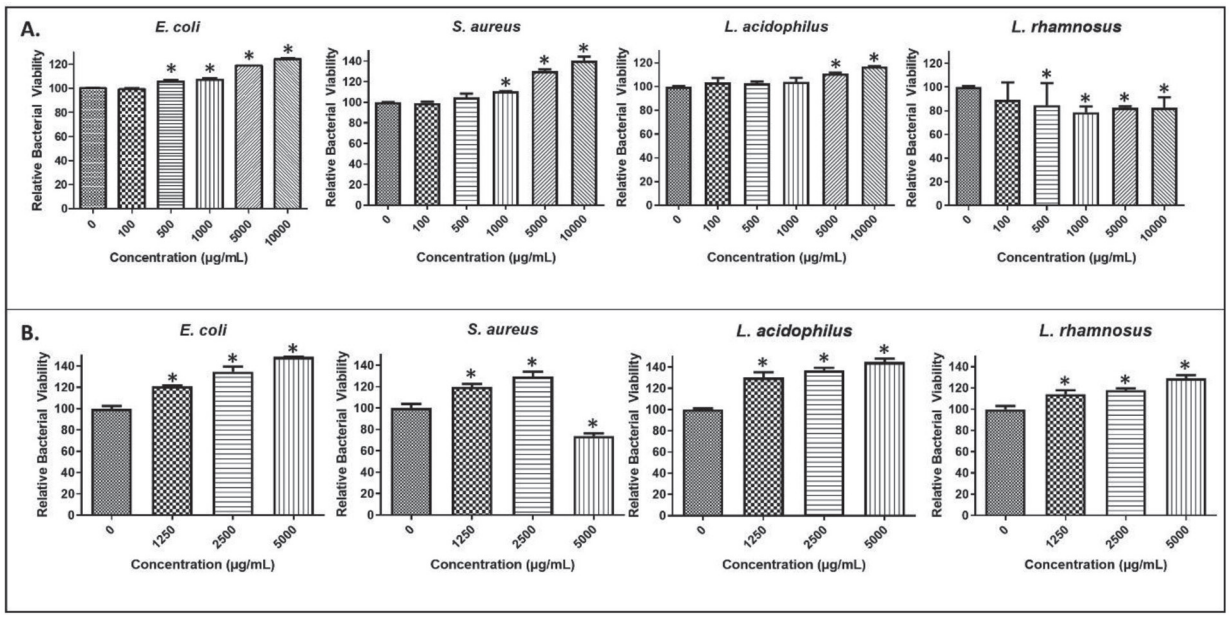

Fig. 1. The effect of chestnut honey on bacterial growth. **A** – Effect of chestnut honey dissolved in water on growth of *E. coli*, *S. aureus*, *L. acidophilus* and *L. rhamnosus.* **B** – Effect of chestnut honey dissolved in 1:1 (v/v) ethanol:water on growth of *E. coli*, *S. aureus*, *L. acidophilus* and *L. rhamnosus.* An asterisk (*) indicates that the difference is statistically significant ($p<0.05$) as compared to the control group according to one-way ANOVA.

Escherichia coli, Staphylococcus aureus, Lactobacillus acidophilus, and *L. rhamnosus.*

Fig. 1A shows the effects of different concentrations of chestnut honey dissolved in water on the examined bacteria. Except for *L. rhamnosus,* the honey significantly increased the growth of bacteria ($p<0.05$) in a dose-dependent manner. For *E. coli,* the bacterial growth at 10000 µg/mL was 24% higher than in the control group, while it was increased by 40% for *S. aureus.* On the other hand, 17% increased growth was observed in *L. acidophilus* LA-5, when 10000 µg/mL honey was used than in the control in which no honey was present. The only decreased growth was observed for *L. rhamnosus,* which exhibited a 17% decrease at 10000 µg/mL.

As there could be some ingredients in the honey that were not water soluble, honey was prepared as an ethanol:water (1:1 v/v) solution. Similar results were obtained as for honey dissolved in water alone, except for *S. aureus* and *L. rhamnosus* (Fig. 1B). Thus, for *S. aureus*, at 5000 µg/mL honey growth was significantly decreased. For *L. rhamnosus,* the ethanol:water solution of honey increased bacterial growth, while the water-dissolved honey decreased it.

Effects of chestnut honey on the probiotic properties of lactobacilli

Chestnut honey was used as 1% (w/v) solution in the growth cultures of probiotic bacteria *L. acidophilus* LA-5 and *L. rhamnosus* GG, and its effects on the probiotic properties of the bacteria were examined. The control group contained 1% glucose as the carbon source instead of chestnut honey.

Autoaggregation is considered an important property of bacteria for residing and adhering to the GIT [22]; thus, in the present study, the autoaggregation abilities of probiotic bacteria when grown on chestnut honey were evaluated for 5 h. Fig. 2A shows that chestnut honey significantly ($p<0.05$) increased the autoaggregation of *L. acidophilus* LA-5 almost 5-fold after 1 h incubation. As the incubation time increased, the aggregation abilities of the two bacterial groups were similar; however, the differences were not significant. For *L. rhamnosus* GG, chestnut honey also increased bacterial autoaggregation in the period of 1 to 3 h; it was about 2-fold higher at the 1st h, about 3-fold higher at the 2nd h, and 10-fold higher at the 3rd h as compared to the control (Fig. 2B).

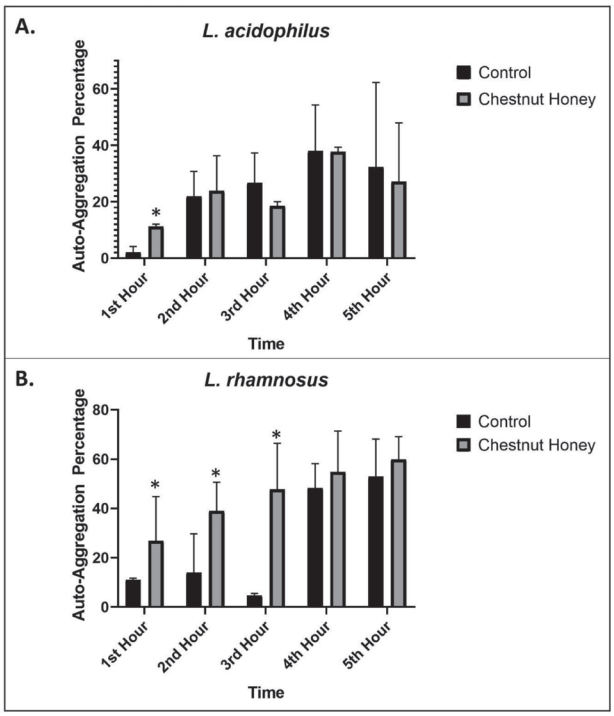

Fig. 2. The effect of chestnut honey on the auto-aggregation of probiotics. **A** – *L. acidophilus* LA-5; **B** – *L. rhamnosus* GG. An asterisk (*) indicates that the difference is statistically significant ($p<0.05$) as compared to the control group according to one-way ANOVA.

Surface hydrophobicity of probiotics was also evaluated as it is another important property. Chestnut honey significantly increased the surface hydrophobicity of *L. acidophilus* LA-5, which was about 50% higher than the control (Fig. 3A). However, it did not show any effects on the surface hydrophobicity of *L. rhamnosus* GG (Fig. 3B).

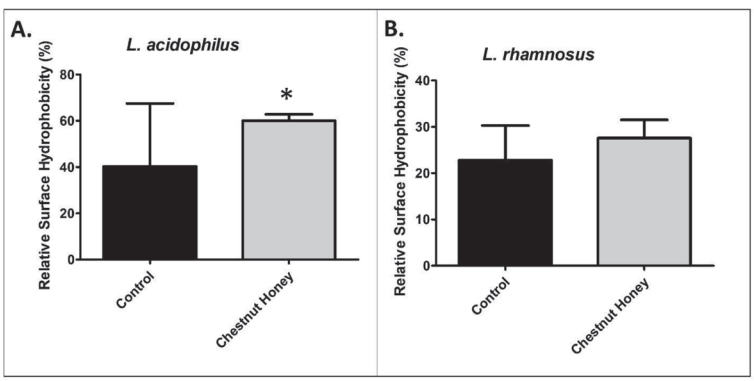

Fig. 3. The effects of chestnut honey on the surface hydrophobicity of probiotics. **A** – *L. acidophilus* LA-5; **B** – *L. rhamnosus* GG. An asterisk (*) indicates that the difference is statistically significant ($p<0.05$) as compared to the control group according to one-way ANOVA.

In vitro cytotoxic effects of probiotic bacteria grown with chestnut honey

The *in vitro* cytotoxic effects of probiotic bacteria *L. acidophilus* LA-5 and *L. rhamnosus* GG grown with chestnut honey on human breast cancer cells (MCF-7) and colon cancer cells (Caco-2) were evaluated using the MTT Assay. The non-cancerous breast epithelial cell line MCF-10A was used to assess whether these bacteria grown with chestnut honey also affected the viability of healthy cells.

After treatment of MCF7, MCF-10A and Caco-2 cells with different dilutions (1/2, 1/5, and 1/10) of the cell-free supernatant of *L. acidophilus* LA-5 for 24 h, the cell viability changes were estimated (Figs. 4 and 5, respectively). Even though the honey alone did not alter the cell viability of MCF-7 at dilutions of 1/10 and 1/5, the cell-free supernatants of *L. acidophilus* LA-5, grown either on glucose (LA-5 Ctrl) or on chestnut honey, significantly reduced the cell viabilities by about 20-30% (Fig. 4A). However, using chestnut honey for growth did not significantly change the cell viability as compared to the glucose group. Moreover, when using 1/2-diluted supernatants, chestnut honey and *L. acidophilus* LA-5 additionally reduced cell viability, which was 2-fold lower than for the LA-5 control. In contrast, in the healthy cell line (MCF-10A), neither chestnut honey alone nor the supernatant of *L. acidophilus* LA-5 affected cell viability at 1/10 and 1/5 dilutions (Fig. 4B), however, the dilution of 1/2 reduced cell viability by about 20%. While the cell-free supernatants of *L. acidophilus* LA-5 either grown on glucose (control) or on chestnut honey reduced the viability of colon cancer cells (Caco-2), there was no difference between these groups (Fig. 4C).

Fig. 5 shows the changes in cell viability of MCF-7, MCF-10A, and Caco-2 cells after treatment with the cell-free supernatant of *L. rhamnosus* GG. When the supernatants were diluted 10-fold with cell culture medium (1/10), only *L. rhamnosus* GG grown with chestnut honey reduced the viability of cancer cells by 10% (Fig. 5A), but when diluted 5-fold, chestnut honey, used as a carbon source, caused more reduction in cell viability than *L. acidophilus* GG grown

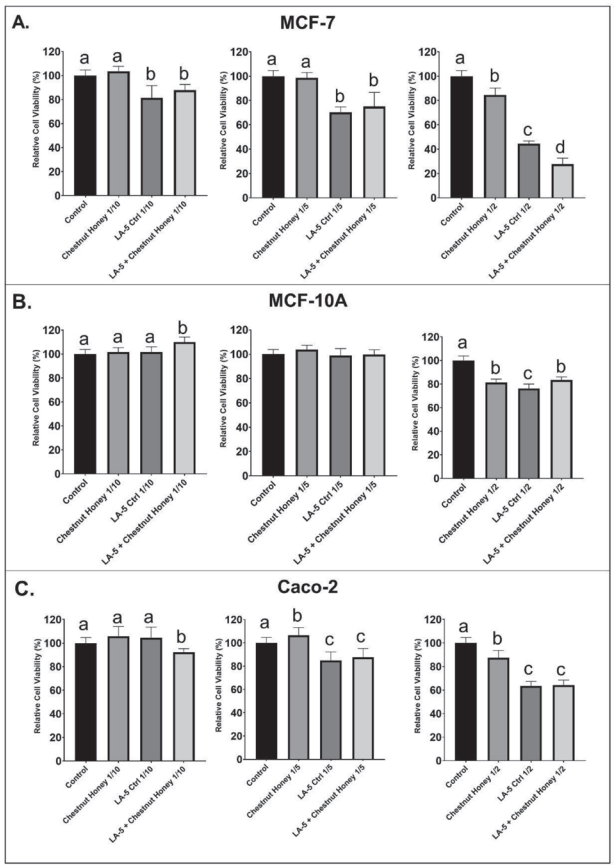

Fig. 4. The *in vitro* cytotoxic effects of *L. acidophilus* LA-5. **A** – Cell-viabilities of breast cancer cells MCF-7 treated with different dilutions of cell-free extracts of *L. acidophilus* LA-5. **B** – Cell-viabilities of breast non-cancerous cells MCF-10A treated with different dilutions of cell-free extracts. **C** – Cell-viabilities of colon cancer cells Caco-2 treated with different dilutions of cell-free extracts. Different lowercase letters indicate that the difference is statistically significant (p<0.05) according to one-way ANOVA.

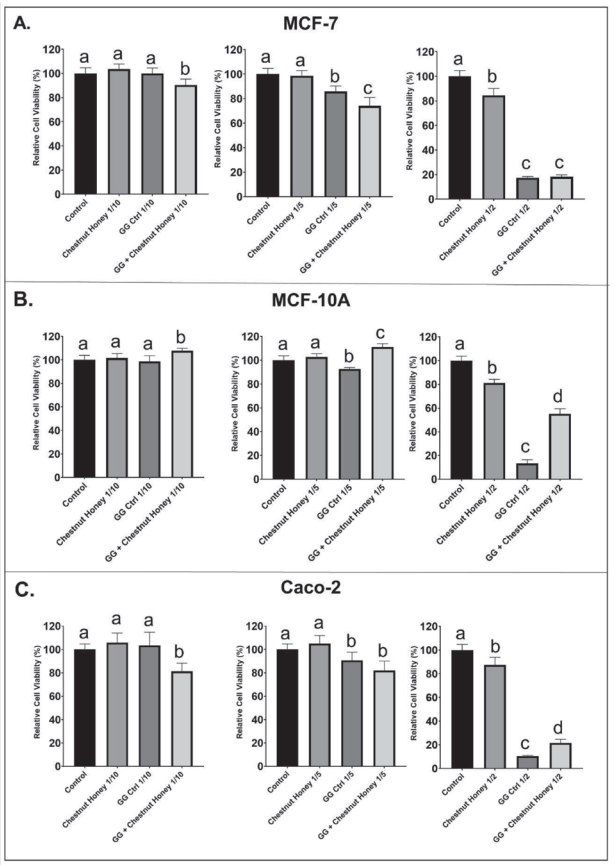

Fig. 5. The *in vitro* cytotoxic effects of *L. rhamnosus* GG. **A** – Cell-viabilities of breast cancer cells MCF-7 treated with different dilutions of cell-free extracts of *L. rhamnosus* GG. **B** – Cell-viabilities of breast non-cancerous cells MCF-10A treated with different dilutions of cell-free extracts. **C** – Cell-viabilities of colon cancer cells Caco-2 treated with different dilutions of cell-free extracts. Different lowercase letters indicate that the difference is statistically significant (p<0.05) according to one-way ANOVA.

on glucose (GG Ctrl). Using cell-free supernatants diluted 2-fold reduced the cell viability of cancer cells by more than 80%. Examination of healthy cells (Fig. 5B) showed that the cell-free supernatant of *L. acidophilus* GG grown on glucose (which served as the control) did not change cell viability at the dilution of 1/10, but reduced it at dilutions of 1/5 and 1/2 by 10% and 85%, respectively. However, using chestnut honey as the carbon source for *L. rhamnosus* GG significantly (p<0.05) increased the viability of healthy cells, as compared to when glucose was the carbon source. The cytotoxicity on Caco-2 cells was slightly increased when *L. rhamnosus* GG was grown on chestnut honey at the dilution of 1/10; however, at dilutions of 1/5 and 1/2, the addition of chestnut honey did not increase the cytotoxicity as compared to *L. rhamnosus* GG grown on glucose (Fig. 5C).

DISCUSSION

Honey has been used as a traditional medicine for centuries, and its positive roles in the treatment of infected wounds, gastrointestinal disorders, burns, asthma, and skin ulcers have been recently studied [4,24]. Chestnut honey is one of the commonly-produced honey types in the Western Black Sea area, and it is characterized by its dark color. Turkish chestnut honey contains 98.26 mg GAE/100 g on average, which is one of the

highest total phenolic contents among honeys. Thus, it has a very high antioxidant capacity, with around 20 mg/mL IC_{50} for DPPH scavenging activity [1,8]. A recent study has determined the phenolic compounds in chestnut honey of Bartin province, which is the same honey used in the present study [25]. It contains gallic acid (0.482 mg/kg), protocatechuic acid (0.034 mg/kg), p-OH benzoic acid (0,433 mg/kg), vanillic acid (1.057 mg/kg), syringic acid (0.541 mg/kg), ferulic acid (0.059 mg/kg) and *t*-cinnamic acid (0.376 mg/kg) [25]. Its protein content is about 0.17 g/100 g, even though most is formed from carbohydrates, predominantly fructose and glucose [4]. Previous studies showed that chestnut honey possesses many *in vivo* and *in vitro* biological activities [26-28]. An *in vivo* study showed that a formulation with chestnut honey protected rats against indomethacin-induced gastric lesions by reducing microvascular permeability, the ulcer index, and myeloperoxidase activity of the stomach [26]. Furthermore, it had *in vitro* antiinflammatory effects, which were observed as the dose-dependent inhibition of the production of lipopolysaccharide-induced NO, as well as *in vitro* antimutagenic activities [27]. Chestnut bee pollen can protect hepatocytes from oxidative stress and promote the healing of liver damage in rats [28].

Even though its antibacterial activity against very common pathogenic bacteria was found to be the highest among other honeys, the activity was moderate and it did not show any inhibition zone against *E. coli* [4]. This finding is also confirmed in the present study, as chestnut honey showed no inhibition against bacteria, and even increased bacterial growth. However, when dissolved in ethanol:water (1:1 v/v), it reduced the growth of *S. aureus,* as shown previously [4,29].

The intestines are covered by a mucus layer, which provides the first contact for microorganisms in the intestine, and is an important site for bacterial adhesion and colonization [30]. Bacterial adhesion is a crucial criterion for probiotics to colonize and reside in the GIT as it can reduce pathogenic colonization, regulate the immune system, and provide a healthy microbiota balance [31], being mediated by different forces on the bacterial surface [32]. Different combinations of proteins and carbohydrates present on the bacterial surface, as well as the physical properties of the surface, are generally responsible for the adhesion activities of probiotic bacteria, which can be different among species [33]. Autoaggregation is one of these forces, allowing probiotics to colonize predominantly the GIT when the bacteria have high aggregation properties [34]. In the present study, chestnut honey increased probiotic autoaggregation, thus exhibiting a potential to reside longer in the GIT and to exert the probiotic activities on the host [29,35].

The hydrophobic characteristic of the bacterial cell surface is another factor that affects bacterial adhesion, and it plays an important role in the contact between a bacterial cell and mucus or epithelial cell [36,37]. Higher surface hydrophobicity of bacterial cells could mean higher hydrophobic interactions between cell surface and mucus components, leading to stronger adhesion [38]. In the present study, chestnut honey increased the surface hydrophobicity of *L. acidophilus* LA-5, but not that of *L. rhamnosus* GG, and could positively affect bacterial adhesion.

In addition to the surface properties of bacteria, the surface proteins that many lactobacilli possess, specifically the surface layer (S-layer) proteins that bind to the cell wall in a non-covalent manner, play significant roles in adhesion [39]. Even though the biological functions of the S-layer include determination of cell shape, protection, and molecular and ion capture to surfaces, it is thought to be responsible for the attachment of a bacterial cell to the mucus layer [35,40-42]. Thus, not only hydrophobicity and aggregation play a role in good adhesion ability, but the S-layer proteins also play a key role [43,44].

Breast cancer is the most common cancer type and the leading cause of cancer-related death worldwide in women [45]. Probiotics have been known to play roles in the prevention of several cancer types or in reducing the risk of cancer, due to the secreted compounds, metabolites and/or proteins [12]. Thus, in the present study, cell-free supernatants containing secreted contents, referred to as the "secretome" of the probiotics, were used to evaluate the cytotoxic activities of *L. acidophilus* LA-5 and *L. rhamnosus* GG grown with chestnut honey on the breast cancer cell line MCF-7, i.e. to see whether this combination of probiotic and honey exerted more positive effects than probiotics grown on glucose or honey alone. The results showed that when the probiotics are grown in the presence of chestnut honey as a carbon source, the

cell viability of breast cancer cells was more reduced more as compared to probiotics grown on glucose or chestnut honey alone. This implies that the addition of chestnut honey to the bacterial growth medium had an additional effect on cytotoxicity. On the other hand, non-cancerous healthy cells were not affected as much as cancer cells, indicating that probiotics and chestnut honey selectively inhibited the growth of cancer. Previously, it was shown that chestnut honey caused about 50% cytotoxicity on the MCF-7 cell line at concentrations of 0-10 μg/mL [46]. However, in the present study, chestnut honey alone produced a cytotoxic effect (about a 20% decrease in cell viability) only at the dilution of 1/2 (5 μg/mL), while the previous study demonstrated more than a 90% decrease in cell viability at this concentration [46]. Another study conducted revealed the anticarcinogenic potential of Greek honeys, but not of chestnut honey [47]. Generally, honeys exhibit anticancer properties against different cancer cells, but no study has described the potential interactions between beneficial microorganisms and chestnut honey and their additional effects on breast cancer [48]. It is thought that honey could reduce tumor cell proliferation by arresting the cell cycle [49]. The cell cycles of colon, glioma and melanoma cancer cell lines have been blocked by honey and its phenolic compounds in G0/G1 phase, which could be due to the downregulation of some cellular pathways involving kinases [50-52]. Furthermore, honey is known to modulate p53, which is involved in tumor suppression [50].

Cytotoxicity results reveal beneficial interactions between probiotics and chestnut honey; thus, when chestnut honey is taken in the diet its phenolic contents are possibly metabolized by the probiotics. Furthermore, new formulations including chestnut honey and probiotics can be more beneficial when compared to either bacteria or honey alone.

CONCLUSIONS

The effects of chestnut honey on the probiotic properties of *Lactobacillus acidophilus* and *L. rhamnosus*, and the effect of the combination of chestnut honey and probiotic bacteria on *in vitro* cytotoxic effects against human breast adenocarcinoma were examined. Chestnut honey promoted the growth of probiotic bacteria, and positively modulated their probiotic properties. When used as a carbon source of probiotics, chestnut honey increased the *in vitro* cytotoxic effects of the probiotic bacteria against MCF-7 cells, while it showed no or little effects on healthy cells. Thus, chestnut honey and probiotic bacteria have the potential to increase the benefits of each other through a synbiotic interaction.

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Author contributions: HUC designed the study, performed the experiments, analyzed the results and wrote the manuscript.

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REFERENCES

1. Can Z, Yildiz O, Sahin H, Akyuz Turumtay E, Silici S, Kolayli S. An investigation of Turkish honeys: Their physico-chemical properties, antioxidant capacities and phenolic profiles. Food Chem. 2015;180:133-41.
2. Hellner M, Winter D, Von Georgi R, Munstedt K. Apitherapy: Usage and experience in German beekeepers. Evidence-based Complement Altern Med. 2008;5(4):475-9.
3. Sahinler N, Sahinler S, Gul A. Biochemical composition of honeys produced in Turkey. J Apic Res. 2004;43(2):53-6.
4. Kucuk M, Kolayli S, Karaoglu S, Ulusoy E, Baltaci C, Candan F. Biological activities and chemical composition of three honeys of different types from Anatolia. Food Chem. 2007;100(2):526-34.
5. Sahin H, Aliyazicioglu R, Yildiz O, Kolayli S, Innocenti A, Supuran CT. Honey, pollen, and propolis extracts show potent inhibitory activity against the zinc metalloenzyme carbonic anhydrase. J Enzyme Inhib Med Chem. 2011;26(3):440-4.
6. Tezcan F, Kolayli S, Ulusoy HSE, Erim FB. Evaluation of organic acid, saccharide composition and antioxidant properties of some authentic Turkish honeys. J Food Nutr Res. 2011;50(1):33-40.
7. Robin C, Morel O, Vettraino AM, Perlerou C, Diamandis S, Vannini A. Genetic variation in susceptibility to *Phytophthora cambivora* in European chestnut (Castanea sativa). For Ecol Manage. 2006;226(1-3):199-207.
8. Sarikaya AO, Ulusoy E, OztUrk N, Tuncel M, Kolayli S. Antioxidant activity and phenolic acid constituents of chestnut (*Castania Sativa* Mill.) Honey and Propolis. J Food Biochem. 2009;33(4):470-81.
9. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11:506-14.

10. González-Rodríguez I, Ruiz L, Gueimonde M, Margolles A, Sánchez B. Factors involved in the colonization and survival of bifidobacteria in the gastrointestinal tract. FEMS Microbiol Lett. 2013;340(1):1-10.
11. Capurso L. Thirty years of *Lactobacillus rhamnosus* GG A Review. J Clin Gastroenterol. 2019;53:S1-41.
12. Gill H, Prasad J. Probiotics, immunomodulation, and health benefits. Adv Exp Med Biol. 2008;606:423-54.
13. Korotkyi O, Vovk A, Galenova T, Vovk T, Dvorschenko K, Luzza F, Abenavoli L, Kobyliak N, Falalyeyeva T, Ostapchenko L. Effect of probiotic on serum cytokines and matrix metalloproteinases profiles during monoiodoacetate-induced osteoarthritis in rats. Minerva Biotecnol. 2019;31(2):68-73.
14. Darsanaki RK, Ghaemi N, Mirpour M. Antimutagenic activity of *Lactobacillus* spp. isolated from commercial yoghurt versus sodium azide, acrylic amide, potassium permanganate and 2-nitrofluorene. Minerva Biotecnol. 2017;29(1):24-9.
15. Górska A, Przystupski D, Niemczura MJ, Kulbacka J. Probiotic Bacteria: A promising pool in cancer prevention and therapy. Curr Microbiol. 2019;76:939-49.
16. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, Ruddy K, Tsang J, Cardoso F. Breast cancer. Nat Rev Dis Prim. 2019;5(1):66.
17. Chuah LO, Foo HL, Loh TC, Mohammed Alitheen NB, Yeap SK, Abdul Mutalib NE, Abdul Rahim R, Yusoff K. Postbiotic metabolites produced by *Lactobacillus plantarum* strains exert selective cytotoxicity effects on cancer cells. BMC Complement Altern Med. 2019;19(1):114.
18. Scott AJ, Merrifield CA, Younes JA, Pekelharing EP. Pre-, pro- and synbiotics in cancer prevention and treatment—a review of basic and clinical research. Ecancermedicalscience. 2018;12:869.
19. Brandt AL, Castillo A, Harris KB, Keeton JT, Hardin MD, Taylor TM. Inhibition of *Listeria monocytogenes* by food antimicrobials applied singly and in combination. J Food Sci. 2010;75(9):M557-63.
20. Celebioglu HU, Delsoglio M, Brix S, Pessione E, Svensson B. Plant polyphenols stimulate adhesion to intestinal mucosa and induce proteome changes in the probiotic *Lactobacillus acidophilus* NCFM. Mol Nutr Food Res. 2018;62(4):1-11.
21. Celebioglu HU, Ejby M, Majumder A, Køble 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. Differential proteome and cellular adhesion analyses of the probiotic bacterium *Lactobacillus acidophilus* NCFM grown on raffinose - an emerging prebiotic. Proteomics. 2016;16(9):1361-75.
22. Kos B, Susković J, Vuković S, Simpraga M, Frece J, Matosić S. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J Appl Microbiol. 2003;94(6):981-7.
23. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55-63.
24. Al-Mamary M, Al-Meeri A, Al-Habori M. Antioxidant activities and total phenolics of different types of honey. Nutr Res. 2002;22(9):1041-7.
25. Çol Ayvaz M, Ömür B, Ertürk E, Kabakçi D. Phenolic profiles, antioxidant, antimicrobial, and DNA damage inhibitory activities of chestnut honeys from Black Sea Region of Turkey. J Food Biochem. 2018;42(3): e12502.
26. Nasuti C, Gabbianelli R, Falcioni G, Cantalamessa F. Antioxidative and gastroprotective activities of anti-inflammatory formulations derived from chestnut honey in rats. Nutr Res. 2006;26(3):130-7.
27. Sipahi H, Aydogan G, Helvacioglu S, Charehsaz M, Guzelmeric E, Aydin A. Antioxidant, antiinflammatory and antimutagenic activities of various kinds of Turkish honey. Fabad J Pharm Sci. 2017;42(1):7-13.
28. Yildiz O, Can Z, Saral O, Yuluğ E, Ozturk F, Aliyazicioğlu R, Canpolat S, Kolayli S. Hepatoprotective potential of chestnut bee pollen on carbon tetrachloride-induced hepatic damages in rats. Evidence-based Complement Altern Med. 2013;2013:461478.
29. Bogdanov S. Nature and origin of the antibacterial substances in honey. LWT - Food Sci Technol. 1997;30(7):748-53.
30. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol 2007;226:1065-73.
31. Van Tassell ML, Miller MJ. *Lactobacillus* adhesion to mucus. Nutrients 2011;3:613-36
32. Servin AL, Coconnier M-H. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Best Pract Res Clin Gastroenterol. 2003;17:741-54.
33. Buck B, Altermann E, Svingerud T, Klaenhammer TR. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol. 2005;71(12):8344-51.
34. Pedersen K, Tannock GW. Colonization of the porcine gastrointestinal tract by lactobacilli. Appl Environ Microbiol. 1989;55(2):279-83.
35. Alp D, Kuleasan H, Korkut Altıntas A. The importance of the S-layer on the adhesion and aggregation ability of lactic acid bacteria. Mol Biol Rep. 2020;47:3449-57.
36. Boonaert CJP, Rouxhet PG. Surface of lactic acid bacteria: Relationships between chemical composition and physicochemical properties. Appl Environ Microbiol. 2000;66(6):254854.
37. Schillinger U, Guigas C, Holzapfel WH. *In vitro* adherence and other properties of lactobacilli used in probiotic yoghurt-like products. Int Dairy J. 2005;15(12):1289-97.
38. Krasowska A, Sigler K. How microorganisms use hydrophobicity and what does this mean for human needs? Front Cell Infect Microbiol. 2014;4:112.
39. Celebioglu H, Svensson B. Dietary nutrients, proteomes, and adhesion of probiotic lactobacilli to mucin and host epithelial cells. Microorganisms 2018;6:90.
40. Sleytr UB, Beveridge TJ. Bacterial S-layers. Trends Microbiol. 1999;7:253-60.
41. Hynonen U, Palva A. *Lactobacillus* surface layer proteins: structure, function and applications. Appl Microbiol Biotechnol 2013;97:5225-43.
42. Sara M, Sleytr UB. S-Layer Proteins. J Bacteriol 2000;182:859-68.
43. Sengupta R, Altermann E, Anderson RC, McNabb WC, Moughan PJ, Roy NC. The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract. Mediators Inflamm. 2013;237921.

44. Waśko A, Polak-Berecka M, Kuzdraliński A, Skrzypek T. Variability of S-layer proteins in *Lactobacillus helveticus* strains. Anaerobe. 2014;25:53-60.
45. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
46. Seyhan MF, Yılmaz E, Timirci-Kahraman O, Saygılı N, Kısakesen Hİ, Eronat AP, Ceviz AB, Bilgic Gazioglu S, Yılmaz-Aydogan H, Ozturk O. Anatolian honey is not only sweet but can also protect from breast cancer: Elixir for women from artemis to present. IUBMB Life. 2017;69(9):677-88.
47. Spilioti E, Jaakkola M, Tolonen T, Lipponen M, Virtanen V, Chinou I, Kassi E, Karabournioti S, Moutsatsou P. Phenolic acid composition, antiatherogenic and anticancer potential of honeys derived from various regions in Greece. PloS One 2014;9(4):e94860.
48. Ahmed S, Othman NH. Honey as a potential natural anticancer agent: A review of its mechanisms. Evidence-based Complement Altern Med. 2013;2013:829070.
49. Tomasin R, Cintra Gomes-Marcondes MC. Oral administration of *Aloe vera* and honey reduces walker tumour growth by decreasing cell proliferation and increasing apoptosis in tumour tissue. Phyther Res. 2011;25(4):619-23.
50. Jaganathan SK. Honey Constituents and their apoptotic effect in colon cancer cells. J ApiProduct ApiMedical Sci. 2009;1(2):29-36.
51. Lee YJ, Kuo HC, Chu CY, Wang CJ, Lin WC, Tseng TH. Involvement of tumor suppressor protein p53 and p38 MAPK in caffeic acid phenethyl ester-induced apoptosis of C6 glioma cells. Biochem Pharmacol. 2003;66(12):2281-9.
52. Pichichero E, Cicconi R, Mattei M, Muzi MG, Canini A. Acacia honey and chrysin reduce proliferation of melanoma cells through alterations in cell cycle progression. Int J Oncol. 2010;37(4):973-81.