Physicochemical and Active Properties of Gelatine-Based Composite Gels Loaded with Lysozyme and Green Tea Polyphenols

Running head: Antilisterial and Bioactive Gelatine-Based Composite Gels

Derya Boyacı¹,², Pelin Barış Kavur¹, Şükrü Gulec³ and Ahmet Yemenicioğlu¹*

¹Department of Food Engineering, Izmir Institute of Technology, 35430 Gulbahce Koyu, Urla, Izmir, Turkey
²School of Engineering, University of Lincoln, LN6 7TS Brayford Pool, Lincoln, United Kingdom
³Molecular Nutrition and Human Physiology Laboratory, Faculty of Engineering, Izmir Institute of Technology, 35430 Gulbahce Koyu, Urla, Izmir, Turkey

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SUMMARY

Research background. The use of gel-based systems, as a novel method for the delivery of natural antimicrobial, antioxidant, and bioactive compounds is a developing innovative solution for the food industry. This research aimed development of multifunctional active edible gels based on gelatine and its composites with improved mechanical properties.

Experimental approach. Antilisterial and bioactive composite gels showing different physical and active properties than classical gelatine gel were developed by loading lysozyme and green tea extract into gelatine/starch and gelatine/wax composite gels. The gels were characterized for their mechanical properties, swelling profiles, colour, release profiles, and antimicrobial and bioactive properties.

Results and conclusions. Gelatine/wax gels showed 1.3 to 2.1-fold higher firmness and cutting strength than gelatine and gelatine/starch composite gels that had similar firmness and cutting
strengths. Work to shear of both composite gels was 1.4 to 1.9-fold higher than that of gelatine gel. The gelatine/starch gel showed the highest water absorption capacity. Green tea extract reduced soluble lysozyme in gels, but composite gels contained higher soluble lysozyme than gelatine gel. All the gels with lysozyme inhibited *Listeria innocua* growth in the broth media while green tea extract showed antilisterial activity only in gelatine/wax gels. Gels with green tea extract showed antioxidant, antidiabetic (α-glucosidase, and α-amylase inhibition), antihypertensive (angiotensin-converting enzyme inhibition), and antiproliferative activities (on Caco-2 human colon carcinoma cells). However, gelatine and gelatine/wax gels showed the highest antioxidant and antidiabetic activity. The gelatine/wax gels prevented phenolic browning while green tea extract in other gels showed moderate or extensive browning.

**Novelty and scientific contribution.** This work clearly showed the possibility of improving mechanical properties, and modifying water absorption and controlled release profiles of gelatine gels using gelatine/starch and gelatine/wax composites. The novel composite gels reduced browning of incorporated polyphenols and showed antilisterial and bioactive properties.

**Key words:** gelatine gel, candelilla wax, rice starch, lysozyme, composite gel, green tea extract

**INTRODUCTION**

The use of gel-based systems for delivery of natural antimicrobials, antioxidants, and bioactive compounds has gained an increased interest since such systems could find innovative applications in food, biomedical and pharmaceutical sectors (1-4). Gelatine is the most indispensable animal source food hydrocolloid that has been extensively used not only for its unique gelation capacity but also for its elasticity, texture, taste, and nutritive value (5). However, the low mechanical stability of gelatine-based food and materials, as well as incompatibility of gelatine with some bioactive polyphenols (e.g. darkens in contact with polyphenols), interferes with its innovative food applications to obtain novel functional gel-based food and bioactive materials (e.g. film, pad, coating or filling materials) suitable for delivery of active compounds (1,4,6). Therefore, different efforts have been spent to develop more mechanically stable composites of gelatine with proteins (e.g. soy protein isolate), polysaccharides (e.g. starch, alginate, xanthan, carboxymethyl cellulose, gellan, sucrose, and inulin), and waxes that could be employed as antimicrobial filling, coating, or pad materials (1, 4, 7-11). Gelatine is also extensively used to obtain numerous gel-based foods such as fruit jellies prepared by fresh fruits, toppings for pâté, and aspic food obtained by glazing, coating, or embedding ready-to-eat animal source foods such as meat, chicken, pork, fish and eggs with gelatine. Microbial
safety of such gel-based products is extremely important since they are cold-stored for some time, and this provides a perfect medium for the growth of pathogenic bacteria like *Listeria monocytogenes* that causes deadly infections in pregnant women, elderly, and immunosuppressed people (12,13). The most severe effects of this bacterium were observed in the past for pâté and pork tongue in aspic with large-scale invasive listeriosis outbreaks in England (1988) and France (1992), respectively (14).

The main objective of the current study is to develop antilisterial and bioactive gelatine-based composite gels having different physical and active properties than those of classical gelatine gels. Such composite gels could be employed to obtain alternative safer and healthier gel-based foods and edible active filling, coating, glazing, or pad materials. The composite gels of gelatine with hydrophilic rice starch and hydrophobic candelilla wax were prepared mainly to improve the poor mechanical properties of gelatine gel, compatibility of gelatine with polyphenols, and to modify its functional, visual, and water absorption properties. The lysozyme and green tea extract were used as model natural compounds to obtain antimicrobial and bioactive properties of the gels. Both lysozyme and green tea extract are proven antilisterial agents (15-19). The gels were characterized for their antilisterial capacities using *L. innocua* as a surrogate of *L. monocytogenes* that contamination might pose serious risks for the laboratory staff. Moreover, green tea extract is a perfect source of catechins (*e.g.* catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate) that have been increasingly used in foods due to their well-characterized molecular structure, bioavailability, and *in-vivo* and *in-vitro* health benefits (20,21). To evaluate their potential health benefits, the developed gels were characterized for well-known bioactive properties of green tea extract such as antioxidant, antidiabetic, antihypertensive, and antiproliferative activities. This work is original in that it is the first study that focused not only on improving antimicrobial and bioactive properties of classical gelatine gel but also on developing its weak physical and mechanical properties as well as incompatibility with polyphenols that limit its various applications.

**MATERIALS AND METHODS**

**Materials**

Bovine skin gelatine (Type B, 225 g Bloom gel strength), rice starch, candelilla wax, lysozyme from hen egg white (Activity by producer: ≥40000 U/mg), angiotensin-converting enzyme from rabbit lung, α-amylase from human saliva, and rat intestine acetone powder were obtained from Sigma-Aldrich (Missouri, USA). Green tea extract (100 %) with a minimum of 22 % total polyphenol content was obtained from Wild Flavours and Specialty Ingredients (Rudolf Wild GmbH & Co. KG, Eppelheim,
Germany. Caco-2 cells used in cytotoxicity assay were obtained from ATCC, USA. *Listeria innocua* (NRRL B-33314) used in antimicrobial tests was obtained from the United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit (Peoria, IL, USA).

**Preparation of gelatine and gelatine-based composite gels**

Gel solutions were prepared by dissolving gelatine at a concentration of 15 % (by mass) in distilled water (55 °C) by stirring at 500 rpm. Rice starch or candelilla wax was added into the gelatine solution at a concentration of 7.5 % (by mass) to produce gelatine/starch and gelatine/wax composite gels, respectively. The gelatine and gelatine/starch solutions were homogenized at 10,000 rpm for 1 min using a homogenizer (Heidolph Instruments, Silent Crusher M with rotor Φ=6.6 mm tip, Schwabach, Germany). All gel forming solutions were heated in a water bath at 85 °C for 30 min. Gelatine/wax mixture was homogenized at 10,000 rpm for 1 min to distribute melted wax within the gelatine solution. After cooling, green tea extract and lysozyme were added to the gel solutions at a concentration of 1 % (by mass). All mixtures were further homogenized at 10,000 rpm for 3 min to distribute active agents homogeneously. The gel-forming solutions were then poured into moulds and incubated for 20 h at 4 °C to achieve complete gelation.

**Mechanical properties (shear test)**

A shear test was performed to determine the mechanical properties of gels using a TA-XT plus texture analyser (Stable Micro Systems Ltd., Godalming, UK) equipped with a blade set (HDP/BS) with knife/guillotine probe (crosshead speed: 200 mm/min, cell load: 5 kg). Test conditions used by Muñoz et al. (22) were applied with slight modifications. The gel samples were prepared by pouring 50 g of gel-forming solution into cubic moulds (5 cm³) and incubating for 20 h at 4 °C. The gel samples were cut into 2.5 cm³ portions and brought to 4 °C before testing. The gels were sheared through the centre, and their cutting strength (N/mm), maximum shear force (firmness) (N), and area under the curve (work to shear) (N·s) were determined from the force vs time curve. The experiments of each sample were replicated twice with four repetitions.

**Water binding capacity**

Water binding capacity (WBC) of the gels was determined according to a gravimetric method (23). For analysis, 10 g of gels casted into plastic Petri dishes (diameter: 6.6 cm, thickness: 0.5 cm) were weighed (m₁) and placed into 100 mL of distilled water, and they were incubated at 4 °C under
shaking at 80 ×g for 15 days. The gels were taken out of the water in every 24 h and weighed \( (m_2) \). Each gel was weighted in triplicate. WBC was calculated as g water/g gel using Eq. 1.

\[
WBC = \frac{(m_2 - m_1)}{m_1}
\]

where WBC is the water binding capacity (in g water/g gel), \( m_1 \) is the initial mass and \( m_2 \) is the mass of gel at the equilibrium.

**Colour of gels**

Colour of the gels were determined using a digital colourimeter (chromometer type, Konica Minolta, CR-400, Tokyo, Japan) standardized with a white plate \((Y=93.80, X=0.3159, y=0.3322)\). For analysis, 50 g of cubic gel samples were used. Results were expressed with CIE (Commission International de l'Eclairage); \( L^* \) \((0=dark, 100=light)\), \( a^* \) \((-a=greenness, +a= redness, 0=neutral)\) and \( b^* \) \((-b=blueness; +b=yellowness, 0=neutral)\).

**Release profiles**

To determine the release profiles of lysozyme and green tea extract polyphenols, discs of gels \((\text{weight} \sim 10 \text{ g}, \text{thickness}: 0.5 \text{ cm}, \text{diameter}: 6.6 \text{ cm})\) were placed into Erlenmeyer flasks that contained 100 mL of distilled water at 4 °C. The flasks were then shaken at 80 rpm, and the samples \((0.1 \text{ mL})\) collected at different time intervals were tested for lysozyme activity or total phenolic content until reaching the equilibrium. The lysozyme activity was measured spectrophotometrically \((\text{Model } 2450, \text{Shimadzu, Tokyo, Japan})\) at 660 nm using Micrococcus lysodeicticus as a substrate as described in Boyaci et al. \((1)\). The released total phenolic content was measured spectrophotometrically \((\text{at } 795 \text{ nm})\) according to the classical Folin-Ciocalteu method \((24)\). Catechin was used as a standard for the determination of phenolic compounds. The measurements were performed as two replicates and three parallels. All calculations were corrected by considering the activity removed by collected aliquots during sampling. The total lysozyme activity and total phenolic content released from each gel corresponded to maximum units \((U)\) and maximum phenolic content \((\text{mg catechin)}\) released per g of gels at the equilibrium, respectively. The release curves were formed by plotting the calculated released lysozyme activities \((U/g)\) or phenolic contents \((\text{mg catechin/g})\) from gels vs time \((h)\). The initial release rates of lysozyme and green tea extract phenolics were determined from the slope of the initial linear portion of release curves as \(U/g/h\) and \(\text{mg catechin/g/h}\), respectively. The recovery of lysozyme and green tea extract from the gels was determined from the formula given in Eq. 2 \((\text{Note: According to the methods described in this study adding } 1\% \text{ lysozyme and green tea})\).
extract in gels resulted in an initial lysozyme activity of 744194 U and initial concentration of 3.29 mg catechin equivalent total polyphenols per g of gels).

Lysozyme or polyphenol recovery (%) = (Total lysozyme activity or total phenolic content released) / (Total lysozyme activity or total polyphenol added into gels) \times 100

**Antimicrobial activity in broth media**

The antimicrobial activity of gel discs (weight: \(~10\) g, thickness: 0.5 cm, diameter: 6.6 cm) was tested against *Listeria innocua* for 48 h incubation period by the shake flask method (at 80 rpm) in 100 mL of nutrient broth at 4 °C (1). Microbiological counts were expressed as logarithm of colony-forming unit per mL (log CFU/mL), and the means and standard errors were calculated. At least three plates were enumerated for the calculations.

**Bioactive properties**

The bioactive properties of gels were expressed based on the total green tea extract-originated polyphenols released from the gels during the release tests. Thus, the release media of the gels reached equilibrium for released polyphenols were used directly to determine the bioactive properties. Only for antiproliferative activity tests, release media equilibrated for polyphenols were lyophilized using a freeze drier (Freezone 6L, Labconco, Kansas City, MO, USA). Then, the stock solutions were prepared freshly by dissolving lyophilized samples in ultrapure water.

**Antioxidant activity**

Antioxidant activity of active compounds released from the gels was determined according to the Trolox equivalent antioxidant capacity (TEAC) (25) and oxygen radical absorbance capacity (ORAC) methods (26). The iron-chelating capacity (ICC) of active compounds released from the gels was determined according to the spectrophotometric method (27). Results of TEAC and ORAC were expressed as \(\mu\text{mol Trolox/g of gel}\) while ICC was expressed as \(\mu\text{mol Na}_2\text{EDTA/g of gel}\). An average of three measurements was used for all calculations.

**Antihypertensive activity**

The antihypertensive activity of active agents released from the gels was determined by measuring their inhibitory effects on the angiotensin-converting enzyme (ACE) (28). ACE inhibition
(%) was determined as described in the method and results were expressed as µg captopril per g of gel. An average of three measurements was used for all calculations.

Antidiabetic activity

The antidiabetic activity of active agents released from the gels against human salivary α-amylase (HSA) and α-glucosidase (AGH) enzymes was determined according to Lee et al. (29). Both HSA and AGH inhibitions were calculated according to Eq. 3.

\[
\%\text{Inhibition} = \left( \frac{(A_{\text{control}} - A_{\text{control blank}}) - (A_{\text{sample}} - A_{\text{sample blank}})}{A_{\text{control}} - A_{\text{control blank}}} \right) \times 100 / 3
\]

where \( A_{\text{control}} \), \( A_{\text{control blank}} \), \( A_{\text{sample}} \), and \( A_{\text{sample blank}} \) are the absorbances measured for the reaction mixtures prepared with a buffer using the active enzyme, with buffer using the inactive enzyme, with the sample using the active enzyme, and with the sample using the inactive enzyme, respectively. An average of three measurements was used for the calculations. Results were expressed as µmol acarbose equivalent per g of gel.

Antiproliferative properties

The Caco-2 cells were cultured in Minimum Essential Medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 15 % fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Brazil), 100 U/mL penicillin-streptomycin solution (Gibco, Thermo Fisher Scientific, MA, USA), 1 % non-essential amino acids (Gibco, Thermo Fisher Scientific, NY, USA), and 1 % sodium pyruvate (Gibco, Thermo Fisher Scientific, NY, USA) in a humidified incubator with 5 % CO\(_2\) at 37 °C. The Caco-2 cells (5000 cells/well) were plated into 96-well plates and allowed to grow for 24 h. Then, different amounts (0, 21, 62, 123.6 and 247.2 µg) of samples were added into the wells that contain 0.2 mL of MEM (0, 105, 310, 618, 1236 µg sample/mL of MEM). The wells were then incubated for 48 h at 5 % CO\(_2\) at 37 °C. The cell toxicity was determined by CCK-8 (Sigma-Aldrich, St. Louis, MO, USA) assay according to kit direction. Colour changes were measured at 450 nm, using a Varioscan plate reader (Multiscan Go, Thermo, USA). The absorbance from non-treated Caco-2 cells (control group) was taken as 100 % viable and the viability of sample-treated cells was calculated as the percentage of the control group. An average of three measurements was used for the calculations.
Statistical analysis

One-way analysis of variance (ANOVA) was performed to process the data of gel samples using Minitab Statistical Software for Windows, version 17 (30). The normal distribution of samples was checked using the Shapiro-Wilk test. For the data that do not fit the normal distribution, the Kruskal-Wallis test was conducted using IBM SPSS Statistics for Windows, version 23.0 (31). Statistical differences among means were compared with multiple range test at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

Effect of composite gel making on mechanical properties of gelatine gels

The mechanical properties of different gels were characterized by shear test and the parameters calculated were given in Table 1. The force vs time graphs of each gel were given in Fig. S1. The gelatine/wax gels showed significantly higher firmness, work to shear, and cutting strength than corresponding gels of gelatine ($p<0.05$). No significant differences were determined between work to shear of control gelatine/wax and gelatine/starch gels ($p \geq 0.05$), but all active gelatine/wax gels showed higher work to shear than corresponding active gelatine/starch gels. Moreover, all gelatine/wax gels (control or active) showed significantly higher firmness and cutting strength than gelatine/starch gels ($p<0.05$). The firmness and cutting strength of gelatine/starch and gelatine gels with green tea extract or lysozyme-green tea extract combination were similar, but all gelatine/starch gels showed higher work to shear than their corresponding gelatine gels. These results clearly showed that the most mechanically stable gel is gelatine/wax followed by gelatine/starch and gelatine gels. Also, the addition of green tea extract did not affect the work to shear of gelatine and gelatine/starch gels, but addition of lysozyme-green tea extract combination increased the work to shear of both gels. In contrast, the addition of green tea extract increased firmness, cutting strength, and work to shear of gelatine/wax gels significantly ($p<0.05$). These results clearly showed that the green tea extract increased the networking of the gelatine/wax gels. It seemed that the amphiphilic green tea phenolic fractions created some interactions both with gelatine and candelilla wax, and this supported the mechanical stability of the gelatine/wax gels. Different reports in the literature related to the affinity of green tea catechins to hydrophobic lipids support this hypothesis (32-34). The hydroxyl groups (–OH) of polyphenols could form extensive hydrogen bonding with carbonyl groups (C=O) of gelatine proteins (35) while at the same time their hydrophobic phenolic groups like aromatic rings (e.g. those of catechins like epigallocatechin gallate and epicatechin gallate of catechins) might create hydrophobic interactions with wax particles distributed within the gelatine gel matrix. Finally, in
all gels the addition of green tea extract or lysozyme-green tea extract combination did not affect firmness and cutting-strength. However, a significant increase in work to shear was observed in all gels when lysozyme-green tea extract combination was added instead of green tea extract \( (p<0.05) \). The capacity of lysozyme to interact with gelatine \((1)\) and polyphenols \((36)\) have been well documented. However, overall results clearly showed that addition of lysozyme-green tea extract combination instead of green tea extract had no considerable effect on major mechanical properties of gelatine gel such as firmness and cutting strength.

**Water binding properties of different gels**

The swelling characteristics of different gels indicated that the addition of hydrophilic rice starch into gels increased their swelling rates while composite making with hydrophobic candelilla wax reduced the swelling rates of gels \((\text{Fig. 1 and Table S1})\). Thus, at the equilibrium, the highest water binding capacity \((WBC)\) was determined for gelatine/starch gel \((0.54 \text{ g/g})\), followed by gelatine \((0.45 \text{ g/g})\) and gelatine/wax gel \((0.31 \text{ g/g})\). These results clearly showed that the composite gels provide not only alternative mechanical properties but also considerably different WBCs that could be exploited to obtain gels with different textural properties.

**Effects of starch, wax and green tea extract on colour of different gels**

The photos of the developed gels and their \(L^*, a^*, b^*\) values determined by a digital colorimeter were presented in \(\text{Fig. S2 and Table 2}\), respectively. The control gelatine gel is transparent and very light-yellow coloured \((\text{Fig. S2a})\) while control gelatine/starch gel is light yellow coloured and non-transparent \((\text{Fig. S2b})\), and control gelatine/wax gel is non-transparent and white coloured \((\text{Fig. S2c})\). The addition of green tea extract turned gelatine gels quite turbid and dark brownish \((\text{Fig. S2d})\) while green tea extract turned the colour of gelatine/starch gels light brownish to greenish \((\text{Fig. S2e})\). Discolorations in colour were also reported by Jamróz \textit{et al.} \((37)\) who added tea extracts into furcellaran-gelatin films. In contrast, green tea extract added gelatine/wax gels turned light yellow and successfully masked the brown colour formation \((\text{Fig. S2f})\). The same results were also observed in photos of green tea extract loaded gels when they were tested to obtain fruit jellies using strawberries as a model \((\text{Fig. S3})\). However, the addition of lysozyme-green tea extract combination gave less dark gelatine and gelatine/starch gels than same gels with green tea extract \((\text{Fig. S2g and S2h})\). A reduction was also observed in yellowness of gelatine/wax gel by use of lysozyme-green tea extract combination instead of green tea extract \((\text{Fig. S2i})\). Thus, it appears that the lysozyme showed some protective effect on oxidation of polyphenols due possible to its amino acid side chains capable
to show antioxidant activity (38). The darkening observed in gels by the addition of green tea extract correlated inversely with their \( L^* \) values. The highest \( L^* \) values were determined for the control gels of gelatine/wax. The addition of green tea extract or lysozyme-green tea extract combination reduced the \( L^* \) values of gelatine/wax gels, but these gels still showed 2 to 4-fold higher \( L^* \) than gelatine and gelatine/starch gels with green tea extract and lysozyme-green tea extract combination. Also, it should be noted that \( L^* \) values of all gels with lysozyme-green tea extract combination were significantly higher than those gels with green tea extract. This result showed parallelism with appearances of gels in photos that suggested a potential protective effect of lysozyme on green tea polyphenols. The overall results suggested that the numerous tiny rice starch and candelilla wax particles within composite gels prevented the passing of light from the gels, and this masked the colour originated from green tea extract in the gels. It also appeared that the limited light contact of green tea polyphenols in composite gels also prevented their darkening with photodegradation in presence of gelatine reactive groups (39). A considerable redness (\( a^* \)) was observed only in the gels of gelatine with green tea extract while other gels were close to neutral or they showed a slightly greenish colour. The yellowness (\( b^* \)) of composite gels increased significantly by the addition of green tea extract while gels of gelatine with green tea extract, and with lysozyme-green tea extract combination showed slight and moderate reductions in their yellowness, respectively.

**Release profiles of lysozyme and green tea extract from different gels**

The release profiles and release parameters of different gels for lysozyme and green tea extract are presented in Fig. 2a-d, and Table 3, respectively. The initial activity of lysozyme and initial green tea phenolic content of gels achieved by loading 1 % of both active compound preparations were 744194 U and 3.29 mg catechin equivalent per g of gels, respectively. The gels incorporated with lysozyme showed similar release profiles (Fig. 2a). However, the recovery of lysozyme from gelatine, gelatine/starch, and gelatine/wax gels changed between 16 and 20 %. This finding indicated that at the pH of gels (~6.0), the negative charges formed by gelatine (pI: 5.0–7.5) bound a significant amount of positively charged lysozyme (pI: 11.4) by charge-charge interactions. The total activities of released lysozyme from gelatine/starch and gelatine/wax gels were similar to each other, and they were 16 % and 23 % higher than that from gelatine gel, respectively. The higher total released lysozyme activities of composite gels might be favoured by the altered structure of the gelatine gel matrix by rice starch granules and candelilla wax particles. It seemed that the rice starch and candelilla wax incorporated into gels created less interaction with lysozyme than gelatine. Thus, this increased the fraction of soluble lysozyme within the gels. In contrast, in the presence of green tea extract, the
gels again showed similar lysozyme release profiles, but the lysozyme recoveries of different gels dropped to between 12 and 14% (Fig. 2b). Thus, it is evident that the combination of lysozyme with green tea extract increased the binding of lysozyme onto the gelatine gel matrix. The -OH groups of phenolic compounds are capable to form extensive H-bonds with carbonyl groups of proteins (40). Therefore, it seemed that the polyphenols in green tea extract caused the formation of crosslinks between combined lysozyme and green tea extract. Moreover, increased crosslinks among gelatine might have also caused physical trapping of lysozyme within the film matrix. The initial lysozyme release rates of gelatine, gelatine/starch, gelatine/wax gels without green tea extract were 1.4, 1.2, and 1.8-fold higher than similar gels containing lysozyme-green tea extract combination, respectively. This finding supported the hypothesis that gel networking increased due to interaction of green tea extract polyphenols with gelatine. This hypothesis complies well with previous reports of Arcan and Yemenicioglu (41) and Zhu et al. (42) who showed that the phenolic crosslinking is effective to sustain lysozyme release from zein and gelatin films, respectively.

The phenolic release profiles of gels are presented in Fig. 2c and Fig. 2d. The release tests clearly showed that gelatine, gelatine/starch, and gelatine/wax gels showed similar green tea polyphenol release profiles with initial release rates changing between 0.069 and 0.087 mg catechin g⁻¹h⁻¹. It was also important to note that the release rates of green tea extract were much faster than those of lysozyme and it reached an equilibrium almost within 24 h. The total amounts of green tea polyphenols released from different gels were also similar and its phenolic recovery corresponded to almost 53-63% of total green tea polyphenols added into the gels. These results showed that the amount of free green tea extract in gels and green tea extract release rates of the gels were not affected by the presence of candelilla wax and rice starch when gels swelled in distilled water. To better realize the significance of phenolic content released by the gels, the results were also expressed by considering the phenolic content of 1 serving portion of green tea. According to Arcan and Yemenicioglu (43) who applied similar testing methods, the 1-serving portion (200 mL) of green tea contains 140 mg gallic acid (or 61.7 mg catechin) equivalent of polyphenols. Thus, the total phenolic content released from 30-35 g portion of green tea extract loaded gelatine, gelatine/starch, and gelatine/wax gels is equivalent to that in 1-serving portion of green tea.

Antimicrobial activity of different gels against L. innocua

The results of antimicrobial tests conducted at 4 °C for 48 h for gelatine, gelatine/starch, and gelatine/wax gels with lysozyme, green tea extract, or lysozyme-green tea extract combination in broth media against L. innocua are presented in Table 4. A 1.5-1.9 log increase in the Listeria load
was observed for control culture and the control gels lacking any active agents within 48 h. Gelatine and gelatine/starch gels with green tea extract were unable to inhibit *Listeria* growth, thus, this caused a significant (0.8-0.9 log) increase in microbial load within 48 h (p<0.05). In contrast, gelatine/wax with green tea extract showed antimicrobial effect and prevented the significant increase of *Listeria* load during the 48 h incubation period (p<0.05). The antimicrobial effect of green tea extract against *L. monocytogenes* has been demonstrated before by different research (44-46). Thus, the lack of significant antimicrobial effect of green tea extract released from gelatine and gelatine/starch gels could be related to its modifications (e.g.; oxidation and polymerization condensation) or interactions within these gels. In contrast, it appeared that the antimicrobial phenolic green tea extract fractions dispersed or solubilized within the hydrophobic wax particles in gelatine/wax gels underwent very limited modifications and/or interactions that diminish their antimicrobial properties. It was also found that all gels with lysozyme and lysozyme-green tea extract combination inhibited the growth of *Listeria* within the 48 h period. No significant differences were found between the antilisterial properties of similar gels with lysozyme and lysozyme-green tea extract combination (p>0.05). Thus, it seemed that the reduced lysozyme release in the presence of green tea extract prevented the detection of any additive or synergetic antilisterial effects between these two active compounds within the gels. However, it is important to note that gelatine/starch gels with lysozyme-green tea extract combination, and gelatine/wax with lysozyme were the only gels that caused a significant reduction (~ -0.5 log) in initial *Listeria* loads within 48 h.

**Bioactive properties of gelatine and its composite gels**

ORAC, TEAC, and ICC based antioxidant activity of different gels

The antioxidant properties of gels are presented in Figs. 3a-c. The control gels of gelatine, gelatine/starch, and gelatine/wax lacking green tea extract showed considerable inherent ORAC values that were equivalent to 54 to 71 % of those ORACs obtained for gels containing green tea extract or lysozyme-green tea extract combination (Fig. 3a). The inherent ORAC of gels could be originated from soluble antioxidant protein and peptide residues released from the gelatine gel matrix (47). In fact, the water-soluble protein contents (assayed by the Bradford method) released from the gels incubated in distilled water at 4 °C changed between 99 and 119 µg/g gel. No considerable TEAC and ICC were measured for the control gels. The addition of green tea extract or lysozyme-green tea extract combination caused a 1.4 to 2-fold increase in ORACs of the gels while this increased TEACs of gels 28 to 32 folds (Fig. 3b). The ORAC values of green tea extract containing gelatine, gelatine/starch, and gelatine/wax gels were not significantly different (p≥0.05) regardless of
the presence of lysozyme. In contrast, the TEACs of the gelatine gel with lysozyme-green tea extract combination, and gelatine/wax gel with green tea extract were 20 % to 41 % higher than those of gels with green tea extract or lysozyme-green tea extract combination. The gels did not show such a great variation in their total soluble phenolic contents (Table 3). However, the green tea extract might contain eight different forms of catechins that vary in antioxidant potential even for epimer pairs (48). Therefore, the variations in TEACs of gels could be due to their compositional and morphological differences that affected their released phenolic profiles. Although the highest TEACs were determined for gels of gelatine with lysozyme-green tea extract combination and gelatine/wax with green tea extract, green tea polyphenols in gelatine/wax gel did not show browning and had better antimicrobial activity than all other green tea extract loaded gels (Table 4). It appeared that the gelatine/wax got a protective effect on functional groups of green tea polyphenols such as galloyl residues that are responsible for antimicrobial and antioxidant activity of catechins (49). Moreover, it seemed that the polyphenols solubilized or dispersed in wax particles are protected from undesired interactions and modifications, thus, they maintained not only their antioxidant and antimicrobial activity but also, they avoid reactions causing discoloration.

The ICC of lysozyme containing gels could not have been determined due to the turbidity formed in the reaction mixture caused by lysozyme. However, measurements with green tea extract containing gels indicated that ICC of gels (ranged between 8.32 and 16.15 µmol EDTA/g gel) increased 2.5 to 5-fold by the addition of green tea extract (Fig. 3c). The overall results showed that green tea extract caused substantial increases in TEACs of gels while it caused limited improvements in ORAC and ICC of gels.

### Antihypertensive and antidiabetic activities of different gels

The results showed that the soluble proteins and peptides in control gelatine, gelatine/starch, and gelatine/wax gels showed inherent antihypertensive activities that are not significantly different from each other (p≥0.05) (Fig. 3d). It seemed that the gelatine peptides, known for their antihypertensive activity, might have dissolved into release media and showed an ACE inhibitory effect (50,51). The addition of green tea extract into gels caused a limited increase in their antihypertensive activities except for gelatine with green tea extract and gelatine/wax with lysozyme-green tea extract combination. However, the IC\textsubscript{50} of captopril determined for ACE inhibition was 0.019 µg/mL (0.089 µM). Thus, it is important to note that the captopril equivalents released from one g of green tea extract or lysozyme-green tea extract combination loaded gelatine, gelatine/starch or gelatine/wax gel were 5 to 8.4-fold higher than IC\textsubscript{50} of captopril for ACE.
Due to the soluble gelatine protein fractions in gels, control gels of gelatine, gelatine/starch, and gelatine/wax showed similar inherent HSA inhibition capacities (p≥0.05) (Fig. 3e). In contrast, the control gels did not show any inhibitory effect on AGH (Fig. 3f). The addition of green tea extract or lysozyme-green tea extract combination into gels caused significant increases in AGH and HSA based antidiabetic activities of all gels. The HSA based antidiabetic activities of green tea extract and lysozyme-green tea extract combination loaded gels did not show significant variations. In contrast, the gels of gelatine with green tea extract, and gelatine/wax both with green tea extract or lysozyme-green tea extract combination showed significantly higher AGH based antihypertensive activities than other gels. This result once more suggested differences among bioactive profiles of phenolics released from different gels. Moreover, the acarbose equivalents released from one g of green tea extract or lyophilized release medium of gelatine/starch and gelatine/wax gels were 6 to 8 and 5 to 11-fold higher than IC_{50} of acarbose determined for HSA (IC_{50}: 2.1 µg/ml or 3.24 µM) and AGH (IC_{50}: 8.07 µg/ml or 12.50 µM), respectively.

Antiproliferative activity of different gels on Caco-2 cells

The antiproliferative activities of pure green tea extract and lyophilized release test mediums from green tea extract containing gels of gelatine, gelatine/starch, and gelatine/wax were tested on Caco-2 cells and the results were given in Fig. 4. Different rates of cellular proliferative activities were observed for lyophilized release mediums of green tea extract loaded gelatine/starch and gelatine/wax gels at 0.1 and 0.3 mg/mL concentrations. However, the lyophilized release medium of gelatine gels with green tea extract did not show a significant proliferative effect on Caco-2 cell growth. On the other hand, when green tea extract or lyophilized release medium concentration (≥0.6 mg/mL) was increased, the growth rate of Caco-2 cells significantly reduced. The pure green tea extract was used as a control of cell culture experiments and its IC_{50} was determined at the level of 1.12 mg/mL. The lyophilized release mediums from green tea extract containing gelatine and gelatine/wax gels showed similar antiproliferative activities with IC_{50} values at 1.67 mg/mL and 1.65 mg/mL, respectively. The lyophilized release medium from green tea extract containing gelatine/starch gel (IC_{50}: 2.37 mg/mL) showed the lowest antiproliferative activity. Thus, it is important to note that 1.2 to 1.5 g of gelatine, gelatine/starch, and gelatine/wax gels could release green tea extract phenolics equivalent to IC_{50} values determined for Caco-2 cells. These results clearly indicated that green tea extract added gels have the ability to reduce Caco-2 cell growth.

CONCLUSIONS
The use of composites of gelatine with candelilla wax caused dramatic increases in the mechanical stability of obtained gels, and these gels effectively prevented the darkening of loaded green tea polyphenols. Moreover, composite gels formed with candelilla wax showed protective effects on antimicrobial, antioxidant, and antidiabetic activities of green tea polyphenols. The composites of gelatine with rice starch had less pronounced positive benefits on colour and mechanical properties of gels than those obtained with candelilla wax, but these gels caused a considerable increase in water-binding properties of gelatine gels. Composite gel making with rice starch also had some positive effects on the antimicrobial performance of gels, but this caused no improvements in the bioactive properties of the gels. This work provided basic data for tailoring of physical properties, release profile and active properties of gels loaded with bioactive compounds. The bioactive gels could be employed for developing alternative functional gel-based foods and edible active filling, glazing, coating, or pad materials.

**FUNDING**

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**SUPPLEMENTARY MATERIAL**

All supplementary material is available at: www.ftb.com.hr.

**AUTHORS’ CONTRIBUTION**

D. Boyaci was in charge of performing the tests, data collection, analysis and interpretation of the results and drafted the manuscript. P.B. Kavur performed data collection and analysis of the shear tests, water absorption tests, and colour measurements. S. Gulec provided material and took part in designing, analysis, interpretation, and supervision of cell culture studies. A. Yemenicioğlu proposed the concept, designed and supervised the research, interpreted the results, and revised the manuscript critically. All authors read and approved the final version to be published.

**ORCID ID**
D. Boyacı https://orcid.org/0000-0002-9897-0616  
P.B. Kavur https://orcid.org/0000-0002-2422-5680  
Ş. Gulec https://orcid.org/0000-0002-6789-1050  
A. Yemenicioğlu https://orcid.org/0000-0002-5356-0058

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   https://doi.org/10.1111/ijfs.14363
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https://doi.org/10.1021/jf0633239

https://doi.org/10.1016/j.foodchem.2006.07.050

https://doi.org/10.1017/S0022029905001639


Table 1. Shear test properties of gels

<table>
<thead>
<tr>
<th>Gel samples</th>
<th>Firmness/N</th>
<th>Work to shear/(N-s)</th>
<th>Cutting strength/(N/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL</td>
<td>(10.94±0.55)c</td>
<td>(33.32±1.28)e</td>
<td>(0.44±0.02)c</td>
</tr>
<tr>
<td>GEL+GTE</td>
<td>(10.82±0.67)c</td>
<td>(31.58±1.25)e</td>
<td>(0.43±0.03)c</td>
</tr>
<tr>
<td>GEL+LYS+GTE</td>
<td>(14.44±0.54)bc</td>
<td>(41.94±1.05)d</td>
<td>(0.58±0.02)bc</td>
</tr>
<tr>
<td>GEL/RS</td>
<td>(13.12±1.23)c</td>
<td>(50.95±2.01)c</td>
<td>(0.52±0.05)c</td>
</tr>
<tr>
<td>GEL/RS+GTE</td>
<td>(13.82±0.67)bc</td>
<td>(49.40±1.44)c</td>
<td>(0.55±0.03)bc</td>
</tr>
<tr>
<td>GEL/RS+LYS+GTE</td>
<td>(14.30±0.73)bc</td>
<td>(57.98±1.38)b</td>
<td>(0.57±0.03)bc</td>
</tr>
<tr>
<td>GEL/CW</td>
<td>(17.51±1.65)b</td>
<td>(50.38±2.09)c</td>
<td>(0.71±0.06)b</td>
</tr>
<tr>
<td>GEL/CW+GTE</td>
<td>(22.83±2.73)a</td>
<td>(60.15±4.38)b</td>
<td>(0.91±0.10)a</td>
</tr>
<tr>
<td>GEL/CW+LYS+GTE</td>
<td>(24.91±1.74)a</td>
<td>(72.50±2.09)a</td>
<td>(0.99±0.07)a</td>
</tr>
</tbody>
</table>

Different superscripted lowercase letters in the same column indicate significant difference (p<0.05). Values in the parenthesis are presented as mean value±S.E. (N=8). (GEL=gelatine, RS=rice starch, CW=candelilla wax, LYS=lysozyme, GTE=green tea extract)
Table 2. Colour properties of gels

<table>
<thead>
<tr>
<th>Gel samples</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL</td>
<td>(37.49±0.22)</td>
<td>(0.62±0.04)</td>
<td>(14.78±0.24)</td>
</tr>
<tr>
<td>GEL+GTE</td>
<td>(19.87±0.27)</td>
<td>(6.28±0.27)</td>
<td>(13.11±0.53)</td>
</tr>
<tr>
<td>GEL+LYS+GTE</td>
<td>(25.04±0.08)</td>
<td>(-0.99±0.06)</td>
<td>(7.90±0.18)</td>
</tr>
<tr>
<td>GEL/RS</td>
<td>(25.68±0.20)</td>
<td>(-0.02±0.01)</td>
<td>(2.10±0.04)</td>
</tr>
<tr>
<td>GEL/RS+GTE</td>
<td>(24.16±0.32)</td>
<td>(-0.44±0.03)</td>
<td>(7.63±0.27)</td>
</tr>
<tr>
<td>GEL/RS+LYS+GTE</td>
<td>(31.71±0.05)</td>
<td>(-1.66±0.007)</td>
<td>(11.10±0.10)</td>
</tr>
<tr>
<td>GEL/CW</td>
<td>(89.41±0.22)</td>
<td>(-0.35±0.02)</td>
<td>(13.29±0.07)</td>
</tr>
<tr>
<td>GEL/CW+GTE</td>
<td>(77.19±0.27)</td>
<td>(-0.74±0.05)</td>
<td>(31.94±0.13)</td>
</tr>
<tr>
<td>GEL/CW+LYS+GTE</td>
<td>(79.81±0.30)</td>
<td>(-0.98±0.03)</td>
<td>(25.14±0.46)</td>
</tr>
</tbody>
</table>

Different superscripted lowercase letters in the same column indicate significant difference (p<0.05). Values in the parenthesis are presented as mean value±S.E. (N=5). (GEL=gelatine, RS=rice starch, CW=candelilla wax, LYS=lysozyme, GTE=green tea extract)

Table 3. LYS and GTE release properties of different gels in distilled water at 4 °C

<table>
<thead>
<tr>
<th>Gel Samples</th>
<th>Max. released activity/(U/g)*</th>
<th>Initial release rates/(U/(g·h))**</th>
<th>Recovery/%****</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL+LYS</td>
<td>(119071±1561)</td>
<td>10055</td>
<td>16.0</td>
</tr>
<tr>
<td>GEL+LYS+GTE</td>
<td>(89283±6204)</td>
<td>7099</td>
<td>12.0</td>
</tr>
<tr>
<td>GEL/RS+LYS</td>
<td>(138314±4016)</td>
<td>8078</td>
<td>18.6</td>
</tr>
<tr>
<td>GEL/RS+LYS+GTE</td>
<td>(94130±2222)</td>
<td>6624</td>
<td>12.6</td>
</tr>
<tr>
<td>GEL/CW+LYS</td>
<td>(147070±4500)</td>
<td>12568</td>
<td>19.7</td>
</tr>
<tr>
<td>GEL/CW+LYS+GTE</td>
<td>(101241±1766)</td>
<td>6746</td>
<td>13.6</td>
</tr>
</tbody>
</table>

Max. phenolics released/(mg catechin/g)* | Initial release rates/(mg catechin/(g·h))*** | Recovery/%****
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL+GTE</td>
<td>(2.05±0.06)</td>
<td>0.087</td>
</tr>
<tr>
<td>GEL+LYS+GTE</td>
<td>(2.03±0.04)</td>
<td>0.083</td>
</tr>
<tr>
<td>GEL/RS+GTE</td>
<td>(1.96±0.02)</td>
<td>0.079</td>
</tr>
<tr>
<td>GEL/RS+LYS+GTE</td>
<td>(1.74±0.03)</td>
<td>0.069</td>
</tr>
<tr>
<td>GEL/CW+GTE</td>
<td>(1.97±0.05)</td>
<td>0.081</td>
</tr>
<tr>
<td>GEL/CW+LYS+GTE</td>
<td>(1.99±0.02)</td>
<td>0.085</td>
</tr>
</tbody>
</table>
Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Different superscripted lowercase letters in the same column indicate significant difference (p<0.05). Values in the parenthesis are presented as mean value±S.E. (N=3). *Maximum released lysozyme activity and phenolics content in distilled water reached at 120th h and 15th days, respectively. **Time periods (h) of data used in best fit were between 0 and 6 h (R²: 0.87-0.99). ***Time periods (h) of data used in best fit were between 0 and 24 h (R²: 0.56-0.71). ****The addition of 1 % lysozyme and green tea extract in gels provided 7447194 U initial lysozyme activity and 3.29 mg catechin equivalent initial total polyphenols per g of gels. (GEL=gelatine, RS=rice starch, CW=candelilla wax, LYS=lysozyme, GTE=green tea extract).

Table 4. The antimicrobial activity of the gels against *Listeria innocua* during 48 h of storage at 4 °C

<table>
<thead>
<tr>
<th>Gel Samples</th>
<th>N/(log CFU/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t/h</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>(3.77±0.2)a,B (4.25±0.17)a,B (5.64±0.46)a,A</td>
</tr>
<tr>
<td>GEL</td>
<td>(3.15±0.6)a,B (3.83±0.15)bde,B (4.79±0.44)bdc,A</td>
</tr>
<tr>
<td>GEL+LYS</td>
<td>(3.2±0.57)a,A (3.52±0.36)ef,A (3.29±0.21)fg,A</td>
</tr>
<tr>
<td>GEL+GTE</td>
<td>(3.65±0.06)a,B (3.87±0.38)bcd,B (4.55±0.18)cda,A</td>
</tr>
<tr>
<td>GEL+LYS+GTE</td>
<td>(3.39±0.12)a,A (3.65±0.13)cd,A (3.44±0.13)d,A</td>
</tr>
<tr>
<td>GEL/RS</td>
<td>(3.59±0.16)a,C (4.08±0.14)ab,B (5.06±0.03)b,A</td>
</tr>
<tr>
<td>GEL/RS+LYS</td>
<td>(3.30±0.43)a,A (3.43±0.26)f,A (3.02±0.08)g,A</td>
</tr>
<tr>
<td>GEL/RS+GTE</td>
<td>(2.99±0.69)a,B (3.98±0.15)abcd,A (3.77±0.16)e,A</td>
</tr>
<tr>
<td>GEL/RS+LYS+GTE</td>
<td>(3.53±0.21)a,A (3.58±0.23)def,A (3.05±0.07)g,B</td>
</tr>
<tr>
<td>GEL/CW</td>
<td>(2.99±0.7)a,B (4.07±0.1)ab,A (4.45±0.2)g,A</td>
</tr>
<tr>
<td>GEL/CW+LYS</td>
<td>(3.56±0.24)a,A (3.58±0.08)d,A (3.05±0.08)g,B</td>
</tr>
<tr>
<td>GEL/CW+GTE</td>
<td>(3.43±0.38)a,A (3.86±0.4)abcd,A (3.03±0.05)g,A</td>
</tr>
<tr>
<td>GEL/CW+LYS+GTE</td>
<td>(3.04±0.62)a,B (3.77±0.21)bcde,A (3.28±0.03)g,AB</td>
</tr>
</tbody>
</table>

Different superscripted lowercase letters in the same column indicate significant difference (p<0.05). A-C Different superscripted capital letters in the same row indicate significant difference (p<0.05). *Values in the parenthesis are presented as mean value ± S.E. (N=3). (GEL=gelatine, RS=rice starch, CW=candelilla wax, LYS=lysozyme, GTE=green tea extract).
Fig. 1. Water binding capacities of the gels. (GEL=gelatine, RS=rice starch, CW=candelilla wax)
Fig. 2. Release profiles of a) lysozyme released from gels with lysozyme, b) lysozyme released from gels with lysozyme-green tea extract combination, c) phenolic compounds released from gels with green tea extract, d) phenolic compounds released from gels with lysozyme-green tea extract combination. (GEL=gelatine, RS=rice starch, CW=candelilla wax, LYS=lysozyme, GTE=green tea extract, CAT=catechin)
Fig. 3. Antioxidant (a=ORAC, b=TEAC, c=ICC), antihypertensive (d), and antidiabetic (e=against HSA, f=against AGH) activities of the gels. a–d Different superscripted lowercase letters indicate significant difference (p<0.05). (GEL=gelatine, RS=rice starch, CW=candelilla wax, LYS=lysozyme, GTE=green tea extract)
Fig. 4. Effect of green tea extract and lyophilized release media of gels with green tea extract on viability of Caco-2 cells. a-d Different superscripted lowercase letters indicate significant difference between the viability values of a single extract at different concentrations (p<0.05). A-C Different superscripted capital letters indicate significant difference between the viability values of a different extracts at single concentration (p<0.05). (GEL=gelatine, RS=rice starch, CW=candelilla wax, LYS=lysozyme, GTE=green tea extract)

Table S1. Water binding capacity (WBC) of gels.

<table>
<thead>
<tr>
<th>Gel samples</th>
<th>WBC/(g/g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL</td>
<td>(0.45±0.003)b</td>
</tr>
<tr>
<td>GEL/RS</td>
<td>(0.54±0.011)a</td>
</tr>
<tr>
<td>GEL/CW</td>
<td>(0.31±0.005)c</td>
</tr>
</tbody>
</table>

*Values are presented as mean value±S.E. (N=3). Data referred to 10 days after water immersion of gels. a-c Different superscripted lowercase letters indicate significant difference (p<0.05). (GEL=gelatine, RS=rice starch, CW=candelilla wax)
Fig. S1. Force vs time graphs of a) control gels, b) gels with green tea extract, and c) gels with lysozyme-green tea extract combination during shear test. (GEL=gelatine, RS=rice starch, CW=candelilla wax, LYS=lysozyme, GTE=green tea extract)

Fig. S2. Photos of the gels: a) gelatine b) gelatine/starch, c) gelatine/wax, d) gelatine with green tea extract, e) gelatine/starch with green tea extract, f) gelatine/wax with green tea extract, g) gelatine with lysozyme-green tea extract combination, h) gelatine/starch with lysozyme-green tea extract combination, i) gelatine/wax with lysozyme-green tea extract combination
Fig. S3. Photos of gels with strawberries, a) gelatine gel with green tea extract, b) gelatine/starch gel with green tea extract, c) gelatine/wax gel with green tea extract)