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, proanthocyanidins 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...
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 (H₂O₂), 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 *L. 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 (0th h) and after (24th 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 OD₆₀₀ 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:

\[
\text{% Autoaggregation} = (1 - \frac{A_t}{A_0}) \times 100,
\]

where \(A_t\) is the absorbance measured after incubation and \(A_0\) 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 KNO₃ (pH 6.2) to OD₆₀₀ 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:

\[
\text{% Adhesion} = (1 - \frac{A_1}{A_0}) \times 100,
\]

where \(A_1\) is the absorbance measured after the incubation and \(A_0\) 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 \(\times\) 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 -80ºC 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 37ºC with 5% CO₂ 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 15x10³ 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
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.

Fig. 1B shows the effects 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.

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).
Surface hydrophobicity of probiotics was also evaluated as it is another important property. Chestnut honey significantly increased the surface hydrophobicity of \textit{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 \textit{L. rhamnosus} GG (Fig. 3B).

\textbf{In vitro cytotoxic effects of probiotic bacteria grown with chestnut honey}

The \textit{in vitro} cytotoxic effects of probiotic bacteria \textit{L. acidophilus} LA-5 and \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{L. rhamnosus} GG. When the supernatants were diluted 10-fold with cell culture medium (1/10), only \textit{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 \textit{L. acidophilus} GG grown.
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 IC₅₀ for DPPH scavenging activity [1,8]. A recent study has determined the phenolic compounds in chestnut honey of Bartın 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 symbiotic interaction.

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