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Development of gel-based pads loaded with lysozyme and green tea extract: Characterization of pads and test of their antilisterial potential on cold-smoked salmon

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ABSTRACT

The objective of this work is to develop antilisterial gel-based pads (g-pad) for active packaging of cold-smoked salmon. Lysozyme (LYS) and/or green tea extract (GTE) (1% w/w) were incorporated into g-pads of gelatin (GEL), and its mechanically improved composite g-pads obtained by mixing GEL with rice starch (GEL/RS) or candelilla wax (GEL/CW). GEL g-pad with LYS and GEL/RS g-pad with LYS or LYS + GTE, and GEL/CW g-pad with LYS + GTE caused ≥ ~1.8 and ~1.7 log reduction in broth media against *Listeria innocua* within 11 and 15 days, respectively. All g-pads with LYS or LYS + GTE inhibited *L. innocua* growth, and gave cold-smoked salmon with 1.5–1.9 log lower Listeria load than controls after 15 days at +4 °C. GEL g-pad with LYS was the most potent on salmon surface since it achieved faster LYS release (1.3–1.8-fold) than other g-pads. Sustained release of GEL/RS g-pads for LYS, and GEL/CW g-pads for GTE was promising for extended storage. Composite g-pads were more compatible with GTE than GEL g-pads since they prevented discoloration of polyphenols. Water absorption capacities showed that GEL/RS g-pads (75%) could prevent drip-loss better than GEL (59%) and GEL/CW (57%) g-pads. Active g-pads provide alternative to active edible films to reduce listeriosis from processed high-risk food.

1. Introduction

Listeria monocytogenes is a critical pathogen that causes deadly infections in susceptible individuals such as pregnant women, elderly people and people with the suppressed immune system (Vázquez-Boland et al., 2001). The control of *L. monocytogenes* in cold-smoked salmon is a challenging industrial problem since this pathogen exists in different zones of smoked-fish processing plants, and it can grow even at the refrigeration temperatures with or without the presence of oxygen (Ausekar & Butler, 2017; Dass, Abu-Ghannam, Antony-Babu, & Cummins, 2010; Rørvik, Aase, Alvestad, & Caugant, 2000). Vacuum packaging (VP) is not considered as an effective inhibitory hurdle against *L. monocytogenes* (Aymerich, Rodríguez, Garriga, & Bover-Cid, 2019; Duffes, 1999; Gram, 2001). The MAP (70% CO_2 and 30% N_2) helps to control *L. monocytogenes* growth in cold-smoked salmon slices during refrigerated storage (Nilsson, Huss, & Gram, 1997), but the protective effect of this packaging method ends after opening of the package by the end-user.

The antimicrobial edible packaging with whey protein, zein and gelatin films has been applied to prevent Listeria development in cold-smoked salmon (Albertos et al., 2017; Boyacı, Korel, & Yemenicioğlu, 2016; Boyacı & Yemenicioğlu, 2018; Min, Rumsey, & Krochta, 2008). However, studies on using precast edible gel-based pads (g-pads) (or hydrogel pads) in antimicrobial packaging of cold-smoked salmon and other food are scarce. A precast sheet of g-pad could be employed not only as an absorbent pad to bind drip-loss fluids from food, but it could also be used as a high capacity reservoir to deliver active compounds (antimicrobials, antioxidants, bioactive substances) onto food surface (Batista et al., 2018). Hydrogels can be precast directly on surface of plastic packaging films, plates or plastic trays that will be used in food packaging. Alternatively, thin slices of hydrogels obtained from large precast gel blocks could be placed at surface(s) of food, or among layers or slices of food.

Gelatin (GEL) is one of the most preferred gelling agents in the food industry since it is abundant, low priced, and it gives highly elastic, transparent and reversible gels (Osorio, Bilbao, Bustos, & Alvarez, 2007; Spizzirri et al., 2009). However, the low mechanical stability of GEL gels interferes with their application as an active precast g-pad (Ali

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& Ahmed, 2018). The main objectives of this study were to develop different antilisterial g-pads from precast GEL and its more elastic and mechanically stable composites obtained with rice starch (GEL/RS) and candelilla wax (GEL/CW), and to test the effectiveness of developed active g-pads on cold-smoked salmon. *L. innocua* was used as surrogate of *L. monocytogenes* in cold-smoked salmon to eliminate contamination risks in the laboratory (Lebow et al., 2017; Sabanadesan, Lammerding, & Griffiths, 2000). Lysozyme (LYS) and green tea extract (GTE) were used alone or in combination to develop antilisterial g-pads since these natural preservatives are proved antilisterial agents (Kim, Ruengwilysup, & Fung, 2004; Lee, Gwon, Kim, & Moon, 2009; Min et al., 2008; Vodnar, 2012; Yemenicioglu, 2017; Ünalan, Arcan, Korel & Yemenicioglu, 2013). The originality of this work is that it uses antilisterial g-pads to reduce risk of listeriosis from cold-smoked salmon, a popular ready-to-eat seafood.

2. Materials and methods

2.1. Materials

GEL (Type B, 225 g Bloom gel strength), RS, CW and LYS from hen egg white (Activity: ≥40,000 U/mg) were obtained from Sigma (St. Louis, MO, USA). Green tea extract (100%) with minimum 22% total polyphenol content (Product information: assayed by photometric method according to ISO14502-1) was obtained from Wild Flavors and Specialty Ingredients (Rudolf Wild GmbH & Co. KG, Eppelheim, Germany). *Listeria innocua* (NRRL B-33314) used in antimicrobial tests was obtained from United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit (Peoria, IL, USA). Vacuum packed cold-smoked salmon (LERÖY Turkey Su Urunleri San.ve Tic.A.Ş., Turkey) was obtained from a local supermarket in Izmir, Turkey.

2.2. Preparation of gel-based pads

The solutions of gels were prepared by stirring the GEL in water (15%, w/w) and heating it up to 55 °C on a hotplate. The solutions for composite gels with RS were prepared by adding RS into GEL solution at a concentration of 7.5% (w/w). The GEL and RS containing GEL solutions were then homogenized at 10,000 rpm for 1 min using a homogenizer (Heidolph, Germany, rotor Φ = 6.6 mm tip). After that, the solutions were immersed into a water bath at 85 °C for 30 min. On the other hand, the solutions of composite gel with CW were prepared by adding wax component into GEL solution after stirring and then immersing in a water bath at 85 °C for 30 min to melt the wax. The mixture was then homogenized at 10,000 rpm for 1 min to distribute melted wax within the gel solution. LYS and GTE were added into the gel solutions at a concentration of 1% (w/w) after cooling it down to room temperature. Final mixtures were further homogenized at 10,000 rpm for 4 min to distribute active agents homogenously. The g-pads used in water holding capacity (thickness:1.3 cm, diameter: 2.2 cm) and rheological measurements (thickness: 0.3 cm, diameter: 2.2 cm) were prepared by pouring the gel solutions into cylindrical polystyrene molds with 2.2 cm diameter. G-pads used in other tests (release tests, antilisterial activity tests and water absorption capacity) (thickness: 0.5 cm, diameter: 6.6 cm) were prepared by pouring 10 g of gel solution into plastic Petri dishes with a diameter of 6.6 cm. The colored photographs and ESEM images were from small cylindrical gel blocks (height: 13 mm, diameter: 22 mm) obtained by pouring gels into plastic cell culture plates. The poured gel solutions were then kept in a refrigerator at 4 °C for 18 h for g-pad formation.

2.3. Physicochemical properties of gel-based pads

2.3.1. Rheological properties

Dynamic oscillatory rheological measurements were conducted using AR-2000 rheometer (TA Instruments, New Castle, DE, USA) with 2.5 cm diameter parallel plates. The discs of g-pads (thickness: 0.3 cm, diameter: 2.2 cm) were loaded between the plates, and stress-sweep test was performed under 10 Pa to determine their linear viscoelastic range. Frequency sweep test was run from 0.1 to 25 Hz, and 1 Hz frequency was selected for the test. Before each measurement, 1 s^{-1} pre-shear and 1 min equilibration at 25 °C were applied. To determine the melting temperatures (T_m), following an equilibration step, samples were heated from 25 °C to 75 °C at a 5 °C min^{-1} heating rate. Storage modulus (G'), loss modulus (G") and Tan δ (G"/G') were determined to evaluate the viscoelastic nature of the gels. T_m values were estimated from the temperature point where Tan δ was equal to 1. Average of three measurements was used in the calculations.

2.3.2. Water absorption capacity

The water absorption capacity (WAC) of g-pads was determined by incubating gels in distilled water at 4 °C and monitoring the percentage increase in their weights. Briefly, discs of gels (thickness: 0,5 cm; diameter: 6.6 cm) were placed into 50 mL of deionized water and they were incubated under shaking at 80 rpm and 4 °C for 8 days, until the equilibrium (samples were weight after 0.25, 1, 2, 5, 7, 8 days). Average of two measurements was used in the calculations.

2.3.3. Morphology

The internal surface morphologies of slices cut from middle parts of gel blocks were observed by environmental scanning electron microscopy (ESEM) (FEI Quanta 250 FEG, Oregon, USA) under 300 Pa and at 1000x magnification. All samples were cut carefully with a sharp razor from internal surface of small cylindrical gel blocks (height: 13 mm, diameter: 22 mm) and examined at room temperature.

2.3.4. Appearance

The visual appearance was evaluated by taking high quality colored photos (Canon EOS 500D digital SLR camera with compact-macro lens EF-S 18–55 mm f3.5–5.6 IS). The cylindrical gel blocs (height: 13 mm, diameter: 22 mm) GEL, GEL/RS and GEL/CW (with or without LYS and/or GTE) were precast for photographing to observe their color and transparency more clearly.

2.4. LYS and GTE release profiles of gel-based pads on packed cold-smoked salmon

Discs (thickness: 0.5 cm, diameter: 6.6 cm) of different g-pads were placed on both sides of the disk-shaped cold-smoked salmon slices (thickness: 4.02 ± 0.4, diameter: 6.6 cm, weight: 10 g). Each of g-pad coated cold-smoked salmon samples was tightly wrapped firstly with a polyvinylidene chloride (PVDC) stretch film and then secondly with an aluminum foil. The samples were stored at 4 °C and tested for their LYS activity and total phenolic content at 5th, 10th and 15th days of cold storage. For LYS and GTE assays, g-pads were removed and salmon samples (10 g) were homogenized for 1 min with 100 mL of 0.05 M PBS (pH 6.0) or with 100 mL of aqueous ethanol (50% v/v) using a blender (Waring, USA), respectively. The homogenates were then centrifuged at 10,000 g for 10 min, and the obtained supernatants were filtered before they were used for analysis. The LYS activity of samples was measured as described in Boyacı et al. (2016). The total phenolic content of samples was determined according to Folin-Ciocalteu method given by Singleton and Rossi (1965). The total LYS activity and total phenolic content released from each g-pad correspond to maximum units (U) and maximum phenolic content (mg catechin (CAT) equivalents) released per g of g-pad, respectively. The initial release rates of LYS and GTE phenolics were determined from the slope of the initial linear portion of the release curves as U/g/h and mg CAT/g/h, respectively. The recoveries were determined from following formula: (Total released LYS activity or phenolic content from g-gels/Total activity of LYS or total GTE content added into g-gels) x 100. The activity of LYS (74,000 U/

mg) and polyphenol content of GTE (340 μg CAT/mg) added into g-gels were determined according to the given methods by dissolving samples in 0.05 M PBS (pH 6.0) and aqueous ethanol (50% v/v), respectively. The measurements were performed as two replicates and three parallels.

2.5. Antilisterial activity of gel-based pads

2.5.1. Antilisterial activity in broth media

The discs of control g-pads and g-pads containing LYS and/or GTE (thickness: 0.5 cm, diameter: 6.6 cm) were placed in 100 mL of nutrient broth inoculated with cold-adapted *L. innocua* culture (10^6 CFU mL^{-1}) in Erlenmeyer flasks under aseptic conditions. The flasks were incubated at 4 °C under continuous shaking at 80 rpm. The enumeration was conducted at 0^{th}, 7th, 11th and 15th days by the spread-plate method, using Oxford Listeria selective agar (Merck, Darmstadt, Germany). Spread plates were incubated at 37 °C for 48 h and counts were expressed as logarithms of colony-forming unit (Log CFU) per mL of nutrient broth (Log CFU·mL^{-1}). At least three plates were enumerated for the calculations.

2.5.2. Antilisterial activity on packed cold-smoked salmon

The cold-adapted inoculum (0.15 mL at 10^6 CFU mL^{-1}) was spread on both surfaces of disk shaped cold-smoked salmon slices (thickness: 4.02 ± 0.4, diameter: 6.6 cm, weight: 10 g) with a sterile plastic rod. The inoculated slices were kept under safety cabinet for 10 min (for each side) for absorption of the inoculum. Discs (thickness: 0.5 cm, diameter: 6.6 cm) of different g-pads were then placed on both sides of inoculated salmon slices. After that, the coated smoked salmon samples were tightly wrapped firstly with PVDC stretch film and then secondly with an aluminum foil. The packed cold-smoked salmon samples, prepared in duplicate, were stored at 4 °C and their *L. innocua* counts were determined at 0^{th}, 5th, 10th and 15th days. For determination of *L. innocua* counts, 10 g of salmon sample (after g-pad was removed) was placed into a stomacher bag with 90 mL sterile 0.1% peptone water, and it was homogenized using a stomacher (BagMixer ® 400, Interscience, France) for 60 s. The serial decimal dilutions from this homogenate were spread-plated (0.1 mL) onto Oxford Listeria Selective Agar (Merck, Darmstad, Germany). The plates were incubated at 37 °C for 48 h and enumerated on triplicate plates. The results were expressed as Log CFU per g of salmon.

2.6. Statistical analysis

Statistical analysis was performed by using MINITAB® release 17 (Minitab Inc., State College, Pa., U.S.A.). The mean values obtained from the analyses were analyzed by one-way analysis of variance (ANOVA). The significance threshold was $P < 0.05$. Results were given as "mean ± standard error".

3. Results and discussion

3.1. Physicochemical characteristics of gel-based pads

3.1.1. Rheological properties

The storage modulus (elastic response) (G'), loss modulus (viscous response) (G"), Tan δ (G"/G') and melting temperature (Tm) of g-pads are given in Table 1. The GEL/RS g-pad showed significantly higher G' and G" values than GEL g-pad ($P < 0.05$). Thus, it is clear that the GEL/RS g-pad was considerably more viscoelastic than the GEL g-pad. It was reported that a significant increase in G' and G" suggests improved solid-like behavior of a gel (Apichartsrangkoon, 2003) as well as association of biopolymers within a composite gel structure (Lee et al., 2003). This was expected since GEL and RS contain different ionic groups capable to form non-covalent interactions (e.g., H-bonds and charge-charge interactions) within the gel network. As a result of these physicochemical changes the GEL/RS g-pad showed higher mechanical stability than the other g-pads. The GEL/CW g-pad also showed a significantly higher G' than GEL g-pad ($P < 0.05$). The viscous response of GEL/CW g-pad was higher than that of GEL g-pad, but differences between G" values of these two samples were not statistically significant ($P > 0.05$). It is likely that the elastic behavior of GEL/CW was introduced by homogeneously distributed tiny CW particles within the gel matrix. These wax particles also partially interrupted the gelatin-gelatin crosslinking. Moreover, they caused a 1.2 °C lower Tm for GEL/CW g-pad than those of GEL and GEL/RS g-pads. On the other hand, there were no significant differences among the Tan δ of the g-pads ($P > 0.05$). The values of Tan δ called loss factor determined for g-pads were below 1.0, and this indicates the solid-like structure of all gels (Liu, Xu, & Guo, 2007).

Table 1
Rheological properties of different g-pads.

Type of g-pad	G'	G"	Tan δ	T_m (°C)
GEL	1037 ± 17^{b}	120 ± 16^{b}	0.116 ± 0.01^{a}	34.2
GEL/RS	4607 ± 549^{a}	620 ± 174^{a}	0.129 ± 0.03^{a}	34.2
GEL/CW	3544 ± 254^{a}	417 ± 117^{ab}	0.116 ± 0.03^{a}	33

a-b Values at each column followed by the same letter are not significantly different ($P > 0.05$).
*G', G" and Tan δ values measured at 27 °C.

3.1.2. Water absorption capacities

The WAC of g-pads showed some variations (Fig. 1). In the first 5 days, WACs of all g-pads were significantly different from each other ($P < 0.05$) and ranked as follows; GEL/RS, GEL and GEL/CW g-pads. These results suggested differences in intramolecular forces and ability of g-pad matrixes to form hydrogen bonding with water (Young, Wong, Tabata, & Mikos, 2005). It should be reported that the GEL/RS g-pad showed 27–30% and 40–55% higher WAC than GEL and GEL/CW g-pads in the first 5 days, respectively. However, the WACs of g-pads reached an equilibrium on 7th day and they showed only slight changes on 8th day. These results clearly showed that the highly hydrophilic RS caused a significant increase in swelling capacity of GEL/RS g-pad. On the other hand, WAC of GEL g-pad reduced by composite making with CW, a very hydrophobic lipid that is capable to limit diffusion of water within the gel-based matrix.

3.1.3. Morphology and appearance

The surface morphologies of slices cut from middle parts of cylindrical gel blocks were observed using ESEM (Fig. 2). The GEL showed a smooth internal gel surface without any visible pores and aggregates (Fig. 2A–B). However, GEL/RS and GEL/CW showed a rough internal

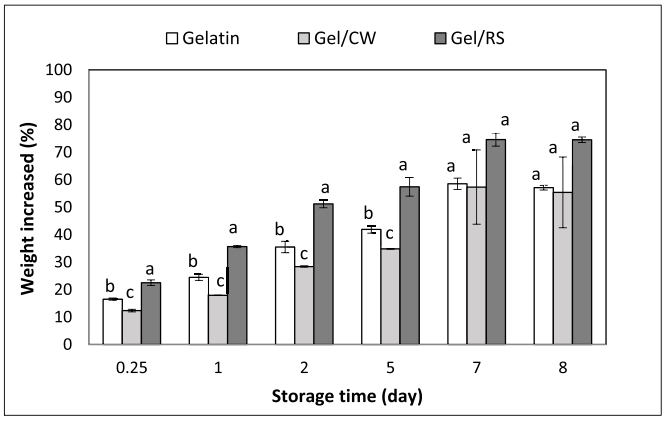

Fig. 1. Water absorption capacity of different g-pads incubated in distilled water at +4 °C.

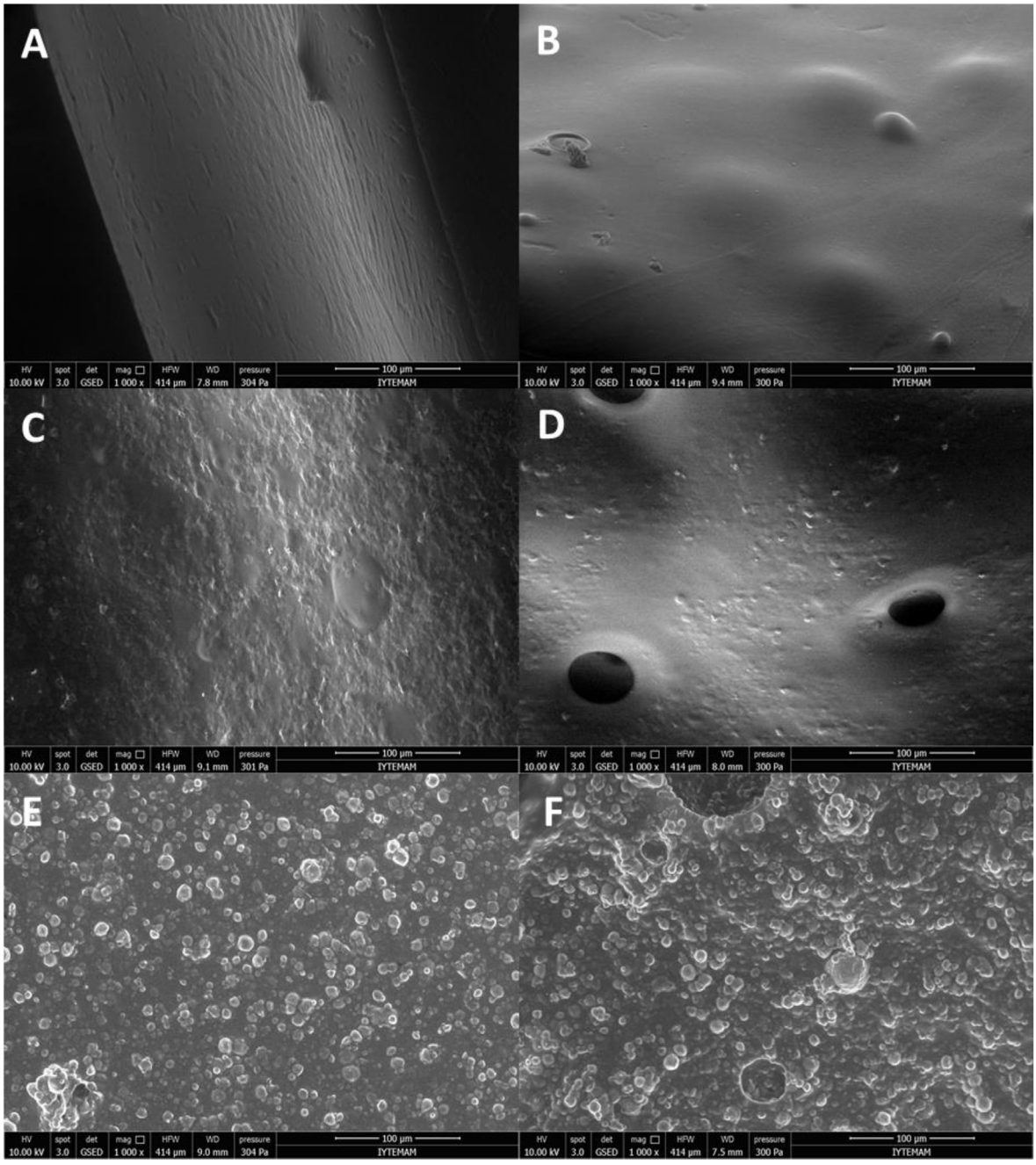

Fig. 2. ESEM surface micrographs for gel slices obtained from internal parts of gel blocks (GEL (A), GEL with LYS + GTE (B), GEL/RS (C), GEL/RS with LYS + GTE (D), GEL/CW (E), GEL/CW with LYS + GTE (F), Magnitude:1000x).

surface due to aggregates/particles formed by starch and wax in these gels (Fig. 2C–F). The RS showed much better distribution in GEL than CW, and it caused a limited roughness within the gel-based structure. In contrast, numerous tiny solid wax particles were observed at GEL/CW surface.

To better reflect colors and transparency of gels employed as g-pad, the photos were also obtained for small cylindrical GEL, GEL/RS and GEL/CW gel blocks (with LYS, GTE or LYS + GTE) (Fig. 3). The control GEL gel block was light brown and transparent while GEL/RS and GEL/CW gel blocks were highly turbid and non-transparent due to large amounts of dispersed RS or CW aggregates/particles within their gel matrix (Fig. 3-A1, B1, C1). The incorporation of GTE or LYS + GTE into GEL gel block caused the formation of a dark greenish to brownish color (Fig. 3-A2, A4) while LYS alone had no detectable effect on the color of GEL gel block (Fig. 3-A3). In contrast, the dark color formation by GTE was not observed in composite gel blocks (Fig. 3-B2, B4, C2, C4). In particular, the color of GTE containing GEL/CW gel blocks that were quite milky and opaque was very light green compared to that of GTE containing GEL gel blocks. It appears that the numerous tiny starch and wax particles within composite gels prevented passing of light from the gels, and this masked the color originated from GTE in gels effectively. Moreover, it seems that the limited light contact of green tea polyphenols in composite gels also prevented their darkening with photodegradation in presence of GEL reactive groups (Grant-Preece, Barril, Schmidtke, & Clark, 2018).

3.2. LYS and GTE release profiles of gel-based pads on packed cold-smoked salmon

The LYS release profiles and parameters of g-pads on cold-smoked

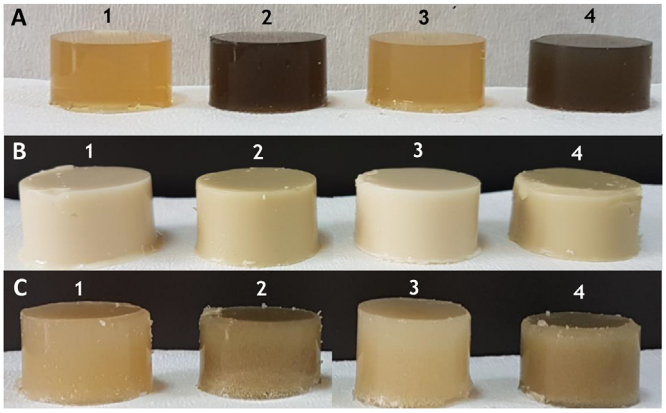

Fig. 3. Photographs of GEL (A1 to A4), GEL/CW (B1 to B4), GEL/RS (C1 to C4) gel blocs. (Control gel blocks: 1; gel blocks with GTE: 2; gel blocks with LYS: 3; gel blocks with LYS + GTE: 4).

salmon are presented in Fig. 4 and Table 2, respectively. The LYS containing GEL g-pad showed the highest initial LYS release rate, and the highest released activity onto salmon samples on the 10th day of storage. The initial LYS release rate of GEL/RS g-pad was lower than that of GEL g-pads. However, both gels delivered similar LYS activity at the end of the storage. This result indicated that the association between RS and GEL, and resulting increased gel networking of GEL/RS g-pad, lowered its initial LYS release rate while maintaining a sustained release for LYS. It should be noted that an equilibrium for LYS release from GEL/CW g-pads was almost reached at the end of storage. On the contrary, the GEL and GEL/RS g-pads did not reach an equilibrium for LYS release within 15 days, hence, they released minimum 1.5-fold higher LYS activity than GEL/CW g-pads between 5th and 15th days. The different release profile of GEL/CW g-pad than the other g-pads could be related to slower swelling rate due to hydrophobic wax particles distributed within its gel matrix. On the other hand, the overall recovery of LYS from all g-pads was low (between 4.8 and 7.6%) since none of the g-pads showed complete swelling on smoked salmon within 15 days. Part of the LYS within g-pads was also retained possibly due to charge-charge attractions between negatively charged GEL (pI: 5.0–7.5) and positively charged LYS (pI: 11.4) at the pH of g-pads (~6.0). In GEL and GEL/RS g-pads, the presence of GTE caused 1.5 and 1.3-fold reduction in LYS release rates, respectively. The addition of GTE also reduced the LYS recovery values. It is known that –OH groups of phenolic compounds could form H-bonds with carbonyl groups of proteins (Damodaran, 1996). Tryptophan (Trp) residues of LYS are capable to bind some green tea polyphenols both by H-bonds and Van der Waals interactions (Ghosh, Sahoo, & Dasgupta, 2008). Therefore, phenolic crosslinking of protein film matrixes and LYS molecules has been previously employed by different workers to achieve sustained release of LYS from GEL (Zhu et al., 2017) and zein films (Arcan & Yemenicioğlu, 2011). Therefore, it appears that the reduced LYS release rates and recoveries of GEL and GEL/RS g-pads with GTE were related with increased networking due to interactions among GTE phenolics, GEL in gel matrixes and LYS. In contrast, initial LYS release rates and recoveries of LYS from GEL/CW g-pads did not change significantly with or without the presence of GTE. This result suggested that the numerous tiny wax particles within these g-pads formed an interrupted gel matrix during swelling (GEL swells while CW particles show no swelling), and this balanced the increased gel networking due to GTE induced crosslinking.

Table 2
LYS and GTE release parameters of g-pads on cold-smoked salmon slices at 4 °C.

	LYS Release Parameters[c]		
Type of g-pad	Max. released activity (U/g)[a]	Initial release rates (U/g/h)[b]	Recovery (%)
GEL + LYS	54,450 ± 3091[a]	4880	7.3
GEL + LYS + GTE	43,052 ± 1915[b]	3216	5.8
GEL/RS + LYS	56,447 ± 3157[a]	3683	7.6
GEL/RS + LYS + GTE	42,007 ± 4310[b]	2742	5.6
GEL/CW + LYS	38,651 ± 1597[bc]	3688	5.2
GEL/CW + LYS + GTE	35,423 ± 2287[c]	3451	4.8
	GTE Release Parameters[c]		
Type of g-pad	Max. phenolics released (mg CAT/g)[a]	Initial release rates (mg CAT/g/h)[b]	Recovery (%)
GEL + GTE	0.28 ± 0.006[bc]	0.020	8.5
GEL + LYS + GTE	0.26 ± 0.002[cd]	0.019	7.9
GEL/RS + GTE	0.19 ± 0.006[e]	0.023	5.8
GEL/RS + LYS + GTE	0.25 ± 0.011[d]	0.018	7.6
GEL/CW + GTE	0.34 ± 0.010[a]	0.025	10.3
GEL/CW + LYS + GTE	0.30 ± 0.008[b]	0.022	9.1

[a-e] Values at each column followed by the same letter are not significantly different ($P > 0.05$).
[a] Maximum LYS activities or GTE content reached at 15th day of storage.
[b] Time periods (h) of data used in best fit were between 0 and 15 days (R^2 for LYS release curve: 0.90–0.99; R^2 for GTE release curve: 0.82–0.97).
[c] The polyphenol content and activity determined for GTE and LYS added into g-pad preparation solutions were 340 μg CAT/mg and 74,000 U/mg, respectively.

The GTE release profiles and parameters of g-pads on cold-smoked salmon are presented in Fig. 5 and Table 2, respectively. The GEL and GEL/RS g-pads showed similar phenolic release profiles. In both GTE and LYS + GTE containing GEL and GEL/RS g-pads, more than half (58–88%) of the total phenolic content released onto salmon within 5 days, but after that phenolic release equilibrated between 5th and 15th days. In contrast, GTE and LYS + GTE containing GEL/CW g-pads showed sustained release properties, and continued phenolic release

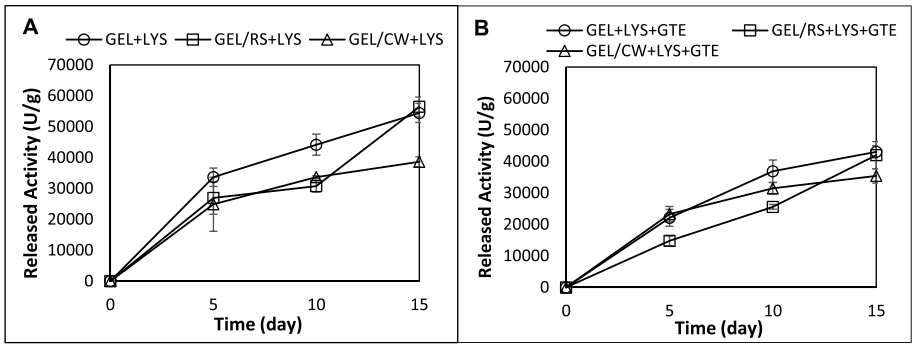

Fig. 4. LYS release profiles of g-pads with LYS (A) and LYS + GTE (B) on cold-smoked salmon slices at 4 °C.

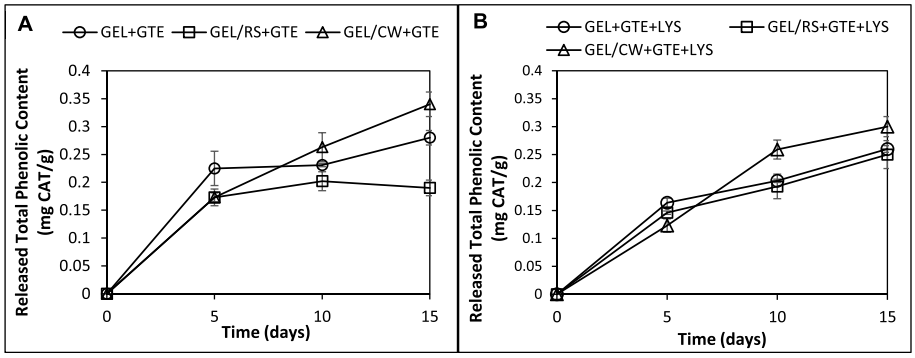

Fig. 5. GTE release profiles of g-pads with LYS (A) and LYS + GTE (B) on cold-smoked salmon slices at 4 °C.

almost at a constant rate for 15 and 10 days, respectively. It seems that the amphiphilic green tea polyphenols retained by the wax particles diffused slowly from matrix of GEL/CW g-pads to cold-smoked salmon surface. Different reports in the literature related to affinity of green tea catechins to hydrophobic lipids support this hypothesis (Barras et al., 2009; Jakobek, 2015; Shishikura, Khokhar, & Murray, 2006). In contrast, the total recoveries of phenolics from GTE and LYS + GTE containing GEL/CW g-pads within 15 days were the highest (10.3%) and second highest (9.1%), respectively. Thus, it appeared that the hydrophobic wax particles also reduce the interaction and binding of GTE by the GEL in composite g-pad matrix.

3.3. Antilisterial activity of gel-based pads in broth media

The results of antimicrobial tests against *L. innocua* are presented in Table 3. No reduction in initial microbial load was observed for control g-pads lacking any active agents, and g-pads with only GTE during 15 days of storage. However, all g-pads with GTE had growth inhibitory activity and showed significantly lower *L. innocua* counts than their own controls for minimum 11 days. The inhibitory effect of GTE on Listeria species was reported in various studies (Theivendran, Hettiarachchy, & Johnson, 2006; Si et al., 2006; Giménez, López de Lacey, Pérez-Santín, López-Caballero & Montero, 2013). However, some workers reported only a reduction in growth rates of Listeria in presence of GTE (Kim et al., 2004; Von Staszewski, Pilosof, & Jagus, 2011). It was reported that the antimicrobial potency of GTE is highly affected from its polyphenol composition as well as presence of interacting constituents in the medium such as proteins (Von Staszewski et al., 2011). On the other hand, the g-pads containing LYS or LYS + GTE showed a potent antilisterial activity and caused minimum 1.5 decimal (D) reduction in initial *L. innocua* counts within 15 days. These results clearly showed that the LYS was a more potent antilisterial agent than the GTE at the test conditions. However, the potency of LYS containing g-pads against *L. innocua* showed a great variation. For example, the *L. innocua* counts in presence of GEL with LYS, GEL/RS with LYS, and GEL/RS with LYS + GTE dropped below 1 log CFU·mL^{-1} within 11 days. The GEL with LYS showed the highest LYS release rates in tests conducted on food surface (see Table 2). Thus, it was possible that these g-pads owed their high antimicrobial activity in broth media also to their rapid LYS release. On the other hand, the high antimicrobial activity of GEL/RS g-pad with LYS or LYS + GTE should be related with their high WAC that enabled more interaction of these g-pads with the broth and suspended bacteria. The GEL g-pad with LYS + GTE, and GEL/CW g-pad with LYS + GTE were less potent than the three g-pads specified above since they dropped *L. innocua* counts below 1 log CFU·mL^{-1} within 15 days. The GEL/CW g-pad with LYS was the least potent of all g-pads with LYS or LYS + GTE. This should be related to the limited interaction of these lipid incorporated hydrophobic g-pads with the broth, and low amounts of LYS release from these g-pads as observed in the release tests. The GTE had no positive contribution on antilisterial activity of LYS in GEL and GEL/RS g-pads. It seemed that the interactions created by GTE within the g-pads also interfered with their soluble LYS content and resulting antilisterial activity in broth. In contrast, GEL/CW g-pads with LYS + GTE showed significantly higher antilisterial activity than GEL/CW g-pads with LYS. GEL/CW g-pads with GTE also showed significantly higher antilisterial capacity than GEL and GEL/RS g-pads with GTE. Thus, it appeared that the hydrophobic environment provided by CW in the composite g-pads

Table 3
Antimicrobial activity of g-pads against *L. innocua* in broth media stored at 4 °C.

	Log cfu/mL			
Type of g-pad	Day 0	Day 7	Day 11	Day 15
Control	2.69 ± 0.08^{a,D}	4.63 ± 0.15^{a,C}	6.57 ± 0.05^{a,B}	7.88 ± 0.04^{a,A}
GEL	2.69 ± 0.11^{a,D}	3.62 ± 0.04^{c,C}	4.31 ± 0.05^{c,B}	5.1 ± 0.03^{c,A}
GEL + LYS	2.76 ± 0.05^{a,A}	1.63 ± 0.15^{f,B}	< 1^{a}	< 1
GEL + GTE	2.75 ± 0.09^{a,C}	3.44 ± 0.03^{c,B}	4.00 ± 0.16^{d,A}	4.29 ± 0.11^{d,A}
GEL + LYS + GTE	2.56 ± 0.05^{a,A}	2.29 ± 0.14^{e,A}	1.72 ± 0.12^{fg,B}	< 1
GEL/RS	2.76 ± 0.04^{a,D}	4.28 ± 0.03^{b,C}	5.35 ± 0.02^{b,B}	6.88 ± 0.02^{b,A}
GEL/RS + LYS	2.77 ± 0.11^{a,A}	1.39 ± 0.06^{f,B}	< 1	< 1
GEL/RS + GTE	2.72 ± 0.11^{a,D}	3.49 ± 0.06^{c,C}	4.39 ± 0.03^{c,B}	5.32 ± 0.03^{c,A}
GEL/RS + LYS + GTE	2.83 ± 0.05^{a,A}	1.53 ± 0.25^{f,B}	< 1	< 1
GEL/CW	2.65 ± 0.07^{a,D}	3.28 ± 0.09^{c,C}	4.01 ± 0.05^{d,B}	4.52 ± 0.10^{d,A}
GEL/CW + LYS	2.74 ± 0.07^{a,A}	2.66 ± 0.40^{d,A}	1.89 ± 0.11^{e,B}	1.15 ± 0.08^{e,C}
GEL/CW + GTE	2.60 ± 0.20^{a,B}	2.87 ± 0.10^{d,B}	3.02 ± 0.02^{f,B}	4.25 ± 0.03^{d,A}
GEL/CW + LYS + GTE	2.72 ± 0.08^{a,A}	2.07 ± 0.12^{e,B}	1.58 ± 0.11^{g,C}	< 1

$^{a-g}$ Values at each column followed by the same letter are not significantly different (P > 0.05).
$^{A-D}$ Values at each row with the same letter are not significantly different (P > 0.05).
^{a} Below the detection limit (10 cfu/mL).

Table 4
Antimicrobial activity of g-pads against *L. innocua* inoculated on cold-smoked salmon slices at 4 °C.

Type of g-pad	Log cfu/g			
	Day 0	Day 5	Day 10	Day 15
Trial#1				
Control	3.19 ± 0.12^{a,C}	3.74 ± 0.09^{a,B}	4.06 ± 0.09^{a,B}	4.61 ± 0.06^{a,A}
GEL	3.08 ± 0.08^{a,B}	3.13 ± 0.06^{b,B}	3.24 ± 0.12^{b,B}	4.61 ± 0.01^{a,A}
GEL + LYS	3.15 ± 0.09^{a,A}	2.93 ± 0.08^{b,AB}	2.33 ± 0.15^{d,C}	2.69 ± 0.09^{c,B}
GEL/RS + LYS	3.27 ± 0.10^{a,A}	2.94 ± 0.16^{b,A}	3.00 ± 0.16^{bc,A}	2.91 ± 0.07^{bc,A}
GEL/CW + LYS	3.19 ± 0.12^{a,A}	2.96 ± 0.11^{b,AB}	2.82 ± 0.05^{c,B}	2.98 ± 0.09^{b,AB}
Trial#2				
Control	4.20 ± 0.03^{a,B}	5.54 ± 0.03^{a,A}	5.36 ± 0.12^{a,A}	5.58 ± 0.07^{a,A}
GEL	4.19 ± 0.01^{a,C}	4.66 ± 0.10^{b,B}	4.29 ± 0.03^{b,C}	4.80 ± 0.08^{b,A}
GEL + LYS + GTE	4.04 ± 0.04^{a,B}	4.44 ± 0.04^{c,A}	4.11 ± 0.04^{c,B}	4.22 ± 0.05^{c,B}
GEL/RS + LYS + GTE	4.10 ± 0.10^{a,AB}	4.17 ± 0.06^{d,A}	4.10 ± 0.02^{c,A}	3.96 ± 0.04^{d,B}
GEL/CW + LYS + GTE	4.07 ± 0.07^{a,ABC}	4.22 ± 0.06^{d,A}	3.93 ± 0.03^{d,C}	4.05 ± 0.04^{cd,B}

$^{a-d}$ For each trial values at each column with the same letter are not significantly different ($P > 0.05$).
$^{A-C}$ Values at each row with the same letter are not significantly different ($P > 0.05$).

helped to protect GTE from interactions or modifications that neutralize its antimicrobial activity. In the literature, antilisterial test results conducted with GEL and/or its composite g-pads containing LYS-polyphenol combinations are scarce. LYS incorporated into fish skin gelatin gel alone showed antimicrobial activity against Gram-positive bacteria such as *Baccillus subtilis* and *Streptococcus cremoris,* but not against Gram-negative bacteria (Bower, Avena-Bustillos, Olsen, McHugh & Bechtel, 2006). Therefore, different workers combined LYS with green tea polyphenols to provide antimicrobial activity on both Gram-positive and Gram-negative bacteria (including *L. monocytogenes, E. coli, L. innocua* and *S. aureus*) as well as to prevent lipid oxidation in food systems (Ku, Hong, & Song, 2008; Rawdkuen et al., 2013; Ünalan, Arcan, Korel, & Yemenicioğlu, 2013).

3.4. Antilisterial activity of gel-based pads on packed cold-smoked salmon

The food packaging applications were conducted with g-pads containing LYS or LYS + GTE since g-pads with GTE alone showed a limited antilisterial activity in broth media. Results for *L. innocua* inoculated cold-smoked salmon samples packed with LYS containing g-pads are presented in Table 4 (Trial#1). There were no significant differences among the *L. innocua* counts of samples on the 0th day of cold-storage. However, *L. innocua* counts of samples packed with LYS containing g-pads were significantly lower than those of control samples packed without g-pads on 5th, 10th and 15th days of cold storage. Moreover, it is important to note that the *L. innocua* counts of samples packed with LYS containing g-pads were 1.5–1.7 D lower than those of samples packed with or without control GEL g-pads at the end of 15-day cold-storage. Significant drops in initial microbial loads of samples were observed on 10th and 15th days (0.5–0.8 D) for those coated with LYS containing GEL g-pads, and on 10th day (almost 0.4 D) for those coated with LYS containing GEL/CW g-pads. In contrast, no significant changes were observed in initial *L. innocua* counts of samples coated with LYS containing GEL/RS g-pads within 15 days. As a result, GEL g-pads with LYS was more effective on *L. innocua* than the composite g-pads with LYS. According to release tests, the GEL g-pads had the highest LYS release rates on salmon samples. Therefore, the potency of GEL g-pad with LYS was possibly due to the rapid increase of antimicrobial activity on salmon surface at the beginning of the storage.

L. innocua counts of salmon samples packed with LYS + GTE containing g-pads are also presented in Table 4 (Trial #2). The samples packed with g-pads containing LYS + GTE had significantly lower *L. innocua* counts than those of uncoated controls and controls packed with GEL g-pads lacking any antimicrobials on 5th, 10th and 15th days of cold-storage. However, there were no considerable differences among the antimicrobial potential of GEL, GEL/RS and GEL/CW g-pads with LYS + GTE. All LYS + GTE containing g-pads prevented the growth of *L. innocua* on salmon surface effectively, but none of these g-pads caused a significant reduction in initial Listeria counts of samples within 15 days. Thus, it is clear that the g-pads with LYS were more effective than g-pads with LYS + GTE at the test conditions. The release tests showed that the presence of GTE reduced the free LYS content of g-pads. Thus, larger amounts of LYS could be used in g-pads that contain GTE.

On the other hand, it is also essential to report that on the 15th day of cold-storage, the GTE containing composite g-pads better maintained their light color than GEL g-pads with GTE that turned brown (see Supplementary file 1 that shows circular g-pads with similar sized cold-smoked salmon at the bottom after 15 days of cold storage). The GEL/CW g-pad with LYS + GTE showed hardly detectable limited browning by storage while GEL/RS g-pad with LYS + GTE showed detectable slight browning. The GEL/CW and GEL/RS g-pads with LYS maintained their initial light color and milky appearance while GEL g-gel with LYS is still transparent and colorless (the transparent GEL g-pad reflected the color of cold-smoked salmon at the bottom). These observations proved that the developed composite g-pads (especially GEL/CW) are effective to mask discolorations by phenolic compounds.

The data related to food application of precast g-pads of GEL or its composites incorporated with natural antimicrobial such as LYS and polyphenols are scarce. In contrast, films or coatings of GEL containing different antimicrobials (e.g. combination of LYS-catechin, LYS-nisin-ethylenediaminetetraacetic acid, Na-lactate-Na-diacetate, and olive leaf extract) have been applied to control Listeria on cold-smoked salmon and other food (Albertos et al., 2017; Gill & Holley, 2000; Kaewprachu, Osako, Benjakul, & Rawdkuen, 2015; Ye, Neetoo, & Chen, 2011).

4. Conclusions

This study clearly showed that the precast edible g-pads loaded with natural active compounds could be alternative tools for food industry to prevent Listeria growth in risky food as cold-smoked salmon. The GEL g-pads loaded with LYS showed the highest potency due to their rapid release properties. However, g-pads based solely on GEL showed low mechanical stability and induced discoloration when incorporated with GTE. The combination of LYS with polyphenols is an emerging trend to support limited antimicrobial spectrum of LYS (especially against Gram-negative bacteria) and minimize oxidative changes in food. The composite technology applied by combining GEL with suitable ingredients such as RS and CW could play a central role in improving mechanical stability, masking undesired polyphenol origin discolorations, sustained release properties or water (drip-loss fluid) absorption capacity of g-pads. However, use of composites need careful

adjustments in concentrations of active agents to balance amount of soluble active agents in g-pads. Further studies are continuing in our laboratory to design ready-to-use precast active g-pads at different sizes and shapes (e.g. suitable for those of a standard plate, plastic trays, fish and fish fillet etc.) and to evaluate their antimicrobial performances with food applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.109471.

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