DOCTORAL THESIS

Biological Activity and Physicochemical Properties of Chitosan Film Cross-Linked with Chestnut Extract for Active Food Packaging Applications

Kristi Kõrge

TALLINNA TEHNIKAÜLIKOOL
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TAL TECH PRESS

TALLINN UNIVERSITY OF TECHNOLOGY
School of Engineering
Department of Materials and Environmental Technology
This dissertation was accepted for the defense of the degree 26/04/2021.

Supervisor: Dr. Katrin Laos
Department of Chemistry and Biotechnology
Tallinn University of Technology
Tallinn, Estonia

Co-supervisor: Dr. Uroš Novak
National Institute of Chemistry
Ljubljana, Slovenia

Opponents: Prof. Rajeev Bhat
Estonian University of Life Sciences
Tartu, Estonia

Dr. Valeria Giosafatto
Department of Chemical Sciences
University of Naples "Federico II"
Naples, Italy

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology, has not been submitted for a doctoral or equivalent academic degree.

Kristi Kõrge _____
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Kastani ekstraktiga seotud kitosaankilede bioloogiline aktiivsus, füüsikalis-keemilised omadused ning rakendatavus toidupakendina

KRISTI KÕRGE

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Contents

LIST OF PUBLICATIONS

The present dissertation bases on the following papers:

I. Novak, U., Bajić, M., **Kõrge, K.**, Oberlintner, A., Murn, J., Lokar, K., Triler, K.V., Likozar, B., From waste/residual marine biomass to active biopolymer-based packaging film materials for food industry applications – A review, *Phys. Sci. Rev.* 5 (2019) 1–24.

II. Bajić, M., Oberlintner, A., **Kõrge, K.**, Likozar, B., Novak, U., Formulation of active food packaging by design: Linking composition of the film-forming solution to properties of the chitosan-based film by response surface methodology (RSM) modeling, *Int. J. Biol. Macromol.* 160 (2020) 971–978.

III. **Kõrge, K.**, Bajić, M., Likozar, B., Novak, U., Active chitosan–chestnut extract films used for packaging and storage of fresh pasta, *Int. J. Food Sci.* (2020) 1–10.

IV. **Kõrge, K.**, Šeme, H., Bajić, M., Likozar, B., Novak, U., Reduction in Spoilage Microbiota and Cyclopiazonic Acid Mycotoxin with Chestnut Extract Enriched Chitosan Packaging: Stability of Inoculated Gouda Cheese, *Foods* 9, 1645 (2020a) 1–18.

The articles I–IV with associated supplementary used are attached to the appendix of this thesis and reproduced with the publishers' permission.

AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

Contribution to the papers in this thesis are:

I. Co-author, gathering of the last 5-year research articles regarding antifungal properties of chitosan-based films, manuscript writing, and proofing.

II. Co-author, performance of the experimental work concerning mechanical and active properties of the films, illustration, interpretation of the data.

III. 1st author, performance of the experimental work, interpretation of the data, illustration, writing of the manuscript.

IV. 1st author, performance of the experimental work, interpretation of the data, illustration, writing of the manuscript.

INTRODUCTION

Biodegradable biopolymer-based materials represent future technologies to reduce pollution suppressing today's ecosystem. When conventional synthetic materials consist of long, linear, tightly packed polymer chains durable for tremendous manipulation, the beneficial features do not allow almost any degradation by nature without additional help. To tackle the problem, the biopolymer-based materials in combination with plant-based extract represent a hundred-percentile bio alternative with integrity comparable to conventional plastics and an additional benefit of active components diffusion. Besides, this approach enables the valorization of abundant biomass looking for applications. Even so, the core limitation with the alternative materials is the biopolymers cyclic structures in need of additional cross-linkers, fillers, etc., to form tightly packed film matrices. It could widen to an increased permeability, physical integrity limitations, which are fundamental requirements in the food industry when packing food. A variation of macromolecular ingredients proposed is essential to improve the biopolymer materials' mechanical properties to meet the demands. The look-out is an ongoing process until today due to the seeming lack of decision-making approaches when downsizing a composition linked to material physicochemical properties. Apart from the promising results reported in the literature, further studies are required to understand film's performance on food products, their stability concerning microbial growth, etc. A wide range of natural value-adding extracts for food is yet to be investigated.

Chitosan is a strong film-forming biopolymer combined with various plant-based extracts to produce bioactive foil materials for packing food [Moustafa et al., 2019; Rodrıguez-Rojas et al., 2019; Sabbah et al., 2019; Rajoka et al., 2019]. It is a derivative of chitin biomass, an abundant seafood waste product (crustaceans) collected worldwide 2.5 million tons annually [FAO, 2017]. On top of many, sweet chestnuts (*Castanea sativa*. Mill) heartwood-derived extract is an abundant polyphenol-rich component with the potential to enhance chitosan film physicomechanical antioxidative/antimicrobial properties, i.e., heighten the biofoil's bioactivity. The abundance of this biomass is estimated up to 2.4 million tons annually [FAO, 2019], and various segments (fruits, leaves, galls, bark, and wood) are used for the extract, although the most accessible one from timber processing refineries is heartwood-based [Adamczyk et al., 2017]. When it comes to biomaterial development for food, safety assurance, and its practicality mainly depend on the formulation. Here, the plant extracts intend to play a prominent role assuring the cross-linking and food safety instead of preservatives added to the food, while chitosan builds the matrix of the material. Those component's various properties, concentrations that bestow into film-forming properties, bioactivity, and absorbance capability, determine the biofoil's overall functionality, and this could be effortlessly, time savingly predicted with the help of statistical-mathematical tools. Furthermore, the material's nature could be highly influenced by the macromolecular composition of food in contact. Intermediate moisture food (IMF) could feature food composition high in fat or high in starch, vary in acidity. Both foods with considerable moisture content are differed by the possible ability of fat to act as a natural protective layer against moisture loss in the first food category. On top, food is nutritional support for microorganisms, a plateau for spoilage, and even toxicity. Thus, it is in high essence to determine how newly developed active packaging provides food safety while holding its integrity and

affects the quality of IMF as it is the biggest category packed in conventional plastics (PE, PA, PET, etc.) and plastics composites such as fiber-reinforced plastic, etc.

This thesis aims to develop an approach to assess biomaterial physicochemical properties in relation to its composition. Tannins and polyphenolic components, with their ability to cross-link biopolymers, offer a new possibility, and therefore, the correlations were performed based on a chestnut-extract incorporated chitosan-based material. Compared to other plant extracts, the main advantage of the chestnut extract is the higher concentration of hydrolyzable tannins, high level of extract purity, and solubility. A film was chosen for intermediate moisture food shelf life studies covering high- and low-fat food based on the results.

ABBREVIATIONS, TERMS, AND SYMBOLS

AC	active component
CE	chestnut extract
CE:CH	chestnut extract chitosan blend
CFU	colony forming unit
CH	chitosan
CPA	cyclopiazonic acid
DA	deacetylation degree
EB	elongation at break
EO	essential oil
FFS	film forming solution
FRP	fiber reinforced plastics
G^-	gram-negative bacteria
G^+	gram-positive bacteria
GLY	glycerol
HHDP	hexahydroxydiphenic
HMF	high moisture food
HTs	hydrolyzable tannins
IMF	intermediate moisture food
LAB	lactic acid bacteria
LMF	low moisture food
MAP	modified atmosphere packaging
MC	moisture content
OH	hydrogen-oxygen bond
PA:PE	polyamide polyethylene packaging
PA:PE/CE:CH	two-layer packaging and chestnut extract chitosan biofoil as an inner layer
PA:PE/CH	two layer packaging and chitosan biofoil as inner layer
PE/BOPP/EVOH	polyethylene-biaxially oriented polypropylene-ethylene vinyl alcohol
RSM	response surface methodology
TA	tannic acid
TA:CH	tannic acid chitosan blend
TPC	total phenolic content
TPP	sodium tripolyphosphate
TS	tensile strength
UHPLC	Ultrahigh Performance Liquid Chromatography
FTIR-ATR	Fourier-transform infrared – Attenuated Total Reflectance

Biopolymer material = chitosan-based material = chitosan-based film = biofoil
Biopolymer material composition = film composition

1 LITERATURE OVERVIEW

1.1. Chemical characteristics of the main biopolymer material constituents

1.1.1 Chitosan

Chitosan (CH) is an N-deacetylated derivative of chitin (deacetylation degree (DA) > 50%) with a chemical structure of two subunits D-glucosamine and N-acetyl-D-glucosamine linked linearly with each other *via* β-(1,4)-glycosidic bonds (Figure 1). The overall structural organization of its molecule consists of a minimum of three rings on the sheath without H-atoms. Three functional groups of primary, secondary hydroxyl groups, and amine groups are present in each repeating molecule [Mujtaba et al., 2019] and prone to undergo chemical modifications, which play a vital role in some essential physicochemical characteristics, including solubility and overall mechanical attributes.

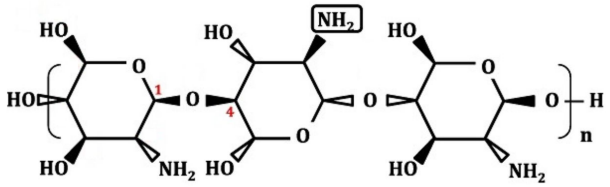

Figure 1. *Chitosan (β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine) chemical structure.*

The source-polymer chitin could be found in arthropods, e.g., shrimps, crabs, lobsters, crayfish, king crabs, insects, as well in mollusks, i.e., squids. It is isolated from raw biomass with chemical-based methods of acidic demineralization and alkaline deproteinization steps. Notably, the constant lookout for a greener solution to extract chitosan is a "hot topic" in research [Publication I]. In nature, there are three allomorphic forms of chitin: anti-parallel α-chitin, parallel β-chitin, and mixed arranged γ-chitin [El Knidri et al., 2018]. Although allomorphic forms of chitosan are not straightforward, they vary after isolation in DA and molecular weights (M_w) [Rinaudo et al., 2006, Mujtaba et al., 2019, Borić et al., 2018]. Throughout the research, chitosan has been described with different average M_w (50–2000 kDa) and DA (40–98%) [Cazón et al., 2019]. Three commercially available derivatives could be distinguished, namely CH_{low} with M_w 50–190 kDa DA 75–85%, CH_{medium} with M_w 190–310 kDa DA 75-85%, and CH_{high} with M_w 310–375 kDa DA >75%. The polysaccharide is one of a kind weak polybase (pKa around 6.5), indicating its charge density variation in the pH range of 6–6.5. This property imparts pH-responsiveness, which is beneficial for various active components (AC) diffusion [Hosseinnejad & Jafari, 2016] when combined with film base material.

1.1.2. Chestnut extract

What makes sweet chestnut heartwood extract (CE) interesting, especially for the food industry [Molino et al., 2020],´ä is its polyphenolic content with a higher share of hydrolyzable tannins [Comandini et al., 2014; Amarowicz et al., 2019] (Figure 2), besides simple sugars and crude fiber [Biagi et al., 2010].

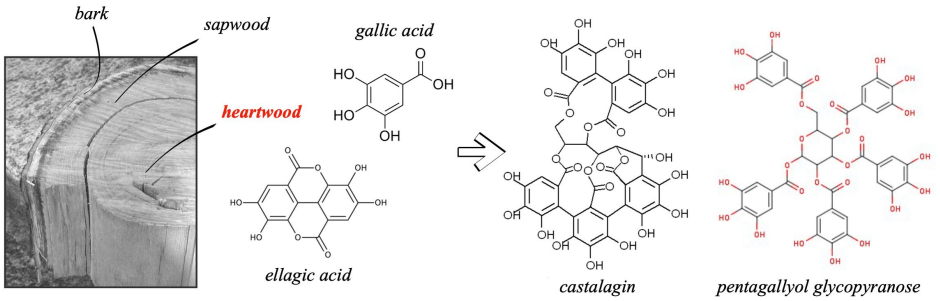

Figure 2. The source of chestnut extract (Castanea sativa Mill.) and its main polyphenolic compounds.

The sweet chestnut (*Castanea sativa*) is the most spread tree species in the Mediterranean area and processed in large quantities in local refineries. The residual wood chipped, steam distilled, and heat-dried into chestnut heartwood tannin extract [Messini et al., 2017]. Analysis of the extracts has shown that the main constituent polyphenolics are gallic and ellagic acid, which forms ellagitannins (castalagin, vescalagin, etc.), latter in turn could form hexahydroxydiphenic acid; and gallotannins (di-, tri-, tetra, pentagalloyl glucopyranose, and di-, trigalloyl-hexahydroxydiphenoyl glucopyranose, etc. The amount and category of those vary with the species, topography, and climate [Sanz et al., 2010].

1.2. Engineering of biopolymer packaging materials

Biopolymer-based packaging materials require the composition of several components and a suitable production method to uphold the expectations of food packaging during shelf life. Aside, biodegradability at the end of the life cycle is crucial [Wang et al., 2018]. Engineering of the chitosan-based films addresses barrier, mechanical, optical, and activity properties in different conditions simultaneously, which leads to a constant search for suitable ingredients for the tasks in the proper ratios.

1.2.1. Conjugations of chitosan and macromolecules

Chitosan bestows beneficial interactions with several macromolecules. For instance, by being able to aggregate with negatively charged molecules of fats (oleic, linoleic, palmitic, stearic, linolenic), it performs ionic complexes (between NH^{2+} and O^{-}), hence strengthens barrier properties [Wydro et al., 2007; Ylitalo et al., 2002]. Chitosan can interact with whey proteins at low pH (< 4.5), where the weak electrostatic attraction plays a dominant role. However, the complexity decreases with the M_w decrease. Depending on the nature of the interaction (co-soluble or associative), the use of proteins can improve heat stability [Ye & Chen, 2019]. Sugars, such as sucrose, sorbitol, glycerol, etc., have multi-hydroxyl moiety structures, and conjugation with chitosan occurs through strong hydrogen bonding. The interactions are known to increase miscibility, ductility, or crystallinity of the blend [Liang et al., 2009; Domján et al., 2009]. Chitosan can be covalently or ionically cross-linked to salts. Covalent cross-linking leads to the formation of permanent structures, while ionic cross-linking forms ionic bridges between the chitosan chains. Both approaches enhance the mechanical properties of the formulated film. One of the widely used ionic cross-linkers is sodium tripolyphosphate, also known as an acceptable food additive. In the presence of monovalent salts (NaCl,

KCl, etc.), the swell ratio of the biopolymer decreases, ascribing to the osmotic pressure in the external solution [Zhang et al., 2013]. Chitosan blending with synthetic polymers (like PVA) results in intramolecular hydrogen bonds promoting the film's fabrication [Wang et al., 2015].

1.2.2. Biopolymers produce hybrid-like materials with polyphenolic constituents

In a similar way to other molecules, cross-linking reactions with natural modifiers (tannins) from plant extracts produce hybrid-like materials. The agents usually used for cross-linking include formaldehyde, glutaraldehyde, and glyoxal, which may pose considerable health hazards [Tomadoni et al., 2019]. Within the main applications regarding polymers, the nanocomposites represent a combination of organic polymer, nanofibers/ -flakes, and tannins [Koopmann et al., 2020]. In the composition, the fibers and organic polymer form a mix-arranged network cross-linked with tannins [Shibata et al., 2010; Wang et al., 2018; Chen et al., 2019]. Interpenetrating networks resemble well-organized organic polymer networks tangled with mix arranged tannins [Kadokawa et al., 2010; Yang et al., 2017]. Most simplistic are the blends of organic polymer and tannins, which are studied on a molecular scale by their ability to form hybrids [Sun et al., 2018; Bajić et al., 2019a]. Layer-by-layer (LbL) multilayers or covalent networks consist of organic polymer/ polyelectrolyte and tannins, prepared according to the name given. The latter is used to produce multilayer microcapsules, network particles, coatings, and even fibers cross-linked physically or covalently [Laos, 2005; Yovcheva et al., 2019; Giosafatto et al., 2020].

Due to their multiple phenolic groups, phenolic acids have been considered cross-linking agents either through Schiff base or Michael-type adducts [Ravi Kumar et al., 2004, Rivero et al., 2010], and the strength of the bonds depends on applications used as brought out earlier.

1.2.3. Film lab-scale fabrication methods and scalability options

Fabrication of the films could be performed through direct casting, coating, dipping, LbL assembly, and extrusion. Due to its simplicity, casting is the most used method for preparing chitosan-based films. With the main instruments of the drying unit/environment and Petri dishes, the film blend is poured with measured volume to achieve uniform thickness and homogeneity of the dried films [Ashrafi et al., 2018; Pires et al., 2018]. Coating technology covers two approaches of spread-coating and spray-coating. These methods can prepare thin layers of chitosan film on the surfaces and protect the chosen objects without excess material. A brush and/or spatula, and tools, such as a compressed air-assisted sprayer, knapsack sprayer, and copper backpack, are used, respectively [Guo et al., 2014]. Dipping or immersing subjects of interest into the chitosan-based solutions has been adopted to develop uniform films on the surface [Han et al., 2005]. The LbL electrostatic deposition technique depends on the electric properties of the components, i.e., polycation chitosan and polyanion, and has been gaining a lot of application by researchers [Yovcheva et al., 2019; Alkekhia et al., 2020]. While applying the method, the pH is an essential factor that affects the formation of LbL films as it can change the electrostatic properties of the materials. The assembly method resembles the immersing process wherein the object is covered with oppositely charged materials several times [Giosafatto et al., 2020; Laos et al., 2006]. Furthermore, biomaterials are durable for melt extrusion where the steps include preparation of different compositions, blending in a mixer with a follow-up blending and mixture in a

twin-screw extruder under designed conditions, cutting the pellets through the pelletizer, drying the pellets in a hot-air oven, and then extruding the pellets into sheets through the twin-screw extruder. According to the literature, the method has been applied successfully on starch: chitosan [Rodriguez Llanos et al., 2021] and fossil-fuel-based polymer plus low amounts of chitosan blends [Wang et al., 2018]. Essential to know is that the chitosan does not have thermoplastic properties and therefore can not be used in the extrusion process, and it needs additional components to be heat-sealable [Cazón et al., 2019].

Overall, the actual appliance of the materials for specific applications (antibacterial films, barrier films, sensing films) is strongly dependent on the detailed characterization of intermediates conjugations during the process and the most feasible production method.

1.3. Characteristic mechanical properties describing biopolymer material

Biopolymer materials may present either escalated stiffness or ductility properties. In the first case, the materials are most suitable for scaffolds or beads when more robust, rigid materials are required, and ductile materials for packaging materials as they would be more enduring to consumer's manipulation [Wahba, 2019; Chen et al., 2018; Matet et al., 2013]. Some materials break very sharply; others may be more pliable, which depends mainly on the composition of the material.

The most important mechanical properties in materials engineering are presented in Figure 3. When the progressive, longitudinal stretching force is applied to the material with specific dimensions (length and width), it starts to resist the workforce or change its molecular structure [Shi et al., 2018]. Chitosan does not have distinguished allomorphic forms, and crystalline or semicrystalline structures are known to form when it is used solely in a film. Resistance of the materials comes from the addition of plasticizers, either glycerol, ionic liquids [Chen et al., 2018], or even deep eutectic solvents [Jakubowska et al., 2021]. Salts (NaCl), in a sense, have been shown to reduce the end-to-end length and increase the flexibility of chitosan chains [Tsai et al., 2017]. This transition is registered with Young's modulus or the elastic modulus (straight line) until the yield point. From that point on, the material is deformed and would not regain its original pre-test form unless the load is removed. The stiffness of the materials could be determined with the secant or the static Young's modulus that can situate initial deformation to the chosen point of the plastic region deformation point. Doing so makes it possible to adjust the process for the particular material and understand the excessive strain and stress that could soften during the loading. That leads to less likelihood of stress fluctuation along with the softening slope near the convergence point [Mahmoud et al., 2020].

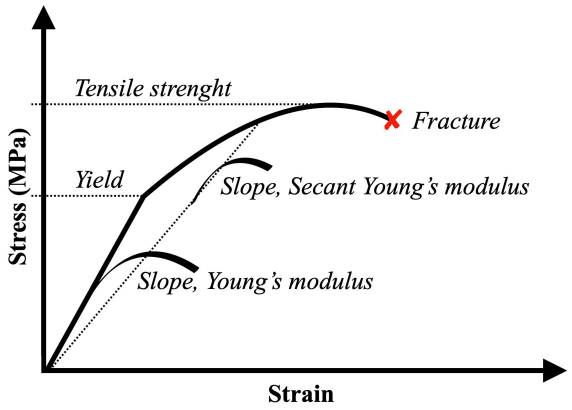

Figure 3. *Stress-strain curve. The stress axes express tensile strength, and the strain axes elongation at break of the material [Chen et al., 2018].*

By creating the stress-strain curves of the material, it can be told rather quickly is the material brittle or not. Brittle materials lack molecular mobility and generate linear curves with fast appearing fracture point without any apparent non-linear part [Wu et al., 2020]. Ductile material, on the other hand, develops a non-linear function and the highest point of it depicts its elongation (EB) percent (aka. strain, that has no unit) [Tilton et al., 2021]. Another imperative parameter is tensile strength (TS) which is the maximum stress that material can withstand while being stretched before braking. For brittle materials, it's close to the yield point (stress load that deforms material for good), whereas, for ductile materials, the point is higher [Wahba, 2019]. Those two parameters (TS, EB) have been the major indicators of mechanical durability throughout the research, more comprehensible than entire stress-strain curves.

1.4. Activity enhancement of the chitosan-based film

Antimicrobial activity and bioactivity of the biofoils that fuel-based materials can not perform are the most distinguishing and pressing arguments for their implementation into the food industry [Kulawik et al., 2020, Lunkov et al., 2018; Quintavalla et al., 2002; Randazzo et al., 2016; Sung et al., 2013; Coma et al., 2002]. So far, the established practices consider adding synthetic preservatives directly into the food to guarantee food safety [Seetaramaiah et al., 2011; Benbettaïeb et al., 2018]. Regardless, this approach may turn out ineffective as inactivation or evaporation or rapid migration of AC into the deeper layers of the food could occur and cause health hazards. Loss of the AC would be higher and, therefore, antimicrobial activity and bioactivity lower [Gülçin et al., 2009; Han et al., 2005]. However, an approach of gradual release of biological antimicrobial agents incorporated into the film's matrix has shown to be a way to safely control food spoilage and extend the shelf life of foods [Muxika et al., 2017, Murali et al., 2020].

1.4.1. Microbial cell-targeted mechanisms of chitosan

The antibacterial activity of the films is applied extrinsically and intrinsically (Figure 4). At first, it is commonly expressed through visual methods as in (i) diameter of the inhibition zone using the disc diffusion method or by (ii) evaluating bacterial burden reduction through counting colony-forming units (CFU). There are several confirmations that the biofoils work against *Escherichia coli* (G^-), *Salmonella typhimurium* (G^-),

Pseudomonas aeruginosa (G^-), *Staphylococcus aureus* (G^+), *Listeria monocytogenes* (G^+), *Bacillus subtilis* (G^+) and *Bacillus cereus* (G^+) species [Table 1 and 2 in Publication I]. For construction reasons, G^+ bacteria are seen to be more susceptible than G^- bacteria [Fisher et al., 2008] as electrochemical properties turn the susceptibility to the other way around [Kong et al., 2010]. The activity has been determined from the fungi kingdom against *Penicillium, Aspergillus, Rhizopus, Botrytis, and Candida* species (Table 1). Notably, not only the films incorporated with AC have the inhibition activity, but chitosan films without any antimicrobial activity have shown inhibition to some extent. However, in many cases, the inhibition zone has been detected only under the film and without actual widening [Bajić et al., 2019a]. The researchers have mooted over several antimicrobial activity mechanisms, although it is still unclear how, precisely, it is applied. From the exterior, (Figure 4, i) chitosan absorbs on the surface of the microbe through electrostatic properties, forms a thick polymeric membrane that causes a blockage, (ii) an oxygen barrier by chitosan hinders the transference of oxygen, eventually inhibiting the respiratory activity and the growth of bacteria in food [Hosseinnejad & Jafari, 2016].

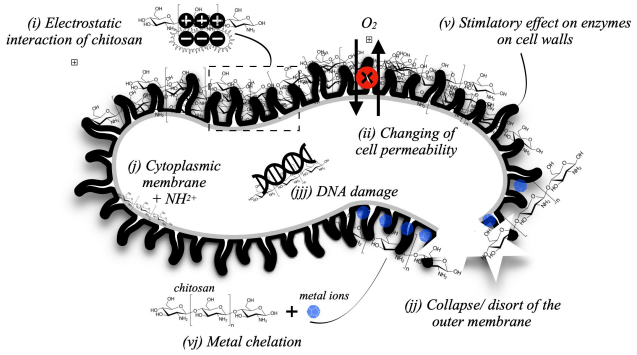

Figure 4. *Schematic representation of antimicrobial mechanisms of chitosan [Benbettaïeb et al., 2018].*

After clear access through the cell wall (Figure 4, j), the negatively charged phosphoryl groups on the cell membrane of the bacteria can be disturbed by chitosan NH^{2+} groups causing distortion and deformation; (jj) a wall diffusion occurs, disrupting the cytoplasmic membrane, affecting bacteria's integrity that leads to leakage of the intracellular electrolytes; (jjj) it can penetrate nuclei, bind DNA, inhibit its replication ability and eventually suppress the synthesis of the RNA; and (vj) it chelates internal nutrients, essential metals that are important for microbes. The building blocks lose their function, the damage could not be utilized by microbes and will be restrained by their growth. Additionally, (v) chitosan may induce enzyme activity (fruits), thus increasing gene expression, which indirectly degrades the cell walls of the microbe [Wang et al., 2018]. All the actions most likely happen in sequence, yet different bacteria have varied sensitivity to the NH^{2+} group, making it possible that the likelihood may deviate.

1.4.2. Chemical interactions of active components bestowed by biopolymer material

Functional properties of the films are majorly enhanced by plant extracts or essential oils (EO) and may direct the received effect towards reducing oxidation processes. Table 1 depicts various ingredients used for the tasks, and nominating compounds in all of those are tannins and volatile compounds. What makes extracts favorable next to EOs is their abundance and high concentration of complex hydrolyzable tannins (HTs) [Comandini et al., 2014; Echegaray et al., 2018; Bargiacchi et al., 2020]. Essential oils, on the other hand, are the essence of plants, concentrated and thus extraordinarily effective yet highly interactive to oxygen and, due to hydrophobicity, show low water solubility. That is why they require higher incorporation, and when adding more, it most potentially affects organoleptic properties. Besides, their extraction is more complicated, and even in the film matrix, they preserve their high volatility [Fisher et al., 2008].

Tabel 1. *Recent studies on the antimicrobial and -fungal activity of chitosan-based films incorporated with active components [Publication I].*

Against bacteria:	**Active component**	**Reference**
Ziziphora clinopodioides EO	carvacrol (65.22%), thymol (19.51%)	Shahbazi 2017
Grape seed extract	flavanols, procyanidins, phenolic acid	
Turmeric extract	curcumin (2–5%), sesquiterpenes, diterpenes, triterpenoids, sterols	Kalaycıoğlu et al. 2017
Litsea cubeba oil	citral (~51%), D-limonene (18.82%), linalool (2.36%)	Zheng et al. 2018
Naringin	naringin, rutin, quercetin, isoquercitrin	Iturriaga et al. 2014
Spirulina extract	caffeic, chlorogenic, salicylic, synaptic, *trans*-cinnamic acids	Balti et al. 2017
Citrus EOs	limonene, linanool, citral	Fisher et al. 2008
Eucalyptus globulus EO	*p*-cymene 18.2% terpineol 8.5%, methyl eugenol 8.8%	Hafsa et al. 2016
Oak extract	castalagin, vescalagin	Bajić et al. 2019
Against fungi:	**Active component**	**Reference**
Anise EO Orange EO Cinnamon EO	anethole limonene cinnamaldehyde	Escamilla-García et al. 2017

Cinnamon EO Ginger EO	cinnamaldehyde (73%) zingiberene (29%)	Noshirvani et al. 2017
Thyme-oregano mixture Thyme-tea tree mixture Thyme-peppermint EO mixture	Oregano: carvacrol (6.37%), thymol (13.70%), *p*-cymene (13.33%), ɣ- terpinene (12.32%) Thyme: thymol (26.04%), *p*-cymene (26.36%), γ-terpinene (16.69%) Tea tree: terpinen-4-ol (38.4%), γ-terpinene (22.6%), α-terpinene (8.1%) Peppermint: menthol (33.38%), menthone (34.31%), 1,8-cineole (6.34%)	Hossain et al. 2019
Cinnamon leaf EO	eugenol (70-95%) cinnamaldehyde (1-1.5%)	Perdones et al. 2014
Eucalyptus globulus EO	*p*-Cymene (18.2%), terpineol (8.5%), methyl eugenol (8.8%)	Hafsa et al. 2016

Tannins are classified as organic non-protein nitrogen compounds and can react to a broad set of organic nitrogen compounds, including chitin and chitosan. They follow chemistry mechanisms similar to protein-tannin interactions, i.e., through electrostatic hydrogen bonding between amide and hydroxyl groups (Figure 5). Beneficial with polymers is that they have high molecular weight, and tannins tend to have higher reactivity towards compounds with abundant amine groups and high molecular weight [Adamczyk et al., 2017]. Mechanisms of EO volatiles interactions towards chitosan are less straightforward. The EO are hydrophobic, and according to that, the droplets have been depicted to be entrapped in between chitosan matrices (Figure 5) instead of actual bonding interactions [Keawchaoon et al., 2011].

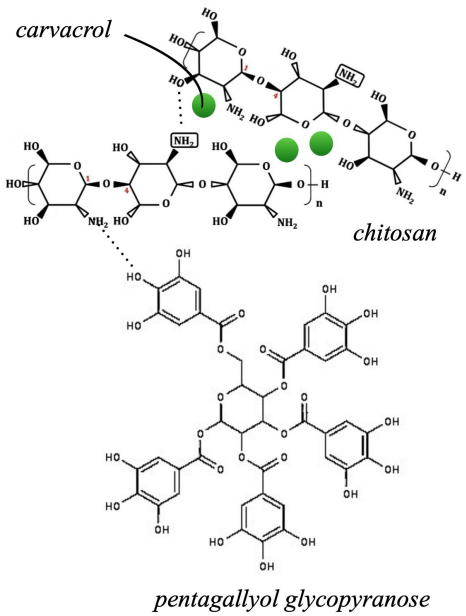

Figure 5. *Possible interaction between chitosan, polyphenol tannic acid, and volatile compound carvacrol droplets as an example [Keawchaoon et al., 2011].*

The agents providing activity against microbial spoilage could be simultaneously empowered with antioxidative properties. Concerning food, browning reactions, protein oxidation, and lipid oxidation are induced by oxygen. Oxygen is a potent oxidant that will create reactive oxygen species and reactive nitrogen species necessary to control scavenging. Additionally, oxygen dissolves freely in organic phases like lipids and sparingly in water [Skibsted et al., 2010; Andersen et al., 2000]. In Publication I with Figure 2, an attempt was made to line up AC used as antioxidants in recent studies and depicting them in increasing order by activity. Extracts bestow higher activity than EO AC in a range of 1–4% w/v. Among all, the propolis extract shows the highest activity, and *Camelina sativa* seed oil the lowest in recent research. Not all antioxidants are equally good that have radical scavenging or reducing activity. Tannins and flavonoids are so-called chain-breaking antioxidants, i.e., they break the chain reaction responsible for oxidation which is not the strongest case with EO-derived AC [Mehta et al., 2015]. Tannins have hydrogen-oxygen (OH) bond energy relatively small and more petite than lipid hydroperoxides. The selection of flavonoids represents OH bond energy with chitosan even smaller than tannins. Nevertheless, in continuous lipid oxidation processes, intermediates phenoxyl radicals are less reactive and act as a dead-end for reactive oxygen species [de Oliveira Neto, 2017]. The extent of the AC release and actual influence on organoleptic properties after or continues interactions with chitosan polymers during the shelf life of stored food products is still yet to be discovered.

1.5. Determination of the feasible biopolymer material composition as food packaging material

One of the follow-up strategies to narrow down biopolymer material's actual composition-based feasibility is to set all the critical process affecting parameters together to a statistical and mathematical tool known as response surface methodology (RSM) [Myers et al., 2016]. After singlet components characterization and basic knowledge of how the components should be mixed, various composition possibilities could exceed the border of simple calculations. The options provided by computing methods [Mäkelä 2017] can downsize the time lost on "wandering" while solving the problem and help reach the end goal of tangible material. RSM explores how a significant factor is in correlation to others important, do they follow linear or quadratic function or do they have cubic-like effects [Myers et al., 2016]. During the first stages of biopolymer film production, the composition is of great importance, an indicator of the film's mechanical properties at the later stages. With numerical inputs from experiments, it could produce an empirical polynomic model that approximates the actual response over a factor region. It seeks the optimal setting for the process factors so it would be possible to maximize, minimize, or stabilize the outcome of interest.

Under the RSM toolkit, there are several design approaches. Most standard, text-book approach Central Composite Design is a five-level design, although in many cases overly complicated and found its use in high-throughput research accounting axial points outside the "cube" (out of the region of interest or impossible to conduct) [Siewe et al., 2021]. The approach Optimal (Custom) Design is customizable for nearly any situation [Mäkelä 2017; Yolmeh and Jafari 2017], but could act poorly as the method accuracy increases when experimental data is set into second-order polynomials [Baş and Boyacı 2007]. A family of efficient three-level Box–Behnken designs [Box & Behnken, 1960] is an appropriate tool for blended chitosan-based films. Few examples are the formulation of chitosan-containing composite films blended with TiO_2 [Tao et al., 2007] or cellulose/polyvinyl alcohol [Cazón et al., 2018] or pea starch [Thakur et al., 2017]. A lot of knowledge has been gathered on drying temperature as an independent variable, either in combination with other process parameters, e.g., relative humidity, storage period [Srinivasa et al., 2007] or with film composition parameters, e.g., CH/GLY level [Singh et al., 2015], glycerol/vanillin level [Tomadoni et al., 2019].

1.6. Physical mechanisms endorsing mobility in biopolymer material

Besides the interactive chemistry between components, physical processes change the biofoil matrix. Back then, chitosan-based films were synthesized to receive more chemically interactive and dissolvable biopolymer material in an aqueous environment. Thus, the ability to absorb, bind and release various active and/or other micro-, macro components were bestowed. Generally, it is assumed that there is no reaction between the food and the biofoil matrix. Nonetheless, the changes happening in biofoils are considered to be driven by mass-transfer processes, and not only Brownian but also convection, transport resistance, or capillary forces reactions [Pereda et al., 2009].

1.6.1. Water diffusion

There is always moisture mobility in the biofoil matrix. Essentially, chitosan has a hygroscopic nature and regularly contains chemically bound, loosely bound, and free water. If water within the film is thermodynamically (humidity, temperature) favored to

move around, it disorders polymer chains making the overall matrix more permeable (water plastification) [Qiao et al., 2019]. Other components enhance the process. The most common plasticizer and hydrophilic-natured glycerol (a humectant) elevate the polymer matrix's water-holding capacity, thus acting as a moisture gateway. Moreover, it is known that the higher concentration results in more substantial phenomena.

The process could be monitored through water absorbance, commonly depicted with the swelling degree. The absorbance of the chitosan without added hydrophobic components could be up to double its initial mass [Bajić et al., 2019a]. Even so, if several mechanisms can shed light on the water sorption of moderately hydrophilic polymers, it is still rather complex as there are specific interactions between water and the polymer backbone. With some certainty, it is thought to be a Brownian type absorption mobility, i.e., part of water molecules are absorbed and other adsorbed to hydrophilic sites, dependent on water vapor pressure and water-affine diffusivities [Pereda et al., 2009].

1.6.2. Mass transfer

Chitosan has been applied as a promising biosorbent for removing various unwanted components, most importantly mycotoxins produced by different fungal species of *Aspergillus, Fusarium,* and *Penicillium* [Yang et al., 2020; Agriopoulou et al., 2020]. Herein, cyclopiazonic acid (CPA) is a mycotoxin with indole and a tetrameric acid structure [Ansari et al. 2016] constantly monitored in cheese and milk samples [Losito et al., 2002] as a causative agent in mycotoxicosis for animals, potentially harmful to humans [Dorner et al., 2001; Costa et al., 2018; Duan et al., 2007]. A simple mass-transfer biosorption-oriented process from a higher moisture content phase to another phase occurs. The process base on selective adsorption, either with thermodynamic and/or kinetic selectivity depending on the affinity between biosorbent and spoiling agent [Crini et al., 2019a; Vakili et al., 2014]. Most food contaminating mycotoxins (aflatoxins, ochratoxins, zearalenone, trichothecenes, fumonisins, and CPA) are anionic under alkaline conditions and have an affinity towards chitosan. According to Juárez-Morales et al., 2017 and Ostry et al., 2018 the conjugation of various mycotoxin to chitosan would be as depicted in Figure 6.

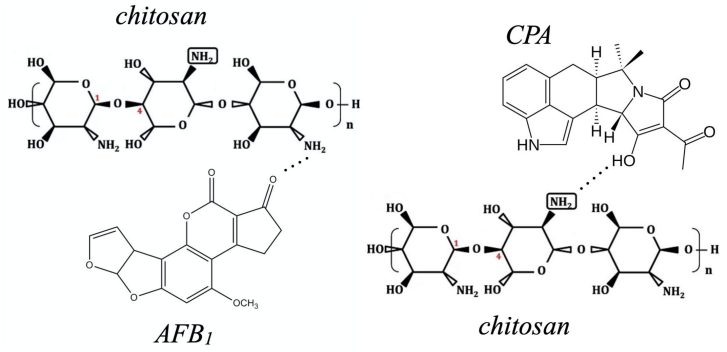

Figure 6. *Exemplary interactions of mycotoxin and chitosan. Computational prediction of aflatoxin B_1 (AFB_1) and chitosan interactions through 6th O and NH_2 groups [Juárez-Morales et al., 2017] (left), and estimated interaction of chitosan and cyclopiazonic acid (right) [Ostry et al., 2018]. Essentially the binding takes place on amine groups and most potential electron donor groups.*

However, chitosan is sensitive to pH (dissolves pH < 3), and according to widespread knowledge, adsorption uptake is strongly pH-dependent when natural sorbents are used. Hence, this creates a need for polymer grafting with functional groups to improve adsorption performance. When implemented, it has been shown to reduce selectivity towards mycotoxin and several of those at once [Zhao et al., 2015; Divya et al., 2018].

1.7. Chitosan packaging influence on food shelf life

The food categorizes into three major categories. Most fresh foods, such as meat, poultry, fruits, vegetables, milk, and bakery products, have high moisture content (MC, starting from 60%) and thus classified as perishable foods. Intermediate moisture food such as cheese, fried food snacks, etc., and low moisture food (LMF) that cover powders, coffee, spices, frozen, canned foods, etc., typically contain 20–50% and <20% w/w of moisture, respectively. Additionally, the shelf life of the perishable foods is estimated to be between one day to one and a half week, whereas for IMF up to six months and LMF six to eighteen months [Qiu et al., 2019]. When the food is packed, it is done according to its usage, first-hand dictated by the MC, leaving IMF as a food category sealed most into petrol-based packaging aside from LMF. Packaging is used against food deterioration (sensory, microbiological) and retains unwanted oxidation, retrogradation, and crystallization in food if not stored appropriately. For example, IMF classified Gouda cheese MC has been reported around 30% and water activity (a_w) around 0.972 while fresh pasta reported for 35% MC and a_w about 0.950 when fresh [Del Nobile et al., 2009a; de Camargo Andrade-Molina et al., 2013; Roos et al., 2018]. The high water activity values (max. a_w is 1) imply that food (cheese) could undergo microbial spoilage, which is an indicator for mycotoxin hazard and lipid oxidation [Kapoor et al., 2008; Lei & Sun, 2019; Sengun et al., 2008; Saravani et al., 2018]. Fresh pasta could be less influenced by microbial spoilage and undergo hardening instead, due to retrograding starch in dough [Walstra et al., 1993; Jideani & Vogt, 2016; Kerch et al., 2010; Sicignano et al. 2015]. This information, patterns used today, is the cornerstone for understanding how and which purposes to develop biofoils.

Preservation of the food is an art in itself. Some of the approaches for IMF preservation are a combination of either drying, osmotic dehydration, a_w lowering agents, electroosmotic dewatering, plasma treatment, high-pressure processing, modified atmosphere packaging (MAP), active packaging, freeze-drying, and biofoils [Qiu et al., 2019]. A single approach has shown not to be effective. The same goes for casted biofoils. Figure 7 depicts the latest research with biofoils and additional packaging to heighten their efficiency on food shelf life. Most of the work has been done with perishable food, and the chitosan biofoils show to enhance the food shelf life up to more than two weeks (mushrooms). The work is scarce with chitosan biofoils abilities to preserve IMF and LMF food lately. Based on the cheese and fresh pasta examples, it has been incorporated into the dough mix and not used as a protective foil.

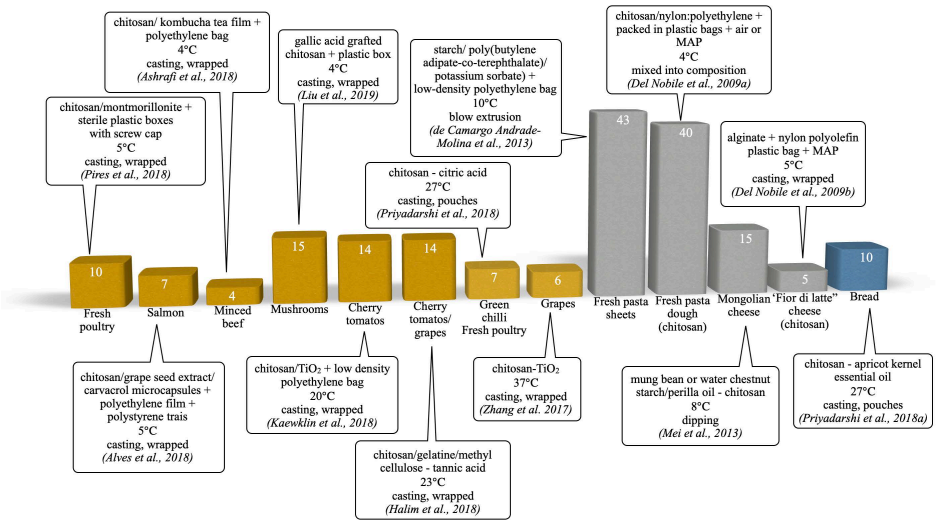

Figure 7. *Selection of latest research on high (yellow), medium (grey), and low (blue) moisture content food shelf life. The numeric values denote the shelf life of the food produce in days. The labels describe the packaging method for shelf life analysis, storage temperature, and preparing biofoil packaging.*

Biofoils can be used to preserve the taste, color, and texture of food products. Chitosan has excellent film-forming properties, and when sprayed on pineapples every 30 days (during seven months), it intensifies its color and softens the texture [Uddin et al., 2020]. Some drugs may have a bitter taste, which can be masked with chitosan microspheres [Bora et al., 2008]. Color is an essential feature of the food, and chitosan helps capsule natural colorants prone to oxidize or polymerize while exposed to excessive light or temperature. One of those natural colorants is saffron, and when encapsulated with a chitosan-based blend, it preserves chewing gum color through gradual diffusion [Chranioti et al., 2015]. Contrary, it may not be suitable for all applications as chitosan-coated strawberries during a week's storage turned out to be astringent in taste [Han et al., 2005]. Overall, it has been proven with many other research articles over a decade that chitosan foils with plant extracts [Zhang et al., 2020; Hromis et al., 2015; Kaya et al., 2018; Zhang et al., 2019b; Li et al., 2019; Priyadarshi et al., 2018b; Sogut & Seydim, 2018; Squillaaci et al., 2018; Sun et al., 2017; Sun et al., 2018] bestow highly potential replacement for single-use petrol-based packaging.

1.8. Legislation and safety of the biopolymer materials

According to European Commission (EC) No 450/2009 legislation definition, "active and intelligent materials extend the shelf-life by maintaining or improving the condition of packaged food, by releasing or absorbing substances to or from the food or its surrounding environment" [EUR-Lex, 2020]. Based on the other EU legislation (EU) 2017/2470, (EU) No 231/2012 [EUR-Lex, 2020], chitosan-based film components are declared as food safe although, at time-being, there are no officially registered food contact materials (FCM) using chitosan hybrid-materials considered safe to use. The confirmed food additives of gallic acid derivatives are propyl gallate (E310), octyl gallate (E311), dodecyl gallate (E312). To the knowledge, the plant extracts containing natural tannins are not regulated by the law when added to food [Fraga-Corral et al., 2020].

1.9. Future perspectives of chitosan

In upcoming years, growing chitosan product applications in cosmetics, food, beverage, pharmaceutical and biomedical, and water treatment industries are expected to drive the market growth. The raw materials are easily obtained from the fishery industry, one of the most important businesses in the Asia Pacific, followed by North America and Europe. China has taken the lead in the global shrimp market and even importing from neighboring counties. The primary product development and end-use industries are situated in Japan, China, India, and South Korea. In Europe, the most extensive activity takes place in Germany, UK, Spain, Italy, and France [CAGR, 2021].

The waste is managed according to the capacity in the country or continent where it is produced. Following the example of Bangladesh seafood waste management, around 43,321 tons of waste produced every year go to local consumption, for aquaculture feed, and the majority is dried and exported to some Asian countries. Accordingly, only half is processed as described. Thus, lately, the overproduction of waste favors new development for silage production [Islam & Peñarubia, 2021]. China is at the crossroad and likely to see seafood consumption outstrip domestic production by 2030, hence influencing all the other concerning segments [Crona et al., 2020]. Latin-American countries export their shrimp in 41% globally [Gómez-Ríos et al., 2017], while Europe converts its waste into compost and later on to fertilizer or imports [Muñoz et al., 2018].

Chitosan production presents drawbacks such as environmentally hostile processes and low volumetric yields regarding raw material and high water requirements [Gómez-Ríos et al., 2017]. Nonetheless, the price for high purity, chemical grade chitosan is 940 Eur *per* kg (Merck KGaA, Germany), while medium-grade chitosan could be purchased for 157 Eur *per* kg (Glentham Life Sciences Ltd, UK) and possibly for even lower from Asia.

Specifically in the food and beverage industry, new ideas are emerging to use chitosan for intelligent nano-food packaging, biosensors, temperature or gas indicators, antimicrobial agents, and derivatives in body-weight management well as for their ability to interact with gut microbiota [Moustafa et al., 2019].

2 AIM OF THIS DISSERTATION

• To blend chitosan with chestnut extract and characterize the films by their physicomechanical properties.

• Implementation of mathematical tool-set to evolve decision-making patterns possible to implement to another biofoil engineering.

• To use the developed film in food shelf life analysis and determine the film's behavior.

3 MATERIALS AND METHODS

3.1. Materials

For the materials development of chitosan-based chestnut extract film, an experimental design with commercial CH derivative of high M_w (0.5–1% w/v Sigma-Aldrich (Steinheim, Germany)) and heartwood CE (0.5–2% w/v, Tanin Sevnica (Sevnica, Slovenia)) was implemented. In this thesis, in total, 15 formulas were produced and analyzed; gathered results of film's mechanical properties (TS, EB) and activity (TPC) fed into the RSM toolkit by 3-level-3-factor Box-Behnken design (Paper II).

For the shelf-life analysis, the fresh pasta was provided by Mlinotest (Ajdovscina, Slovenia), and the Gouda cheese was purchased from a local supermarket in Slovenia. The fresh pasta was chosen to pack into 1.5% CH: 1% CE film and the Gouda cheese into 1.5% CH: 1% CE and 1.5% CH: 1% TA film, latter as ultimate reference aside CE tannins effect.

Film formulations besides CH and CE (≥ 75% of tannins and < 4% of ash) included lactic acid (LA) (purity N 85%; density 1.206 g mL^{-1}) from Sigma-Aldrich (Steinheim, Germany) and glycerol (GLY) from Pharmachem Susnik (Ljubljana, Slovenia)). Milli-Q water was used throughout all experiments. The main chemicals used in the analysis could be found in Paper II, III, and IV.

3.2. Analysis used for film characterization

Paper II, III, IV, additional for thesis.

3.2.1. Film-forming solution preparation

The preparation of the film-forming solutions was carried out with the same procedure within every original research. In short, CH (1.5% w/v) and GLY (plasticizer; 30% w/w based on the mass of CH) were dissolved in an aqueous solution of LA (1.0% v/v) and placed onto magnetic stirrer IKA RCT (IKA, Staufen, Germany) at 1000 r.p.m. and room temperature (24 °C) till homogeneous. Afterward, a filtration step with medical gauze cleared the solution from all the larger impurities. The clear chitosan solution was divided and finalized with the addition of chestnut extract by weighing it straight onto the base mixture. For the faster coacervation, the blend of the base mixture and chestnut extract was homogenized (at 6000 r.p.m. during 2 min) with Ultra-Turrax T50 (IKA, Staufen, Germany) and left overnight. The next day, some sticky foaming occurred, which was carefully removed from the mixtures using a laboratory spatula.

3.2.2. Film casting

Right after film-forming solution (FFS) adjustment (foam collecting), the solutions were cast on rectangular polyurethane 12 cm x 12 cm Petri dishes (~0.32 mL of FFS per cm^2 of a Petri dish) and left to dry for one day in a drying oven Kambic (Semic, Slovenia) with continuous ventilation (24 °C, RH 40%) or with larger production under laminar airflow box (Microbium d.o.o, Ljubljana, Slovenia) in ambient conditions. Obtained films were peeled off from the Petri dishes, separated with baking paper to prevent direct contact from each other, and stored in an airtight container (24 °C, no exposure to light) before the analysis.

3.2.3. Thickness

Thicknesses were measured at five different film locations using a calibrated ABS Digital Thickness Gauge (Mitutoyo, Aurora, USA), and the results were averaged.

3.2.4. Mechanical properties

Chitosan-films were mechanically characterized according to guidelines of ASTMD 882 standard method, 2002. TS and EB were determined simultaneously by testing rectangular film samples (length × width = 8 cm × 2 cm; gage length segment 6 cm) on the XLW Auto Tensile Tester (Labthink® Instruments, Jinan, China) equipped with a 100 N load cell, at a crosshead speed of 25 mm min^{-1}. The calculations for both parameters were performed by Lystem™ Lab Data Sharing System (Paper II, IV).

3.3. Shelf life analysis of intermediate moisture food

Paper III, IV.

3.3.1. Samples and sampling routine

After receiving freshly packed fresh pasta straight from the industry, it was re-packed into chitosan-chestnut extract (CE:CH) sachets (eight pieces). Two sets of samples were prepared for the shelf life study, one with biofoils and another in original industry packaging (PE/BOPP/EVOH plastic). The sachets were additionally packed into paper envelopes to prevent biofoil packaging's direct contact. To avert ambient microbial spoilage, the packaging took place under a laminar airflow box with constant ventilation, 24 °C, 40% RH, and the final samples placed in refrigerator storage (8 °C, RH of 60%) for 60 days.

The sachets were produced out of two 12 cm × 12 cm sheet of chitosan film by heat sealing (165 °C, 700 Pa, 7 s) on HST-H6 heat seal tester PARAM® (Labthink®, Jinan, China). After repacking the food, a more adjustable heat/vacuum sealer (Status d.o.o, Metlika, Slovenia) was used to close the sachets.

For the cheese, a similar procedure to fresh pasta was applied. In short, purchased cheese was unpacked and cut under a laminar airflow box, contaminated, and re-packed (described in detail in the microbiology section). Three sets of biofoils and a reference packaging using polyamide polyethylene (PA:PE) vacuum bags purchased from a local supermarket were used. All the cheese:biofoil systems were additionally packed into an extra layer of PA:PE vacuum bags and vacuum-sealed to avert environmental impact.

A 4:4:2 factorial experiment design was implemented during this study, meaning four different packaging sets (CE:CH, TA:CH, CH, PA:PE, two of each) × four time points (time zero, 7 day, 14 day, 37 day) × two temperatures (4 °C, 25 °C) over 37 days of storage. The spoilage microbiota was chosen to be *Pseudomonas fluorescens*, *Escherichia coli*, *Penicillium commune*. Hench, the first set according to factorial design was placed into storage without the spoilage and a second set with the spoilage making it in total 48 samples *per* time point (48 x 3 = 144). It must be emphasized that the blank samples including (non-inoculated, in total 8 *per* time point and two samples at time zero) were only studied under 4 °C conditions.

Additional experiments with fresh pasta and Gouda cheese for this thesis were performed. The storage and sample preparation was done as described previously. Two sets with two foods were prepared. To study fresh pasta changes during storage without the environmental impact, it was packed into CE:CH/PA:PE packaging. The cheese was

studied with the environmental impact and packed into CE:CH biofoil. Both sample sets were placed in 4 °C storage for 30 days to receive reproducible results. Sampling was performed in three time points (time zero, 14 day, 30 day) for pH analysis and in two time points (time zero, 30 day) for MC and TPC.

3.3.2. Microbiology

Microbiological analysis was conducted with 4 ± 0,5 g of fresh pasta samples, which was aseptically transferred into a sterile plastic homogenizer bag and blended with 36 ± 4.5 mL of 0.1% (w/v) sterile buffered Peptone water (BPW) using Masticator Basic blender (IUL Instruments, Barcelona, Spain) for 30 s, 1500 r.p.m. at 24 °C. The dilution of the sample followed with 0.1% BPW and 100 µL of each inoculated into plate count agar growth medium. Analysis of total count of yeast and molds (Dichloran Rose Bengal Chloramphenicol Agar (Biokar diagnostics, Allonne, France)), total bacterial count (Tryptic Glucose Yeast agar, (Merck KGaA, Darmstadt, Germany)) and total count of bacterial group Enterobacteriaceae (Violet Red Bile Lactose Agar (Merck KGaA, Darmstadt, Germany)) were conducted.

For deliberate spoilage of Gouda cheese, the *Escherichia coli* K12 and *Pseudomonas fluorescens* NRRL B-253 were grown overnight at 30 °C in 2TY medium. The medium was discharged and grown culture resuspended in sterile saline solution to approximately 10^7 CFU/mL for *E. coli* and 10^9 CFU/mL for *P. fluorescens*. The *Penicillium commune* NRRL 894 was grown on malt extract agar plates for 10 days to obtain spores. A saline solution with collected spores homogenized with a stomacher (Lawson Scientific, Ningbo, China) to approximately 10^5 CFU/mL.

The cheese was pre-cut into rectangular pieces of 50 × 25 × 8 mm (approximately 20 g) and inoculated with 100 uL of bacterial or fungal inoculum. After spreading the inoculum uniformly over the sample surface (on the side only) the slices were wrapped by biofoils or packed into reference packaging. At the analysis time point, the cheese sample was opened aseptically and cut in half. The one half (approximately 10 g) mixed with 90 mL of sterile saline solution and homogenized. Received suspensions were diluted in sterile saline solution and plated on a selective medium. 2TY medium and incubation at 37 °C for 24h was used for *E. coli* count, *Pseudomonas* selective agar and incubation at 30 °C for 48h was used for *P. fluorescence* count, and malt extract agar and incubation at 25 °C for 5 days was used for *P. commune* enumeration.

3.4. Analysis for films and food characterization during shelf life

Paper II, III, IV.

3.4.1. Moisture content

In the film development stage (*Paper II*), the MC of the samples was determined gravimetrically at room temperature using a precisely adjustable Kambic oven (Semic, Slovenia). Briefly, the rectangular samples (~1 cm^2) were weighed on an analytical balance (Kern & Sohn, Balingen, Germany) to get the initial mass (M_1) and then dried for 24h at 105 °C to achieve the dry mass (M_2). The dried samples were weighed again and gathered results of both masses expressed as the percentage of water content in the films following the equation (1):

$$MC = \left(\frac{M1 - M2}{M1}\right) \times 100\% \quad (1)$$

The food samples and biofoils for food packaging were analyzed during the shelf life with a rapid MC analysis using Moisture analyzer HE 53 (Mettler Toledo, Wien, Austria). The samples were received from cold storage, reduced into smaller pieces using a knife or scissors, and immediately placed onto a measuring plate for analysis (Paper III, IV).

3.4.2. pH

The pH values were measured from fresh pasta, Gouda cheese, and packaging biofoils for both foods during shelf life using a 781 pH/ion meter (Metrohm AG, Ionenstrasse, Switzerland). Approximately 4 g of fresh pasta and 10 g of cheese sample was mixed with deionized water and homogenized. Biofoil samples (2 x 2 cm) were cut, immersed in MilliQ water for 2 h, and removed from the solvent. The pH was measured from the remaining water solvent (Paper IV, additional data).

3.4.3. Total phenolic content

Total phenolic content (TPC) as an activity indicator was determined from fresh pasta, Gouda cheese, and packaging biofoils for both foods during shelf life by Folin–Ciocalteu's (FC) phenol reagent. For food sample TPC measurements, extraction of the phenols was performed as a first step. For that, pasta samples (200 mg) were pre-powdered in liquid nitrogen and the same amount of cheese sample cut using a knife. Both weighed on an analytical balance (Kern, Balingen, Germany) and covered with 1 mL extraction solution of methanol: acetone: water (30:30:40 mL). The prepared mixture was agitated on Tehtnica Vibromix 10 (Domel, Zelezniki, Slovenia) vortex for 5 min and centrifuged (3293 g; 10 min) using MiniSpin centrifuge (Eppendorf, Hamburg, Germany). Received supernatants were separated into separate e-tubes, and the extractions repeated. Obtained extracts were mixed, and 20 µL added to 120 µL water. New samples underwent FC reaction by successive addition of FC phenol reagent and Na_2CO_3 (10% w/v) aqueous solution 10% w/v and 20% w/v based on the volume of the sample, respectively. Those samples were diluted with water up to 1 mL and incubated for 2 h in the dark at room temperature. The film samples for the analysis were weighed (~5 mg) on an analytical balance and added into water. Film samples followed the FC reaction in the same manner as the food samples. After the incubation, all the samples were applied to absorbance analysis using SynergyTM 2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA) at 765 nm. Gallic acid was used as the standard, and the results were expressed as the mass of gallic acid equivalent (GAE) per mass of the pasta (Paper II, III, IV).

3.4.4. Fourier Transform Infrared – Attenuated Total Reflectance

The Fourier-transform infrared – Attenuated Total Reflectance (FTIR-ATR) spectrums were recorded for fresh pasta samples, CE:CH sachets at every time point used for packing fresh pasta, and for film samples in the material development stage. Spectrums were gathered at the wavenumbers from 4000 to 700 cm^{-1} with the resolution of 4 cm^{-1} using Spectrum Two FTIR-ATR spectrometer (PerkinElmer) and normalized before carrying out the statistical analysis (Paper II, III).

3.4.5. Ultrahigh Performance Liquid Chromatography

Following analysis was used to analyze Gouda cheese samples and the biofoils used for packing the cheese. The analysis was modified for the cheese samples and developed for the film samples according to Zambonin et al., 2001. Comprehensively, the cut Gouda cheese sample (0.5 g) underwent extraction with 1.5 ml of methanol, and the received

solution was sonicated for 10 min. The sample was filtered using PTFE-20/13 HPLC filter (Sartorius, Goettingen, Germany) and evaporated under N_2 flow to receive residue restored with 1.5 ml of phosphate buffer (5mM, pH 2.8). The film samples (2 × 2 cm) were cut and placed next to an e-tube wall, covered by 1 mL of methanol, shaken on a thermoshaker TS-100C (Biosan, Riga, Latvia) for 1 h, evaporated, and restored in the same manner to food samples. Every sample preparation took place at ambient room temperature. For the detectible results, the samples (400 µl) were spiked with 100 µl of CPA standard solution (0.1 mg mL^{-1}). Based on the results, recovery calculations were done. The standardization curve with seven dilution levels was prepared (CPA concentrations range between 50 ng–0.025 mg mL^{-1}).

The chromatographic separation was followed and performed on an Ultrahigh Performance Liquid Chromatography (UHPLC) Ultimate 3000 (Thermo-Fisher Scientific UltiMate™ 3000, Waltham, MA USA) system consisting of a pump, UV-VIS detector. Sample separation was achieved using Hypersil GOLD™ Amino, 150 x 4.6 mm, 5 µm (Thermo Scientific, city, country) column with the mobile phase of acetonitrile and ammonium acetate buffer (50 mM, pH 5) 80:20 v/v (linear gradient, 25 min.). Other technical parameters were as follows: flow rate was set as 0.6 mL min^{-1}, oven 30 °C, sampler 5 °C, and detection wavelength settled on 282 nm (Paper IV).

3.4.6. Box-Behnken design

To understand the FFS-dependent high-performance physicochemical factors for biofoils a 3-level-3-factor Box-Behnken-Design (BBD) was applied with 15 experimental runs, i.e., prepared samples. For this purpose, the FFS three main variables (i) concentration of chitosan (CH, x_1, % w/v), (ii) concentration of chestnut extract (CE, x_2, % w/v), and (iii) concentration of glycerol (GLY, x_3, % w/w, calculated per mass of chitosan) were inserted and coded with three levels of low (–1), medium (0), and high (+1). The low level has the interpretations for the scarce addition of raw materials, resulting in mechanically unstable materials, while high-level materials made out of very viscous FFS are perceived as inapplicable, too rigid (Paper II).

3.4.7. Statistical analysis

The data underwent a one-way analysis of variance with a confidence level of 95% ($P \leq 0.05$), followed by Tukey's test. Each sample was taken from a random location in triplicate and values averaged (Paper II, III, IV). The clustering was performed by single-linkage K-mean calculations ($K = 3$).

4 PRESENT INVESTIGATION

A summary of the major results from Paper II, III, and IV is provided below, with some additional findings discussed in the following sections.

In this doctoral thesis, biofilms with two raw materials from major natural and renewable sources of marine- and wood-based biomass were prepared and characterized for potential active food packaging. One of the underpinnings of the approach in this research is the use of statistical and mathematical tool RSM for rapid design, development, and optimization of durable chitosan-based chestnut extract cross-linked food packaging. The developed biomaterial was used to pack fresh pasta (low-fat food) and Gouda cheese (high-fat food) with the main focus on the material's activity when influenced by extrinsic and intrinsic factors; to gain insight in terms of being safe and applicable packagings in natural, also induced, microbial spoilage conditions.

4.1. Production and characterization of the chitosan-based chestnut extract biofoils

4.1.1. Preparation of film-forming solutions and chitosan-based films

An FFS composition of four components - a matrix-forming biopolymer CH, plasticizer GLY, active component/cross-linker CE, and solubility enhancer LA was used to prepare biofoils. Initially, when FFSs were blended, modifications changed solutions behavior, which reflected on biofoils properties. The CH with CE increases the viscosity of the solution, and CE more than 1% exceeds the saturation point in the solution. Processing of the components led to brown-shaded foils (Figure 8), and the color intensified when CE concentration increased. Without additives, the foils received a transparent, slightly beige-colored appearance (Appendix 1, Figure A3).

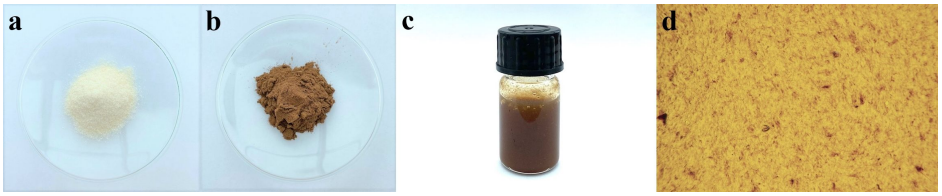

***Figure 8**. Photographs of the appearance of the material: a) chitosan, b) chestnut extract, c) film-forming solution, d) 1.0% (w/v) chestnut extract film.*

The film's texture formed coarse/ mat on one side and smooth/ shiny on the other side due to the plates used in the casting method. Steadily casted films were bubble-free and uniform in thickness with an average of 120 ± 30 µm, approximately twice as thick compared to conventional plastics such as PE/BOPP/EVOH (70 µm). Nevertheless, the values are still in the range of other chitosan-based films [Mir et al., 2018; Rambabu et al., 2019]. Films without additives were measured 80 ± 10 µm thick. (Paper III). The morphological evaluation revealed the film's compact structure without any significant microscopic pores or cracks (Appendix A in Paper II).

4.1.2. Chitosan-based film cross-linking efficiency with chestnut extract

The efficiency of tannins cross-linking in CE:CH film was studied with the FTIR-ATR analysis. It showed decrease in transmittance when chestnut extract (0.5%, 0.75%, 1% w/v) as a cross-linker concentration increased [Appendix A, Figure A1] referring to heightened cross-linking. The neat CH film indicative absorption bands were seen in the wavelength region of 3600 cm^{-1} and 3000 cm^{-1} (attributed to O–H and N–H stretching), followed by two weak bands between 2950 cm^{-1} and 2850 cm^{-1} (attributed to C–H stretching), and the peaks around 1640 cm^{-1} and 1550 cm^{-1} attributed to C=O stretching (amide I) and N–H bending (amide II), respectively. The addition of chestnut extract altered these spectrum regions to higher intensity, indicating an additional amount of O–H groups and interactions with amide groups (pronounced amide II bending). Similar changes in the FTIR-ATR spectrums were recorded with oak extract in chitosan film [Bajić et al., 2019b], which belongs to the same class as CE [Tondi & Petutschnigg, 2015]. The cross-linking effect is even comparable to a few of the best radical donor cross-linkers. In peak-wise, the spectrums have been found similar to the cross-linking of chitosan by glutaraldehyde [Galan et al., 2021] and formaldehyde [Singh et al., 2006]. Some correlations could be drawn with conventional plastic materials.

Plasticizers had an influential role in the micro-changes of the film and cross-linking. According to specters of unplasticized, plasticized, and plasticized with 1% CE film [Figure 2 in Paper II], glycerol with its three OH groups *per* structure impacts the macromolecular composition by giving extra functional groups to the film. The component generated a new peak at wavelength 2950 cm^{-1} and showed high affinity to CH amide groups. It was seen with the widening and shifting to higher wavelengths (specific to CH) before adding the chestnut extract. A similar specter outline has been reported by Liu et al., 2013. The same was seen with the peak 2930 cm^{-1}, leading to the conclusion that glycerol supports cross-linking of chitosan and making films more ductile.

4.1.3. Physicochemical and mechanical properties of the films

CE:CH film's mechanical and chemical properties were measured to determine film's durability and activity as food packaging foils. Moisture content is one of the critical parameters and, in many cases, measured attentively as it affects the barrier and mechanical properties of the chitosan-based films the most. A set of 15 different blends (out of which three the same for consistency evaluation) and their experimental moisture measurements revealed that moisture was ranging from 20.2% to 42.8% [Table 2 in Paper II] for formula 6 (CH 2%, CE 0.75%, GLY 30%, LA 1%) and 11 (CH 1.75%, CE 0.5%, GLY 90%, LA 1%), respectively. The result indicated that GLY content has the highest impact on moisture. A similar correlation of GLY and MC in chitosan-based films has been shown before and corresponds to the study findings [Ma et al., 2019]. Nonetheless, the effect of all three independent components on MC was seen. MC was almost constant with all the tested CH concentrations, but the values increased with the decreasing amount of CE. Subsequent addition of CE adds HTs that possess multiple interaction sites to cross-link polymer chains. Consequently, the HTs saturate hydrogen bonding sites of CH and further lead to retention of the water molecules within the matrix. The CE concentration was increased double, and it was able to retain moisture intake about ⅕ based on the mass. The other plant-based ACs have been reported to reduce the water absorption capacity of chitosan-based films. Akin effectiveness was shown by mango leaf extract biofoil (0.209 ± 0.02 μm thick) rich in gallic acid, glucosides, and other phenolic compounds, although it was used in 5% w/v and abled to retain moisture absorption only

up to 3.5% compared to its reference [Rambabu et al., 2019]. A more complex foil (only 61 ± 5 μm thick) with the tea polyphenols (0.1% v/v) in combination with silver nanoparticles was able to retain MC in 6% [Zhang et al., 2017]. Based on these examples, moisture reduction is concentration-related and more to the source of polyphenolic cross-linkers.

Tensile strength and elongation at break are the mechanical properties that refer to film's resistance to failure at elevated deformation and stretching capacity, i.e., extensibility. The produced CE:CH films acquired TS values ranging from 3.2 MPa to 21.6 MPa and EB values between 10.3 and 86.7%. It was seen that GLY concentration elevation to triple decreased TS of the film while CH or CE concentration effect was seen to be less influential. Every 0.5-percentile CH increase had a considerable decreasing effect on EB when plasticized with 30% GLY and not that significant when 90% GLY was used. Every 0.5-percentile CE elevation in 60% GLY-plasticized formulas increased TS and EB simultaneously. For comparison, the presented biofoils cross-linked with CE had higher EB values than polystyrene (PS; 1%–4%) and polyamide (PA; 5%–10%), smaller than low-density polyethylene (LDPE; 200%–900%), polyvinyl alcohol (PVA; 220%–250%) and ethylene vinyl alcohol (EVOH; 180%–250%), while their EB values fell in the range of polyethylene terephthalate (PET; 20%–300%) and polyvinyl chloride (PVC; 40%–75%) materials [Luzi et al., 2019]. From the chosen examples, the TS values could be considered equivalent only to LDPE (7–17 MPa) [Brostow, 2007].

4.1.4. Activity properties of the films

The activity of the 15 biofoils in aqueous solvents is depicted in Table II in Paper II. With the increase of the CE, the antioxidative activity increased gradually. Accordingly, the range of TPC in the studied films was from 9.6 to 23.7 $mg_{GAE}\ g_{film}^{-1}$. With the maximum concentration of CE (1%), the TPC values were received between 15 to 24 $mg_{GAE}\ g_{film}^{-1}$. TPC tended to be high with a maximal concentration of CH (1.75%) and the minimal concentration of GLY (30%). In comparison to other reports from the literature, TPC values in chitosan-based films prepared from different FFSs containing a banana peels extract, a hop extract, and a mango leaf extract has been reported to be up to ~4.8 $mg_{GAE}\ g_{film}^{-1}$ [Zhang et al., 2020], up to ~12.7 $mg_{GAE}\ g_{film}^{-1}$ [Bajić et al., 2019b], and up to ~12.8 $mg_{GAE}\ g_{film}^{-1}$ [Rambabu et al., 2019], respectively.

4.2. Distinguishing of packaging foil

The definition of desired attributes of the final foil-like material is a subjective matter made by a decision-maker. Thus, whether the CE:CH formulations are akin, the partitioning based on their physicomechanical properties was carried out and depicted in Figure 9. Three significant clusters were formed, including outliers belonging to the clusters. It could be seen that EB dependence on MC:TPC shuffled the formulas into clusters giving out borderline blends 11 (CH 1.75%:CE 0.5%:GLY 90%) and 10 (CH 1.75%:CE 1%:GLY 30%), indicating these films' excessive flexibility although low activity with high MC and opposite, respectively (Figure 9, a). Elongativity of the material is a requirement for wrappings as these are easily shaped. Hence, the food suitable for the packaging would be somewhat complicated to pack, moderate in MC, and thus spoilage hazardous. For example, similar film have been used on bread [Noshirvani et al., 2017]. Regarding rigidity dependence – TS on MC:TPC (Figure 9, b), the outliers were the same with the difference of scattered clusters showing films' divergent natures. TS turned out to be a more decisive parameter when choosing active material to tell the difference. Materials

with higher rigidity are ideal for cheese and meat [Mei et al., 2013; Ashrafi et al., 2018; Arslan et al., 2018]. Guided by the latter dependence, MC dependence on mechanical properties was constructed, and this was able to distinguish three clusters of high, medium, and low MC films (Figure 9, c). Here, the food categorization into three major clusters (HMF, IMF, LMF) and the defined physics law describing equilibrium of the system, helps to outline the biofoil packaging that could be the utmost [Aguirre-Loredo et al. 2016]. Nonetheless, most important with the biopolymer packaging materials is their activity. With TPC dependence on mechanical properties, a slight difference within the same blends (13, 14, 15) received repeatability, although, with this outline, many combinations do not cluster (Figure 9, d). As a result, when a film is chosen by its activity measurement in the first-bases, all the blends are characteristic in their unique way and tend to lead to "wandering".

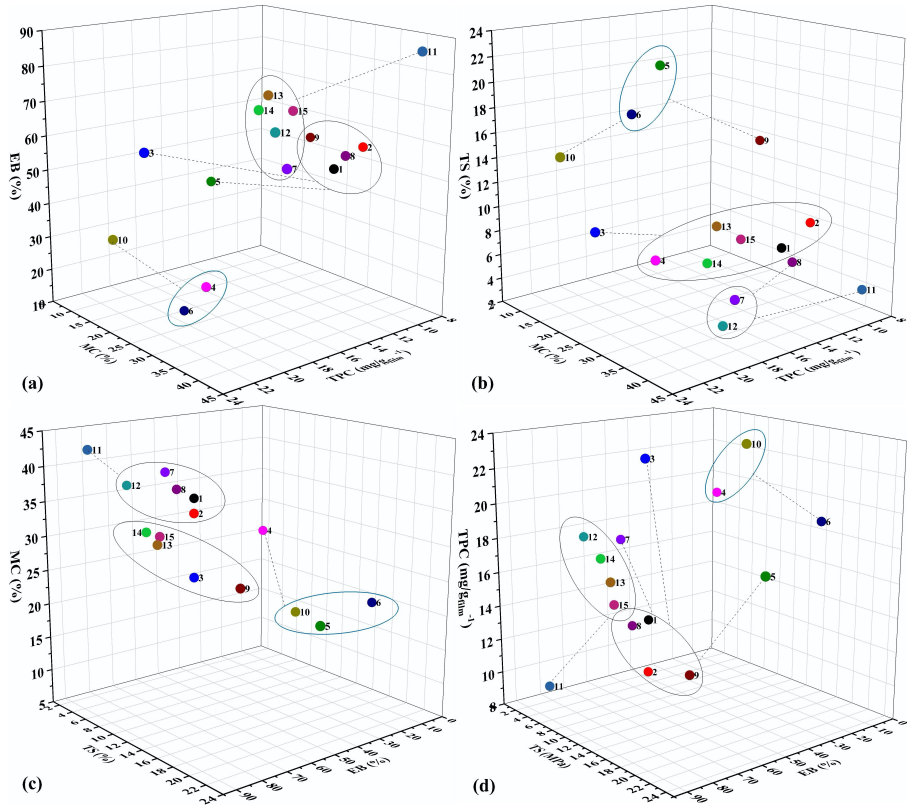

Figure 9. *3D partitioning of the blended mixtures and the dependencies of (a) EB on MC:TPC, (b) TS on MC:TPC, (c) MC on TS:EB, and (d) TPC on TS:EB. Films MC is depicted based on eq. (1). The clusters formed by single-linkage K-mean calculations (K=3) are connected to groups' formulas. Blue circles represent the preliminary choice of packaging for shelf life studies.*

According to response plots in Figure 3 in Paper II, the model to describe auxiliary components' effect on biofoils properties aligned well with experimental measurements. The relationship of independent variables to MC, TS, EB was quadratic and linear for TPC. In the case of MC, the model proposed equation matched at least 96.85% of the total variations. The polynomial equation used for TS was able to describe the foils' stiffness

in accordance to three different components (CH, CE, GLY) at least 98.27% of total variations. Calculations for extensibility showed even higher accuracy with EB, precisely 99.24% of the total variations. This was indicated by lack of model: F-value fit ran for each input, meaning a good fit and high significance of the model was gained. Statistical parameters of ordinary and adjusted R^2 showed good agreement for physicomechanical properties, hence supporting the applicability of the model. It was revealed that the activity attribute TPC fits best into a two-factor interaction model. The latter approach does not have quadratic terms, and its simplicity indicated that CH–CH, CE–CE, and GLY–GLY interactions did not affect TPC. The accuracy of the calculations was in line with models generated for other polymer-based films [Saberi et al., 2016; Thakur et al., 2017].

The most suitable packaging foil for the shelf-life analysis of the food was distinguished based on the partitioning and RSM plots. Criteria for the material were placed according to food MC (30-35%), affinity to spoilage, and physical attributes implying to packaging method. Figure 9 with blue circles depicted the potential blends. According to conventional plastic packaging food-dependent classification, packagings for permeable foods are chosen as more rigid, less elastic (lower EB) [Luzi et al., 2019]. Although, most of the biomaterials were more elastic, less rigid. Thus, with the robust, criteria-based choosing, the blends 4, 5, 6, and 10 were found to be potential. Due to the high number of choices, RSM was able to downsize the similar formulation for the single blend. The optimal composition of the FFS in terms of actual values of the raw materials was determined to be 1.93% (w/v) of CH, 0.97% (w/v) of CE, and 30.0% of GLY (w/w; per mass of chitosan) by Design-Expert® software calculations.

4.3. Biofoil interactions with food and changes during shelf life

Two food:biofoil systems were monitored of which, the first overlooked low-fat food (5%; fresh pasta) packed in CE:CH sachets and another high-fat food (26%; Gouda cheese) packed in CE:CH, TA:CH, and CH sachets, and latter three additionally into PA:PE vacuum packaging. All the samples were sided with reference samples (see Materials and methods). The CE:CH sachet foil was chosen with the composition of 1.5% (w/v) of CH, 1% (w/v) of CE, and 30.0% of GLY (w/w; per mass of chitosan), where CH concentration was chosen to be lower in terms of cost-efficiency.

4.3.1. Physicochemical changes of developed biofoil when in contact with food

4.3.1.1. Water transfer

With approximately the same MC content for both food products, although one with low (fresh pasta) and the other with a high-fat content (Gouda cheeses,e), the moisture transfer was slightly faster for high-fat food stored in 4 °C with and without the environmental impact. The food products were held in different conditions up to 60 days and 37 days, though, for the comparison's sake, the following data will be presented for 0 and 30/37 day of storage (4 °C, with and without environmental impact) (Table 2).

Table 2. *The effect of biofoil (CH, TA:CH, CE:CH) on pasta/cheese moisture content compared to moisture accumulation in films (both in % per 0.5 g). Values are means (n = 2) with standard errors, which within a column are significantly different (p < 0.05). Asterisk marked samples represent additional data for the thesis.*

Sample	*0. Day*	*30. Day*
Pasta CE:CH	33.1±1.90	7.19±0.34
Pasta PA:PE/CE:CH*	31.1±1.17	23.5±1.80
Pasta PA:PE*	31.1±1.17	31.9±1.37
Sample	*0. Day*	*37. Day*
CHE CH*	31.5±1.50	1.06±0.10
CHE TA:CH*	31.5±1.50	1.36±0.25
CHE CE:CH*	31.5±1.50	1.72±0.25
CHE PA:PE/CH*	33.5±1.80	20.1±2.16
CHE PA:PE/TA:CH	33.5±1.80	24.6±1.82
CHE PA:PE/CE:CH	33.5±1.80	25.5±2.03
CHE PA:PE	33.5±1.80	29.5±1.97
Sample	*0. Day*	*30. Day*
F Pasta CE:CH	13.00±0.12	9.32±0.17
F Pasta PA:PE/CE:CH		39.9±0.35
Sample	*0. Day*	*37. Day*
F CHE CH*	4.03±0.35	6.74±1.59
F CHE TA:CH*	2.73±0.41	5.67±0.65
F CHE CE:CH*	3.21±0.19	6.83±0.46
F CHE PA:PE/CH		62.4±1.51
F CHE PA:PE/TA:CH		51.0±1.55
F CHE PA:PE/CE:CH		52.2±2.33

Herein, fresh pasta moisture loss was measured for 27.0% (from 32.1% to 5.10%) when packed in CE:CH biofoil and 8.60% (from 32.1% to 23.5%) when packed in PA:PE/CE:CH packaging. On the other hand, Gouda cheese had a moisture loss of 31.2% (from 32.5% to 1.35%) and 6.90% (from 32.5% to 25.6%) packed in CE:CH and

PA:PE/CE:CH, respectively. A staling process [Bressani, 2014 and Chillo et al., 2009] was seen. While the food yielded moisture, the biofoil absorbed it and was revealed to be highly permeable. The higher fat content of food had no additional barrier ability even if interaction should be efficient [Rodrigues et al., 2020]; thus, the biofoils acted similarly for both foods. Initially, the CE:CH film MC was measured at 13.0%, and when applied for fresh pasta, the final MC was measured 9.32%. When used for the cheese, the value was 6.83% (initially 3.21%), being slightly less. With fresh pasta study, an equilibrium point from where biofoil stops absorbing moisture was seen to be at day 6. At that time MC of the foil was 19.0%, meaning it had a 6.00% moisture uptake. From that point on, the permeability of the biofoils started to increase as both matrices lost their moisture rapidly (Figure 4 in Paper III). The results were recorded when the samples were affected by the ambient environment. Results for biofoils moisture uptake were 26.9% (fresh pasta; from 13.0% to 39.9%) and 38.8% (cheese; from 13.0% to 51.8%) in conditions without the environmental impact. Food at the same time showed lesser moisture loss to its surrounding biofoil. Precisely, fresh pasta yielded moisture in (from 32.5% to 25.6%) 8.60% and the cheese 7.90%. Clearly, the moisture is gained by the ambient environment when additional packaging is lacking. Although, if present, it was seen that food yielded less than it was measured for biofoils' uptake. This revealed that micro-level changes occur, and additional water molecules are released in the biofoil matrix, i.e., bound water is released. The phenomenon was stronger with the cheese.

Additional findings without environmental impact regarding MC were recorded. Chestnut extract contains tannins as cross-linkers, therefore, the comparative film with pure compound tannic acid was produced, which has been used as a cross-linker previously [Rivero et al., 2010]. Moisture content as an indicator supported FTIR-ATR results by showing slightly enhanced cross-linking of CE tannis next to TA. This was revealed by a cheese study, where the cheese packed in TA:CH/iTA:CH yielded moisture in 7.9%, more than the cheese packed in CE:CH/CE:CH, as in 6.9%. The cross-linking and overall protection to moisture loss was confirmed by the neat film (CH) MC observation. Accordingly, cheese yielded 12.4% (from 32.5% to 20.1%) of moisture by day 30 of storage (4 °C) and surrounding CH biofoil in time being shown to have a moisture uptake in 59.3%. Besides, higher temperature (25 °C) did not have a differentiative influence on MC when environmental impact was removed.

Water transfer in the world of conventional packaging is interpreted to water vapor permeability and water vapor transfer rate (WVTR). The fresh pasta used in this study was originally packed in PE/BOPP/EVOH packaging with the WVTR 0.6 g/m^2/24h by the specification. It is unknown for the Gouda cheese. What was seen, the food MC in the packaging remained unchanged within 30 days of storage for both foods, and would even keep the food fresh up to two months according to the factories. A permeability test for the *Quercus* polyphenol rich chitosan film showed the result with the WVTR value 17 g/m^2/1h [Oberlintner et al., 2021]. The films were prepared in the same laboratory as those used in this study. Hitherto, the novel packaging materials, including the materials from the study, are roughly 680 times more water permeable compared to conventional plastic.

4.3.1.2. pH change

In comparison to reference packaging (PA:PE/CH), a conversion of food to more acidic while the packaging foil altered to less acidic was observed. The food products were stored in different conditions, and for the comparison's sake, the following data will be

presented for 0, 7 and 30/37 day of storage (4 °C, with and without environmental impact). Additional data has been generated to understand low-fat food (fresh pasta) and high-fat food (cheese) pH change with/without and without environmental impact, respectively (Table 3).

Table 3. *The effect of biofoil (CH, TA:CH, CE:CH) on pasta/cheese pH compared to pH in films (per 8±1 g and 2x2 cm, respectively). Values are means (n = 2) with standard errors, which within a column are significantly different (p < 0.05). Asterisk marked samples represent additional data for the thesis.*

Sample	*0. Day*	*7. Day*	*30. Day*
Pasta CE:CH*	6.115±0.020	5.803±0.001	5.538±0.070
Pasta PA:PE/CE:CH*	6.115±0.020	5.162±0.459	4.709±0.151
Pasta PA:PE*	6.115±0.020	6.109±0.021	6.079±0.036
Sample	*0. Day*	*7. Day*	*37. Day*
CHE CH*	5.858±0.121	5.454±0.113	5.318±0.051
CHE TA:CH*	5.858±0.121	5.474±0.035	5.296±0.008
CHE CE:CH*	5.858±0.121	5.484±0.001	5.338±0.054
CHE PA:PE/CH	5.586±0.056	5.504±0.026	5.322±0.004
CHE PA:PE/TA:CH	5.586±0.056	5.590±0.037	5.392±0.052
CHE PA:PE/CE:CH	5.586±0.056	5.428±0.061	5.338±0.002
CHE PA:PE*	5.858±0.121	5.801±0.068	5.975±0.016
Sample	*0. Day*	*7. Day*	*30. Day*
F Pasta CE:CH*	3.142±0.007	3.181±0.047	3.100±0.029
F Pasta PA:PE/CE:CH*	3.142±0.007	3.553±0.117	3.507±0.094
Sample	*0. Day*	*7. Day*	*37. Day*
F CHE CH*	3.561±0.069	4.454±0.127	3.753±0.102
F CHE TA:CH*	3.479±0.022	4.468±0.312	4.220±0.323
F CHE CE:CH*	3.142±0.007	4.006±0.181	4.100±0.046
F CHE PA:PE/CH	3.892±0.036	0.000±0.000	4.832±0.003
F CHE PA:PE/TA:CH	3.801±0.008	0.000±0.000	4.768±0.033
F CHE PA:PE/CE:CH	3.193±0.026	0.000±0.000	4.502±0.168

Initially, both foods were recorded with different pH values, as in for fresh pasta pH 6.1 and cheese pH 5.6–5.9. Even if the initial values varied for cheese, the values between different environment samples at the end of the analysis were not significantly different ($p > 0.05$) and measured 5.32 on average. Opposite to cheese results, the pH results of the fresh pasta were varying and shifted to lower values at the end of storage. Herein, the changes in the pH are mainly attributed to the electrostatic and hydrophobic interactions between chitosan (cationic) and lipids (anionic) [Wydro et al., 2007, Ham-Pichavant et al., 2005]. The conformity of final results is most likely due to hasty close-up interactions within the first days of storage. Fresh pasta lacks those interactions being low in fat, and therefore, the environmental impact seems to be decisive (i.e., PA:PE/CE:CH packed pasta was more acidic). Moreover, it was learned that when food (cheese in this case) is spoiled, the pH drop delays, most likely due to default LAB antagonistic action towards spoilage bacteria. When cheese inoculated with *E. coli* and packed in TA:CH foil, its initial pH remained up to two weeks, indicating a potential microbial battle provided by TA:CH film favorable conditions. The phenomenon was not noticed when cheese was packed in CE:CH (Figure 4 in Paper IV). Food composition influence on pH was proven by change of pH of films used for packing the food. A jump of pH was observed after 6 days, which was seen with every biofoil under the influence of the ambient environment. Here, moisture mobility influence can be considered, and the samples are experiencing a so-called buffering effect. The latter reflects matrix degradation, which leads to free water transfer with a result of elevated pH values [Amjadi et al., 2019; Fajardo et al., 2010]. Total shift to steady pH was depicted with an extra layer of packaging that cut off the environmental impact. Overall, the pH change in the films was more pronounced when used for packing cheese.

The pH trend in samples stored at 25 °C took a similar course in every sample set, with a difference in the films containing tannins, being both lower and identical in values (acidic) ($p < 0.05$) compared to the CH film.

When food is packed in conventional plastic, it is unnecessary to consider whether the packaging composition affects food acidity. The factors accounted for are the permeability to gases, mechanical properties, transparency of the material, and, most importantly, the atmosphere used for storage [Costa et al., 2018]. Food processes will moderate the acidity of the food with the inner atmosphere due to solid materials dethatching the environmental impact. Khoshgozaran et al. 2012 have described MAP influence on cheese pH packed in high-barrier bags. The review brings out several examples of how MAP modifies cheese pH into a drop, although majorly increasing. Accordingly, an example based on CO_2 usage presumably led to the formation of carbonic acid, etc., during proteolysis and lipolysis that increased pH in whey cheese from 6.8 to 6.6. Another study showed a pH increase in industrially made, smoked semi-hard cheese from 5.24 to 5.46 associated with the formation of amines and ammonium. The pH increase has also been associated with lactic acid consumption by molds and yeast in sliced Kashar cheese from 5.48 to 5.88. It is common to use MAP to preserve fresh pasta, and according to the literature, it has changed the pH similar to cheese. Sanguinetti with co-authors found that the presence of CO_2 had a dropping effect on the pH values (6.07 to 5.76) of fresh filled pasta [Sanguinetti et al., 2011] until they changed the dough of the pasta to gluten-free and saw pH to increase from 5.17 to 5.45 [Sanguinetti et al., 2016]. In conclusion, the pH drop described by the MAP effect is similar to the drop shown by CE:CH biofoil used in this study, and the change is strongly related to the type of food like when using conventional packaging.

4.3.2. Activity changes of chitosan-based chestnut extract biofoil when in contact with food

4.3.2.1. Antioxidative properties

The total phenolic content assessment is the approach through how the activity of the biofoils next to food was determined. The main mechanism was considered moisture transfer-induced diffusion [Benbettaïeb et al., 2019], supported by the results in Table 4 and Figure 4 in Paper III. Next to low-fat food, the CE:CH foil diffusion was seen somewhat existent when it had its utmost efficiency without environmental impact at 4 °C compared to a blank sample in PA:PE in the same conditions. Received results were gained by a small amount (200 mg) of pasta sample and stayed calculatingly in the range 0.09–0.67 $mg_{GAE}\ g_{pasta}^{-1}$ *per* 2 to 5 g reported by Gull et al., 2018. The biofoil activity, based on cheese samples TPC analysis, was determined to be stronger and not only related to AC diffusion from biofoils. An elevated TPC value was recorded from the cheese sample packed in blank PA:PE packaging on the 37th day with the average 19.9 $mg_{GAE}\ g_{cheese}^{-1}$ close to the values measured from samples exposed to biofoils. The cheese was repacked before the experiment, and that may have had an inducing effect on catabolism, hence, changing protein structure and releasing bound phenolic compounds from cheese that are then detected by the Folin–Ciocalteu assay. The test has shown to be sensitive to several interfering agents, even sugar components (oligosaccharide and lactose) present in the cheese [Rashidinejad et al., 2013]. Moreover, CE:CH-packed cheese with the additional packaging showed an approximately 6% increase in TPC value, as in 21.0 $mg_{GAE}\ g_{cheese}^{-1}$ and while exposed to the environment, in value of 15.6 $mg_{GAE}\ g_{cheese}^{-1}$. This confirmed that CE:CH foil could be highly efficient next to high-fat food products without additional packaging. It must be taken into account that CE:CH biofoil activity is less than expected due to seemingly occurring catabolism reactions. Lastly, most potential catabolism did impact the cheese samples exposed to the environment, despite lower values measured from the same samples packed in diffusive CE:CH foil. This was confirmed by visual changes of cheese surface color depicted in Appendix 1, Figure A2, A3, A4, A5.

Table 4. *The effect of biofoil (CH, TA:CH, CE:CH) on pasta/cheese TPC compared to TPC in films (per 0.2 g and 5 mg, respectively). Values are means (n = 2) with standard errors, which within a column are significantly different (p < 0.05). Asterisk marked samples represent additional data for the thesis.*

	TPC, mg_{GAE} g_{pasta}^{-1}	
Sample	*0. Day*	*30. Day*
Pasta CE:CH	0.017±0.002	0.021±0.001
Pasta PA:PE/CE:CH*	0.016±0.001	0.021±0.002
Pasta PA:PE*	0.016±0.001	0.016±0.000
Sample	*0. Day*	*37. Day*
CHE CH*	0.016±0.002	11.7±1.00
CHE TA:CH*	0.016±0.002	26.6±1.20
CHE CE:CH*	0.016±0.002	15.6±2.30
CHE PA:PE/CH*	0.016±0.002	12.2±0.90
CHE PA:PE/TA:CH*	0.016±0.002	33.0±0.00
CHE PA:PE/CE:CH*	0.016±0.002	21.0±1.40
CHE PA:PE*	0.016±0.002	19.9±0.60
	TPC, mg_{GAE} g_{film}^{-1}	
Sample	*0. Day*	*30. Day*
F Pasta CE:CH	21.8±0.20	21.6±0.40
F Pasta PA:PE/CE:CH	16.0±1.60	7.50±1.80
Sample	*0. Day*	*37. Day*
F CHE CH*	1.10±0.20	1.30±0.00
F CHE TA:CH*	20.0±0.10	11.3±1.60
F CHE CE:CH*	14.2±0.10	8.90±2.80
F CHE PA:PE/CH*	1.10±0.20	1.30±0.00
F CHE PA:PE/TA:CH*	20.0±0.10	7.30±0.10
F CHE PA:PE/CE:CH*	14.2±0.10	3.40±0.30

Gradual release of the biofoil AC was observed to increase till equilibrium point of moisture of two side-by-side low-fat food and CE:CH foil matrices, but to return to the value measured at the beginning of shelf life (Figure 4 in Paper III). Meaning, there is a relatively small migration of phenolic content towards pasta surface when stored with environmental impact. No return was observed with the biofoil samples used to pack cheese in both environments. Unfortunately, comparative research related to biofoils in recent years was close to impossible to find. A kinetic release study of chitosan [Yeamsuksawat et al., 2019] has shown similar behavior of chitosan-based film towards food simulant 50%-ethanol that covers dairy and non-dairy fatty foods. Therefore, conventional packaging material kinetic studies of mass transport were able to shed some light. A study of model chemicals migration from LDPE packaging into and within cheeses containing different amounts of fat and water (Gouda cheese, soft cheese, cottage cheese) was conducted [Cruz et al., 2008]. The results showed the highest diffusion coefficient for Gouda cheese due to the highest fat/water content. It was registered 10^3 times faster in the food itself compared to LDPE-cheese migration, indicating that the mass transport of a chemical from film to cheese is a rate-limiting factor. Strong fat/water content influence on the migration was confirmed by Goulas et al., 2000. Additionally, they measured chemical plasticizer migration from food-grade PVC into Edam cheese and observed 222.5 ± 6.9 mg kg^{-1} migration after 10 days. The distinct literature would concur that gradual release of the conventional packaging does align with the behavior of the biofoil towards tested food in this study.

4.3.2.2. Antimicrobial properties

Chitosan-based chestnut extract biofoils met the antimicrobial requirements when preserving low-fat food or high-fat food, while latter deliberately contaminated by spoilage bacteria. According to the results, fresh pasta (conditioned with 8 °C, 60% RH) did not develop microbial growth during 60 days of storage. The total count of yeast, molds, bacteria, and *Enterobacteriaceae* were all under detection limits.

Significant changes in microbiology on Gouda cheese are depicted in Figures 1 and 2 in Paper IV. Most interestingly, two different Gram-negative bacteria depleted in two ways. Precisely, *E. coli* count (approx. 6.5 $\log_{10}$ CFU/g) decreased in 2 $\log_{10}$ CFU/g and stagnated after, while *P. fluorescens* (approx. 8.2 $\log_{10}$ CFU/g) count gradually reduced by 6 $\log_{10}$ CFU/g in CH biofoil (conditioned without environmental impact at 4 °C, 37 days storage). With the additives (CE, TA biofoil) the reduction was seen 1 $\log_{10}$ CFU/g and approximately 4 $\log_{10}$ CFU/g, respectively, for *E. coli* and *P. fluorescens* in the same conditions. After all, the recommended microbiological maximum limit is 7 $\log_{10}$ CFU/g, stated by the International Commission on Microbiological Specifications for Foods [ICMSF, 1996]. The literature complies with the results. A comprehensive study by Hu & Gänzle, 2019 shows chitosan's bactericidal effect towards several pathogenic microbes on artificially contaminated IMF and states that the lethality is limited to 5 $\log_{10}$ CFU/g. Several other biofoils successful inhibition of *E. coli* range between 1–19.6 $\log_{10}$ CFU/g (Table 1 in Publication I) placing the CE incorporated chitosan film among the least effective ones. Moreover, a clear understanding of biofoils effective inhibition over MAP modified conventional packaging on cheese products has been shown – attempts to inhibit microbial spoilage with MAP report the opposite outcome [Khoshgozaran et al. 2012]. Instead, the count of *P. fluorescens* in polystyrene-MAP packaging [Moir et al., 1993], *E. coli* in polyamide-MAP packaging [Arvanitoyannis et al., 2011], coliforms plus

Pseudomonas spp. in high-barrier bags [Gammariello et al., 2009] was decreased compared to air atmosphere, although still increased during storage at refrigeration conditions.

There are several mechanisms of the antimicrobial activity described in the literature, although straightforward action based on this study was not distinguished. Higher reduction by CH film could be encountered for less complex matrix and hydrogen bonds forming with a higher number of active sites [Liu et al., 2004]. After 7 days, the films next to food become less acidic (pH 4+), and *E. coli* pH tolerance for survival is between pH 4 to 5, indicating a more susceptible environment. Moreover, it is a facultative anaerobic bacterium; otherwise, the CE:CH film would have gradually decreased the count. On the contrary, CE has been demonstrated to contain reducing sugars, providing adenosine triphosphate (ATP) and thus most potentially supporting the bacteria's survival [Blaiotta et al., 2013]. However, TA:CH films had a similar effect to CE:CH film, disputing the sugar's effect and proving the effect of predominant HTs to be destructive to spoilage bacteria, *E. coli*, to some extent. Furthermore, *P. fluorescens* pH tolerance remains in a range of 6–7, and it is an aerobic bacterium. Next to the fact of food changing biofoil's pH, the biofoil stayed acidic enough (due to tannins) to induce bacterium depletion gradually. On top, it was seen that the count dropped virtuously in reference material, suggesting anaerobic environmental impact to be a firsthand factor causing the change. All existent microbiota was outruled with non-inoculated samples of the study that showed also no *E. coli* or *P. fluorescens* presence.

The study of cheese inoculation with fungi *P. commune* spores (approximately 4 $\log_{10}$ CFU/g) showed spoilage reduction at refrigeration conditions (4 °C) less than 1 $\log_{10}$ CFU/g regardless of the biopackaging and at the room temperature (25 °C) approximately 2 $\log_{10}$ CFU/g on all the samples, except on CH samples, where the *P. commune* count had dropped only for 1 $\log_{10}$ CFU/g. Evidently, the higher temperature with the support of LAB [Cheong et al., 2014] contributes to the film's AC diffusion process, which has also been shown by Ouattara et al., 2000. Additionally, by Esposito et al., 2019, CE polyphenols inhibit fungus, both in mycelial and spore form. Overall, it was resulted that CE:CH film antifungal effect exceeds the effect of several EO incorporated biofoil, except cinnamon [Table 1 in Publication I].

4.3.2.3. Assessment to mycotoxin cyclopiazonic acid absorption

Induced spoilage on the cheese with fungi was expected to be reduced by active biofoils, which it has. With the high fungal spoilage, it was also expected that hidden hazards by mycotoxin CPA production would occur and grant an assessment of additional safety-guarantee of active biofoils. The presence of tannins in the packaging material at 25 °C hindered the CPA production in cheese samples and had an endorsing effect while held at 4 °C. As seen in Figure 3 in Publication IV, the cheese samples packed in biopackaging CE:CH or TA:CH showed CPA concentration decrease from 6400 to 533 µg/kg and 3800 to 1400 µg/kg respectively, invariability (200 µg/kg) when packed in CH packaging and increase from 800 to 1400 µg/kg when packed in reference PA:PE packaging at 25 °C. The refrigeration temperature (4 °C) counter-effective influence resulted in an elevation from 5900 to 8367 µg/kg, 1867 to 7600 µg/kg, 700 to 5800 µg/kg and demotion from 1400 to 267 µg/kg when packed in CE:CH, TA:CH, CH, and PA:PE packaging, respectively. The levels measured in the cheeses are relatively higher compared to levels measured in white mold cheese 1.83–3610 µg/kg [Ansari & Häubl, 2016] or in inoculated cheddar cheese stored under MAP as in 4–280 µg/kg [Taniwaki et al., 2001]. It is difficult to say if the levels are hazardous to humans due to only

accessible literature on animal trials, although they do remain in the levels of non-toxicity based on the 50% (LD50) lethal dose in rats by oral ingestion (36,000 μg/kg) [Purchase, 1971].

There are several reasons for the outcome, one of them being tannin-CPA conjugations [Juárez-Morales et al., 2017; Thipe et al., 2020] explaining the sharpest CPA reduction displayed by CE:CH film cheese results at 25 °C. This is supported by the fact that CPA is insoluble in water [Ostry et al., 2018], and according to FTIR-ATR results, 1% CE addition into biofoil deposit OH groups to interact with. Another reason being the supremacy of spoilage over LAB [Barr, 1976] and continuous mycotoxin production when looking at 4 °C results. After all, the reduction of fungal spoilage was seen less than 1 log10 CFU/g, and based on a thorough screening by Leyva Salas et al., 2018 LAB has been shown to delay *P. commune* spoilage in cheese products with low starter inoculation (6 log10 CFU/g) up to 6 days only. Peculiar jump at both temperatures, day 14, could be described by the action of water mobility inducing mass transfer [Crini et al., 2019a] as a soluble matter of similar chitosan films have been shown approximately 18% [Bajić et al., 2019a]. Moreover, CPA synthesis increases at higher temperatures [Ansari & Häubl, 2016; Hamdi et al., 2019] and is seen to be stable when stored in refrigeration temperature [Taniwaki et al., 2001; Ostry et al., 2018]. In spite, it has been stated that fungal growth cannot be correlated with inhibition of mycotoxin production because plant extracts fungistatic activity could trigger secondary metabolism (phenolics inhibit enzymes) as a response to stress [Cabral et al., 2013], meaning the latter could continue. A potential catabolism reaction was considered when going over TPC results at 4 °C. All the previous is pin-pointed by PA:PE-packed cheese results stored at 4 °C. The cheese had a CPA elevation recognized at day 14, referring to even pourer LAB activity and catabolism, likely to start working against mycotoxin synthesis as the PA:PE material would not undergo water transfer and was not exposed to promoting tannins. Almost similar action with cheese was seen at 25 °C, with the difference at the endpoint, affirming CPA production at higher temperatures.

Concurrently, the packaging foils depicted opposite results with adsorption of CPA. With the help of UHPLC, it has been found that CE:CH has the most substantial adsorption (3200 μg/kg, 2 × 2 cm^2, day 37). Clearly, the diversity of AC is an advantage as the film received higher efficiency compared to pure compound tannic acid. Theoretical multiple mycotoxin absorption by cross-linked chitosan polymer has been reported to be 5.67 g/kg [Zhao et al., 2015] supporting the possible protective feature of chitosan films used for packing cheese in this study.

CONCLUSIONS

Based on an original study intertwining biomaterial production, characterization, and application as food packaging, many conclusions can be made.

Chitosan cross-linking with chestnut extract produces ductile materials resistant to consumer's manipulation. Developed biofoils using a casting method receive brown-shaded color, the texture of coarse on one side, smooth on another without microscopic pores or cracks, and with the thickness twice as thick compared to conventional fossil-fuel based packaging. According to physicochemical parameters, the films present elongativity comparable to commercial polyethylene terephthalate and polyvinyl chloride commonly used as wrapping films. Tensile strength does not meet the requirements.

Basic formulation suggestions could be followed according to the study. Biofoil increases rigidity with GLY less than 30% and every 0.5-percentile CH or CE addition to 1% LA: water solution. Glycerol addition over 60% acts overly plasticizing. Limitations occur at film-forming solution preparation where more than 1% CE addition exceeds the saturation point while more than 1.5% CH complicates mixing. The optimal formulation for satisfactory moisture, mechanical stability, and antioxidant capacity would be 1.93% (w/v) of CH, 0.97% (w/v) of CE, and 30.0% of GLY (w/w; per mass of chitosan).

Heartwood origin chestnut extract cross-link chitosan biofoil matrix with an efficient simultaneous AC diffusion, i.e., activity. An increasing concentration of chestnut extract contributed to cross-linking, being influenced by glycerol (plasticizer) increase the most. Activity expressed by phenolic compounds reactivity in the film sample has been determined to be twice as effective overtopping oak, banana peel, and several essential oils.

The response surface methodology has been successfully applied in the decision-making of active food packaging. By linking the film-forming solution composition to the final material's physicomechanical properties, the accuracy of the polynomial calculation revealed predicted values alignment with those obtained by experimental evaluations. This indicated high reproducibility of homogeneous material and confirmed the time-saving applicability of the approach. The outcome was supported with prior clustering of the formulation showing the possibility to narrow down options based on optimal requirements (high TS, low MC, etc.) stated uphand.

The chitosan-based chestnut extract biofoil is affected by food macromolecular composition. Developed biofoils moisture permeability slows down when used for packing high-fat food without any additional packaging. In ultimate efficiency (vacuum packed), moisture content is elevated in the material when used to pack high-fat food. Chitosan-based chestnut extract biofoils shows remarkable activity dependent on moisture mobility by decreasing when next to food, and with a comparable result to biofoil in its full capacity. Quite acidic biofoils turn into less acidic while the food slightly modifies its acidity, indicating that the biofoils could act as benign acidity regulators.

The biofoil, with its diverse polyphenolic content, grants food safety. With induced bacterial and fungal spoilage, it does it selectively with gram-negative bacteria and endorses it at higher temperatures. Food safety is granted even by mycotoxin reducing more effectively at higher temperatures. A potential mechanism for mycotoxin reduction is absorption with LAB-promotion and mass transfer when applied on cheese products.

The shelf life of food is majorly moderated by moisture mobility, less with the material's possible taste-altering high acidity. The two main factors determined that both

foods are organoleptically acceptable for 7 days. Thus, suitable food for packing into chitosan-based chestnut extract biofoil should remain within the MC under 35% and between 5–24% fat content. The food may be liable to oxidation degradation and microbial/fungal spoilage, latter even in higher storage temperatures. The food may be asymmetrical, pose weight up to 20g *per* 12 x 12 cm^2 in 45 cm^3 volume and advisably present darker color.

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ABSTRACT

Biological activity and physicochemical properties of chitosan film cross-linked with chestnut extract for active food packaging applications

The main goal of this thesis was to characterize chestnut extract cross-linked chitosan-based materials with physicochemical parameters and link the outcome to the compositional disparities based on the limits given using the time-saving mathematical tool RSM. To take the decisions made and implement a material with the characteristics calculated into IMF food shelf life analysis. The shelf life study goals were designated to determine packaging behavior next to food. To evaluate its efficiency by granting food safety against induced microbial spoilage dependent on physicochemical properties while storing the food. To depict and time-frame the changes happening with food while packed in developed biofoil.

The materials were composed with ingredients ranging in between CH 1.5–2% w/v, CE 0.5–1% w/v, GLY 30–60% w/w; *per* mass of chitosan, giving a total of 15 blends. After the fabrication, the biofoils were measured with TS 3.2–21.6 MPa, EB 10.3-86.7%, MC 20.2–42.8%, and TPC in the range 9.6-23.7 mg_{GAE} g_{film}^{-1}. The plasticizer (GLY) level had the most substantial impact on moisture content and mechanical properties, while CE was most influential on the activity capacity. The GLY use in films emphasizes cross-linking with CE, and the increase of CE increased the linking.

Implementation of mathematical tool RMS showed a time-saving approach to compose a formula of FFS. The satisfactory composition based on polynomial calculations was received 1.93% (w/v) of CH, 0.97% (w/v) of CE, and 30.0% of GLY (w/w; per mass of chitosan) supported by clustering of the formulas narrowing down most tangible. The method could be considered predictive due to the high alignment between predicted and experimentally obtained evaluations.

Two different foods of low fat (fresh pasta) and high fat (Gouda cheese), similar in moisture content, were re-packed into biofoil packaging and stored in two conditions featuring two extremes of allowing the environmental impact, other eliminating the exposure (additional vacuum foil). The moisture transfer in biofoil was seen faster and non-gradual when used on fresh pasta and gradually increasing when used to pack the cheese while exposed to the environment. The biofoils act as absorbers with the final MC higher than yielded by food, referring to released bound water from the biofoil. At the end of storage, MC of fresh pasta and the cheese packed CE:CH biofoil was 40% and 52%. The activity of the biofoil was seen in correlation with moisture mobility, more efficient next to high-fat food regardless of the environmental impact. The efficiency of biofoil increases 5% when packed in an additional vacuum bag. Conversion of food to more acidic while the packaging foil altered to less acidic was observed.

The antimicrobial properties of CH:CE biofoil were recognized while preserving fresh pasta and with induced spoilage on Gouda cheese during 60 and 37 days, respectively. The total count of yeast, molds, bacteria, and *Enterobacteriaceae* remained under detection limits when stored with the environmental impact at 4 °C. The reduction of induced spoilage on Gouda cheese with CE:CH biofoil was seen selective towards gram-negative bacteria decreasing *E. coli* count 1 $\log_{10}$ CFU/g and *P. fluorescens* 4 $\log_{10}$ CFU/g at 4 °C. Antifungal activity of CE:CH biofoil with induced *P. commune* spoilage on Gouda cheese was more efficient at higher temperature (25 °C) as in, the fungal growth

was reduced in 2 $\log_{10}$ CFU/g. The results were supported with mycotoxin development evaluation in cheese matrix and biofoil matrix storing the food, depicting potential tannins interaction with CPA, thus potentially posing an additional safety mechanism towards food.

The brown-shaded, 120 ± 30 μm thick CE:CH biofoils could grant a 7-day shelf life for IMF food with an average MC of 33% and varied fat content. A strong coloring effect emerged on both foods, emphasized by vacuum packaging and higher temperature. The integrity of the biofoil remains throughout the storage compared to reference packagings.

LÜHIKOKKUVÕTE

Kastani ekstraktiga seotud kitosaankilede bioloogiline aktiivsus, füüsikalis-keemilised omadused ning rakendatavus toidupakendina

Käesoleva doktoritöö eesmärgiks oli kirjeldada kastani ekstraktiga seotud kitosaan kilede füüsikalis-keemilisi muutusi sõltuvalt kilemoodustava lahuse (KML) komponenidest, kasutada sõltuvuste modelleerimiseks asendusmudeleid ning saadud kalkulatsioonide põhjal valmistada pakendid keskmise niiskussisaldusega toitude säilitamiseks. Säilivuskatsete peamisteks eesmärkideks oli uurida biopakendi omaduste muutust toiduga kokkupuutel, määrata pakendi bioaktiivsus toiduohutuse tagamiseks sõltuvalt pakendi füüsikalis-keemilistest parameetritest ning uurida pakendi mõju toidu säilivusajale.

Pakendite väljatöötamiseks valmistati 15 KML, mille põhikomponentideks olid kitosaan (1.5–2% w/v), kastani ekstrakt 0.5–1% w/v ja glütserool 30–60% w/w (vastavalt kitosaani massist). Pärast kilede kuivatamist saadi nende niiskusesisalduseks 20.2–42.8%, tõmbetugevuseks 3.2–21.6 MPa ja suhteliseks pikenemiseks purunemisel 10.3-86.7%. KML-te üksikkomponentide mõju hindamisel leiti, et plastifikaator glütserool mõjutab kõige enam kilede mehaanilisi omadusi ja niiskusesisaldust ning kastani ekstrakt aktiivsust. Ristsidestamise tõhusus kasvas kastani ekstrakti kontsentratsiooni suurenemisel ja glütserooli juuresolekul.

Väljundsõltuvuste modelleerimiseks kasutatud asendusmudelid näitasid, et KML on võimalik valmistada kiiresti ja matemaatilisi valemeid saab edukalt rakendada koostisest sõltuvate füüsikalis-keemiliste parameetrite tuletamiseks, mida kinnitasid kõrged kokkulangevused tuletatud ja katseliste andmete vahel. Koostise piiride etteandmisel võimaldas kahe andmestiku sõltuvus tuletada optimaalne KML, mis sisaldas kitosaani 1.93% w/v, kastani ekstrakti 0.97% w/v, ja glütserooli 30.0% w/w (vastavalt kitosaani massist). Tulemusi toetas klasterdamise meetod, andes võimalikud KML-id sobivaima leidmiseks.

Väljatöötatud biokilesse pakendati madala rasvasisaldusega (5%) värske pasta ja kõrge rasvasisaldusega (24%) Gouda juust, mille niiskussisaldused olid sarnased (34%). Toidutooteid säilitati +4 °C juures kahes säilituskeskkonnas – õhukeskkonnas ja vaakumis. Niiskuse liikumine pakendis toimus õhukeskkonnas säilitatud värske pasta puhul kiiremini ja mitte-graduaalselt ning juustu puhul aeglasemalt ja graduaalselt. Kastani ekstraktiga kitosaanist pakkematerjalid käituvad absorbendina ja omavad säilivusaja lõppedes suuremat niiskussisaldust kui toidust eemalduda võiks, mis viitab seotud vee vabanemisele kile maatriksites. Kilede aktiivsus sõltub niiskuse liikuvusest ning on tõhusam kõrge rasvasisaldusega toote puhul sõltumata säilituskeskkonnast. Vaakumpakendis suureneb biokilede efektiivsus 5%. Täheldati, et kui pakend muudeti vähemhappeliseks, muutusid toiduained happelisemaks.

Kastani ekstraktiga kitosaanist pakkematerjali antimikroobseid omadusi vaadeldi nii värske pasta kui ka riknemisorganismidega kaetud Gouda juustu pakendamisel vastavalt 60 ja 37 päeva jooksul. 4 °C juures õhukeskkonnas säilitatud värske pasta pärmide, hallitusseente, bakterite ja *Enterobacteriaceae* koguarvarv jäi alla määramispiiri. Juustu säilitamisel alandas biopakend gram-negatiivsete bakterite kolooniat moodustavate üksuste arvu (*in. k.* CFU) selektiivselt, *E. coli* puhul 1 $\log_{10}$ CFU/g ja *P. fluorescens* puhul 4 $\log_{10}$ CFU/g 4 °C juures. Kile antifungitsiidse mõju määramisel nähti Gouda juustu

P. commune saastamisega korral tõhusamat tulemust kõrgemal temperatuuril (25 °C), kus seenekasv alanes 2 $\log_{10}$ CFU/g ulatuses. Mükotoksiini tootmise hindamistulemused juustu ja biopakendi maatriksitest kinnitasid mikrobioloogia tulemusi, näidates võimalikku tsüklopiasoonhappe sidumist tanniinidega, mehanism, mis tagab täiendava ohutuse toidule.

Pruunikad, keskmiselt 120 ± 30 µm paksusega kastani ekstraktiga kitosaankiled tagasid keskmise niiskussisaldusega toidule (33% niiskus, varieeruv rasvasisaldus) keskmiselt 7-päevase säilivusaja. Esines tugev toidu värvumine, mis võimendus vaakumpakendi kasutamisel ning kõrgemal temperatuuril. Välja töötatud pakendi terviklikkus püsis kogu säilivusaja vältel.

APPENDIX 1

Doctoral thesis additional data:

Kõrge, K., Biological activity and physicochemical properties of chitosan film cross-linked with chestnut extract for active food packaging applications, *TalTech* (2021) 1–57.

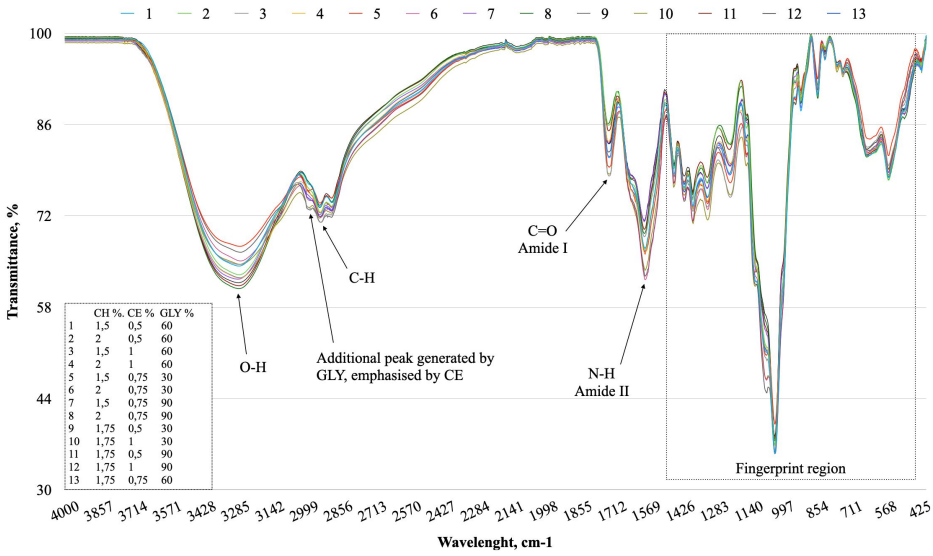

Figure A1. *The FTIR-ATR specters depict cross-linking of chitosan with chestnut extract according to the concentration of the blend in the table.*

1. Chestnut extract chitosan film induce the color change of the food

The Gouda cheese and Fresh pasta underwent photographic observation, and it was seen that the color is strongly affected by biomaterial packaging, which is emphasized with the temperature (25 ℃). Notably, high temperature changes cheese color from yellow to dark solid brown when packed in iCE:CH packaging (similar observation in low temperature, but less). Fresh pasta color at 4 ℃ conditions changed slightly brownish.

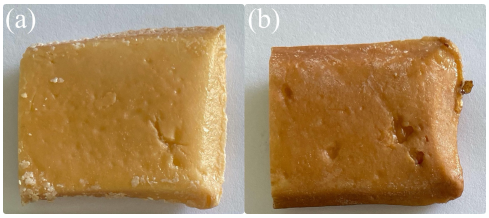

Figure A2. *Gouda cheese after storage on day 37 (a) at 4 ℃ and (b) at 25 ℃.*

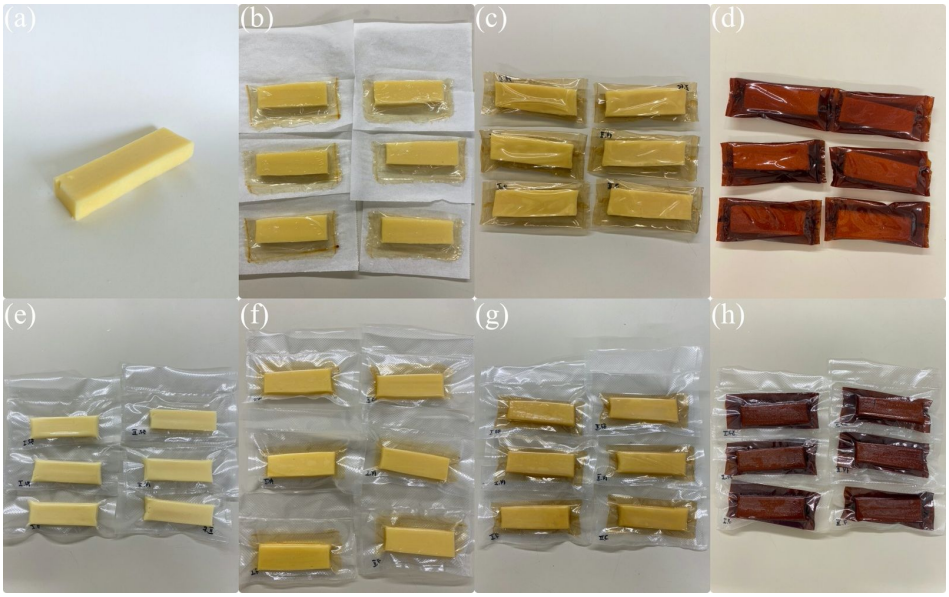

Figure A3. *The Gouda cheese packaging on day 0 at 4 ℃. Upper row, from the left corner, the pictures represent: (a) fresh Gouda cheese from original packaging, (b) cheese packed in CH (c) TA:CH, (d) CE:CH biofoil. The bottom row depicts Gouda cheese packed in (e) PA:PE, (f) PA:PE/CH, (g) PA:PE/TA:CE, (h) PA:PE/CE:CH double packaging. The CH biofoil packages were separated from each other with baking paper.*

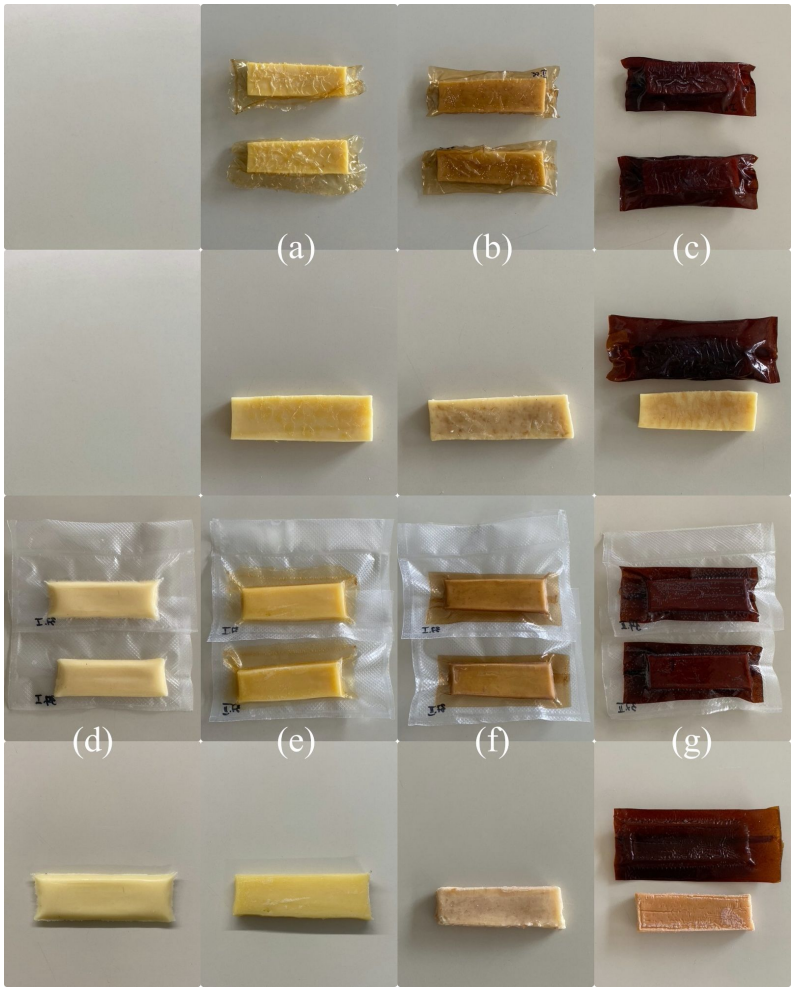

Figure A4. *The Gouda cheese after storage on day 37 at 4 ℃. Upper row, from the left corner, the pictures represent: (a) cheese packed in CH, (b) TA:CH, (c) CE:CH biofoil, and unpacked cheese under each. The bottom row depicts Gouda cheese packed in (d) PA:PE, (e) PA:PE/CH, (f) PA:PE/TA:CE, (g) PA:PE/CE:CH double packaging, and unpacked cheese under each. The only chitosan-based material that kept its uniformity was CE:CH, while others had to be removed in pieces.*

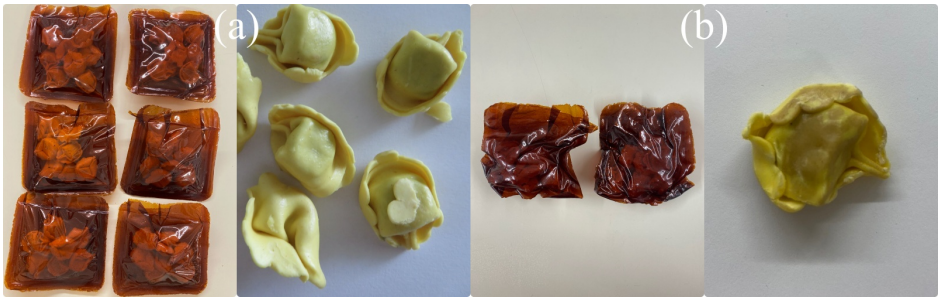

Figure A5. *The Gouda cheese before and after storage at 4 ℃. Upper row, from the left corner, the pictures represent fresh pasta packed in CE:CH at (a) day 0 and (b) day 30 with the fresh pasta under each at the same time points.*

APPENDIX 2

Publication I:

Novak, U., Bajić, M., **Kõrge, K.**, Oberlintner, A., Murn, J., Lokar, K., Triler, K.V., Likozar, B., From waste/residual marine biomass to active biopolymer-based packaging film materials for food industry applications – A review, *Phys. Sci. Rev.* 5 (2019) 1–24.

Uroš Novak[1] / Marijan Bajić[1] / Kristi Kõrge[1] / Ana Oberlintner[1] / Janja Murn[1] / Krista Lokar[1] / Karin Veronika Triler[1] / Blaž Likozar[1]

From waste/residual marine biomass to active biopolymer-based packaging film materials for food industry applications – a review

[1] Department of Catalysis and Chemical Reaction Engineering, National Institute of Chemistry Slovenia, Hajdrihova 19, Ljubljana 1000, Slovenia, E-mail: uros.novak@ki.si, marijan.bajic@ki.si, kristi.korge@ki.si, ana.oberlintner@ki.si, janjamurn@gmail.com, krista.lokar@gmail.com, karin.triler@gmail.com, blaz.likozar@ki.si. https://orcid.org/0000-0003-0561-8427, https://orcid.org/0000-0002-9283-244X, https://orcid.org/0000-0001-7226-4302.

Abstract:
Waste/residual marine biomass represents a vast and potentially underexplored source of biopolymers chitin/-chitosan and alginate. Their isolation and potential application in the development and production of bio-based food packaging are gaining in attractiveness due to a recent increment in plastic pollution awareness. Accordingly, a review of the latest research work was given to cover the pathway from biomass sources to biopolymers isolation and application in the development of active (antimicrobial/antioxidant) film materials intended for food packaging. Screening of the novel eco-friendly isolation processes was followed by an extensive overview of the most recent publications covering the chitosan- and alginate-based films with incorporated active agents.

Keywords: active food packaging materials, antimicrobial and antioxidant agents, biopolymers isolation, chitosan- and alginate-based active films, green processes, marine biomass

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1 Introduction

Our civilization is built on plastic, and according to The World Economic Forum, its amount is expected to triple by the year 2050 [1]. Yet, less than 15 % of it is currently being recycled. The highest use of plastic materials is intended for the packaging in the food industry, which represents up to 40 % of the total plastic consumption within the European Union [2]. Thus, the need for alternatives has recently got a lot of boost in the research of using bio-based or biodegradable materials.

Food processing and packaging are the most important parts of the food industry [3]. Due to increasing environmental burden, there is a growing effort to replace synthetic petroleum-based packaging materials with biodegradable and consumable materials synthesized from natural polymers. These changes are probably less related to any depletion of nonrenewable resources, but rather to increased interest in addressing sustainability aspects related to resource efficiency as well as waste disposal and treatment [4]. In this regard, governments, industries, and consumers are very much concerned about the impact of the products consumed. A recent review presents the valorization of abundant and available bio-wastes with high potential to manufacture value-added products, creating the first step to close the loop between waste and consumption in line to attain the main goal of the circular economy [5]. More processed and packaged food is consumed as a proportion of the total in better-off, urbanizing, and industrializing economies [6]. In the specific field of food packaging, there are clear trends with regard to the sourcing and use of raw materials.

Food is the main nutritional support for organism, hereby unsafe and contaminated food presents an unceasing health risk for billions of people all over the world. According to a comprehensive estimation of the global burden of foodborne diseases led by the World Health Organization (WHO), a consummation of contaminated food caused a hundred million cases of illnesses and thousands of deaths in 2010 [7]. Since microbial contamination can easily occur at every exposure of food to the external environment, conventional food preservation techniques (drying, fermentation, thermal processing, etc.) are often not enough to ensure high quality of food and efficient extension of food shelf life [8]. Referring to the aforementioned facts, it is obvious that new alternatives for limiting the microbial contamination and overall food deterioration are needed.

Marijan Bajić is the corresponding author.

Development of novel food packaging materials with antimicrobial and/or antioxidant activity is getting a broad research interest, whereby this kind of materials can be provided by the incorporation of various active agents (AAs) in the packaging formulations [9–12]. Since direct incorporation into food leads to a reduced antimicrobial activity over a short period of time, the incorporation into packaging matrix ensures greater agents stability. Providing slow but constant migration of AAs from the packaging material is another advantage of the active packaging systems since the control over microbial growth and antioxidant protection can be ensured for a prolonged period of time [8, 13–15].

The current review focuses on the biopolymers that are obtainable from the waste/residual marine biomass and that are potentially applicable in the preparation of active food packaging materials. In this regard, chitin (together with its derivative chitosan) and alginate are the most promising for this purpose due to their non-toxicity and good film-forming abilities leading to the production of mechanically stable films [16, 17]. Therefore, recent studies covering the most commonly used marine biomass as sources of the aforementioned biopolymers, extraction methods for their isolation, and consequent utilization in the preparation of active film materials are scrutinized.

2 Marine-based biomass as a source of chitin/chitosan and alginate

Chitin is known as the crucial structural polymer which constitutes a big portion of crustaceans' exoskeletons, whereby its content varies not only between different sources but also between different species [18]. In nature, there are three allomorphic forms of chitin: α-chitin (anti-parallel arrangements of polymer chains), β-chitin (parallel arrangements of polymer chains), and γ-chitin (with alternated arrangements of polymer chains; distinct, yet closer in structure to the previous two forms) [18, 19]. The most common α-chitin is found in crabs and shrimps (also in fungi, yeast, and insects), β-chitin is found in a combination with proteins (mostly in squid pens), while γ-chitin is found in the stomach of squids (and in the cocoon of moths and beetles) [19, 20]. Researchers have revealed the presence of chitin from other types of marine organisms as well (e.g. diatoms, corals, sponges) [21–24], further confirming its use in biological structures formations in nature. In terms of its availability, chitin is (next to cellulose) available to the extent of over 10 gigatons annually [25]. Besides, chitin is a precursor of chitosan, i.e. its N-deacetylated derivative whose chemical structure consists of D-glucosamine and N-acetyl-D-glucosamine sub-units linearly linked *via* β-1,4-glycosidic bonds [18, 26].

Alginates are naturally occurring, indigestible polysaccharides that are commonly produced by and refined from various brown seaweed (mainly from *Laminaria hyperborea*, *Macrocystis pyrifera*, *Ascophyllum nodosum*; in lesser extent from *Laminaria digitata*, *Laminaria japonica*, *Eclonia maxima*, *Lessonia nigrescens*, *Sargassum* sp.). The molecular structure of alginate is composed of unbranched, linear binary copolymers of α-D-mannuronic acid (M) and α-L-guluronic acid (G) residues linked *via* 1,4-glycosidic bonds. An algal-based alginate structure could be separated into three fractions (three uronic acid blocks): homopolymeric regions of M blocks, homopolymeric regions of G blocks, and alternating MG blocks containing both polyuronic acids [16]. The M:G ratio varies amongst brown seaweed taxonomic ranks (i.e. orders), and it is typically reported to be in the range between 0.8 and 2.2 [27]. Alginates isolated from *Laminaria hyperborea* generally have the highest guluronic acid content, whereas those extracted from *Laminaria japonica* and *Ascophyllum nodosum* are low in guluronic acid content [28–31]. Percentages of mannuronic and guluronic acids as well as M:G ratios of alginates from various commercial brown seaweeds are listed elsewhere in the literature (Table 2.1 in [32]).

2.1 Isolation of chitin/chitosan

Many different methods have been proposed for chitin (and hence chitosan) isolation, but no standard method has been adopted yet. Traditional methods are chemical-based and they rely on acidic demineralization and alkaline deproteinization as two major steps. Therefore, green technologies that are cost-effective and sustainable are being presented as a good choice [33]. A few novel alternative methods, such as those that are using enzymes and fermentation, deep eutectic solvents, ionic liquids, and plasma-based extraction, have been proposed as well (Figure 1).

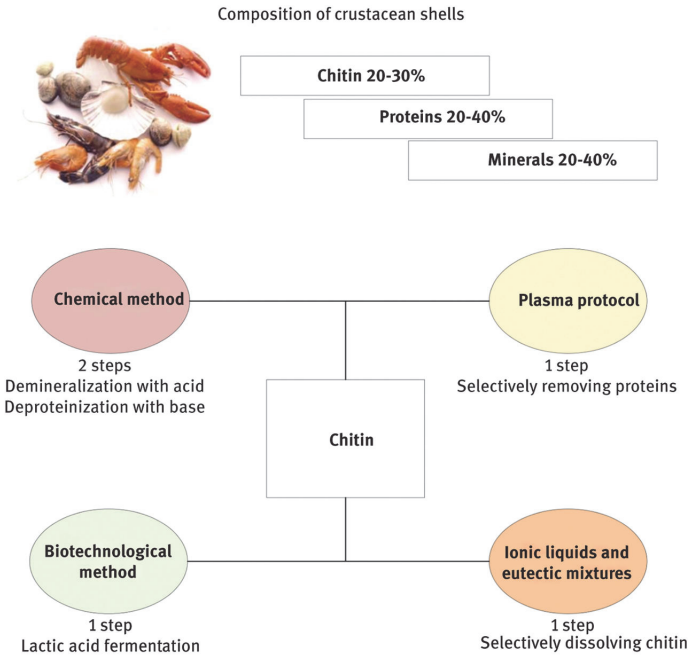

Figure 1: Schematic representation of available methods for the isolation of chitin from crustacean shells.

Chitin/chitosan have been successfully isolated from different marine organisms (e.g. shrimps [34–44], lobsters [43, 45], squid [46], crabs [38, 43, 47], crayfish [38], prawn and krill [43], etc.) by using methods summarized in Sections 2.1.1–2.1.4. Molecular weight (M_W) and degree of (de)acetylation of the final product(s) highly depend on the source, isolation methods, and deacetylation protocols, whereby more information on this topic can be found in other review articles dealing with chitin/chitosan extraction and characterization (e.g. Table 3 in [18] and Table 1 in [48]).

2.1.1 Chemical methods

The simplest and the most effective industrial method for the extraction of highly pure chitin is a chemical-based one, while other less efficient methods are more work- and time-consuming [49]. However, some chemical methods have several drawbacks: (i) large volume of corrosive acidic and basic wastewater hazardous to the environment, (ii) energy-consuming extraction and purification, and (iii) negative effect of strong acids on the physicochemical properties (lowering M_W). Although chemical methods are efficient, they do not grant full control over physical characteristics (crystallinity, purity, polymer chain arrangement, etc.), and besides other biomolecules (like proteins, lipids, carotenoids) are discarded [45, 50]. The chemical extraction of chitin followed by its derivatization into chitosan is conducted in three major steps: (i) demineralization, (ii) deproteinization, and (iii) deacetylation.

Demineralization: In this step calcium carbonate ($CaCO_3$) and other minerals are converted into water-soluble calcium salts (easily removed by washing) and carbon dioxide (CO_2) as a by-product. The most frequently used acids are hydrochloric (HCl), nitric (HNO_3), sulfuric (H_2SO_4), acetic (CH_3COOH), and formic (HCOOH), whereby HCl is being the most represented one. Parameters in this step (time, temperature, particle size, acid concentration, solid to liquid ratio) are determined empirically. Solid to liquid ratio is important since two molecules of HCl are needed for one molecule of $CaCO_3$, so acid intake should be equal or higher to the stoichiometric amount of minerals in order to achieve the complete reaction [50]. Usually up to 10 % of the acid is used with constant stirring at room temperature for about 2–3 h. To minimize depolymerization and deacetylation of chitin, HCl can be replaced with ethylenediaminetetraacetic acid (EDTA; $C_{10}H_{16}N_2O_8$), sulfurous acid (H_2SO_3), or CH_3COOH, but their usage increases the ash content [50]. Contrarily, the extraction of chitin from shrimp shells using mild conditions has been studied as well [34].

Deproteinization. This step is usually performed by chemical methods which assume the use of different deproteinization reagents such as sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), potassium hydroxide (KOH), calcium bisulfite ($CaHSO_3$), potassium carbonate (K_2CO_3), calcium hydroxide ($Ca(OH)_2$), sodium

bicarbonate ($NaHCO_3$), sodium sulfite (Na_2SO_3), sodium bisulfite ($NaHSO_3$), trisodium phosphate (Na_3PO_4), and sodium sulfide (Na_2S), among which NaOH is the most used one [50]. Instead of NaOH, cheaper calcium oxide (CaO) can be used to increase the ionic strength and to extract proteins. Anyhow, high ratios of solid to alkali (1:10 or 1:20) are suggested for the uniform reaction [34]. Alkali reagents can cause partial depolymerization and deacetylation of chitin due to continuous hydrolysis, therefore a change in the mechanical properties and lower M_W of chitin has been observed [50]. Reaction conditions vary considerably, and mean use of 0.125 M to 5 M NaOH with temperatures up to 160 °C and time from a few minutes up to several days. Longer times (up to 24 h) result only in a slight drop in the ash content, but on the other hand can cause polymer degradation [34]. It has been suggested that solid sodium chloride (NaCl) treatment followed by demineralization and deproteinization better preserves chitin structure [51]. Tolaimate et al. have proposed a new approach with successive baths with lower concentrations of HCl (0.55 M) and NaOH (0.3 M) for good preservation of the native chitin (100 % acetylated) [46]. Furthermore, a shorter alkaline process at room temperature has been suggested to avoid chitin depolymerization [52]. A simple fractionation method using hot water for deproteinization and carbonic acid (H_2CO_3) for demineralization with high efficiency and chitin purity in a short time (within hours) has been addressed as well [35]. After these steps, chitin can be still colored so the sample can be bleached, but it is neither really needed nor advised since it causes a decrease in the viscosity (i.e. M_W) of chitin [53].

Deacetylation: In the last step, acetyl groups are partially removed from chitin leaving behind chitosan with highly reactive amino groups. Acids or alkalis can be used, but the latter ones are preferred since the glycosidic bonds are sensitive to acids. Deacetylation can be divided into two categories: (i) heterogeneous (producing insoluble chitosan), and (ii) homogenous (producing soluble chitosan). Concentrated solutions of NaOH or KOH can be used, but the latter one is less effective [50]. In the methods that use highly concentrated NaOH (50–60 %) at high temperatures (130-150 °C), deacetylation is very fast (within 2 h) but a balance must be found between time and depolymerization [54]. The chemical deacetylation has some environmental disadvantages such as large energy input, large waste of concentrated alkaline solution as well as heterogeneous deacetylation range of soluble and insoluble products with different M_W [37, 50].

Several studies have been aiming to improve the chemical method yield and impact on the environment. One has been conducted to determine if the modifications in the production sequence have any effect on yield, physicochemical, and functional properties [39]. It was found that demineralization and deproteinization steps can be reversed, but for higher yields deacetylation is preferred to be performed the last one. The highest chitosan yield is obtainable with a sequence of demineralization, deproteinization, deacetylation, and decolorization [39]. Besides, chitin and chitosan can be modified into many products with desired novel attributes and functions suitable for different applications [18, 55, 56].

A new method using 3 % of sodium hypochlorite (NaClO) for 10 min before demineralization and deproteinization for time and energy saving has been proposed by Kaya et al. [38]. Furthermore, a soft alkaline treatment with much lower chemical use (and with possible NaOH and water recovery) has been suggested in order to improve the negative influence on the environment [57]. In addition to this, designers from the Royal College of Art (London, UK) and the Imperial College of London (London, UK) have developed a small-scale desktop chitin extractor from seafood waste called "Shelly", which allows automated control over each parameter in order to obtain different grades of chitosan [58].

2.1.2 Biotechnological methods

Green isolation methods have been promoting the use of enzymes and microorganisms. Biotechnological-based extraction of chitin holds higher reproducibility, shorter processing time, lower solvent/energy consumption, and higher preservation of the native form [50]. Nevertheless, this method is still bound to the laboratory scale due to disadvantages such as low chitin yield, costly enzymes, challenging scale-up (entire process requires sterile conditions), and long cycles in the microbial fermentation [45, 50].

Chemical and biotechnological methods involve analog steps: (i) demineralization (using lactic acid bacteria in case of biotechnological method), (ii) deproteinization (with commercial enzymes or with proteolytic bacteria), and (iii) deacetylation (with chitin deacetylase or lactic acid bacteria), or by hydrolysis (using chitinolytic enzymes) [59]. A comparative study between chemical and biotechnological methods for chitin extraction has been performed by Khanafari et al. [41]. A biorefinery-based method, which means crustacean shells fractionation to the main components and their transformation into value-added materials, is still in the developing stage but it could create a new and profitable market with its multiple applications [60].

Enzymes can be used for deproteinization, therefore avoiding the application of strong alkaline treatments. Procedures with enzymes are fast, production conditions are mild, complicated equipment is not required, and lower deacetylation and depolymerization (in regard to the chemical method) have been reported [37, 61]. Due to lower efficiency, an additional NaOH step may be needed to achieve higher purity. Since minerals can limit

proteases access and lower efficiency, demineralization should be performed first [50]. To enhance accessibility, a pre-treatment can be used with physical or chemical methods such as sonication, grinding, and heating [62]. A cheaper alternative to commercially purified enzymes is crude proteases, which are also more efficient and eco-friendly [50].

Sustainability assessment of chemical and enzymatic processes has been done by Lopes et al. [61]. It has been shown that even the production of enzymes and chemical reagents in small quantities requires more energy and raw materials. The energy and enzyme consumption is high due to a low yield, but the overall enzymatic process is in overall 20 % more favorable to the environment, as compared to the chemical one. The chemical process has high production costs and requires waste management, but a higher yield of chitin increases profit. However, a more homogenous biocatalytic production of chitosan with defined size and degree of acetylation (DA) has been conducted under mild conditions with recombinant chitin deacetylase [37].

The enzyme cost can be lowered if deproteinization is performed by a fermentation process. This can be achieved by endogenous microorganisms (auto-fermentation) or by the addition of selected microbial strains. In a microbial fermentation, deproteinization and demineralization steps are processed simultaneously [50]. Proteins and minerals are removed by a combination of enzymatic activity and mineral solubilization by organic acid produced during bacterial growth [42]. Fermentation process (deproteinization and demineralization) by protease and organic acid bacteria followed by deacetylation with chitin deacetylase is an example of the alternative and economical method [63]. For industrial requirements, a combined chemical and biotechnological (fermentation) methods with the application of seawater for chitin extraction could be used as well [43].

Fermentation of crustacean shells can be performed by bacterial strains that consume proteins and decompose $CaCO_3$, or with *Lactobacillus* strains, which produce lactic acid and proteases, whereby lactic acid reacts with $CaCO_3$ and forms a precipitate. Rao et al. have studied the effect of different fermentation parameters on deproteinization and demineralization [44]. In non-lactic acid fermentation, both bacteria and fungi can be used for crustacean shells fermentation. A one-pot fermentation has been reported for the production of chitin where fungi proteases hydrolyze proteins into amino acids that present a nitrogen source for fungal growth [36]. The biotechnological process can be also followed by mild chemical treatment to remove the residual protein and minerals [50].

2.1.3 Ionic liquids- and deep eutectic solvent-based methods

The use of ionic liquids (ILs) in chitin extraction is a relatively new approach, thus most of the studies are still at the laboratory scale. ILs are salts with unique properties, being composed of a wide range of raw and renewable materials such as organic salts, sugars, and amino acids. Their infinite anion/cation combinations give rise to the favorable designer solvent character, allowing them to be tailor-made according to the final applications [33].

Chitin extraction with ILs has many advantages: (i) less energy, time, and chemicals are used in comparison to the chemical methods, (ii) high M_W is achieved, (iii) direct chitin extraction from marine waste is possible, (iv) broad range of usage, (v) possibility for recycling/reuse, and (vi) more sustainable alternative to organic solvents due to higher thermic and chemical stability and low vapor pressure [64, 65]. Therefore, ILs can be recycled, which presents an important economic aspect. Nevertheless, they cannot be purified by distillation, so recycling with vacuum treatment, supercritical fluids, and soxhlet extraction can be used [64]. In contrast, IL extraction seems to be a promising method, but some disadvantages (moisture sensitivity, difficult recycling, high cost) challenge large-scale production [50, 60]. ILs are considered to be green solvents, although their effect on the environment has not been entirely understood yet [66].

The extraction process for chitin isolation requires only IL which dissolves chitin leaving the proteins and minerals undissolved, coagulation solvent (water or alcohols), and direct heating [33, 65]. For chitin extraction with ILs only a few studies exist, but ammonium-based and choline-based ILs with acetate and chloride are considered as the most promising and safe [64]. Series of ILs have been synthesized and their chitin-dissolution ability has been evaluated under mild condition [67]. Low-cost ILs with highly acidic and basic ions, such as $[NH_3OH][OAc]$, can be used to pulp shrimp shells with high chitin yield and purity. An aqueous solution of this IL was found to be effective solvent for chitosan at room temperature even in the presence of water [68]. A high DA of chitosan can be attained by a simple hydrothermal treatment in the 1-butyl-3-methylimidazolium acetate-chitosan-water system without alkali use, which also allows recovery and reuse of IL [69]. A pre-treatment with ionic liquids can also weaken chitin structure and decrease its crystallinity for better efficiency of double chitinase hydrolysis [47].

Deep eutectic solvents (DESs) are novel sustainable solvents that can replace organic solvents or ILs. DESs present a mixture of hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) that self-associate

through H-bonds and can be used to dissolve poorly soluble chitin. Chitin can be selectively isolated by breaking strong H-bonds in the reaction between chitin NH_2 and donors of substituents in a DES [70]. DESs are considered to be superior over ILs because they are biodegradable, but also have low toxicity and relatively low price. On the other hand, they share low volatility and wide polarity range with ILs [70–72]. A DES allowed a downstream protocol that enables multiple extractions in a sequence without the need to isolate minerals and proteins [73].

Natural deep eutectic solvents (NADESs) are able to produce chitin in a single, fast, and eco-friendly step to minimize water and toxic chemicals consumption in deproteinization and demineralization. The most promising commercially available NADESs are choline chloride lactic acid (CCLA), malonic acid (CCMA), urea (CCUR), citric acid (CCCA), thiourea (CCT), and glycerol (CCG) [45, 71, 73]. In a single step, NADESs have to play three roles: (i) demineralization − organic acid (HBD) must be used since $CaCO_3$ removal occurs under acidic conditions (in the same time minerals are partially degraded), (ii) deproteinization and chitin dissolution by breaking H-bonds with choline chloride (HBA) which is then precipitated with water. Bradić et al. have studied temperature and time influences on chitin extraction process for higher yield and purity [73]. In CCUR (alkaline pH) chitosan had the highest solubility, but that is not necessarily good, since proteins and minerals have to be removed first. By using CCMA, chitin can be divided into two parts (supernatant and precipitate) with different crystallinity and thermal stability. CCMA could successfully remove $CaCO_3$, so it could replace acid in chemical methods [45]. The alternative green approach to synthesize a permanently positively charged *N*-methylated chitosan for a better solubility has been introduced in order to avoid using organic solvents in alkaline conditions with non-selective methyl iodide (CH_3I) [74].

2.1.4 Plasma-based method

The first solvent-less protocol using atmospheric pressure dielectric barrier discharge plasma-based separation method as a pretreatment process for deproteinization in chitin production has been reported by Borić et al. [75]. Although the pre-treatment process was very fast (1.5 min – 6 min), proteins had been intensively removed while preserving the native structure of chitin. This method does not require any solvents or produces hazardous waste, and scale-up is possible due to operating at atmospheric pressure. This alternative method for chitin extraction uses plasma, which can break C–C or C–H bonds, but inorganic materials remain inert. The method is carried out by placing a whole shell body part in the gap between the electrode and the quartz tube. Therefore, plasma in combination with different gasses (N_2 and O_2) can be used for selective protein removal from the shrimp shells [75].

2.2 Isolation of alginate

Alginate is isolated from the cell walls of brown seaweed (about 40 % of dry weight), where it is responsible for the strength and flexibility. In the natural state, it is bonded with seawater ions, mainly Ca^{2+} and smaller amounts of Na^+, Mg^{2+}, Sr^{2+}, and Ba^{2+} [76]. Alginate is mostly used in a sodium form due to its better solubility in cold water. The aim of the extraction method is to turn water-insoluble alginate salts into water-soluble sodium salts, whereby cellulose remains undissolved [76]. Alginate is subsequently recovered as alginic acid or calcium alginate, and there are two isolation methods which start with similar extraction procedures but vary in the intermediates formed during precipitation. In the first one (which is also commercially used), calcium alginate and alginic acid are the main intermediates, and in the second one only alginic acid is formed [32]. The first method is usually preferred when alginic acid forms an insoluble gel that can plug filters. The chemical process can also be performed by a hot (50 °C) or cold (25 °C) method [77].

The chemical method usually has five relatively simple steps: (i) raw material fragmentation and ethanol treatment to remove pigments and lipids for easier processing, (ii) transforming alginate salts into insoluble alginic acid with acid pre-treatment (HCl or H_2SO_4) which also breaks the cell walls, (iii) transforming alginic acid into soluble sodium alginate (SA) with alkaline extraction (Na_2CO_3 or NaOH), (iv) precipitation (H_2SO_4, HCl, alcohol, or $CaCl_2$) followed by filtering, (v) and drying (if precipitated with alcohol) [32, 78]. Most of the unwanted substances (fucoidans, laminarins, and polyphenols) can be also removed by acid treatment. Polyphenols can oxidize into brown substances under alkaline conditions; therefore, a mild pre-treatment with formaldehyde is needed to make them insoluble by polymerization [32, 78]. Pre-treatments and alternative solvents (ethanol, methanol, acetone) that would allow alginate extraction and retrieve polyphenol-rich fraction have been investigated [79]. For pigment removal, formaldehyde can be avoided by using photobleaching, which was reported for agar but could also be tested on alginate [76]. The alkaline (or main extraction step) is time-, water-, and reactant-consuming, and is usually carried out as 2 % $CaCO_3$ with pH 10 at 80 °C, inde-

pendently of species. On the other hand, acid treatment conditions vary greatly [80]. The alginate-influencing extraction parameters have been studied by Fertah et al. [81], while Davis et al. have shown that alginate yield is independent of the temperature or the extraction method employed [27]. Since alginate includes a lot of contaminants, it needs to be purified with ethanol, methanol, and acetone for medical use [81].

The chemical method for alginate isolation is not eco-friendly or cost-effective due to: (i) high energy, water, and solvent use, (ii) quite expensive alcohol, (iii) need for wastewater treatments, and (iv) lower yields caused by degradation (since alginate cannot be precipitated) [80, 82]. On the contrary, a study dealing with alkaline extraction kinetics has reported that alginate depolymerization in the alkaline step could reduce extraction time in order to obtain better rheological quality [83]. There has also been found a relation between extraction yield and algal destruction [83]. The chemical method has become traditional for industrial extraction, but still holds certain limitations such as efficiency and product consistency. On the contrary, some novel and greener extraction methods have been proposed, but many of them are still under development on the laboratory scale, so the most environmentally sustainable one has not been identified yet [76].

A continuous and green method for the industrial isolation of alginate might use reactive extrusion with a twin-screw extruder to avoid using the alkaline extraction step [83]. By using this method, yield can be increased by 15 %, time-scale shortened from hours to minutes, water and reactants use can be reduced two-fold, while the purity remains high in comparison to the chemical method. Besides, alginate of a high M_W and superior rheological properties can be obtained due to shorter processing time (which reduces depolymerization), while costly equipment could be a drawback of the method [83].

An alternative method might be a microwave-assisted extraction (MAE) since it could overcome drawbacks like alginate thermal instability, long processing time, cost-ineffectiveness, and low yield [84]. Although this isolation method is used for other compounds, hardly any reports have been published with MAE for alginate extraction. On the contrary to the chemical method, which only heats up the surfaces from where heat is conducted to the core of the particles, MAE works by heating up the system with microwave energy [85]. Acid pre-treatment with 0.1 M HCl for MAE has also been optimized for shorter times and lower solvent usage [80].

There are a few studies for ultrasound-assisted extraction (UAE) of alginate capable of replacing the alkaline step, and whose advantages encompass: (i) extraction in only minutes, (ii) high reproducibility, (iii) lower solvent consumption, (iv) high purity, (v) simple process, (vi) no wastewater treatment, (vii) very low energy use, and (viii) easy scale-up [86–88]. Ultrasound allows better solvent penetration into the sample, and hence increasing contact area and reducing extraction time without influencing the chemical structure or M_W [89]. Youssouf et al. have studied the effect of temperature, pH, and ultrasound power for optimal extraction [90]. UAE can be also coupled with microwave (UMAE), which is considered to be the most promising hybrid technique for fast and cost-effective extraction, but has not been applied by many authors yet. UAE could be also combined with supercritical fluid extraction or extrusion extraction [86].

For alginate extraction, complex algae cell walls need to be broken, therefore enzyme assisted extraction (EAE) method that applies enzymes such as proteases and carbohydrases, might be used [85]. EAE holds several advantages: (i) eco-friendliness, (ii) low cost, (iii) high yield, and (iv) ability to make water-soluble materials. A pre-treatment with cellulase or alcalase might as well be applied instead of the acidic step before the extraction with Na_2CO_3 [91, 92]. With cellulase, it is possible to achieve a high yield of highly pure alginate, which possesses immunostimulatory and weak antioxidant activity. Commercial enzymes might be used instead of the acid step [93], but also other compounds could be extracted after digesting the cell wall [94].

Compounds obtained with supercritical fluid extraction (SFE) show very high purity without any residual solvents. SFE method is (i) eco-friendly, (ii) low cost, (iii) non-flammable, and (iv) time-saving since the sample concentration is not needed [85]. Widely available, low cost, and eco-friendly water or CO_2 can be used as supercritical solvents. To the best of your knowledge, there is no report of the alginate extraction by this method. Nevertheless, a pressurized solvent extraction (PSE) in an extraction method that uses temperatures in the range from 50 °C to 200 °C and pressures in the range between 35 bar and 200 bar. A high temperature combined with increased pressure causes an increase of solubility and penetration of solvent into the sample and therefore enhancing the extraction process. This method is very similar to the soxhlet extraction, but the solvents employed are in subcritical state and thus have high extraction abilities. The advantages of PSE are high extraction efficiency, simple instruments, and relatively short extraction time [95, 96].

Finally, it is important to point out that biological and physicochemical (M:G ratio, M_W) properties of alginate are dependable on the extraction method. For instance, by applying different extraction methods it was possible to produce alginates from *Colpomenia peregrina* and *Sargassum angustifolium* with M_W ranging from ~247×10^3 g/mol to ~354×10^3 g/mol and from ~ 356×10^3 g/mol to ~557×10^3 g/mol, respectively [91, 92].

3 Active chitosan- and alginate-based films

According to G.L. Robertson, active packaging can be defined as "packaging in which subsidiary constituents have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system" [97].

Most foods are susceptible to microbial contamination. A way to tackle this problem could be to add antimicrobial compounds directly in food products, but that in turn might lead to the reduction of the active compounds' efficiency and change foods' organoleptic properties. On the other hand, the application of antimicrobial films has shown to overcome these problems as well as preserve quality and increase the shelf life of various food products [9, 98–100]. The enhancement in the quality of food products is achieved throughout the inhibition of the target microorganisms. In addition to the chemical agents, a broad variety of natural antimicrobial components (essential oil, plant extracts, enzyme, bacteriocins, and probiotics) might be incorporated into packaging materials to boost their antimicrobial activity [12, 101–103]. Antioxidant protection of perishable foods also plays a crucial role [12, 104, 105], and therefore the improvements in the films' antioxidant activity after the incorporation of natural-based compounds is of paramount importance.

3.1 Antimicrobial activity of chitosan- and alginate-based films

Chitosan possesses antimicrobial activity against a wide range of bacteria, yeast, and fungi [106–108]. The most accepted hypothesis of its antimicrobial activity is based on the presence of positively charged amino groups (NH_3^+) of glucosamine (chitosan molecule becomes polycationic in the acidic environment, i.e. when pH is below the pKa of chitosan) which might react with negatively charged molecules on the microbial cell surface [109]. Such electrostatic interactions cause extensive alterations to the cell surface and leakage of intracellular components or inhibition of nutrient penetration into the cell, which eventually leads to cell death [109]. The charged amino groups interact either with lipopolysaccharides on the cell surface of Gram-negative (G–) bacteria or with teichoic acids on the cell surface of Gram-positive (G+) bacteria. A similar mechanism of action might be possessed against fungi, although chitosan's antifungal efficiency is shown to be low [110]. The key factors that affect chitosan's antimicrobial activity include environmental factors (pH, T), microbial factors (the type of microorganism and phase of the cell growth), and intrinsic factors (M_W, DA, derivate form, concentration, etc.) [111].

In spite of the fact that chitosan has inherent antimicrobial activity, chitosan-based films are usually incorporated by different AAs in order to boost it up (Sections 3.1.1 and 3.1.2). On the contrary to chitosan, alginate does not have inherent antimicrobial activity, but alginate-based films with incorporated AAs do have (Section 3.1.3).

3.1.1 Antibacterial activity of chitosan-based films with incorporated active agents

In all herein reviewed studies, the antibacterial activity of chitosan-based films was tested *in vitro*. The antibacterial efficiency is often expressed as a diameter of the inhibition zone using the disc diffusion method or by evaluating bacterial burden reduction through counting colony-forming units (CFU) or measuring the optical density of a sample. The tests were accomplished against the most common foodborne pathogens and representatives of (G+) and (G–) bacteria. Among (G–) bacteria, *Escherichia coli* and *Salmonella typhimurium* are reported as a leading cause of many severe and fatal foodborne outbreaks mostly related to meat and meat products [112]. In the majority of studies, *Staphylococcus aureus* was used as a representative of (G+) bacteria since it is a major public health concern worldwide as well as the most common cause of foodborne disease in the United States [113]. Another very concerning (G+) bacteria is *Listeria monocytogenes*, responsible for disease listeriosis associated with a high mortality rate [114].

Generally speaking, control chitosan-based films (i.e. without incorporated AAs) have showed certain antibacterial activity in the majority of overviewed studies (Table 1). However, in many cases a lack of the inhibition zone has been reported, whereby growth inhibition has been observed only in the area that is in direct contact with a film [131]. This is mostly on account of chitosan's solid-state possessed in the form of a film, which disallows efficient diffusion of chitosan into the agar medium and therefore to pathogenic microorganisms. Anyway, a quest for new methods and active components that could improve the antibacterial activity of chitosan-based films has appeared as a "hot topic" in recent times (Table 1).

Table 1: Recent studies on the antibacterial activity of chitosan-based films with incorporated active agents.

Active agent (AA)	AA concentration in the film-forming solutions	Microorganism	Expression of antibacterial activity	CH[a]	CH-AA[b]	Ref.
Ziziphora clinopodioides EO (ZEO)	1 % (v/w)	*Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Bacillus subtilis*, *Bacillus cerus*	Inhibition zone, Log reduction of CFU/mL	–	+(i)	[115]
Grape seed extract (GSE)	1 % (v/w)			–	+(i)	
Turmeric extract (TEE)	1:2 (v/v) dilution of CH solution with TEE in ethanol (25 mg_{TEE}/mL)	*Staphylococcus aureus*, *Salmonella typhimurium*	Log reduction of CFU/g sample	+	+(i)	[116]
Zinc oxide (ZnO)	0.1–0.5 % (w/v)	*Escherichia coli*	Inhibition zone	+	+(i)	[117]
Neem oil	0.5 % (v/v)			+	+(i)	
Litsea cubeba oil (LEO)	4 – 16 %; total weight	*Escherichia coli*, *Staphylococcus aureus*	Inhibition zone	+	+(i)	[118]
Citrus extract	0.5 % (v/v)	*Listeria innocua*	Inhibition zone	–	+(i)	[119]
Naringin	0.05 – 1 % (w/v)			–	–(d)	
ε-Polylysine	ε-polylysine:chitosan mixtures (weight ratios 1:5–1:15) dissolved in distilled water	*Escherichia coli*, *Staphylococcus aureus*	Inhibition zone	–	+	[120]
β-Cyclodextrin (β-CD)/EO complex	0.25 – 1 % (v/v)	*Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*	Log reduction based on optical density measurement	+	+(i)	[121]
Zinc oxide nanoparticles (Zn-NPs)	0.5 – 2 % (w/v)	*Escherichia coli*, *Staphylococcus aureus*	CFU counting	–	+(i)	[122]
Zinc oxide (ZnO)	2 – 8 %; total weight	*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cerus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*	Inhibition zone	–	+(i)	[123]
Montmorillonite-copper oxide (MMT-CuO)	1 – 5 % (w/w); based on CH mass	*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeroginosa*, *Bacillus cerus*	Mortality rate based on CFU counting	+	+	[124]
			Inhibition zone	+	+	
Spirulina extract (SE)	2.5 – 20 % (w/v)	*Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Bacillus subtilis*, *Bacillus cereus*	Inhibition zone	–	+(i)	[125]
Cinnamon bark oil (CBO)	1 – 3 % (w/w)	*Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*	Inhibition zone	–	+(i)	[126]
Citrus EOs	0.5 % (v/v)	*Listeria monocytogenes*	Log reduction of CFU/cm^2	+	+(i)	[127]
Eucalyptus globulus EO	1 – 4 % (v/v)	*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*	Inhibition zone	–	+(i)	[128]

Caraway EO/beeswax	1 % (v/v) (caraway)/18 – 90 km/m^3 (beeswax)	*Escherichia coli*, *Staphylococcus aureus*	Reduction of CFU/mL	+	–	[129]
Hop extract (HE)	0.1–1.5 % (w/v)	*Escherichia coli*, *Bacillus subtilis*	Inhibition zone/Contact inhibition	+	+(i)	[130]
Oak extract (OE)/algal extract (AE)	0.1 % (w/v)	*Escherichia coli*, *Bacillus subtilis*	Inhibition zone/Contact inhibition	+	+	[131]
Propolis extract (PE)	2.5 – 20 % (w/w); based on CH mass	*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*	Inhibition zone/Contact inhibition	–	+(i)	[132]
Syringic acid	0.125–0.5 g in 50 mL of film-forming solution	*Escherichia coli*, *Staphylococcus aureus*	Inhibition zone	+	+(i)	[133]

[a] CH − chitosan-based films.
[b] CH-AA − chitosan-based films with incorporated AA: (–) no antibacterial activity; (+) antibacterial activity; (i) antibacterial activity increased after the incorporation of AA; (d) antibacterial activity decreased after the incorporation of AA.

Nanocomposites are based on natural polymer matrix incorporated with nanoparticles (NPs) [134]. Since some NPs have shown convincing antibacterial and antioxidant properties, nanotechnology has emerged as a good alternative for the improvement of chitosan-based films' antibacterial activity [135]. For instance, stable ZnO-NPs are classified as Generally Recognized as Safe (GRAS), and therefore represent one of the most frequently studied nano-based materials for the development of active food packagings. Consequently, ZnO-bionanocomposite-blended chitosan films have been used as pouches to study antimicrobial activity and effectiveness in extending the shelf life of meat, cheese, and carrots [117, 122, 123]. Next to it, montmorillonite-copper oxide (MMT-CuO) nanocomposite was incorporated into chitosan matrix as a reinforcement and antibacterial agent [124]. MMT also serves as a stabilizer of copper ions by preventing their uncontrolled leaching and toxicity. Since it has been shown that MMT-CuO nanocomposite significantly improves antibacterial activity against (G+) and (G–) bacteria, a chitosan-MMT-CuO nanocomposite film was considered as a promising novel active food packaging [124]. However, there is a growing concern related to the application of NPs because they might have different physicochemical properties than their larger counterparts, and therefore might cause health problems [134].

A growing awareness of food safety and increasing life standard have led to even higher public disapproval and negative perception of the application of synthetic additives as food preservatives. Essential oils (EOs), which are natural compounds, i.e. secondary metabolites of aromatic plants produced for their protection against pathogens and herbivores, present a good substitute [103, 136]. Nowadays, there are many studies that approve EOs' broad antibacterial activity against bacteria, yeast, and molds [136]. Although the exact mechanism of their action is still unknown, the most common explanation is related to hydrophobic nature of their main compounds which might contribute to a disruption of the cell membrane, cytoplasmatic leakage, cell lysis, and eventually cell death [136]. In general, (G+) bacteria are more susceptible than (G–) bacteria, which is attributed to differences in the cell wall structure of (G+) and (G–) bacteria. The latter is due to dense hydrophilic lipopolysaccharide covering, which prevents diffusion of hydrophobic compounds more resistant to the EOs [136]. *Ziziphora clinopodioides* EO (ZEO) and grape seed extract (GSE) [115], turmeric extract (TE) [116], *Litsea cubeba* oil (LEO) [118], *Eucalyptus globulus* EO [128], and caraway EO [129], present examples of a successful application of EOs as antibacterial agents in chitosan-based films (Table 1).

However, there are some drawbacks that limit the use of EOs as food preservatives. Low water solubility demands their incorporation in higher amounts in the film-forming formulations, which can negatively affect food organoleptic properties due to their intense aroma and potential toxicity. High extraction costs and a quick and significant decrease in their effectiveness due to relatively high volatility are just other restrictions to the extensive application of EOs. Sun et al. have developed β-cyclodextrin-EOs complexes that increase the water solubility of EOs and hence enable their use in lower concentrations [121]. Increased water solubility might also lead to the increased contact surface between pathogens and EOs, thus effectiveness is also improved [121]. EOs are also known to cause the formation of particular structures in the chitosan-based films that scatter visible light, whereby this problem was overcome by the incorporation of microemulsions of cinnamon bark oil and soybean oil [126].

The antibacterial activity of chitosan-based films can be also improved by the incorporation of ε-polylysine − a water-soluble, biodegradable, and non-toxic homo-poly-amino acid characterized by the peptide bond between the carboxyl and ε-amino groups of L-lysine [120]. Its antibacterial activity is related to the polycationic amino groups that are responsible for ε-polylysines electrostatic adsorption to the cell surface leading to the disruption of the outer cell membrane [120]. Besides, the extract from cyanobacterium *Spirulina* incorporated in chitosan-films has shown a positive antibacterial effect, because it is a good source of various active polyphenolic compounds [125].

3.1.2 Antifungal activity of chitosan-based films with incorporated active agents

Fungi present one of the major causes of post-harvest decay of various agricultural foods (such as cereal crops, fruits, vegetables), and are responsible for a big portion of food waste and thus large economic losses in agriculture [137, 138]. Besides, it could lead to serious life threats if fungi-contaminated food is consumed. By that, mycotoxin-producing fungi present the major health concern and a leading cause of acute poisoning. In general, *Aspergillus*, *Fusarium*, and *Penicillium* have been reported to be the most commonly responsible for mycotoxin food contamination [139]. The most recent publications of chitosan-based films with antifungal activity have been collected and presented in Table 2.

Table 2: Recent studies on the antifungal activity of chitosan-based films with incorporated active agents.

Active agent (AA)	AA concentration in the film-forming solutions	Microorganism	Expression of antifungal activity	CH[a]	CH-AA[b]	Ref.
Anise, oregano, cinnamon EOs	250 ppm	*Penicillium* sp., *Rhizopus* sp.	Inhibition zone	–	+(i)	[140]
Quince juice, cranberry juice	lyophilised cranberry juice:water weight ratios: 1:19, 2:18, 3:17; lyophilised quince juice:water weight ratios: 1:16, 2:15, 3:14	*Penicillium expansum*	Inhibition zone	–	+	[141]
Cinnamon (CEO) and ginger (GEO) EOs	4.4–13.2 % (w/w) (CEO) and 3.5–10.6 % (w/w)(GEO); based on CH mass	*Aspergillus niger*	Inhibition zone	+	+(i)	[142]
Thyme-oregano; thyme-tea tree, or thyme-peppermint EOs mixtures	0.13 and 0.19 % (w/w)	*Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Penicillium chrysogenum*	Log reduction of CFU/g sample	–	+(i)	[143]
Cinnamon leaf EO	0.25 – 1 % (w/w)	*Aspergillus niger*, *Botrytis cinerea*, *Rhizopus stolonifer*	Inhibition zone	–	+(i)	[144]
Eucalyptus globulus EO	1 – 4 % (v/v)	*Candida albicans*, *Candida parapsilosis*, *Botrytis cinerea*	Inhibition zone	–	+(i)	[128]

[a] CH − chitosan-based films.
[b] CH-AA − chitosan-based films with incorporated AA: (–) no antifungal activity; (+) antifungal activity; (i) antifungal activity increased after the incorporation of AA.

Incorporation of EOs has significantly improved the antifungal activity (usually increases with increasing concentration of EOs) of blended films, since pure chitosan films barely show any antifungal activity (Table 2). However, it highly depends both on fungi species and on the type of EO, whereby the following plant extracts have been tested so far: cinnamon [140, 142, 144], oregano and anise [140], ginger [142], quince and cranberry juice [141], thyme [143], and *Eucalyptus globulus* EOs [128]. For instance, cinnamon EO is more effective against *Aspergillus niger* than ginger EO [142]. Besides, it has been shown that antifungal activity could be improved by the incorporation of EOs mixtures instead of single sort of EOs [143]. A combination of EOs from thyme and oregano, tea tree, or peppermint has reduced fungal growth of *Aspergillus* and *Penicillium* species by 51 % – 77 % [143].

3.1.3 Antibacterial and antifungal activity of alginate-based films with incorporated active agents

Contrary to chitosan, SA has no inherent antimicrobial activity and thus fails to provide a barrier against microbial infections, which could restrict its application. However, it has been increasingly regarded as a promising food packaging material due to its water-solubility, non-toxicity, biocompatibility, biodegradability as well as capability of forming films with incorporated different AAs.

According to the recent publications related to the development of alginate-based films, SA is rarely used as a sole component. To improve mechanical and water-resistance properties, a formation of composite or nanocomposite films is preferred. Composite films are mostly gained through blending with other biopolymers, such as chitosan, carboxymethyl cellulose (CMC), or microfibrillated cellulose (MFC). Nanocomposites are formed through the incorporation of NPs, like nano-sized clay in SA matrix. Composites or nanocomposites also serve as a good matrix for the incorporation and stabilization of various antimicrobial agents [145]. The most recent studies dealing with the improvement of antimicrobial activity of alginate-based composites are summarized in Table 3.

Table 3: Recent studies on the antimicrobial activity of alginate-based films with incorporated active agents.

Active agent (AA)	AA concentration in the film-forming solutions	Microorganism	Expression of antimicrobial activity	SA	SA-AA	Ref.
Ag-nanoparticles, grape seed extract (GSE)	10 % (w/w) of GSE; based on polymers mass	*Escherichia coli*, *Listeria monocytogenes*	Log reduction of CFU/ml	–	+(i)	[146]
Pyrogallic acid (PA)	0.01–0.04 % (w/w); based on polymers mass	*Escherichia coli*, *Staphylococcus aureus*	Inhibition zone	–	+(i)	[147]
Au-TiO_2-nanoparticles	up to 2.5 %; total weight	*Escherichia coli*, *Staphylococcus aureus*	Survival rate based on CFU counting	–	+(i)	[148]
Lactococcus lactis	0.5–2.5 % (w/w)	*Escherichia coli*, *Staphylococcus aureus*	Log reduction of CFU/cm^2	–	+(i)	[149]
Microfibrillated cellulose/chitosan-benzalkonium chloride complex (MFC/C–BC)	2 – 14 % (w/w); based on SA mass	*Escherichia coli*, *Staphylococcus aureus*	Inhibition zone	–	+(i)	[150]
Carboxymethyl chitosan-ZnO nanoparticles	0.005–0.05 % (w/w); based on SA mass	*Escherichia coli*, *Staphylococcus aureus*	Bactericidal ratio based on CFU counting	–	+(i)	[151]
Clove, coriander, caraway, marjoram, cinnamon, and cumin EOs	0.5–1.5 % (w/v)	*Listeria monocytogenes*	Log reduction of CFU/cm^2	–	+(i)	[152]
Lemongrass oil microcapsules (LMO)	1250 – 5000 ppm	*Escherichia coli*, *Listeria monocytogenes*	Growth inhibition based on optical density measurement	–	+(i)	[153]
Elicriso, chamomile blue, cinnamon, lavender, tea tree, peppermint, eucalyptus, lemongrass, lemon EOs	16 – 66 % (w/w); based on dry film mass	*Escherichia coli*, *Candida albicans*	Inhibition zone	–	+(i)	[154]

[a] SA: alginate-based film.
[b] SA-AA: alginate-based film with incorporated AA: (–) no antimicrobial activity; (+) antimicrobial activity; (i) antimicrobial activity increased after the incorporation of AA.

Regarding antibacterial activity, the majority of tests have been accomplished against *Staphylococcus aureus* as a representative of (G+) bacteria and as a representative of *Escherichia coli* as (G–) bacteria (Table 3). Antibacterial activity has been enhanced with the application of metal NPs (Ag, Au, ZnO, TiO_2) or organic salts complexes. For instance, it has been developed a TiO_2-nanocomposite-incorporated alginate-based film whose antibacterial activity stems from the photocatalytic activity of TiO_2 and reactive oxygen species (ROS) production upon illumination of the film with UV light [148]. In the same study, the antibacterial activity was further improved with the incorporation of plasmonic NPs such as Au in TiO_2 nanostructures what led to enhanced light absorption in the visible light region and more intensive ROS production [148]. A film that consists of a chitosan-based outer layer, an SA-based inner layer, and incorporated carboxymethyl chitosan-ZnO NPs has been developed, whereby the proposed mechanism of action was ROS production as well [151]. Moreover, the incorporation of biocomposite synthesized from chitosan-benzalkonium chloride (C-BC) complex and micro fibrillated cellulose (MFC) in SA formulation has shown improved antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* [150].

Regarding increasing public demands for natural preservatives, some plant extracts and EOs have been also tried as the antibacterial agents in alginate-based composite films. For example, the antibacterial activity of pyrogallic acid (PA) was tested through its incorporation in a sodium alginate/carboxymethyl cellulose (SA/CMC) composite formulation [147]. Furthermore, Alboofetileh et al. have prepared a functional bionanocomposite film based on sodium alginate/montmorillonite (SA/MMT) formulation, whose antibacterial activity against *Listeria monocytogenes* was provided with the addition of either marjoram (MEO), cinnamon (CIEO), or clove (CEO) EOs [152]. All EOs have shown a significant reduction in the microbial count, whereby MEO has appeared as the most successful one [152]. The alginate-based films with microencapsulated lemongrass EO were able to inhibit the growth of *Escherichia coli* and *Listeria monocytogenes*, and therefore such films could also have a potential for the practical application in the food shelf life extension [153].

Composite hydrogel films containing Ag-NPs or GSE have been developed using three biopolymers: agar, SA, and collagen [146]. Ag-NPs-containing films and GSE-containing films have showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, respectively, whereby differences in the activity are explained by different cell wall characteristics of (G+) and (G–) bacteria [146]. *Lactococcus lactis*, a probiotic strain that inhibits pathogenic bacteria in the digestive tract by producing lactic acid and bacteriocin, has been also successfully incorporated in an SA/CMC composite film [149]. All films with added *Lactococcus lactis* have shown significant antibacterial activity, although it depends mostly on the amount of added bacteria, types of packaged food, and the initial amount of pathogens [149].

In the field of alginate-based films with antifungal activity, only one study has been found so far. Namely, nine different EOs (elicriso, chamomile blue, cinnamon, lavender, tea tree, peppermint, eucalyptus, lemongrass, lemon) were applied in increasing concentrations and the antifungal activity was tested against fungi *Candida albicans*, whereby the films with incorporated cinnamon, peppermint, and lemongrass EOs showed the highest inhibition zones [154].

3.2 Antioxidant activity of chitosan- and alginate-based films with incorporated active agents

Due to a bad image of the chemical-based additives, there is a growing interest in the application of natural antioxidant activity-enhancing components [155]. While blank chitosan-based films show some antioxidant activity itself, the antioxidant activity of alginate films is mostly due to the incorporation of AAs in the film matrix.

One of the oldest synthetic radicals used to test antioxidant activity is 2,2-diphenyl-1-picrylhydrazyl (DPPH) [156]. This (frequently used) method means that the films are soaked in methanol, ethanol, or water and allowed to interact with stable radical DPPH, whereby its disappearance is followed by measuring the absorption at 515 nm [157]. The antioxidant properties are quantified by the amount of antioxidant required to decrease initial DPPH concentration by 50 %, and by the time required to reach constant DPPH concentration [156, 157]. A potential drawback of the method is that DPPH interacts with other radicals (such as alkyl), and time needed to reach steady state of DPPH concentration is not linear with changing the antioxidant/DPPH ratios [103, 158]. In addition to this, the following antioxidant activity methods are also prevalent in the literature: reducing power assay [103], ferric reducing antioxidant power (FRAP) assay [103, 158], Trolox®-equivalent antioxidant capacity (TEAC) assay [159–162], ferrous ion chelating activity (FIC) assay [103, 163, 164], etc. The antioxidant activity of films is in a correlation with their total phenolic content (TPC) [128], which can be estimated by means of Folin-Ciocalteu (FC) reagent [130]. In this method, the reduction of reagent is associated with a colour change (from yellow to blue) detected spectrophotometrically, whereby gallic acid is used as a standard and the results are expressed as the mass of gallic acid equivalent (GAE) per mass of the film [130, 144, 165].

The most frequently added class of bioactive antioxidants is polyphenols, which can be incorporated in biopolymer-based films in different ways. Table 4 summarizes the most recent studies on the antioxidant activity of chitosan- and alginate-based films with incorporated AAs.

Table 4: Recent studies on the antioxidant properties of chitosan- and alginate-based films with incorporated active agents.

Biopolymer	Active agent (AA)	Method	Ref.
Chitosan	Black and purple eggplant extract	DPPH	[166]
Chitosan	Gallic acid	DPPH	[167]
Chitosan	Black soybean seed coat extract	DPPH	[168]
Chitosan	Mango leaf extract	TPC, DPPH, TEAC, FRAP	[165]
Chitosan	Purple-fleshed sweet potato extract	DPPH	[169]
Chitosan/gelatin	Nanoemulsions encapsulating active compounds	DPPH, TEAC, FRAP	[170]
Chitosan	Grape seed extract	FC, DPPH	[171]
Chitosan	Kombucha tea	DPPH	[172]
Chitosan	Apple peel polyphenols	DPPH, TEAC	[173]
Chitosan	*Camelina sativa* seed EO	FRAP	[174]
Chitosan	*Nigella sativa* seed extract	FC, DPPH, FRAP	[175]
Chitosan/gelatin	Eugenol and ginger EOs	TEAC	[176]
Chitosan	Extracts of peanut skin/pink pepper residues	FC, DPPH, TEAC, ORAC, superoxide anione	[177]
Chitosan	Citric acid	H_2O_2 radical scavenging assay	[178]
Chitosan	Apricot kernel oil	DPPH, H_2O_2 radical scavenging assay	[179]
Chitosan	*Lepidium sativum* seedcake extract	FC, DPPH	[180]
Chitosan/starch	*Litsea cubeba* oil	DPPH	[118]
Chitosan	Clove essential oil, halloysite nanotubes	FC, DPPH, reducing power assay, migration studies	[181]
Chitosan	Capsaicin	DPPH	[182]
Chitosan	Oregano and thyme essential oils	DPPH	[183]
Chitosan	Olive pomace	DPPH	[184]
Chitosan/starch	Cranberry, blueberry, beetroot, pomegranate, oregano, pitaya/dragon fruit, resveratrol	FC	[185]
Chitosan	Blueberry and blackberry pomace extract	FRAP, TPTZ	[186]
Chitosan	Hop extract	FC (TPC)	[130]
Chitosan	Oak and algal extracts	FC (TPC)	[131]
Chitosan	Chestnut extract	FC (TPC)	[187]
Chitosan	Protocatechuic acid	FC, DPPH	[188]
Chitosan	Dimeric α,β-peptoids	DPPH	[189]
Chitosan	*Origanum vulgare* ssp. *gracile* EO	DPPH	[190]
Chitosan	*Carum copticum* EO	DPPH	[191]
Chitosan	Hydroxybenzoic acid	DPPH	[192]
Chitosan	Young apple polyphenols	DPPH	[193]
Chitosan/starch	Thyme extract	TEAC	[194]
Chitosan	*Eucalyptus globulus* EO	TPC, DPPH, NO-scavenging activity, H_2O_2 radical scavenging assay	[128]
Chitosan	Nettle (*Urtica dioica* L.) extract	DPPH	[195]
Chitosan	*Thymus* species EOs	DPPH, FRAP	[196]
Chitosan	Caraway EO/beeswax	DPPH	[129]
Chitosan	*Lycium barbarum* fruit extract	DPPH	[197]
Chitosan	Maqui berry	DPPH, FRAP, FIC	[198]
Chitosan	Carvacrol and pomegranate peel extract	TPC, FRAP	[199]
Chitosan	Cinnamon leaf oil or oleic acid	TEAC	[144]
Chitosan	Caraway EO	DPPH	[129]
Chitosan	Propolis extract	TPC, DPPH	[132]
Chitosan	*Pistacia terebinthus* extracts	DPPH	[200]
Alginate	Protein hydrolysates	TPC	[201]
Alginate /gelatin	Tea polyphenols	TPC	[202]
Alginate	Cinnamon leaf oil or cinnamon bark oil	DPPH	[203]
Alginate	Black chokeberry extract	TPC	[204]
Alginate	Green tea extract/grape seed extract	TEAC	[205]

A good way of increasing the antioxidant activity *via* natural additives is by using extracts such as apple peel extract [173], *Nigella sativa* seed [175], thyme extract [194], peanut skin extract [177], *Lepidium sativum* seedcake extract [180], purple-fleshed sweet potato extract [169], tea extracts [172, 206], mango leaf extract [165], carvacrol and pomegranate peel extracts [199], thinned young apples polyphenolic extract [193], grape seed extract [171], hop extract [130], oak extract [131], chestnut extract [187], *Pistacia terebinthus* (stem, leaf, and seed) extracts [200], etc. Moreover, the antioxidant activity can be enhanced by the incorporation of EOs obtained from *Thymus* species [196], apricot kernel [179], oregano and thyme [183], *Origanum vulgare* ssp. *gracile* [190], clove [181], *Camelina sativa* [174], *Litsea cubeba* [118], *Eucalyptus globulus* [128], *Carum copticum* [191], black soybean seed coat extract [168], and ginger [176]. Furthermore, the antioxidant activity can be enhanced by the incorporation of berries, as reported in the case of maqui berry [198], and cranberry/blueberry [185]. It has been reported that agro-industrial residuals and olive pomace flour have enhanced the antioxidant activity of the films as well [177, 184].

Priyadarshi et al. have reported the incorporation of citric acid as an active ingredient for the extension of green chili shelf life [178]. Examples of grafting/incorporating chitosan-based films with hydroxybenzoic acid [192], protocatechuic acid [188, 207], gallic acid [167], or even capsaicin − an active substance isolated from chili peppers [182], have been also reported throughout the literature. However, the highest improvements of the antioxidant activity have been observed after the incorporation of AAs such as protein hydrolysates and propolis extract (Figure 2).

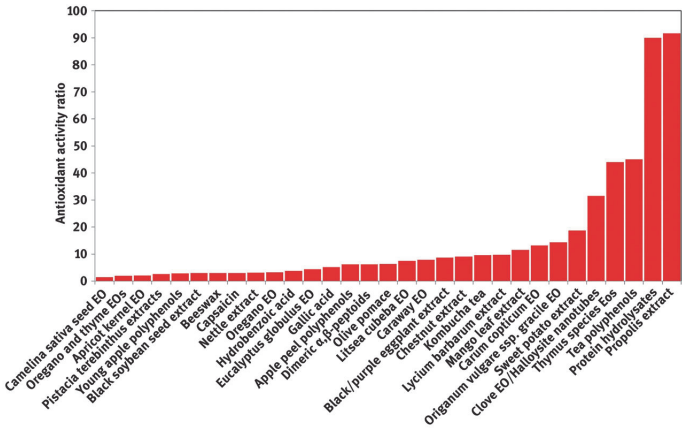

Figure 2: The effect of different active agents on the antioxidant activity of some chitosan- and alginate-based films. The effect was evaluated through the ratio between antioxidant activities of the film samples with and without the incorporated active agent.

4 Future perspectives and conclusions

Waste/residual marine biomass is a valuable source for the isolation of biopolymers such as chitin/chitosan and alginate. Their isolation can be followed by the development and production of advanced biopolymer-based packaging materials in order to create business for food industries, at the same time being aware of both the food quality (and safety) demanded by consumers and the environmental care demanded by the institutions and society. Therefore, this review aims to show that the food packaging films can be successfully prepared from biomass-derived chitosan and alginate as well as that the films' properties can be tailored in terms of antimicrobial and antioxidant activities by the incorporation of a wide variety of components.

Nevertheless, special attention should be devoted to the invention of advanced eco-friendly processes for both isolation of biopolymers and preparation of active agents in sufficient quantities at relatively acceptable costs and low ecological footprint. Besides, the preparation of film materials is a multi-task problem that should be carefully considered and planned. This is because the incorporation of active agents affects not only antimicrobial and antioxidant properties of the films, but simultaneously their mechanical (strengths, stiffness, elasticity) and barrier (against UV-vis light and gases) properties. The release of active agents from the films and their potential side effects on the organoleptic properties of food should be of paramount importance for further development of packaging materials with advanced properties as well. Last but not least, the film's

biodegradability should be sufficient to strengthen the main concept of the circular economy and to make them competitive to other eco-unfriendly materials.

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Formulation of active food packaging by design: Linking composition of the film-forming solution to properties of the chitosan-based film by response surface methodology (RSM) modelling

Marijan Bajić [a,*], Ana Oberlintner [a], Kristi Kõrge [a,b], Blaž Likozar [a,c], Uroš Novak [a]

[a] Department of Catalysis and Chemical Reaction Engineering, National Institute of Chemistry, Hajdrihova 19, 1000 Ljubljana, Slovenia
[b] Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia
[c] Faculty of Chemistry and Chemical Technology, University of Ljubljana, Večna pot 113, 1000 Ljubljana, Slovenia

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ABSTRACT

An active chitosan-based film, blended with the hydrolysable tannin-rich extract obtained from fibrous chestnut wood (*Castanea sativa* Mill.), underwent a simultaneous engineering optimization in terms of measured moisture content (*MC*), tensile strength (*TS*), elongation at break (*EB*), and total phenolic content (*TPC*). The optimal product formulation for a homogeneous film-forming solution was sought by designing an empirical Box–Behnken model simulation, based on three independent variables: the concentrations of chitosan (1.5–2.0% (w/v)), extracted powder-form chestnut extract (0.5–1.0% (w/v)) and plasticizer glycerol (30.0–90.0% (w/w); determined per mass of polysaccharide). Obtained linear (*MC*), quadratic (*TS* or *EB*), and two-factor interaction (*TPC*) sets were found to be significant ($p < 0.05$), to fit well with characteristic experimental data ($0.969 < R^2 < 0.992$), and could be considered predictive. Although all system parameters were influential, the level of polyol played a vital continuous role in defining *EB*, *MC*, and *TS*, while the variation of the chestnut extract caused an expected connected change in affecting *TPC*. The component relationship formula of chemical mixture fractions (1.93% (w/v) of chitosan, 0.97% (w/v) chestnut extract and 30.0% (w/w) of glycerol) yielded the final applicable material of adequate physico-mechanical properties (*MC* = 17.0%, *TS* = 16.7 MPa, *EB* = 10.4%, and *TPC* = 19.4 $mg_{GAE}\ g_{film}^{-1}$). Further statistical validation of the concept revealed a sufficient specific accuracy with the computed maximal absolute residual error up to 22.2%. Herein-proposed design methodology can thus be translated to smart packaging fabrication generally.

1. Introduction

The history of chitosan dates back to the mid-19th century, although its industrial production and utilization start from the 1970s [1]. Nowadays, a quest for novel methods of chitosan extraction, modification, and characterization is in full swing [2,3]. This trend is based on the facts that chitosan is a non-toxic, biodegradable, and biocompatible biopolymer with a good film-forming capacity as well as inherent antimicrobial and antioxidant activities [4,5]. Following this, chitosan has been placed in focus as prospective raw material for the preparation of eco-friendly films for food packaging applications [6–8]. Chitosan-based films, however, may require the incorporation of certain auxiliary components to ameliorate functional properties. In the first instance, plasticization is done to minimize the films' rigidness and brittleness [9], whereby polyols (most of all glycerol) stand as the most widely used plasticizers [10,11]. On the other hand, the incorporation of various active components can lead to the production of chitosan-based films with the enhanced mechanical, barrier, and antimicrobial and/or antioxidant properties [3,7,12].

Extracts obtained from different parts of terrestrial plants are frequently used active components in eco-friendly films [13]. Recent studies on chitosan-based films highlight the utilization of extracts obtained from banana [14], mangosteen [15], mango [16], black plum [17], pine/peanut/jujube [18], turmeric [19], hop [20], oak [21], and chestnut [22]. The extract obtained from chestnut wood is quite complex in composition but its major fraction consists of hydrolyzable tannins (HTs), while other components include water, ellagic/gallic acids, simple sugars, proteins, mineral substances, and crude fibre [23]. HTs are secondary plant metabolites, but also phenolic compounds by their chemical nature [24]. Due to their prominent biological activity, HTs-rich chestnut extracts have found applications beyond the border of their traditional use in tanning, e.g. as an active agent in the food industry [25,26].

The above-mentioned auxiliary components (plasticizers, active agents) are usually pre-added in the film-forming solutions (FFSs),

* Corresponding author at: Department of Biochemical Engineering, University College London, Gower Street, London WC1E 6BT, United Kingdom.
E-mail addresses: marijan.bajic@ki.si, m.bajic@ucl.ac.uk (M. Bajić).

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which could be considered as the films' semi-finished products. The further strategy of the film production means casting of homogeneous FFSs on a flat surface followed by a time-limited process of solvent evaporation at moderate temperatures [6]. The auxiliary components affect the biological and physico-chemical characteristics of resulting films interdependently, and thus the formulation of FFSs should be carefully optimized in terms of achieving desired properties of the final products. A set of statistical and mathematical tools known as RSM is of great importance in the design, development, and optimization of new as well as in the improvement of existing products [27]. For instance, a family of efficient three-level Box–Behnken design, BBD [28], has been applied for the development of either pure or blended chitosan-based films in several studies. Drying temperature seems to be the most frequently studied independent variable, either in combination with other process parameters (e.g. relative humidity, storage period [29]) or with film composition parameters (e.g. chitosan/glycerol level [30], glycerol/vanillin level [31]). On the other hand, BBD was used to optimize only the formulation of chitosan-containing composite films blended with TiO_2 [32], pea starch [33], and cellulose/polyvinyl alcohol [34].

This study brings the optimization of novel active chestnut extract-incorporated chitosan-based film intended for food packaging into the foreground, and therefore represents an extension of our previous work on it [22]. The simultaneous optimization was done by linking the composition of the FFS containing matrix-forming biopolymer (chitosan), active component (chestnut extract), and plasticizer (glycerol) to the properties of the active film material. Individual and interactive effects of the independent variables (concentrations of the FFS components) on the response variables (*MC*, *TS*, *EB*, and *TPC* of the film material) were evaluated by RSM. Moreover, model validation was done by comparing predicted values of the response variables with those obtained from the experimental measurements.

2. Materials and methods

2.1. Materials

High molecular weight chitosan (M_w = 310–375 kDa; deacetylation degree ≥ 75%), lactic acid (purity > 85%; density 1.206 g mL^{-1}), Folin-Ciocalteu's phenol reagent, magnesium nitrate, and gallic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate and glycerol were from Merck (Darmstadt, Germany) and Pharmachem Sušnik (Ljubljana, Slovenia), respectively. All chemicals except lactic acid were of analytical grade. Milli-Q® water was used throughout all experiments.

Tanin Sevnica (Sevnica, Slovenia) kindly donated a commercially available chestnut extract obtained from individual parts of chestnut wood. The extract contained ≥75% of tannins and <4% of ash [22], according to the manufacturer's specifications.

2.2. Experimental design

A 3-level-3-factor BBD with three replicates at the central point, which gives a total of 15 experimental runs [29,31–33], was used to study the effect of FFS composition on the physico-mechanical properties of chitosan-based films (prepared according to the protocols described in Section 2.3). The levels of three main components of the FFS were chosen as independent variables: (i) concentration of chitosan (CH, x_1, % w/v), (ii) concentration of chestnut extract (CE, x_2, % w/v), and (iii) concentration of glycerol (GLY, x_3, % w/w, calculated per mass of chitosan); whereby each variable was tested at three different coded levels: low (−1), medium (0), and high (+1). The low level was limited by mechanical properties of the final materials (films prepared from the FFSs containing a low amount of raw materials tend to be mechanically unstable and brittle), while the high level was limited by physical properties of the FFSs (the addition of raw materials in high concentrations gives very viscous and inapplicable FFSs). The experimental design matrix, in terms of actual (x_i) and coded (X_i) levels of the independent variables, is presented in Table 1.

The coded values are related to the actual values by the relation indicated in Eq. (1):

$$X_i = 2 \times (x_i - x_m)/d_i \quad (1)$$

whereby x_i, x_m, and d_i denote variable value in the actual units of the ith observation, mean of the highest and the lowest variable value, and the difference between the highest and the lowest variable value, respectively [30]. After conducting all experimental runs (Table 1), a second-order polynomial equation was applied to fit the experimental responses to the coded variables, as denoted in Eq. (2):

$$Y_n = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{\substack{i=1 \\ i<j}}^{3} \sum_{i=1}^{3} \beta_{ij} X_i X_j + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \varepsilon \quad (2)$$

where,

Y_n denotes predicted response;
X_i (X_j) denotes a dimensionless coded value of the independent variable x_i (x_j);
β_0 denotes the model constant (intercept);
β_i denotes linear regression coefficient;
β_{ij} denotes cross-product regression coefficient;
β_{ii} denotes quadratic regression coefficient.

2.3. Film-forming solutions and chitosan-based films

2.3.1. Film-forming solutions

All FFSs formulations (Table 1) were prepared by adding predetermined amounts of CH (% w/v) and GLY (% w/w, calculated per mass of CH) in the solvent (1% (v/v) aqueous solution of lactic acid) followed by continuous stirring (1000 rpm; 12 h; room temperature, 24 °C) on RCT magnetic stirrer (IKA, Staufen, Germany) and vacuum-filtration through two sheets of medical gauze [20]. The predetermined amounts of CE were added subsequently after the filtration step and the mixtures were homogenized (6000 rpm; 2 min) on Ultra-Turrax® T50 (IKA) and left overnight to get rid of the air bubbles formed during this process. A small amount of stable sticky foam that

Table 1
Box–Behnken experimental design matrix.

Run	Actual values[a]			Coded values[b]		
	x_1	x_2	x_3	X_1	X_2	X_3
1	1.50	0.50	60	−1	−1	0
2	2.00	0.50	60	+1	−1	0
3	1.50	1.00	60	−1	+1	0
4	2.00	1.00	60	+1	+1	0
5	1.50	0.75	30	−1	0	−1
6	2.00	0.75	30	+1	0	−1
7	1.50	0.75	90	−1	0	+1
8	2.00	0.75	90	+1	0	+1
9	1.75	0.50	30	0	−1	−1
10	1.75	1.00	30	0	+1	−1
11	1.75	0.50	90	0	−1	+1
12	1.75	1.00	90	0	+1	+1
13	1.75	0.75	60	0	0	0
14	1.75	0.75	60	0	0	0
15	1.75	0.75	60	0	0	0

[a] Actual values: x_1 – concentration of chitosan (CH, % w/v); x_2 – concentration of chestnut extract (CE, % w/v); x_3 – concentration of glycerol (GLY, % w/w; calculated per mass of chitosan). The concentration of lactic acid was kept constant, as stated in Section 2.3.1.

[b] Coded values: X_1 – the coded level of CH; X_2 – the coded level of CE; X_3 – the coded level of GLY.

was formed on the top of the mixtures due to the presence of CE was eventually removed by using a laboratory spatula [22]. A potential loss of CE during this procedure was presumed to be insignificant and without influence on the qualitative and quantitative composition of the FFSs.

2.3.2. Chitosan-based films

Prepared FFSs were cast in polyurethane Petri dishes (approximately 0.32 mL cm^{-2}) and left in drying oven Kambič SP-55 C (Kambič, Semič, Slovenia) at 40 °C for the next 48 h. Obtained films were peeled off from Petri dishes, treated by ABS Digital Thickness Gauge (Mitutoyo, Aurora, USA) to measure their thicknesses (the measurements were performed at ten randomly selected positions and the results were averaged), and stored in a glass humidity chamber containing a saturated aqueous solution of $Mg(NO_3)_2$ (relative humidity, RH = 53–55%; room temperature, 24 °C) until further analysis.

2.4. Fourier transform infrared spectroscopy analysis

The Fourier transform infrared (FT-IR) spectra (wavenumbers range from 4000 cm^{-1} to 450 cm^{-1}; resolution 4 cm^{-1}) were recorded at room temperature using Spectrum Two FT-IR spectrometer (PerkinElmer, Waltham, USA). The tested film samples were prepared from the FFSs containing 1.5% (w/v) of CH (unplasticized film), 1.5% (w/v) of CH and 30.0% (w/w; per mass of chitosan) of GLY (plasticized film), and 1.5% (w/v) of CH, 30.0% (w/w; per mass of chitosan) of GLY, and 1.0% (w/v) of CE (plasticized film with incorporated CE). The scans were done in triplicates on random positions of each tested sample and the resulting curves were averaged.

2.5. Response variables

2.5.1. Moisture content

MC was determined gravimetrically, according to the previously described protocol [20]. In short, rectangular samples (~1 cm^2) were weighted on an analytical balance (Kern & Sohn, Balingen, Germany) to get the initial (M_1) and dry mass (M_2; obtained after drying at 105 °C for 24 h), and the results were expressed as the percentage of water content in the films following that $MC = (M_1 - M_2 / M_1) \times 100\%$.

2.5.2. Tensile strength

TS was determined by testing rectangular film samples (length × width = 8 cm × 2 cm; gage length segment 6 cm) on the XLW Auto Tensile Tester (Labthink® Instruments, Jinan, China) equipped with a 100 N load cell, at a crosshead speed of 25 mm min^{-1}. *TS* was calculated by dividing the maximal load with the average original cross-sectional area in the sample gage length segment [20].

2.5.3. Elongation at break

EB was tested on the same samples and using the same equipment as stated in Section 2.5.2. *EB* was calculated as the ratio between increased length after breakage and the initial gage length [20].

2.5.4. Total phenolic content

TPC was estimated using Folin-Ciocalteu's (FC) phenol reagent, as described elsewhere [20]. Small rectangular film samples of known masses were placed in glass vials, and water was added to reach the final film concentration of 5 mg mL^{-1}, followed by successive addition of FC phenol reagent and 10% (w/v) aqueous solution of Na_2CO_3 (added 10% and 20% based on the volume of water, respectively). After the sample incubation for 2 h (dark conditions; room temperature), the absorbance of the solutions was measured at 765 nm using Synergy™ 2 Multi-Detection Microplate Reader (BioTek, Winooski, USA). The results were expressed as the mass of gallic acid equivalent (GAE) per mass of the film.

2.6. Simultaneous optimization and model validation

A desirability function-based approach [27] was used for the simultaneous optimization of response variables (Eq. (S1), Appendix A). The optimization was done based on the following goals: (i) minimization of *MC*, (ii) maximization of *TS*, (iii) minimization of *EB*, (iv) maximization of *TPC*. An algorithm was then applied to maximize the overall desirability (*D*; ranging from 0 to 1), defined as the geometric average of the individual desirability functions [31]. Model validation was performed by comparing predicted values of the response variables and those obtained from experimental measurements using a set of films prepared from the optimized FFS.

2.7. Statistical analysis

All measurements (Sections 2.5.1–2.5.4) on the film samples prepared for tested FFSs (runs 1–15, Table 1) were performed in triplicates and the mean values were used in the analysis. Statistical analysis and simultaneous optimization were done by a trial version of Design-Expert® software (Stat-Ease, Minneapolis, USA; version 12.0.0.6; serial number: 7614-9562-2103-EVAL). Response surface graphs obtained from the regression equations in terms of coded values were visualized using a Python plotting library Matplotlib.

3. Results and discussion

3.1. Preparation of film-forming solutions and chitosan-based films

Two raw materials used in this study – the matrix-forming biopolymer CH (Fig. 1a) and the active component CE (Fig. 1b) – are obtainable from natural and renewable sources such as marine- and wood-based biomass, respectively. The third raw material, plasticizer GLY, was added in a low concentration relative to CH and CE, but it was very influential on the film properties (Sections 3.3–3.5). Processing of the raw materials led to the preparation of brown-shaded FFSs (Fig. 1c), whose formulation was further optimized to get films of satisfying mechanical integrity and desired physico-mechanical properties (Fig. 1d).

It has been visually observed that variation in the concentrations of raw materials significantly affects the physical properties of the FFSs. In this regard, the viscosity of FFSs was mainly affected by variations in the concentrations of CH and CE, while their visual appearances were affected by variations in the concentration of CE. A brownish shade of FFSs became more intense upon increasing the concentration of CE (Fig. S1, Appendix A), whereby this trend was replicated in the visual appearance of chitosan-based films as well (Fig. S2, Appendix A).

The morphological evaluation of film materials has revealed their compact structure without any significant microscopic pores or cracks at the films' cross-sections and surfaces (Fig. S2, Appendix A). The mean thickness of all tested film samples (Section 2.2) was 120 ± 30 μm, which is slightly above the values obtained for our previous chitosan-based films prepared from the FFSs containing up to 1.5% (w/v) of hop extract [20]. Nevertheless, it is still within the range of values for other chitosan-based films with incorporated active components [16,18].

3.2. Fourier transform infrared spectroscopy analysis

Towards a better understanding of the relationship between the qualitative composition and possible interactions/structural changes after the film formation, the FT-IR analysis of unplasticized, plasticized, and plasticized films with incorporated CE has been done (Fig. 2).

The most characteristic absorption bands in unplasticized films are as follows: a broad band in the wavelength region between 3600 cm^{-1} and 3000 cm^{-1} (attributed to O–H and N–H stretching), two weak bands located approximately between 2950 cm^{-1} and 2850 cm^{-1} (attributed to C–H stretching), and the peaks appearing at around 1640 cm^{-1} and 1550 cm^{-1} attributed to C=O stretching (amide I) and

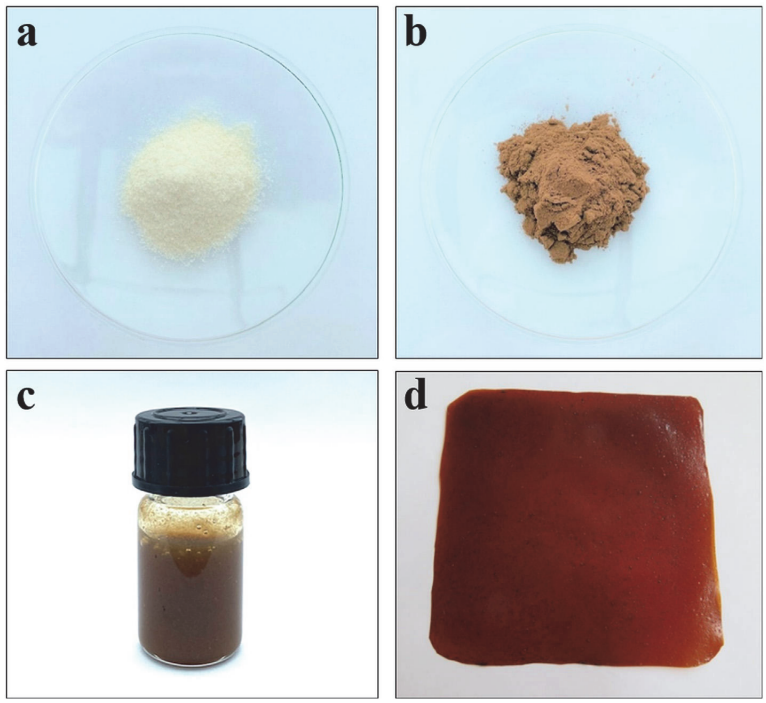

Fig. 1. The physical appearance of: a) chitosan powder, b) chestnut extract powder, c) film-forming solution containing chestnut extract, and d) chitosan-based film prepared from the film-forming solution containing the chestnut extract.

N–H bending (amide II), respectively (Fig. 2). This spectrum is generally in keeping with the spectrum of a similar neat chitosan-based film [35].

Plasticization of chitosan film utilizing GLY and its further incorporation by CE brought about certain alterations in the FT-IR spectra. The most distinctive changes are visible in the wavelength regions between 3600 cm^{-1} and 3000 cm^{-1} (higher intensity of broad bands), 3000–2800 cm^{-1} (higher intensity of peaks at approx. 2930 cm^{-1}), and 1800–1490 cm^{-1} (higher intensity of sharp peaks at approx. 1728 cm^{-1} and 1570 cm^{-1} followed by a slight shift towards higher wavenumbers as compared to the unplasticized film). Such changes might indicate the interactions of chitosan's O–H, C=O (amide I), and N–H (amide II) groups with the main functional groups of auxiliary components. For instance, GLY has been commonly reported to promote hydrogen bonding with chitosan due to a strong hydrogen bond capacity promoted by the presence of three O–H groups [9,36], which is per its role as a plasticizer. On the other hand, the main fraction of CE is composed of HTs – a mixture of structurally distinct compounds (such as simple gallic acid derivatives, gallotannins, and ellagitannins)

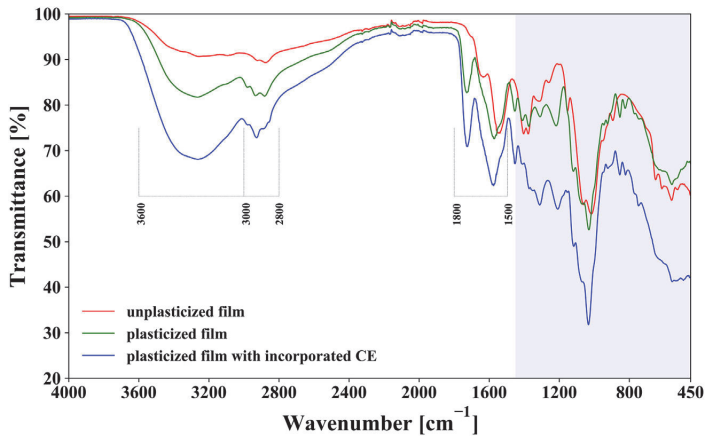

Fig. 2. FT-IR spectra of chitosan-based films prepared from the film-forming solutions containing 1.5% (w/v) of CH (unplasticized film; red line), 1.5% (w/v) of CH and 30.0% (w/w; per mass of chitosan) of GLY (plasticized film; green line), and 1.5% (w/v) of CH, 30.0% (w/w; per mass of chitosan) of GLY, and 1.0% (w/v) of CE (plasticized film with incorporated chestnut extract; blue line). The grey-shaded area represents the fingerprint region.

Table 2
Experimental responses of dependent variables.

Run[a]	Experimental response[b]			
	MC	TS	EB	TPC
1	35.1	7.7	53.7	13.0
2	33.4	8.7	56.9	10.1
3	25.1	10.4	62.4	22.9
4	28.5	7.8	23.4	19.9
5	21.7	21.6	48.1	17.4
6	20.4	18.0	10.3	19.0
7	38.6	5.6	59.3	17.7
8	36.3	6.6	57.6	12.6
9	24.8	14.7	57.1	11.0
10	20.2	15.7	36.7	23.7
11	42.8	4.4	86.7	9.6
12	36.5	3.2	68.3	17.8
13	30.4	9.7	75.0	16.2
14	31.1	7.1	71.8	17.1
15	30.7	8.2	69.6	14.5
Min.	20.2	3.2	10.3	9.6
Max.	42.8	21.6	86.7	23.7
Mean	30.4	10.0	55.8	16.2
Std. dev.	6.7	5.1	19.2	4.2

[a] The actual and coded values of independent variables in each experimental run are presented in Table 1.
[b] *MC*, moisture content (%); *TS*, tensile strength (MPa); *EB*, elongation at break (%); *TPC*, total phenolic content ($mg_{GAE}\ g_{film}^{-1}$).

that possess a huge number of O–H (and C=O) functional groups [24]. It could be assumed that these functional groups are also involved in the non-covalent interactions with CH molecules. This assumption might be supported by similar changes in the FT-IR spectrum of a chitosan-based film with incorporated ellagitannins-rich oak extract [21], which belongs to the same class as CE [37].

General conclusions derived from the FT-IR spectra of herein presented three representative samples of chitosan-based films might point to good incorporation of the auxiliary components in the polymer matrix established over non-covalent interactions of GLY and HTs with O–H, N–H, and C=O (in acetylated monomers) groups of CH. Nevertheless, the FT-IR analysis has not been intended to play a decisive role in whether (and how) the interactions between components reflect at the final properties of chitosan-based films. This analysis actually should play a supportive role, i.e. the results from this section should be interpreted in context with the models discussed below. For those who seek a more detailed spectroscopic analysis, the FT-IR spectra of raw materials are enclosed in Appendix A (Fig. S3), while a more in-depth interpretation can be found elsewhere in the literature [10,35,37].

3.3. Moisture content

MC notably affects the barrier, mechanical, and thermal properties of chitosan-based films, which is of paramount importance for their application in food preservation [38]. Therefore, a set of 15 experimental measurements conducted on this variable revealed that *MC* was ranging from 20.2% to 42.8% (Table 2).

Further processing of the experimental data has led to the development of a linear mathematical model which efficiently described the relationship between independent variables and *MC* [$Y_{MC} = 30.37–0.24X_1 - 3.23X_2 + 8.39X_3$]. An insight into the ANOVA summary statistics implied the model was significant (Table 3), whereby the proposed equation matched at least 96.85% of the total variations. Furthermore, a small difference between R^2 and adjusted R^2 (R^2_{adj}) indicated there were no unnecessary model terms included [27].

In this case, concentrations of CE and GLY were significant model terms ($p < 0.05$). The effect of all three independent variables on *MC* can be seen in Fig. 3a.1–3a.3. *MC* was almost constant along with the entire range of CH concentrations, but the values increased along with decreasing amount of CE (Fig. 3a.1), and the increasing amount of GLY (Fig. 3a.2). Plotting the concentrations of active component and plasticizer showed the highest values of *MC* were in the films produced from the FFS containing the minimal concentration of CE and maximal concentration of GLY (Fig. 3a.3).

Such a response might be a consequence of the hydrogen bonding between CH and auxiliary components. Namely, GLY establishes intermolecular hydrogen bonds with adjacent chains of CH (Section 3.2), causing changes in the spatial conformation of the film three-dimensional matrix [9,36]. This might as well expose hydrogen bonding sites of CH to interact with water molecules and retain them within the polymer matrix. The fact that GLY is hygroscopic by itself due to the presence of three –OH could be contributive to *MC* as well [33]. A positive correlation between the level of GLY and *MC* in chitosan-based films is in line with the findings reported elsewhere [10,33]. On the other hand, CE was added in much higher concentrations relative to GLY. Its major components (HTs) possess multiple interaction sites that crosslink polymer chains and therefore tend to saturate hydrogen bonding sites of CH. This might further prevent the retention of water molecule within the matrix and lead to lower values of *MC*. The incorporation of other plant-based active components has also been reported to reduce the water absorption capacity of chitosan-based films [15,16,20].

3.4. Tensile strength

TS represents one of the most studied mechanical property of chitosan-based films intended for food packaging, and it refers to the films' resistance to failure at elevated loads or deformation. The values of *TS* should be as high as possible because food packaging must preserve mechanical integrity and therefore provide sufficient protection of the food during transportation and storage. The *TS* values of tested film samples were ranging from 3.2 MPa to 21.6 MPa (Table 2), which is in agreement with the values of many other biodegradable packaging films with incorporated plant extracts [13], but slightly below the values of some frequently used commercial materials [39]. The relationship between the independent variables and *TS* was found to be quadratic [$Y_{TS} = 8.33–0.53X_1 + 0.20X_2 - 6.28X_3 - 0.90X_1X_2 + 1.15X_1X_3 - 0.55X_2X_3 + 1.88X_1^2 -$

Table 3
ANOVA summary statistics.

Response[a]	Source	*SS*[b]	*df*[b]	*MS*[b]	*F*-value	*p*-Value	R^2	R^2_{adj}[b]	Remark
MC	Model	646.46	3	215.49	112.81	<0.0001	0.9685	0.9599	Linear model
	Lack of fit	20.77	9	2.31	18.71	0.0518			
TS	Model	378.68	9	42.08	31.51	0.0007	0.9827	0.9515	Quadratic model
	Lack of fit	3.27	3	1.09	0.64	0.6572			
EB	Model	5494.74	9	610.53	72.91	<0.0001	0.9924	0.9788	Quadratic model
	Lack of fit	27.12	3	9.04	1.23	0.4786			
TPC	Model	255.82	6	42.64	43.78	<0.0001	0.9704	0.9483	2FI model
	Lack of fit	4.30	6	0.7174	0.4115	0.8314			

[a] *MC*, moisture content (%); *TS*, tensile strength (MPa); *EB*, elongation at break (%); *TPC*, total phenolic content ($mg_{GAE}\ g_{film}^{-1}$).
[b] *SS*, the sum of squares; *df*, degrees of freedom; *MS*, mean square; R^2_{adj}, adjusted R^2.

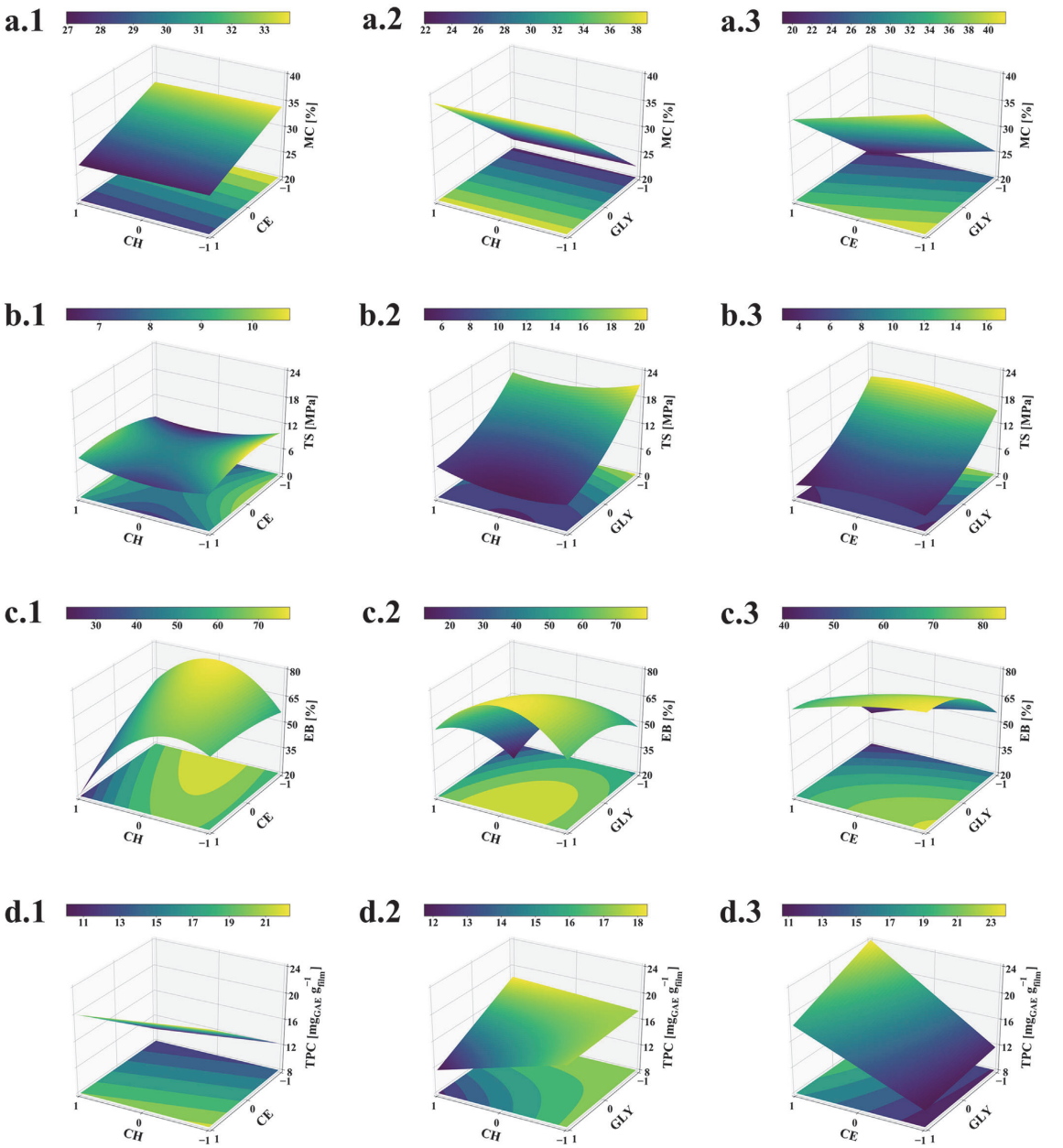

Fig. 3. Response surface plots showing the impact of independent variables on: a) moisture content, b) tensile strength, c) elongation at break, and d) total phenolic content of chitosan-based films. The numbers 1, 2, and 3 denote the mutual effect of CH–CE while the level of GLY was held constant, the mutual effect of CH–GLY while the level of CE was held constant, and the mutual effect of CE–GLY while the level of CH was held constant, respectively. Corresponding two-dimensional contour plots can be seen in Appendix A (Fig. S4).

$1.57X_2^2 + 2.73X_3^2]$. As can be seen from Table 3, this equation could describe at least 98.27% of the total variations, while the model and lack of fit *F*-values imply significance and good fit of the model, respectively.

TS of the samples was likely governed by physical interactions established between CH molecules, the vast majority of CE components, and GLY (as discussed in Section 3.2). It was found that *TS* was significantly ($p < 0.05$) affected by GLY level (in linear and quadratic terms), while the levels of CH and CE found to be influential as well, but less dramatically and mostly in quadratic terms. This can be observed from the response plots — *TS* slightly varied along with the variations of CH and CE levels (Fig. 3b.1), but it steeply decreased with increasing the concentration of plasticizer, with the values below 10 MPa in the films prepared from the FFSs containing maximal concentrations of GLY (Fig. 3b.2 and b.3). This was in line with a well-known fact about the remarkable

plasticization effect of GLY due to high hydrogen-bonding capacity [9,10]. However, the level of GLY significantly affected *MC* as well (Section 3.3), and in return, *MC* affects the mechanical properties of chitosan-based films [38]. So we might assume there were more water molecules absorbed in the polymer matrix than it would have happened in the absence of GLY. It can be further concluded that these water molecules exhibit a joint plasticization effect with GLY. This effect is very likely 'masked' in this model, and should not be neglected or excluded when considering mechanical properties of chitosan-based films.

3.5. Elongation at break

EB is another important mechanical property of chitosan-based films, and it stands for their stretching capacity (i.e. extensibility) before breaking. The obtained values were between 10.3% and 86.7% (Table 2), which falls in the range reported for other plant extract-blended biopolymer films [13]. For comparison's sake, the presented chitosan-based films had higher *EB* values than polystyrene (PS; 1%–4%) and polyamide (PA; 5%–10%), smaller than low-density polyethylene (LDPE; 200%–900%), polyvinyl alcohol (PVA; 220%–250%) and polyvinyl alcohol-co-ethylene (EVOH; 180%–250%), while their *EB* values fell in the range of polyethylene terephthalate (PET; 20%–300%) and polyvinyl chloride (PVC; 40%–75%) [39].

The model, which could describe at least 99.24% of the total variations (Table 3), revealed a quadratic relationship between *EB* and independent variables [$Y_{EB} = 72.13 - 9.41X_1 - 7.95X_2 + 14.96X_3 - 10.55X_1X_2 + 9.03X_1X_3 + 0.50X_2X_3 - 20.70X_1^2 - 2.33X_2^2 - 7.60X_3^2$]. A perusal of Table 3 shows there was a reasonable agreement between the ordinary and adjusted R^2, while observation of the *F*-values implies the model was significant. All three independent variables exerted significant ($p < 0.05$) influence on *EB* in linear, interaction (excepted CE and GLY), and quadratic (excepted CE) terms. As evident from the model equation, levels of CH and CE were in negative correlations with *EB* in all three terms, i.e. the flexibility of films decreased in a non-linear manner at elevated concentrations of CH and CE — most probably due to extensive cross-linking between CH polymer and HTs from chestnut extract (Section 3.2). This could be seen on the response plot, where the lowest *EB* values were predicted for the films produced from the FFSs with maximal concentrations of CH and CE (lower-left corner of the plot in Fig. 3c.1). On the other hand, the plasticization effect of GLY was evident since the films' elongation capabilities increased along with increasing the concentration of GLY, with the highest *EB* values around mid-level of CH (Fig. 3c.2) and the minimal level of CE (Fig. 3c.3). This trend was in line with the results of *TS* (Section 3.4) since it is expected that *TS* and *EB* have a linear, inversely proportional relationship due to plasticization by GLY [9]. The same relationship was confirmed in this study by plotting model-predicted *TS* and *EB* values as a function of GLY level (Fig. S5, Appendix A).

3.6. Total phenolic content

Natural antioxidants are common active components in sustainable food packaging systems due to their prominent role in the scavenging of oxidation inducers ([21] and ref. therein). HTs from CE retained their antioxidant activity after the incorporation in chitosan-based film, while their potential for scavenging free radicals was in a positive correlation with *TPC* [22]. This is the reason why the *TPC* of the given chitosan-based films was chosen as a relevant parameter to be optimized together with *MC* and mechanical properties. Following this, the *TPC* values in the studied film samples were ranging from 9.6 $mg_{GAE}\ g_{film}^{-1}$ to 23.7 $mg_{GAE}\ g_{film}^{-1}$ (Table 2). For instance, *TPC* values in chitosan-based films prepared from different FFSs containing a banana peels extract, a hop extract, and a mango leaf extract were up to ~4.8 $mg_{GAE}\ g_{film}^{-1}$ [14], up to ~12.7 $mg_{GAE}\ g_{film}^{-1}$ [20], and up to ~12.8 $mg_{GAE}\ g_{film}^{-1}$ [16], respectively.

It was revealed that the relation between *TPC* and independent variables was best described by a two-factor interaction (2FI) model [$Y_{TPC} = 16.17 - 1.18X_1 + 5.07X_2 - 1.68X_3 - 0.03X_1X_2 - 1.68X_1X_3 - 1.12X_2X_3$]. The 2FI model does not have quadratic terms which indicate that CH–CH, CE–CE, and GLY–GLY interactions did not affect *TPC*. Based on the parameters presented in Table 3, it was possible to conclude the proposed equation fitted the experimental data very well and the model was significant. As expected, the most influential variable was the concentration of CE (linear term), which exhibited a strong positive correlation with *TPC*. A steep slope in the response plot was observed along with the increase of the extract concentration, whereby the films prepared from the FFSs with the maximal concentration of CE tended to have *TPC* values higher than 15 $mg_{GAE}\ g_{film}^{-1}$ (Fig. 3d.1 and d.3). Considering the effect of CH and GLY levels, the highest values of *TPC* tended to be in the films prepared from the FFSs with the maximal concentration of CH and the minimal concentration of GLY (Fig. 3d.2). This is most likely because chitosan-based films can exhibit certain *TPC* values due to the reaction of FC reagent with $–NH_2$ groups from the polymer molecules [20 and ref. therein]. These functional groups were probably occupied upon the increase of GLY level and therefore became inaccessible for FC regent resulting in a decline of *TPC* at higher concentrations of plasticizer eventually.

3.7. Simultaneous optimization and model validation

The results presented throughout Sections 3.3–3.6 testified that the evaluated independent variables were mutually competitive. Simultaneous optimization of four variables that govern the films' physico-mechanical properties is thus not always an easy task since it implies a compromise between different attributes of the final material. Besides, the definition of desired attributes of the final material is a subjective matter made by a decision-maker, whereby the authors of this study were guided by the fact this material might be used for the production of sachets for packaging and storage of food [22]. Following this, the process of simultaneous optimization was done to find the optimal formulation of FFS that could be used to get film material of high gas barrier capabilities, mechanical stability, and antioxidant capacity. In the light of hereto evaluated film properties, it implies minimization of *MC* and *EB* as well as maximization of *TS* and *TPC* as the optimization criteria (Section 2.7).

The numerical solution within the constraints of the model was calculated by Design-Expert® software employing desirability function-based approach. The optimal composition of the FFS in terms of actual values of the raw materials was determined to be 1.93% (w/v) of CH, 0.97% (w/v) of CE, and 30.0% of GLY (w/w; per mass of chitosan), whereby the overall desirability was 0.912. Validation of the model was made by comparison of predicted values with those obtained by experimental evaluation of a new set of film materials prepared from the optimal FFS according to the protocol stated in Section 2.3.2. As evident

Table 4
Predicted and experimental responses of the films prepared using the optimal FFS formulation.

Response[a]	Predicted value[b]	Experimental value ($n = 3$)[c]	Absolute residual error (%)[d]
MC	18.9	17.0 ± 0.6	11.2
TS	16.0	16.7 ± 1.3	4.2
EB	10.3	10.4 ± 4.0	1.0
TPC	23.7	19.4 ± 0.1	22.2

[a] *MC*, moisture content (%); *TS*, tensile strength (MPa); *EB*, elongation at break (%); *TPC*, total phenolic content ($mg_{GAE}\ g_{film}^{-1}$).
[b] Predicted values obtained from the model equations.
[c] Experimental values obtained for the chitosan-based film prepared using the optimal FFS (according to the protocol stated in Section 2.3.2).
[d] Absolute residual error (%) = [(experimental value − predicted value) / experimental value] × 100 [40].

from Table 4, the absolute residual errors were between 1.0% and 11.2%, which is in line with the accuracy of the models generated for other polymer-based films [31,33,40].

4. Conclusions

The response surface methodology has been successfully applied in the formulation of active food packaging by linking composition of the film-forming solution to the physico-mechanical properties of the final material. The results revealed that, among the tested independent variables, the level of plasticizer was the most influential on moisture content and mechanical properties, while the level of the active component was the most influential on the antioxidant capacity of chitosan-based films. Further insight into the optimization and validation of the model-based results showed that it was possible to produce a material with satisfactory moisture, mechanical stability, and antioxidant capacity from the optimized film-forming solution under defined fabrication process parameters. The achievements of this study undoubtedly proved the response surface methodology as a time-saving and cost-efficient tool in design, development, and optimization of active chitosan-based films intended for food packaging.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2020.05.186.

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Appendix A. Supplementary data

Formulation of active food packaging by design: Linking composition of the film-forming solution to properties of the chitosan-based film by response surface methodology (RSM) modelling

Marijan Bajić [a,*], Ana Oberlintner [a], Kristi Kõrge [a,b], Blaž Likozar [a,c], Uroš Novak [a]

[a] *Department of Catalysis and Chemical Reaction Engineering, National Institute of Chemistry, Hajdrihova 19, 1000 Ljubljana, Slovenia*

[b] *Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia*

[c] *Faculty of Chemistry and Chemical Technology, University of Ljubljana, Večna pot 113, 1000 Ljubljana, Slovenia*

[*]Corresponding author at: Department of Biochemical Engineering, University College London, Gower Street, London WC1E 6BT, United Kingdom.
Email address: marijan.bajic@ki.si, m.bajic@ucl.ac.uk (M. Bajić)

The desirability function (D), which reflects the desirable ranges for each response (d_i), is defined as follows (Eq. (S1))[†]:

$$D = (d_1 \times d_2 \times \ldots \times d_n)^{\frac{1}{n}} = \left(\prod_{i=1}^{n} d_i\right)^{\frac{1}{n}} \quad \text{(S1)}$$

where n is the number of responses in the measure.

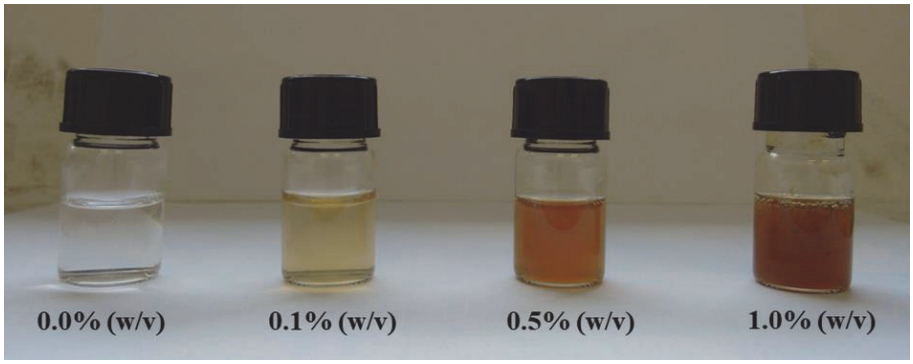

Fig. S1. Visual appearances of the film-forming solutions containing a) 0.0% (w/v), b) 0.1% (w/v), c) 0.5% (w/v), and d) 1.0% (w/v) of chestnut extract.

[†] Retrieved from: https://www.statease.com/docs/v11/contents/optimization/desirability-details/ (Accessed on 04/04/2020).

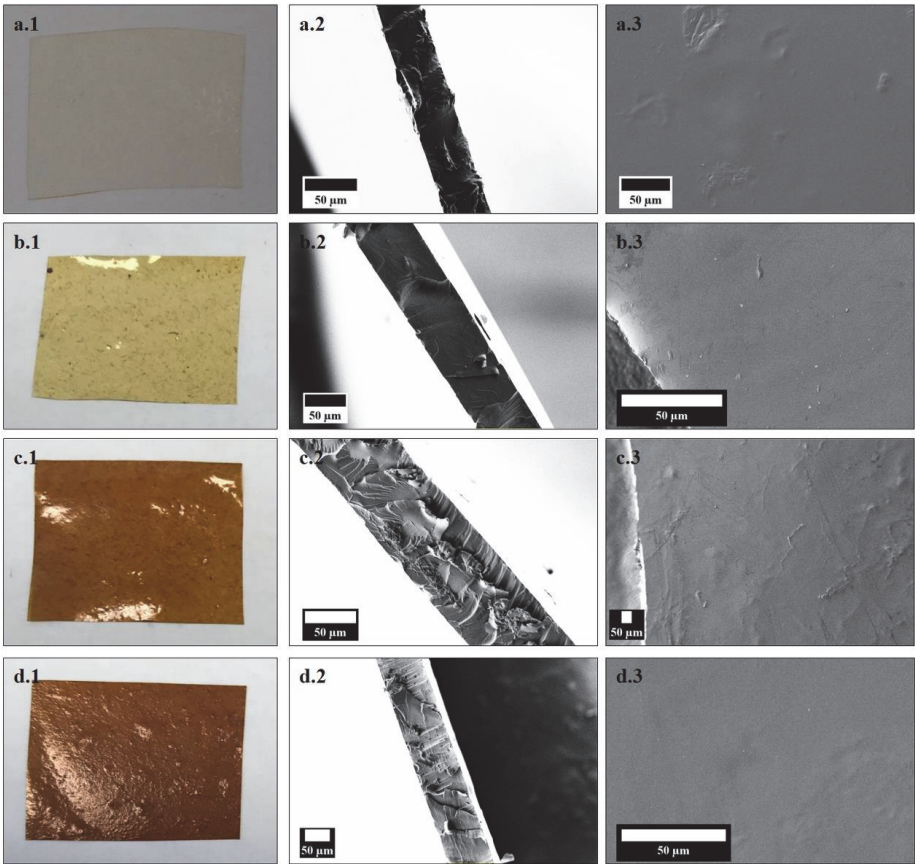

Fig. S2. Effects of the chestnut extract concentration on morphological properties of chitosan-based films prepared from the film-forming solutions containing a) 0.0% (w/v), b) 0.1% (w/v), c) 0.5% (w/v), and d) 1.0% (w/v) of CE. The numbers 1, 2, and 3 denote macroscopic images of the samples, SEM images of their cross-sections, and SEM images of their surfaces, respectively. The SEM images were captured using the scanning electron microscope SUPRA 35 VP (Carl Zeiss, Jena, Germany), while the films were prepared according to the protocol stated in Section 2.3.2.

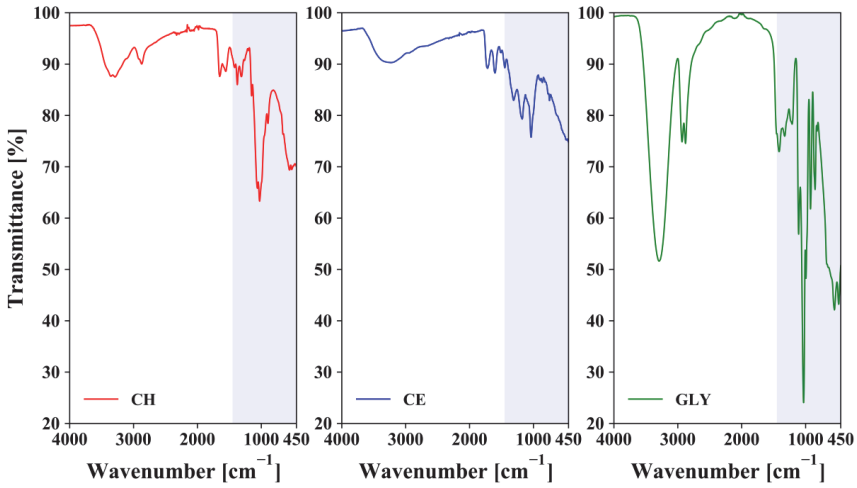

Fig. S3. FT-IR spectra of the raw materials: a) chitosan powder; b) chestnut extract powder; c) glycerol. The FT-IR spectra were obtained according to the protocol stated in Section 2.4. The blue-shaded area represents the fingerprint region.

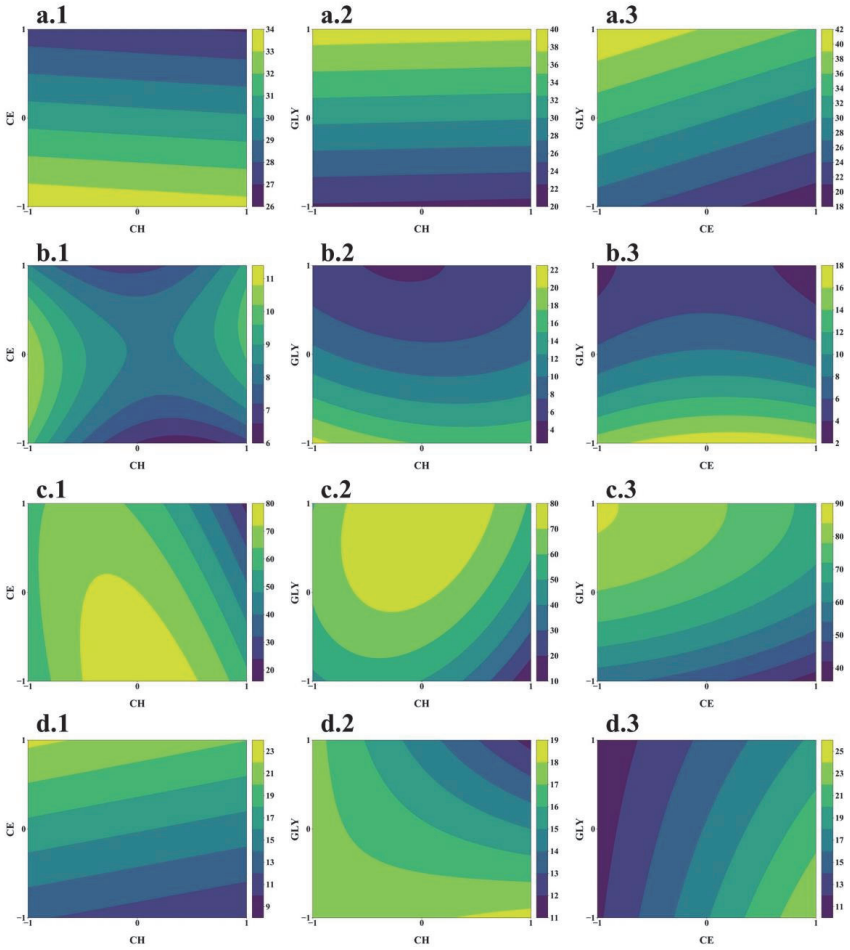

Fig. S4. Two-dimensional contour plots showing the impact of independent variables on a) moisture content, b) tensile strength, c) elongation at break, and d) total phenolic content of chitosan-based films. The numbers 1, 2, and 3 denote the mutual effect of CH–CE while the level of GLY was held constant, the mutual effect of CH–GLY while the level of CE was held constant, and the mutual effect of CE–GLY while the level of CH was held constant, respectively.

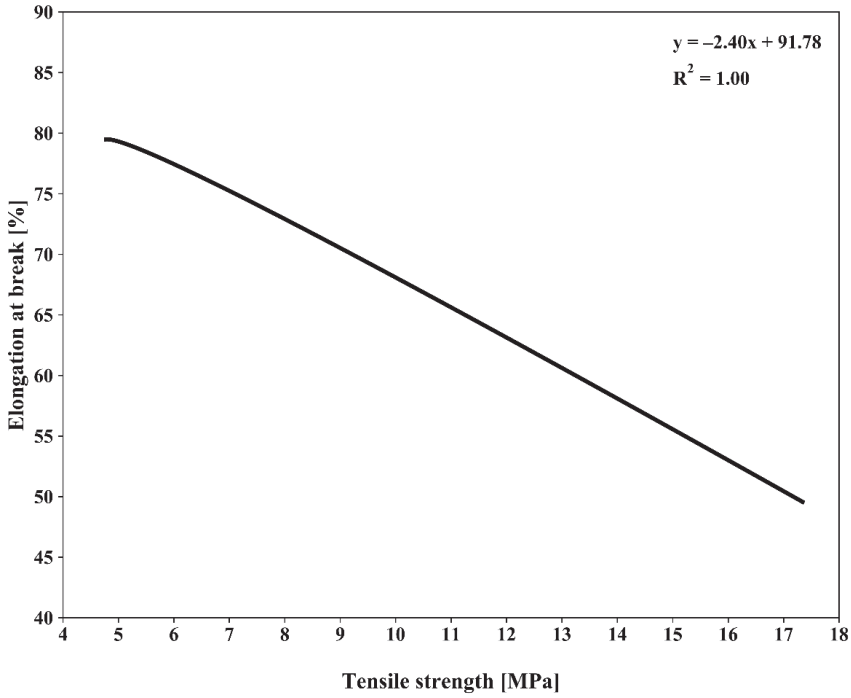

Fig. S5. The relationship between tensile strength and elongation at break of chitosan-based films as a function of glycerol content. The plot was generated from the model equations by varying glycerol level from −1 to +1, while two other independent variables were kept constant at mid-level (0 in terms of coded variables).

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Active chitosan–chestnut extract films used for packaging and storage of fresh pasta

Kristi Kõrge,[1,2] Marijan Bajić,[1] Blaž Likozar[1] & Uroš Novak[1]*

1 Department of Catalysis and Chemical Reaction Engineering, National Institute of Chemistry, Hajdrihova 19, 1000, Ljubljana, Slovenia
2 Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618, Tallinn, Estonia

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Summary A tannins-rich chestnut extract was used to enhance the antioxidant and antibacterial properties of chitosan-based film materials. The favourable mechanical properties of the novel material enabled its application in the preparation of the sachets that were used for packing and storage of filled fresh pasta. The fresh pasta ageing progressed in conventional refrigerated storage conditions of 8 °C with 60 ± 2% relative humidity in the absence of light for 60 days. The rapid moisture mobility between a starchy food and sachets during the first 9 days of storage induced retrogradation of the fresh pasta, whereby total phenolic content show dependency on moisture throughout the shelf life. Active components within the sachet prevented microbial growth on the food surface during the entire 60 days.

Keywords Active sachet, antibacterial and antioxidant activity, chestnut extract, chitosan-based film, fresh pasta, shelf life.

Introduction

Active biodegradable materials have recently emerged as an alternative capable of replacing conventional fossil fuel-based plastic packaging in the food industry (Moustafa *et al*., 2019; Rodríguez-Rojas *et al*., 2019; Sabbah *et al*., 2019). In addition, antimicrobial properties of such materials can be enhanced by the incorporation of various antimicrobial agents. Providing slow but constant migration of the active agents from the packaging material is an advantage in favour of the control of microbial growth over a longer period of time (Quintavalla & Vicini, 2002; Sung *et al*., 2013; Jideani & Vogt, 2016).

With global production of 14.3 million tons, pasta is one of the most consumed food products worldwide (International Pasta Organisation, 2017). Fresh pasta is classified as intermediate moisture food with moisture content (MC) of 35% and a reported shelf life 37–60 days (Del Nobile *et al*., 2009; de Camargo Andrade-Molina *et al*., 2013). Several studies rely on modified atmosphere to enhance shelf life of perishable foods packed in conventional (fossil fuel-based) plastic packaging (Sanguinetti *et al*., 2011; Kõrge & Laos, 2019), but notably, scarce literature covers packaging of fresh pasta into biopolymer-based sachets (de Camargo Andrade-Molina *et al*., 2013). However, replacing this pliable and easily shaped material is quite challenging because novel biodegradable materials have high water affinity and sometimes do not have sufficient mechanical properties or water vapour barrier capabilities. Therefore, mastering the structural modifications of the current biodegradable materials is an essential step for their practical use in everyday life.

Chitosan is a biocompatible, biodegradable, nontoxic and partly deacetylated, derivative of chitin, whereby its characteristics highly depend on its degree of deacetylation and molecular weight (Borić *et al*., 2018). Chitosan is also endowed with a good film-forming capacity (Chillo *et al*., 2009) that makes it one of the most favourable biopolymers for the preparation of environmentally friendly films intended for food packaging and protection (Divya *et al*., 2018; Wang *et al*., 2018). The inherent antioxidant and antimicrobial properties of chitosan-based films can be significantly improved with the incorporation of various plant-based active components and, therefore, help in the extension of the shelf life of perishable foods (Priyadarshi *et al*., 2018b; Wang *et al*., 2018). Among others, it has been shown that essential oils of citrus (Randazzo *et al*., 2016), caraway (Hromiš *et al*., 2015) and *Litsea cubeba* (Zheng *et al*., 2018) as well as different extract like turmeric (Kalaycıoğlu *et al*., 2017), spirulina (Balti *et al*., 2017), hop and oak (Bajić *et al*., 2019b) can be used for this purpose. In this context, industrial tannin extracts obtained from the chestnut

*Correspondent: E-mail: uros.novak@ki.si

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wood (*Castanea sativa*) have prominent antioxidant activity (Squillaci *et al*., 2018; Molino *et al*., 2020) and as such could be very interesting candidates as active components in chitosan-based films.

The aim of this study was to explore the possibility to use chestnut extract (CE) as an active component in chitosan-based film materials, which can be further implemented for the preparation of bio-based packaging. Specifically, the sachets prepared from chitosan-based films with incorporated CE were used for packing of fresh pasta and a comparative analysis was conducted to assess the sachet's influence on microbial growth, moisture mobility (in relation to total phenolic content) and microstructural alterations of packed material.

Materials and methods

Materials

High molecular weight chitosan (CH) (acetylation degree ≥75%, 310–375 kDa), lactic acid (LA) (purity ≥85%), gallic acid (GA) and Folin–Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (Steinhem, Germany), while methanol and acetone were from Avantor Performance Materials (Gliwice, Poland) and Honeywell (Hannover, Germany), respectively. Sodium carbonate was obtained from Merck (Darmstadt, Germany) and glycerol (GLY) from Pharmachem Sušnik (Ljubljana, Slovenia). Commercially available CE (≥75% tannins; <4% of ash) was provided by the company Tanin Sevnica (Sevnica, Slovenia). All chemicals except LA were of analytical grade. Milli-Q® water was used throughout all experiments.

Preparation and characterisation of active film packaging

Preparation of film-forming solutions

Film-forming solutions (FFSs) were prepared according to a slightly modified protocol described in Bajić *et al*. (2019a). In short, CH (1.5% w/v) and GLY (plasticiser; 30% w/w based on the mass of CH) were dissolved in an aqueous solution of LA (1.0% v/v). The mixture was agitated overnight on a magnetic stirrer IKA® RCT (IKA, Staufen, Germany) at 1000 r.p.m. and room temperature (24 °C) until homogenisation and then vacuum-filtered through two sheets of medical gauze. Afterwards, the mixtures were supplemented with 0.0% (reference sample), 0.5%, 1.0% and 1.5% w/v of CE in order to study its effect on the active properties of film materials, homogenised (at 6000 r.p.m. during 2 min) with Ultra-Turrax® T50 (IKA, Staufen, Germany) and left overnight. Finally, a formed sticky foam and remaining bubbles were carefully removed from the mixtures using a laboratory spatula, and the mixtures were used for the film preparation.

Preparation of chitosan-based film material

Prepared FFS was cast on rectangular polyurethane 12 cm × 12 cm Petri dishes (~0.32 mL of FFS per cm^2 of a Petri dish) and left to dry in a drying oven Kambič (Semič, Slovenia) with a continues ventilation (24 °C, RH 40% for 48 h) (Bajić *et al*., 2019a). Obtained films (mean diameter 120 ± 2 μm; measured by ABS Digital Thickness Gauge, Mitutoyo, Aurora, IL, USA) were peeled off from the Petri dishes, separated with baking paper to prevent direct contact from each other and stored in an airtight container (24 °C, no exposure to light) prior the analysis.

Total phenolic content of chitosan-based film material

Total phenolic content (TPC) in chitosan-based film material was determined using Folin–Ciocalteu's (FC) phenol reagent, according to the protocol outlined in our previous study (Bajić *et al*., 2019a). Briefly, small rectangular film samples were added in water (~5 mg of film per mL of water), followed by the successive addition of FC phenol reagent and aqueous solution of Na_2CO_3 (10% w/v) in the amount of 10 vol% and 20 vol% based on the volume of water, respectively. After the incubation of samples (2 h in dark, 24 °C), the absorbance was measured at 765 nm using Synergy™ 2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Gallic acid was used as the standard, and the results were expressed as the mass of gallic acid equivalent (GAE) per mass of the films.

Radical scavenging activity of chitosan-based film material

Determination of the antioxidant activity of chitosan-based films was done using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The rectangular film samples were added in a freshly prepared 60 μM DPPH methanolic solution (~5 mg of film per mL of the solution) and incubated for 2 h in a dark place and room temperature. The absorbance for each methanolic extract was measured at 517 nm on Lambda 40 UV/Vis spectrophotometer (Perkin Elmer, Waltham, MA, USA). The inhibition activity ($\%I$) of each sample was calculated as $\%I = ((A_{control} - A_{sample})/A_{control}) \times 100$ (Kaya *et al*., 2018), where $A_{control}$ and A_{sample} denote the absorbance of DPPH solution and the absorbance of solutions containing the films samples, respectively.

Antibacterial properties of chitosan-based film material

Antibacterial properties of chitosan-based films were tested against *Escherichia coli* K12 (*E. coli*) and *Bacillus subtilis* DSM 402 (*B. subtilis*). A fresh bacterial culture suspension in the exponential growth phase (OD_{600} = 0.5) was spread over the Luria-Bertani agar

culture medium, and the UV light-sterilised (at 254 nm for 15 min; both sides) rectangular film samples (~1 cm^2) were placed on the plate surfaces and incubated (37 °C, 24 h) (Bajić *et al*., 2019a). Clear areas that appeared around the films were considered as the inhibition zones (I_z).

Optical properties of chitosan-based film material
Rectangular film samples were cut to fit the size of a cuvette and carefully placed in it using tweezers. The samples were always kept to the same wall of the cuvette to provide equal distance from the light source in each measurement. The absorbance (A) was recorded at room temperature using Lambda 40 UV/Vis spectrophotometer under the wavelengths region ranging from 250 to 800 nm, whereby an empty cuvette (i.e. air) was used as the referent. The opacity (O) was calculated as $O = A_{600}$/film thickness (mm) (Sun *et al*., 2017), while the per cent of transmittance ($\%T$) was calculated as $\%T = 10^{-A} \times 100$ (Bajić *et al*., 2019a).

Preparation of sachets

Each sachet was prepared from two sheets of chitosan-based film material (prepared from the FFS containing 1% w/v of CE according to protocols explained in section Preparation and characterisation of active film packaging) by heat sealing (165 °C, 700 Pa, 7 s) on HST-H6 heat seal tester PARAM® (LabThink, Jinan, China). The first set (sachet in contact with food) was used to pack fresh pasta, and the other was heat-sealed without the pasta (reference sachet).

Preparation and packing of fresh pasta

Preparation of fresh pasta
Fresh pasta (from here onwards pasta) was provided by Mlinotest (Ajdovščina, Slovenia). In short, the production of pasta involves filling/dough preparation, shaping, pasteurisation (98 °C), drying/cooling and packaging. The pasta ingredients were as follows: wheat meals, eggs with the filling (cottage cheese and spinach) with nutritional values calculated on the label: carbohydrates 45% (sugars 1.4%), protein 11%, fat 5.1% (saturated fat 2.5%), dietary fibres 2.1% and salt 0.7%. Material that was used to pack pasta is a combination of polyethylene (PE), biaxially oriented polypropylene (BOPP) and ethylene vinyl alcohol (EVOH) (thickness 70 μm).

Packing of fresh pasta
The factory-packed pasta in modified atmosphere (MAP) conditioned PE-BOPP-EVOH plastic packaging (reference pasta) was re-packed into chitosan-chestnut extract (CH-CE) sachets (eight pieces) and heat sealed from the open end. Received sachets were inserted into paper envelopes to prevent their contact with each other. The entire process was conducted under laminar flow hood conditions (constant ventilation, 24 °C, 40% RH, ultraviolet light) to prevent microbial contamination. Samples were stored in a refrigerator at 8 °C and RH of 60 ± 2% during 60 days.

Characterisation of the CH-CE sachet-pasta system
All the samples (reference sachets, reference pasta, sachets with pasta – eventually analysed as a separate sample, i.e. pasta and CH-CE sachet) were tested for water activity, moisture, TPC, microbiology (pasta only), Fourier-transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM) at days 0, 3, 6, 9, 12, 30 and 60. Final CH-CE sachet-pasta systems were analysed in three biological replicas.

Water activity
LabMaster-aw neo (Novasina, Lachen, Germany) was applied to measure reference samples, pasta's and CH-CE sachets' water activity (a_w). After the packaging was removed from storage, it was opened under ventilating laminar flow hood. A pasta piece was cut and immediately placed into a measuring cup to prevent moisture loss. For the film analysis, a sample was cut from the sachets' middle section.

Moisture content
Moisture analyzer HE 53 (Mettler Toledo, Wien, Austria) was used to measure the moisture content of reference samples, pasta and CH-CE sachets. Pasta samples were analysed by receiving a pasta piece from cold storage, cut into smaller particles and immediately placed onto a measuring plate for analysis. Sachet samples were handled in a similar manner, using scissors for cutting.

Total phenolic content

Total phenolic content of sachets
A TPC of active sachets (~5 mg) was determined by Folin–Ciocalteu's (FC) phenol reagent according to protocol as described in section Total phenolic content of chitosan-based film material.

Total phenolic content of fresh pasta
To measure TPC in pasta samples, extraction of phenols was done prior to analysis according to the protocol outlined in a study of Vignola *et al*. (2018). The samples (200 mg) were pre-powdered in liquid nitrogen, weighted on an analytical balance (Kern, Balingen, Germany) and covered with 1 mL extraction solution of methanol:acetone:water (30:30:40 mL). The prepared mixture was agitated on Tehtnica Vibromix

10 (Domel, Železniki, Slovenia) vortex during 5 min and centrifuged (3 293 ***g***; 10 min) using MiniSpin centrifuge (Eppendorf, Hamburg, Germany). Green-coloured supernatant was pipetted into separate e-tube, and the extraction was repeated. After the double extraction process, supernatants were mixed, and 20 μL of the sample (supernatant) added to 120 μL water, followed by successive addition of FC phenol reagent and Na_2CO_3 (10% w/v) aqueous solution 10% w/v and 20% w/v based on the volume of the sample, respectively. Received samples were diluted with water up to 1 mL. After the incubation of samples (2 h in dark, 24 °C), the absorbance was measured at 765 nm using Synergy™ 2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Gallic acid was used as the standard, and the results were expressed as the mass of gallic acid equivalent (GAE) per mass of the pasta.

Microbiological analysis of fresh pasta

For the microbiological analysis, 4 ± 0.5 g of pasta sample was aseptically transferred into a sterile plastic homogeniser bag and blended with 36 ± 4.5 mL of 0.1% (w/v) sterile buffer Peptone water (BPW) using Masticator Basic blender (IUL Instruments, Barcelona, Spain) for 30 s, 1500 r.p.m. and at 24 °C. Thereon, dilutions were prepared with 0.1% BPW and 100 μL of each dilution was inoculated into plate count agar growth medium. Dichloran Rose Bengale Chloramphenicol Agar (Biokar diagnostics, Allonne, France) was used for a total count of yeast and moulds, Tryptic Glucose Yeast agar was used for a total bacterial count determination and Violet Red Bile Lactose Agar (Merck KGaA, Darmstadt, Germany) was used for total count of bacterial group *Enterobacteriaceae*.

Scanning electron microscopy

Scanning electron microscopy SUPRA 35VP (Carl Zeiss, Jena, Germany) was used to visualise morphological changes in sachets and pasta dough cross section during shelf life. All the samples were mounted on metal stubs with carbon tape and observed using a voltage of 1.00 kV and magnifications 1.00 K, 500 for the sachet film and pasta, respectively.

Fourier-transform infrared spectroscopy

The Fourier-transform infrared spectra of prepared films and pasta were recorded at the wavenumbers ranging from 4000 to 700 cm^{-1} and resolution of 4 cm^{-1}, using Spectrum Two FT-IR spectrometer (PerkinElmer). The sample (cheese and film) spectres were normalised before carrying out the statistical analysis by averaging each value of the spectrum values. Each sample was scanned in triplicate.

Statistical analysis

The data were subjected to the one-way analysis of variance (ANOVA) with a confidence level of 95% ($P \leq 0.05$), followed by Tukey's test. All the results in triplicate are expressed as the mean ± standard deviation.

Results and discussion

Preparation and characterisation of active film packaging

The FFSs containing 0.0% (control sample), 0.1%, 0.5% and 1.0% (blended samples) were successfully used for the preparation of chitosan-based film material. The prepared films were preliminary characterised regarding the effect of the incorporated extract on their active (antioxidant-, antibacterial- and optical-related) properties (Table 1).

The films exhibited favourable antioxidant properties, whereby the TPC values (up to 18.7 $mg_{GAE}\ g_{film}^{-1}$) were in a positive correlation with films' radical scavenging activities (up to 81.8%). Besides, the incorporation of CE endowed the films by antibacterial activity against *E. coli* (I_z was up to 17.8 mm) and *B. subtilis* (I_z was up to 15.5 mm) (Fig. 1).

Finally, evaluation of the films' optical properties has revealed that samples prepared from the FFSs containing CE had opacity values of up to 5.71 mm^{-1} (Table 1), while the per cent of transmittance in the UV and visible wavelength range (from 250 to 800 nm) can be seen in Fig. 2.

The evaluated properties are important in providing sufficient protective capabilities of chitosan-based film materials. Besides, the trends of antioxidant-, antibacterial- and optical-related properties observed after increasing the concentration of CE were in line with trends reported for other chitosan-based films blended with different types of plant-originating extracts (Sun *et al.*, 2017; Kaya *et al.*, 2018; Priyadarshi *et al.*, 2018a; Bajić *et al.*, 2019b; Rambabu *et al.*, 2019). Therefore, the incorporation of CE notably improved properties of chitosan-based films, making them appropriate for further use as active packaging materials, as discussed in the following chapters.

Characterisation of the CH-CE sachet-pasta system during shelf life

Moisture mobility

Mobility of the moisture was evaluated under conventional refrigerated storage conditions at 8 °C with 60 ± 2% relative humidity in the absence of light for

Table 1 The effect of the chestnut extract concentration on the properties of chitosan-based films

	Antioxidant-related properties		Antibacterial-related properties		Optical-related properties	
Film sample*	**TPC† [mg_{GAE} g_{film}^{-1}]**	**I† [%]**	**I_Z (*E. coli*)† [mm]**	**I_Z (*B. subtilis*)† [mm]**	**A_{350}† [%]**	**O† [mm^{-1}]**
CE_0.0	0.4 ± 0.1^a	9.0 ± 6.0^a	0.01 ± 0.1^a	0.01 ± 0.1^a	59.9 ± 5.0^a	0.85 ± 0.2^a
CE_0.1	3.4 ± 0.2^b	35.3 ± 11.0^b	2.50 ± 0.5^a	1.90 ± 1.0^a	5.50 ± 1.0^b	1.53 ± 0.3^a
CE_0.5	11.2 ± 2.0^c	76.2 ± 5.0^c	2.50 ± 2.0^a	7.50 ± 1.0^a	0.01 ± 0.1^b	3.73 ± 0.4^b
CE_1.0	18.7 ± 3.0^d	81.8 ± 3.0^c	17.8 ± 2.0^b	15.5 ± 5.0^b	0.01 ± 0.1^b	5.71 ± 0.4^c

*Labels CE_0.0, CE_0.1, CE_0.5 and CE_1.0 indicate chitosan-based films prepared from the FFSs containing 0.0% (control sample), 0.1%, 0.5% and 1.0% (w/v) of CE, respectively.

†Different subscript letters in each separate column indicate the samples with significantly different mean values ($P < 0.05$).

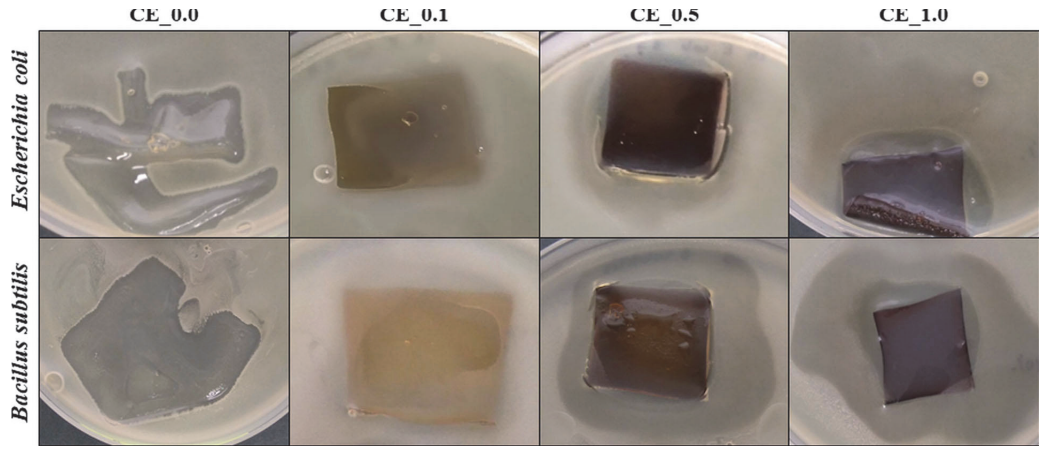

Figure 1 The appearance of inhibition zones visible around chitosan-based films after the incubation in a medium containing *E. coli* (upper row) and *B. subtilis* (lower row). Labels CE_0.0, CE_0.1, CE_0.5 and CE_1.0 indicate chitosan-based films prepared from the FFSs containing 0.0% (control sample), 0.1%, 0.5% and 1.0% (w/v) of CE, respectively.

60 days. Figure 3 depicts results of moisture desorption from pasta to CH-CE sachet and adsorption of the sachet *vice versa*. Reference pasta and reference sachet results are visualised for comparison.

According to the moisture mobility in CH-CE sachet-pasta system, pasta bestowed 9% of its moisture when sachet reached to its moisture equilibrium. From this point on, CH-CE sachet-pasta system depleted its capability to maintain the shelf life of pasta accordance to commercial expectations. A rapid MC desorption of pasta was observed during first 3 days (8%), slowing down over next three (1%) and then a strong decrease (19%) over the remaining time. Tested pasta MC results represented the described behaviour of Bressani (2014) where pasta, during its shelf life goes through three stages: (i) formation of natural crust, (ii) drying and (iii) stabilisation. A retrogradation process took place. A similar outcome has been reported in other studies with a starchy food, in particular with bread staling. Accordingly, moisture starts to move from a crumb to crust and does not move back from crust to crumb because of inability to form new hydrogen bonds in tightened structure (Kerch *et al.*, 2010).

At the same time, Fig. 3 depicts that CH-CE sachet acted as expected regards to pasta changes. During the first 3 days, 6% MC increase in CH-CE sachet was observed. In comparison, bread has been shown to lose 3% of moisture to carboxymethyl cellulose-chitosan sachet after 3 days, but unfortunately, they do not show moisture intake of the sachet (Noshirvani *et al.*, 2017). This is attributed to two theories: (ii) plastification properties of moisture, that is water from the food that relaxes the matrix of the sachet making absorption easier, (ii) due to CE that elevates solubility with its hydrophilic groups through interactions with water, also plasticiser (GLY) (Alves *et al.*, 2018; Sun *et al.*, 2018). The slowing down of moisture mobility during 3–6 days apprised that the turnover point of the sachet moisture intake was approaching. Pereda *et al.* (2010)

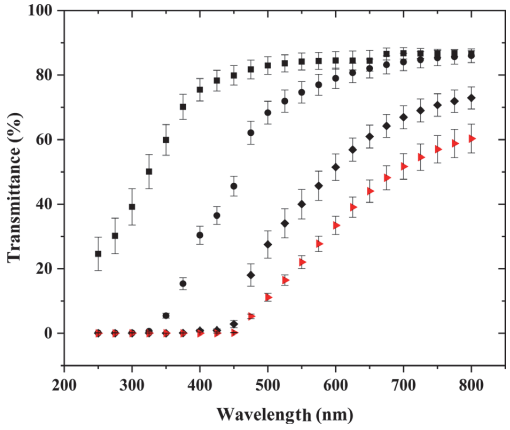

Figure 2 Optical properties of chitosan-based films of CE 0% (■), 0.1% (●), 0.5% (♦) and 1% (►) in the UV-Vis wavelength range. Labels CE_0.0, CE_0.1, CE_0.5 and CE_1.0 indicate chitosan-based films prepared from the FFSs containing 0.0% (control sample), 0.1%, 0.5% and 1.0% (w/v) of CE, respectively.

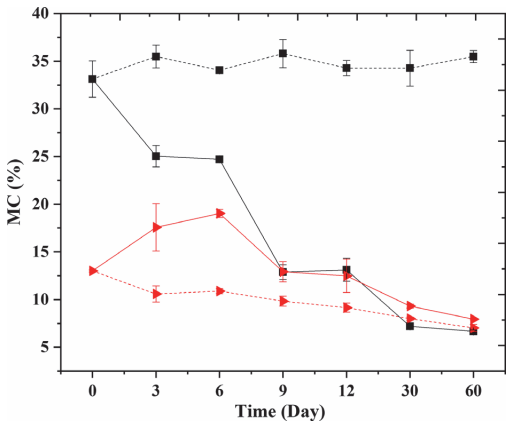

Figure 3 Moisture mobility of the pasta (■) and CH-CE sachet (►) during storage where dashed lines represent reference sample values.

showed moisture equilibrium of the chitosan film at 20% with 1 day (25 °C, 75% RH). In our study, CH-CE sachet matrix reached its highest MC capacity of 19% (4 °C, 60% RH) on the sixth day when started to reveal properties that allowed easy water molecule passage. Subsequent to the turnover point, pasta MC drop gets steeper and reaches from 25% to 10% by the ninth day compared with CH-CE sachet that lost its MC with slower pace from 19% to 12%.

Total phenolic content relation to water activity

Total phenolic content of bio-based films has been established as a reliable indicator of antioxidant capacity. By observation of moisture mobility and its effect as an intrinsic factor on phenolic content change, its dependence on MC was noted in both matrices. Moisture loss highlighted the concentration of already existing phenolic content in pasta and at the same time dilution in CH-CE sachet. Figure 4 depicts a TPC relation to bound water while the retrogradation process was in progress.

Phenolic content of pasta was measured 0.14 $mg_{GAE}\ g_{pasta}^{-1}$ with a_w 0.950 and 0.18 $mg_{GAE}\ g_{pasta}^{-1}$ with a_w 0.606 and MC 5.5% initially and after storage, respectively. Our results were in the range of 0.09–0.67 $mg_{GAE}\ g_{pasta}^{-1}$ previously stated by Gull *et al.* (2018) who worked with pasta that had 9% of moisture. Moreover, phenolic content in reference pasta (data not shown) remained stable (0.14 $mg_{GAE}\ g_{pasta}^{-1}$, average a_w 0.961) during the entire shelf life indicating, when a_w is stable TPC is unchanged. In CH-CE sachets, the TPC was measured 21.8 $mg_{GAE}\ g_{film}^{-1}$ at the beginning of shelf life. A peculiar operation to marine biopolymer sachet, regards to a_w, describes decrease and after a while an increase in TPC near to initial value. This phenomenon indicates that there is likely no migration of phenolic content to the pasta surface.

Microbiology

The CH-CE sachet met the antimicrobial requirements and pasta, conditioned with 8 °C, 60% RH, did not develop microbial growth during 60 days of storage. Total count of yeast, moulds, bacteria and *Enterobacteriacea* were all under detection limits (data not shown).

A short impact time (before retrogradation) for microbial activity was out ruled by a_w measurement in pasta. Figure 3 depicts that CH-CE sachet reaches a_w 0.730, making it a good surface for microbiology and if with heightened vapour permeability to an unprotective barrier against the hazard. But in opposite, even if a_w decrease in pasta was statistically significant accordance to a_w sorption isotherm graph (Roos *et al.*, 2018), it does not leave the range of moderate mould activity. In light of these results, it can be concluded that (under given conditions) antioxidant CH-CE sachet prevented microbial hazard and with high a_w.

Scanning electron microscopy

Scanning electron microscopy images of pasta and CH-CE sachet cross sections are shown in Fig. 5 to assess their visible change during storage. The protein–starch matrix in the pasta samples is well formed throughout the shelf life with strong and continuous

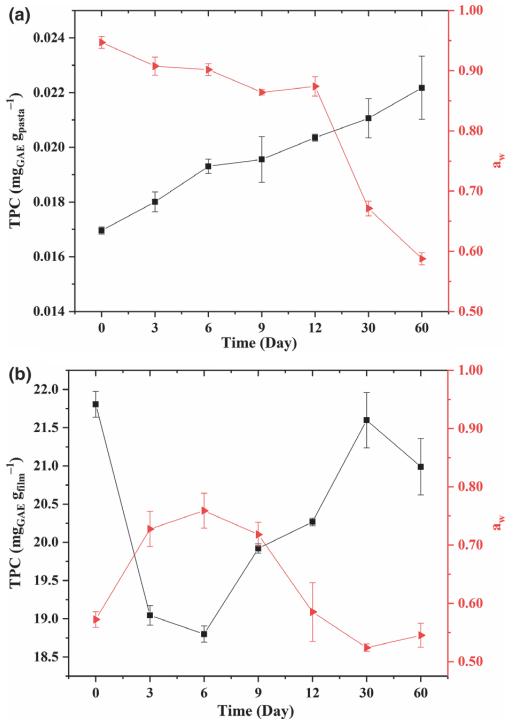

Figure 4 Total phenolic content and water activity changes in the a) pasta and b) CH-CE sachet in contact with one another during storage where the ■- marks represent TPC values and ►- water activity (a_w).

protein matrix entrapping uniform starch granules (Fig. 5b). Focus on single starch granule showed that in the beginning of shelf life lenticular starch granules were slightly covered with amorphous network-type coating giving the indication of gelatinised starch via pasteurisation during manufacture and presence of moisture (Tudorică *et al.*, 2002; Sicignano *et al.*, 2015). Although the observation of amorphous coating was not seen in the end of the shelf life, starch granules within the CH-CE sachet packed samples showed no signs of swelling and deformation (which could lead to additional gelatinisation), indicating that they have been affected by moisture loss. Moreover, the protein–fibre matrix within reference pasta after 60 days appears to be have less tightened than in the samples from CH-CE packaging, resulting distinguishable A and B-type granules (Sicignano *et al.*, 2015). SEM microphotographs of different size granules and open spaces in between the matrix refer to more fresh-like texture of pasta.

The CH-CE sachet show a homogenous and evenly distributed structure on the third day when its moisture capacity was highest compared with the initial texture at day 0 during shelf life (Fig. 5a). When moisture is evaporating from CH-CE sachet, it starts to show uneven cross section, indicating weakened intermolecular interactions, as reported by Sogut & Seydim (2018). The CH-CE sachets did not develop any pores or visible thinning.

Fourier-transform infrared spectroscopy

It is known that the active packaging is capable of releasing its antioxidants or other macrocomponents onto food surface and *vice versa* (Noshirvani *et al.*, 2017; Sogut & Seydim, 2018; Sun *et al.*, 2018). Figure 6 describes FT-IR spectres used to determine specific absorption bands of pasta and CH-CE sachet. In overall, peaks (Fig. 6a) on pasta spectra in the region 800–1150 cm^{-1} refer to residual starch–protein interactions. The IR region from 1175 to 1450 cm^{-1} provides information about sugars and organic acids. Presented are amide I band at 1650 cm^{-1} that results from C=O stretching and amidic band II from vibrations of N-H group with IR 1550 cm^{-1}. C–H band vibrations at 1700 cm^{-1} till 3000 cm^{-1} refer to ester carbonyl functional groups of the triglycerides. Peakwise, our results incline with the results of Priyadarshi *et al.* (2018a) and Durazzo *et al.* (2018) groups. The analysed CH-CE sachets produced same characteristic absorption bands to pasta.

All the spectres with CH-CE sachets showed O–H stretching vibrations in accordance with moisture mobility. Pasta, being more heterogeneous, received stronger O–H band intensity during the sixth day, but otherwise showed a similar trend to MC results. No peak shifts or new peaks were detected on FT-IR spectres generated for the reference pasta and sachet during shelf life. Changes were detected on transmittance intensities. The IR regions of 800–1150 cm^{-1} where residual starch is detected do not influence the same region on CH-CE sachet spectra. We could not differentiate CH-CE sachet components influence on pasta surface.

Conclusions

In this study, chitosan-based active film materials incorporated with CE were used to prepare sachets that were further used to pack fresh pasta. The antimicrobial activity of the film and its interactions within an active CH-CE sachet-pasta system during shelf life were evaluated during the shelf life. The results have shown that a retrogradation process of pasta has occurred, giving a hard-like texture after 9 days of preservation in CH-CE sachet. Changes in

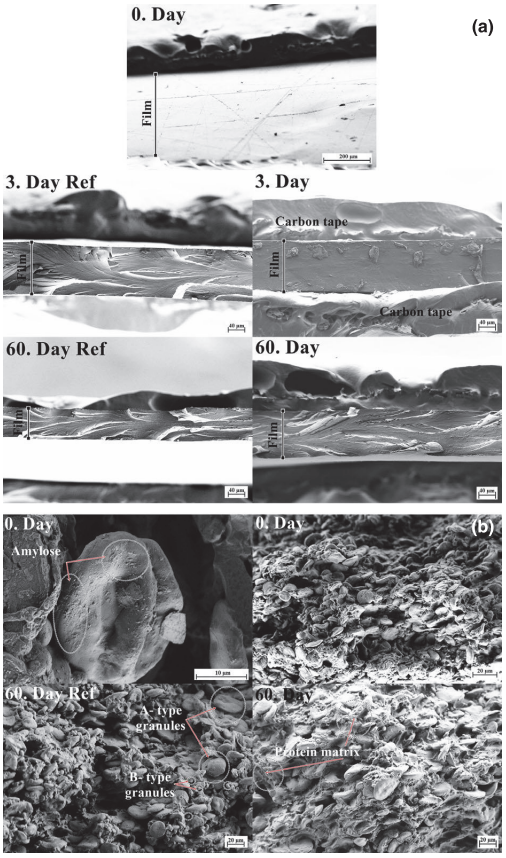

Figure 5 SEM images of (a) CH-CE sachet and (b) pasta on days 0, 3 and 60. From left to right, pasta pictures visualise amylose amorphous phase covering single starch granule and cross-sectional structure on days 0 and 60. Reference sample pictures are sided for comparison.

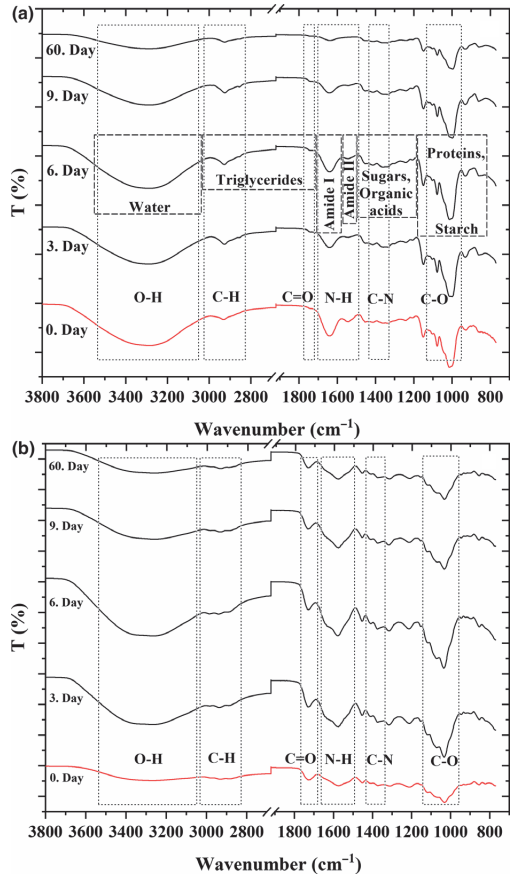

Figure 6 FT-IR results of the (a) pasta and (b) CH-CE sachet in contact during shelf life. Dashed boxes represent an approximate area of macrocomponents.

a_w show total phenolic content concentration or dilution, respectively, to pasta and CH-CE sachet in contact. Regardless, pasta remained free of microbial spoilage in CH-CE sachet during the entire 60-day shelf life. According to FT-IR and SEM results, active packaging ingredients did not affect food surface and its microstructure. In spite of the antimicrobial properties, fresh pasta shelf life was not realised during the 60 days because of the unacceptable texture. Therefore, further research is required to improve the chitosan-based film permeability properties and/or to investigate alternative food products with a higher natural moisture barrier. Moreover, the possible colouring effect of the films (extracts) onto fresh pasta, which will require additional sensory analysis that relates directly to customers acceptance, was not part of our current studies. The sensory analysis studies will be investigated in the future.

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Conflict of interest

The authors declare no competing interests.

Ethics approval

Ethics approval was not required for this research.

Data availability statement

Research data are not shared.

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Publication IV:

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Article

Reduction in Spoilage Microbiota and Cyclopiazonic Acid Mycotoxin with Chestnut Extract Enriched Chitosan Packaging: Stability of Inoculated Gouda Cheese

Kristi Kõrge [1,2], Helena Šeme [3], Marijan Bajić [1], Blaž Likozar [1] and Uroš Novak [1,*]

[1] Department of Catalysis and Chemical Reaction Engineering, National Institute of Chemistry, Hajdrihova 19, 1000 Ljubljana, Slovenia; kristi.korge@ki.si (K.K.); marijan.bajic@ki.si (M.B.); blaz.likozar@ki.si (B.L.)
[2] Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia
[3] Acies Bio d.o.o., Tehnološki park 21, 1000 Ljubljana, Slovenia; helena.seme@aciesbio.com
* Correspondence: uros.novak@ki.si

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Abstract: Active chitosan-based films, blended with fibrous chestnut (*Castanea sativa* Mill.) tannin-rich extract were used to pack Gouda cheese that has been contaminated with spoilage microflora *Pseudomonas fluorescens*, *Escherichia coli*, and fungi *Penicillium commune*. A comprehensive experimental plan including active chitosan-based films with (i) chestnut extract (CE), (ii) tannic acid (TA), and (iii) without additives was applied to evaluate the film's effect on induced microbiological spoilage reduction and chemical indices of commercial Gouda cheese during 37 days while stored at 4 °C and 25 °C, respectively. The cheese underwent microbiology analysis and chemical assessments of ultra-high-performance liquid chromatography (UHPLC) (cyclopiazonic acid), pH, and moisture content. The biopackaging used for packing cheese was characterized by mechanical properties before food packaging and analyzed with the same chemical analysis. The cheese microbiology showed that the bacterial counts were most efficiently decreased by the film without additives. However, active films with CE and TA were more effective as they did not break down around the cheese and showed protective properties against mycotoxin, moisture loss, and pH changes. Films themselves, when next to high-fat content food, changed their pH to less acidic, acted as absorbers, and degraded without plant-derived additives.

Keywords: active chitosan-based film; chestnut extract; gouda inoculated cheese; cyclopiazonic acid; tannins; antimicrobial and antifungal food packaging; biopolymers

1. Introduction

In chemical attributes, the main macromolecules in Gouda cheese are lipids (24%) followed by proteins. With the nutritious media, this semihard cheese can be highly susceptible to microbial hazard in ambient conditions (room temperature, high O_2), including the mold's ability to stay vital at refrigeration temperature, but also at low values of O_2, pH, and water activity [1]. The water activity of the Gouda cheese itself has been reported as an average of 0.972 [2]. When contaminated with pathogenic bacteria or toxigenic fungi, a rapid growth fosters deterioration of the texture and mycotoxin production [3,4]. There is a list of cheese contaminators, but a few concerning ones that can be named are bacteria *Pseudomonas fluorescens* and *Escherichia coli* and fungi *Penicillium commune* [5,6]. The latter is known for producing mycotoxin cyclopiazonic acid (CPA), strongly implicated as a causative agent in

mycotoxicosis for animals and is potentially harmful to humans [7–9]. For these reasons, various types of cheese require a different kind of packaging concepts that meet the demand for prolonged shelf life.

Within current technologies to protect cheese from spoilage, it is either paraffined or packed in flexible film, including conventional polyethylene (PE) or similar polymer materials such as polyethylene terephthalate (PET) or polypropylene (PP) [10]. Both materials represent good barrier properties, although PE (or similar) material act as a single-use packaging and thus in most cases ends up as a non-degradable waste. The wax layer favors bacterial growth, causing off-flavors and gas formation when not applied to the cheese surface properly [2]. On the other hand, films using chitosan constitute thin layers of materials that have been successfully tested to substitute synthetic packaging and prolong the shelf life of cheese in regard to safety [11–13]. Numerous natural substances have been tested and interpreted through modeling to design applicable biomaterials [14,15]. However, to our knowledge, none of the created compositions take into account adjacent food macromolecular composition and its characteristic microbial population effect on the films themselves [16]. Therefore, additionally to food analysis in the biopackaging, it is necessary to monitor how the films are changed in times when they are in contact with certain food macromolecules/microbes. The simultaneous analysis of two matrices is required input to the ongoing engineering of the films, which makes choosing suitable storage material for food more efficient.

Engineering of the bio-based film starts with the matrix-forming biomaterial. Chitosan is a long-chain polymer with reactive OH^- and β-(1–4) positioned NH_2^+ groups, mainly chemically converted biomaterial from chitin, and used as one among others [17]. Due to the conversion, it receives higher solubility in a mildly acetic aqueous solution, which is vital for the film-forming solution (FFS) homogeneity. By being able to aggregate with negatively charged molecules of fats (oleic, linoleic, palmitic, stearic, linolenic), it performs antihyperlipidemic action through ionic complexes (between NH_2^+ and O^-) [18]. In the form of film, it bestows antimicrobial properties through either electrostatic interaction with the cell wall by changing the cell permeability, or metal chelation with the outcome of collapsing/distorting the outer membrane. In the end, the DNA of bacteria will be damaged and depleted [19]. The biopolymer deacetylation (DA) level, molecular weight, and pH are essential factors to the antimicrobial activity [20,21]. In fact, values of DA and molecular weight (M_w) are in correlation. The high numeric value of these two parameters gives chitosan enhanced binding affinity and uptake capacity; employs chitosan into a non-degradable, less penetrable film matrix formation, which is essential when preserving food and plays a role when applying against certain spoilage bacteria. Furthermore, since chitosan amine groups are becoming ionized at pH < 6, antimicrobial activity improves at low pH [19].

The above-mentioned properties of chitosan are well-aligned with properties of plant derivative extracts, frequently used as active additives to engineered biofilms [22]. In this regard, the chestnut extract is one of the widespread derivatives that is also known for enhancing the film's permeability properties [23,24]. Various plant segments (fruits, leaves, galls, bark, and wood) are used for the extract. It is a multifunctional component mainly consisting of starch (40–60%), followed by condensed tannins with flavonoid core and, most importantly, hydrolyzable tannins (HTs). HTs are a mix of simple phenols, which also have the coloring effect. Their chemistry is broad, covering interactions not only with proteins but several other organic nitrogen compounds, including arginine, chitin, and chitosan. The higher reactivity is related to sufficient amine groups and higher M_w [25]. Studies report chestnut extract tannins interactions with abdominal cholesterol and thus lowering adiposity in mice [26], a decrease in *Cladosporium cladosporioides* on sheep cheese rind [7], *L. monocytogenes* in Emmental cheese [27], *E. coli*, *P. fluorescens* in mozzarella cheese [28], and reduction in mycotoxins [29]. Yet even though the chestnut extract is widely spread, there seems to be a gap in knowledge of how the component combines with chitosan to prevent food spoilage.

This work aimed to prepare new food packaging material, as in chitosan-based films with chestnut extract, to enhance the chitosan-based film's antimicrobial activity by incorporating active HTs from chestnut extract (CE). For better realization of the activity, the films were coupled with reference

(tannic acid:chitosan (TA:CH) and CH) films. Enhanced film's antimicrobial activity was tested on induced Gouda cheese spoilage, chosen to study the impact on high lipid food. To do so, comparative analysis of microbiology, ultra-high-performance liquid chromatography (UHPLC), pH and moisture mobility were applied in two conditions (4 °C and 25 °C) for 37 days. To our knowledge, there are no reports on CPA measurements in biopackaging, which was one of the objectives of this study. Biopackaging changes and food changes are described and presented as a two-way system.

2. Materials and Methods

2.1. Materials

High molecular weight chitosan (CH) (acetylation degree ≥ 75%, 310–375 kDa), lactic acid (LA) (purity ≥ 85%), and tannic acid (TA) were purchased from Sigma-Aldrich (Steinheim, Germany), while methanol and acetonitrile were purchased from Avantor Performance Materials (Gliwice, Poland) and Honeywell (Hannover, Germany), respectively. Sodium dihydrogen phosphate dihydrate and ortho-phosphoric acid (purity ≥ 85%) were obtained from Merck (Darmstadt, Germany), ammonium acetate from Kemika (Zagreb, Croatia), and glycerol (GLY) from Pharmachem Sušnik (Ljubljana, Slovenia). Commercially available CE (≥75% tannins; <4% of ash) was provided by the company Tanin Sevnica (Sevnica, Slovenia). All chemicals except LA were of analytical grade. According to EU legislations (EU) 2017/2470 (chitosan) 2017, (EU) 2017/66 (tannic acid) 2016, (EU) no 231/2012 (lactic acid and glycerol) 2012 [30] chestnut extract specification (provided by producer company), all the substances can be considered as food additives. Milli-Q® water was used throughout all the experiments.

2.2. Film-Forming Solutions and Chitosan-Based Films

2.2.1. Film-Forming Solutions

All FFSs formulations were prepared at ambient conditions by adding predetermined amounts of CH (% *w*/*v*) and GLY (% *w*/*w*, calculated per mass of CH) in the solvent (1% (*v*/*v*) aqueous solution of lactic acid) followed by continuous stirring (1000 rpm; 12 h; room temperature, 24 °C) on RCT magnetic stirrer (IKA, Staufen, Germany) [31]. The predetermined amounts of CE or TA were added subsequently after the mixing step, and the mixtures were homogenized (6000 rpm; 2 min) on Ultra-Turrax® T50 (IKA) and left overnight to get rid of the air bubbles formed during this process. A small amount of stable foam that was formed on the top of the mixtures was eventually removed by using a laboratory spatula.

2.2.2. Chitosan-Based Films

Prepared FFSs were cast in polyurethane Petri dishes (approximately 0.32 mL/cm) and left under constant airflow box (Microbium d.o.o, Ljubljana, Slovenia) at room temperature 24 ± 2 °C for the next 24 h. Obtained films were peeled off from rectangular Petri dishes (12 cm × 12 cm), and stored in an airtight container (24 °C, no exposure to light) until further analysis.

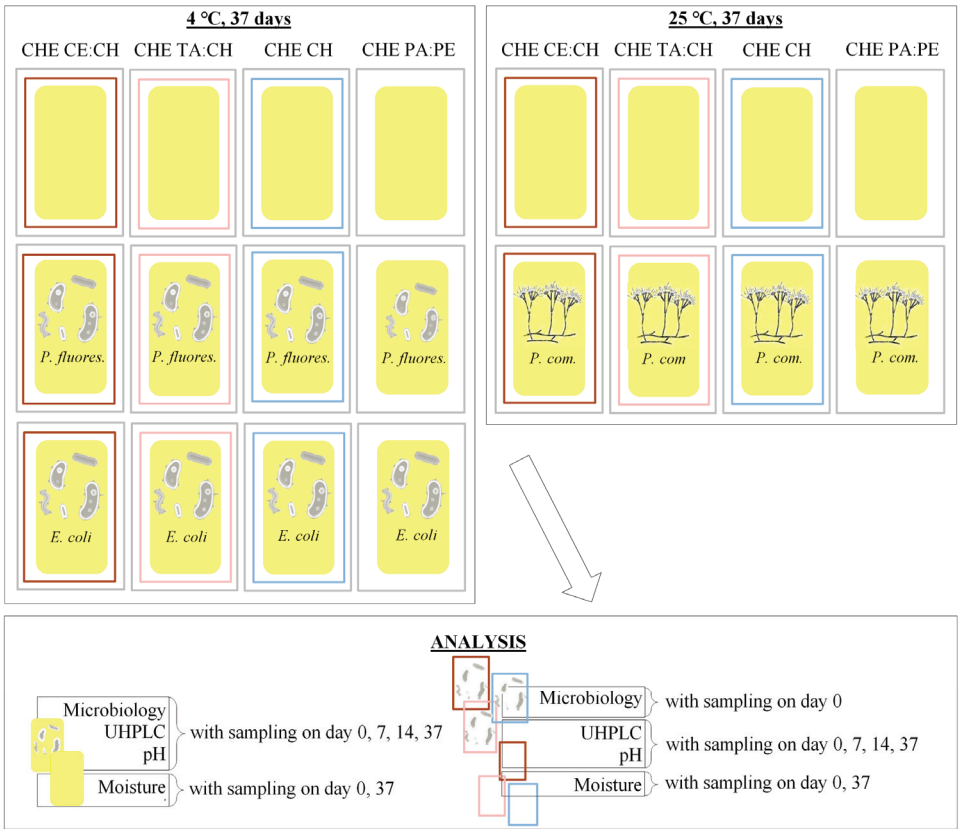
2.3. Gouda Cheese Preparation

The Gouda cheese was purchased from a local supermarket in Slovenia, Ljubljana. Before repacking, the cheese blocks were cut under the sterile constant airflow box into uniform pieces with an average weight of 22 g.

2.4. Experimental Design

A 4:4:2 factorial experiment design (4 different packaging sets × 4 time points × 2 temperatures) was implemented during this study (Scheme 1). Accordingly, three sets of chitosan-based films, namely chestnut extract:chitosan (CE:CH), tannic acid:chitosan (TA:CH), and blank chitosan (CH) with

extra layer of polyamide:polyethylene (PA:PE) vacuum bags (Status d.o.o Metlika, Slovenia) were prepared. A reference set with PA:PE was prepared next to biopackaging sets.



Scheme 1. Experimental design. The cheese (■—CHE) was packed in three different sachets (□—chestnut extract (CE), □— tannic acid (TA), □— chitosan (CH)) and additionally into vacuum packaging (□—polyamide:polyethylene (PA:PE)).

The precut cheese was placed onto a Petri dish, inoculated with chosen spoilage microorganisms (*P. fluorescens*, *E. coli*, *P. commune*), and packed into a sachet of one 12 cm × 12 cm sheet of chitosan film. Each sachet was prepared by heat sealing (165 °C, 700 Pa, 7 s) on HST-H6 heat seal tester PARAM® (Labthink, Jinan, China) along the long and then short edge. After the insertion of the spoiled food, the sachet was heat-sealed once more from a short open edge. The same process was completed with the unspoiled food and all the procedures were conducted under the sterile conditions of a constant airflow box. All the cheese:biofilms sample sets were additionally packed into an extra layer of PA:PE vacuum bags and vacuum sealed. This was achieved using a vacuum sealer (Status d.o.o, Metlika, Slovenia) with a purpose of preventing environmental effects and to study the ultimate mutual impact of the matrices inside the sets. The named sample sets with spoilage bacteria were stored in 4 °C conditions while samples with spoilage fungi in environmental conditions of 4 °C and 25 °C for 37 days (day 0, 7, 14 and 37). The full name of the samples and their abbreviations are presented in Table 1.

Table 1. Abbreviations used in the manuscript.

Name	Abbreviation
Inoculated cheese chitosan	iCHE CH
Inoculated cheese chestnut extract: chitosan	iCHE CE:CH
Inoculated cheese tannic acid: chitosan	iCHE TA:CH
Inoculated cheese polyamide: polyethylene	iCHE PA:PE
Cheese chitosan	CHE CH
Cheese chestnut extract: chitosan	CHE CE:CH
Cheese tannic acid: chitosan	CHE TA:CH
Cheese polyamide:polyethylene	CHE PA:PE
Inoculated chitosan	iCH
Inoculated chestnut extract:chitosan	iCE:CH
Inoculated tannic acid:chitosan	iTA:CH
Chitosan	CH
Chestnut extract:chitosan	CE:CH
Tannic acid:chitosan	TA:CH

The letter i represents inoculated cheese sample, and when the note is used next to the film sample, it means the film used for packing inoculated cheese. The cheese (CHE), chestnut extract (CE), tannic acid (TA), chitosan (CH) and polyamide:polyethylene (PA:PE).

2.4.1. Bacterial Inoculation

E. coli K12 and *P. fluorescens* NRRL B-253 were grown overnight at 30 °C in 2x YT medium. The medium was discarded, and the culture was resuspended in sterile saline solution. Cheese bricks were aseptically cut to a rectangular dimension of 50 mm × 25 mm × 8 mm (~22 g). Then, 100 µL of bacterial inoculum (*E. coli* 6.5 $\log_{10}$ CFU/g and *P. fluorescens* 8.3 $\log_{10}$ CFU/g) was spread onto the surface of the cheese slice and covered with biofilm or in case of control, no biofilm was used. The sachet was put into PA:PE bag and vacuum-sealed (Status d.o.o, Metlika, Slovenia) and stored at 4 °C.

2.4.2. Fungal Inoculation

P. commune NRRL 894 was grown on malt extract agar plates for 10 days to obtain spores. Spores from the plate were collected in sterile saline solution and homogenized with vortexing. Then, 100 µL (4.3 $\log_{10}$ CFU/g) of fungal inoculum was spread onto the surface of the cheese slice and covered with chitosan film, or in case of control, only a vacuum bag was used. The packet was put into PA:PE bag and vacuum-sealed (Status d.o.o, Metlika, Slovenia) and stored at 4 °C or 25 °C.

2.5. Microbiological Analysis

On days 0, 7, 14, and 37, each cheese sample was opened aseptically and cut in half. One half of the sample (approximately 10 g) was mixed with 90 mL of sterile saline solution and homogenized with a stomacher (Lawson Scientific, Ningbo, China). The suspensions were appropriately diluted in sterile saline solution and plated on selective medium. The 2x YT medium with incubation at 37 °C for 24 h was used for *E. coli* count, Pseudomonas selective agar and incubation at 30 °C for 48 h was used for *P. fluorescens* count, and malt extract agar with incubation at 25 °C for 5 days was used for *P. commune* enumeration.

2.6. Chemical and Physical Analysis

2.6.1. UHPLC

Analysis of the liquid samples was performed by using ultra-high-performance liquid chromatography—UHPLC (Thermo-Fisher Scientific UltiMate™ 3000, Waltham, MA USA)—equipped with a 5.0 µm; 4.6 × 150 mm Hypersil GOLDTM Amino column (ThermoFisher Scientific, Waltham, MA USA), heated to 30 °C, and equipped with a DAD detector. Cyclopiazonic acid was identified by retention time and UV-Vis spectra comparison to reference standards using UV–VIS spectra between

282–283 nm. The compound was quantified by external calibration standards. The mobile phase with a flow rate of 0.6 mL/min consisted of a 20:80 aqueous phase (50 mM ammonium acetate buffer solution with pH 5) and organic phase (acetonitrile), respectively. Then, 20 μL of the sample was injected, and a stationary method of a single mobile phase was applied. All the peaks shown in the chromatogram (Supplementary Materials) were identified and quantified. The retention time of cyclopiazonic acid was 3.68 min.

For the determination of the CPA concentrations in the samples, a calibration curve with seven dilution levels was prepared (concentrations range in between 50 ng/mL–0.025 mg/mL). The linear calibration curve was created by plotting the ratio of the peak area of CPA versus the CPA concentrations in the standards. To reduce the measurement errors, the standard series was measured at least in duplicate.

The chitosan-based film sample preparation for UHPLC took place accordingly: the sample (2 × 2 cm) was placed next to a wall of a 1 mL plastic vial, covered with 1.5 mL of methanol, and the received solution was shaken on a thermoshaker TS-100C (Biosan, Riga, Latvia) for 1 h. Afterward, the film was removed from the methanol, and the excess solvent was evaporated under a stream of N_2. Received solid matter was restored with 1.5 mL of phosphate buffer (5 mM, pH 2.8). After 10 min of the diffusion process, the solvent sample was subjected to UHPLC. Spiking of film samples (400 μL) with 100 μL of CPA standard solution (0.1 mg/mL) was carried out to ensure the concentration to be within the concentration range used in the calibration curve. The Gouda cheese sample preparation took place according to Zambonin et al. with a slight modification [32] as follows: the cheese sample (0.5 g) was previously cut into small pieces with a knife and weighted into a vial, 1.5 mL of methanol was added and sonicated for 10 min. Reactive methanol was separated from the cheese sample by filtering through the PTFE-20/13 UHPLC filter (Sartorius, Goettingen, Germany) and then evaporated under a stream of N_2 at ambient conditions (25 °C). Received solid matter was restored with 1.5 mL of phosphate buffer (5 mM, pH 2.8) and then subjected to UHPLC. Recovery calculations were done by spiking cheese samples (400 μL) with 100 μL of CPA standard solution (0.1 mg/mL).

2.6.2. Moisture Content

The moisture analyzer HE 53 (Mettler Toledo, Wien, Austria) at room temperature was used to measure the moisture content (MC) of cheese and films. Cheese samples were analyzed by receiving a cheese piece from cold storage, cutting it into smaller particles, and immediately placing it onto a measuring plate for analysis. The film samples were handled similarly, using scissors for cutting.

2.6.3. pH Value

The pH value of the cheese and film samples were measured at room temperature using a benchtop 781 pH/ion meter (Metrohm AG, Ionenstrasse, Switzerland). The cheese samples were aseptically homogenized in sterile saline solution with a stomacher (Lawson Scientific, Ningbo, China) and analyzed directly. The film pH values were received by measuring the pH of water solvent. For the exact results, the film samples (2 × 2 cm) were immersed into water for two hours and then removed from the water to finalize film activity in the solvent.

2.6.4. Mechanical Properties

Mechanical characterization of chitosan-based films was performed by following the guidelines from the American Society for Testing and Materials (ASTM) D 882 standard method [33]. Rectangular film samples (8 cm × 2 cm) were tested on the Multitest 2.5-i universal testing machine (Mecmesin, Slinfold, UK) equipped with a 100 N load cell, at a crosshead speed of 5mm min^{-1}. Tensile strength (TS) was calculated by dividing the load with the average original cross-sectional area in the gage length segment (6 cm) of the sample, while elongation at break (EB) was calculated as the ratio between increased length after breakage and the initial gage length.

2.6.5. Active Properties

The total phenolic content (TPC) of active sachets (~5 mg) was determined by Folin-Ciocalteu's (FC) phenol reagent according to the protocol outlined in our previous study [34]. Briefly, the small rectangular samples were added into water, followed by the successive addition of FC phenol reagent and aqueous solution of Na_2CO_3 (10% *w*/*v*) in the amount of 10 vol% and 20 vol% based on the volume of water, respectively. After the incubation of samples (2 h in dark, 24 °C), the absorbance was measured at 765 nm using the Synergy TM 2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Gallic acid was used as the standard, and the results were expressed as the mass of gallic acid equivalent (GAE) per mass of the films.

2.7. Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA) with a confidence level of 95% ($p \leq 0.05$). All the results in triplicate are expressed as the mean ± standard deviation.

3. Results and Discussion

3.1. Characterization of the Chitosan-Based Films

3.1.1. Mechanical Properties

The mechanical properties of the packaging material used in this study were measured prior to their use as follows: TS (CH) = 6.7 MPa, TS (TA:CH) = 15.0 MPa, TS (CE:CH) = 15.6 MPa, TS (PA:PE) = 27.5 MPa, and EB (CH) = 75.9%, EB (TA:CH) = 28.5%, EB (CE:CH) = 22.9%, EB (PA:PE) = 40.0% (Figure S1). This correlates with what has been shown previously in a comprehensive modeling study by Bajić et al. [14]. Overall statistical difference according to the analysis of variance showed that the films TS property can be considered as different ($p < 0.05$) when active components are added into films, wherein TA and CE showed similar results. In regard to EB, all the films were considered different ($p < 0.05$). Thus, the TS and EB of the biopolymer films were deemed significantly lower ($p < 0.05$) than conventional PA:PE packaging. The values of the tensile strength (TS) and elongation at break (EB) should be adequate when acceptable integrity of good packaging material is requested. The materials produced show strong integrity, considering that heavy food, e.g., fresh pasta, has been packed into similar material and the material withstood the load during the 2 month shelf life [34].

3.1.2. Activity

Along with mechanical properties, the activity of the films was determined and expressed through the total phenolic content (TPC) value. Films used for packing Gouda cheese in this study received TPC values of CH = 0.5 $mg_{GAE}\ g_{film}^{-1}$, TA:CH = 3.2 $mg_{GAE}\ g_{film}^{-1}$, CE:CH = 17.2 $mg_{GAE}\ g_{film}^{-1}$ (Figure S2). Almost six-fold higher ($p < 0.05$) activity by CE was seen and thus indicates the pure compound's (TA) lack of efficiency as an active component. The higher activity of the CE in the film was expected due to its abundant, diverse phenolic content. Its adverse effect has shown to be even higher than of similar extracts such as oak (9.0 $mg_{GAE}\ g_{film}^{-1}$) and hop (12.7 $mg_{GAE}\ g_{film}^{-1}$) [31,35].

3.2. Gouda Cheese Spoilage Microbiota Reduction with the CE:CH Film

3.2.1. Bacteria Reduction with Chitosan-Based Films

The cheese was inoculated with approximately 6.5 $\log_{10}$ CFU/g of *E. coli* and wrapped in different types of chitosan films, as seen in Figure 1a. The *E. coli* count on inoculated cheese (iCHE PA:PE), stored at 4 °C, remained throughout the 37 days incubation period at the same value (±0.6 $\log_{10}$ CFU/g) as it was at the beginning of the experiment. Between the different tested chitosan films, the *E. coli* count dropped most dramatically with the inoculated chitosan (iCH) film ($p < 0.05$). Accordingly, without the additives (CE or TA), the reduction in *E. coli* count was approximately 2 $\log_{10}$ CFU/g. The primary

reduction in bacterial count in these samples happened during the first 14 days, while from 14 to 37 days, the *E. coli* count stagnated. In the cheese samples wrapped with inoculated chestnut extract:chitosan (iCE:CH) and inoculated tannic acid:chitosan (iTA:CH) films, the reduction in *E. coli* count was 1 $\log_{10}$ CFU/g, and it happened in the first 7 days of the cheese storage.

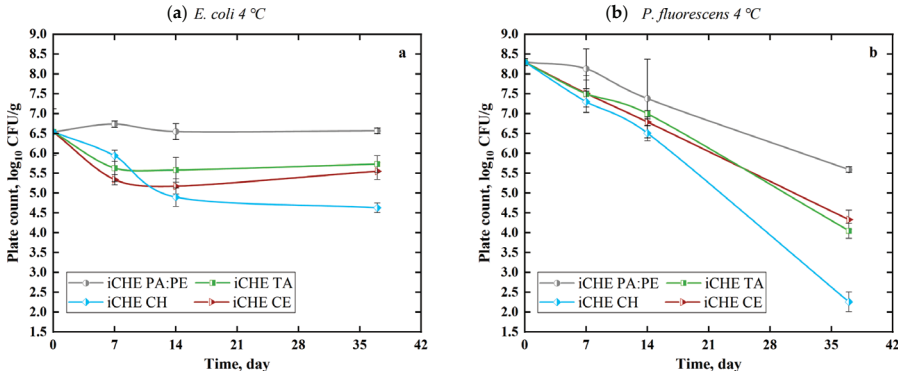


Figure 1. The effect of biopolymer films (CH, TA:CH, CE:CH) on bacterial count on cheese during storage at 4 °C inoculated with: (**a**) *E. coli* and (**b**) *P. fluorescens*. Values are means ($n = 2 \times 3$) with standard errors that are significantly different within columns ($p < 0.05$; Table S1). tannic acid (TA), chitosan (CH), chestnut extract (CE), polyamide (PA), polyethylene (PE), inoculated cheese (iCHE).

According to the evidence of Blaiotta et al. [36], Gram-positive lactic acid bacteria (LAB) cell protection could be retrieved when immobilized in CE fiber in an acidic environment. Considering that *E. coli* is Gram-negative, and positively charged chitosan-based materials disrupt the cell membrane [37], it could be considered that CE fiber has the protective effect on the intact cells. Although, it is believed that the effect is not merely related to the fiber. The CE is demonstrated to contain reducing sugars, provides adenosine triphosphate (ATP), and improves the bacteria's survival [36]. Accordingly, TA:CH films have a similar effect to CE:CH films (Figure 1a), disputing the sugars or fiber effect and indicating the effect of hydrolyzable tannins, and indicating there were destructive effects on the spoilage bacteria, *E. coli*, but only to some extent (until day seven). The lysing result of the neat iCH film stands out in terms of less complexity and its ability to form hydrogen bonds with a higher amount of active cites [37].

The second spoilage bacteria, *P. fluorescens*, was inoculated on cheese in approximately 8.2 $\log_{10}$ CFU/g (Figure 1b). The cheese was stored at 4 °C. Conformably, the drop of *P. fluorescens* count was most dramatic with the iCH film, where the count dropped for 6 $\log_{10}$ CFU/g, and samples with the iCE:CH and iTA:CH films showed bacteria count reduction for approximately 4 $\log_{10}$ CFU/g ($p < 0.05$). Non-inoculated cheese samples were also included in the analysis to exclude the contamination (not shown on graph) and the samples did not show any *E. coli* or *Pseudomonas* spp. presence. Prior the experiment initiation, the count of *P. fluorescens* at inoculation was slightly higher than the *E. coli* count, but it seems that the latter bacteria species is more susceptible to long term storage at low temperatures or the LAB, naturally present in the cheese, was a habitual defense system. The count of *P. fluorescens* dropped even in the samples that were not wrapped in biopolymer films. Herein, one of the main differences between the two bacteria is in their survival conditions. Specifically, *P. fluorescens* is unable to grow under anaerobic conditions, hence the drop. *E. coli*, on the other hand, withstands anaerobic conditions to some extent.

3.2.2. Fungi Reduction with Chitosan-Based Films

For cheese inoculation with fungi, approximately 4 $\log_{10}$ CFU/g of *P. commune* spores were used and incubated at two different temperatures. At refrigeration conditions (4 °C), the drop of *P. commune*

count was minimal (less than 1 $\log_{10}$ CFU/g) in all the samples regardless of packaging (Figure 2a), which confirms the good survival of the mold spores over time at low temperatures. This shows that the *P. commune* spores are susceptible to components of the chitosan films, temperature conditions, and naturally present LAB only until day 14.

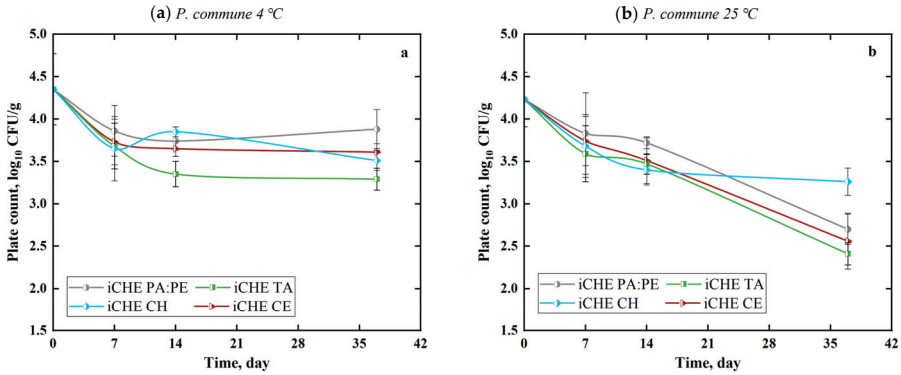


Figure 2. The effect of biopolymer films (CH, TA:CH, CE:CH) on *P. commune* count on cheese during storage at: (**a**) 4 °C and (**b**) 25 °C. Values are means ($n = 2 \times 2$) with standard errors that are significantly different within columns ($p < 0.05$; Table S1).

On the other hand, the room temperature conditions (25 °C) affected *P. commune* survival on the cheese samples over time (Figure 2b). After 37 days, the count of *P. commune* had dropped for approximately 2 $\log_{10}$ CFU/g on iCHE TA:CH, iCHE CE:CH and iCHE PA:PE samples, while on iCHE CH samples, the *P. commune* count had dropped for only 1 $\log_{10}$ CFU/g ($p < 0.05$). Contrary to our study, the study by Duan et al. [28] revealed that the mold count increased (highest at 5.12 $\log_{10}$ CFU/g) during 30 day incubation at 10 °C in control (untreated) samples of the cheese, while the count reduction (highest at 1.90 $\log_{10}$ CFU/g) was seen when the mozzarella cheese was wrapped in chitosan-based films or coatings. Different species of mold and cheese were used in the study. Evidently, the higher temperature contributes to the film's active components diffusion process, which has also been shown by Ouattara et al. [38]. Additionally, in a study by Esposito et al. [23], CE, with its polyphenols, was shown to have an inhibiting effect on fungus, both in mycelial and spore form. We also expected that the mold count would increase on cheese wrapped in iCHE PA:PE at room temperature conditions, which are ideal for mold growth. On the contrary, the *P. commune* count dropped during the 37 days long incubation period. Being strictly aerobic mold, most likely, the reason for the reduction is the activity of the LAB. The study of Cheong et al. [39] confirms our theory, where the antifungal effect of LAB was shown on several mold species, *P. commune* being one of them.

3.2.3. Influence of Chitosan-Based Films on Mycotoxin CPA from Cheese

The cheese was inoculated with *P. commune* and repacked into chitosan-based antimicrobial films to retain induced mold growth and secondary metabolite cyclopiazonic acid (CPA) formation. The presence of tannins in the packaging material at a higher temperature (25 °C) hindered the CPA production in cheese samples and had an endorsing effect while held at low temperature (4 °C).

Based on the analysis of variance, which was employed on spiked and non-spiked sample results prior to the subtraction to observe the difference in CPA production, the sample groups were identified as different ($p < 0.05$) while stored in two temperatures (Table S2).

As seen in Figure 3b, mycotoxin CPA concentration in cheese samples at 25 °C significantly decreased from 6400 (day seven) to 533 µg/kg (day 37) when packed in iCE:CH film, and from 3600 to 1300 µg/kg in iTA:CH film. Furthermore, the values presented are the subtraction of inoculated cheese

with the *P.commune* and non-inoculated samples, which gave the direct comparison of the CE and TA effect on growth, eliminating the need for the partitioning between phases.

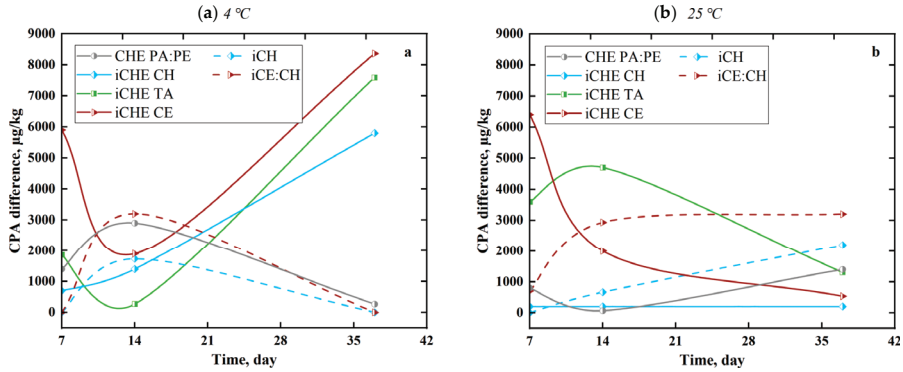


Figure 3. The effect of biopolymer films (CH, TA:CH, CE:CH) on cyclopiazonic acid (CPA) production in cheese (0.5 g), compared with mycotoxin accumulation in films (2 × 2 cm) at: (**a**) 4 °C and (**b**) 25 °C. Dashed lines represent packaging material results. Values are gained by subtraction of spiked non-inoculated sample mean from the spiked inoculated sample mean values, which are significantly different within rows ($p < 0.05$; Table S2).

The cheese packed with iCH film remained constant (200 µg/kg), and the CHE PA:PE sample (without protective film) showed an increase (800 to 1400 µg/kg) in CPA concentration. Likewise, packaging materials themselves depicted opposite outcomes pointing to interaction with CPA and thus possible protective features in regard to food safety (Figure 3).

The mycotoxin concentrations measured from films were low but kept appearing. In the presence of the CE in the film, CPA concentration increased up to 3200 µg/kg (day 37) while with TA, the used UHPLC method enabled the identification of any CPA due to shadowing complex formation with TA (Figure S3). Accordingly, the film without any additives (CH) tended to continue accumulating the mycotoxin, having a CPA concentration of 2200 µg/kg on day 37.

The CPA concentration levels measured in refrigerated cheese (4 °C) elevated from 5900 (day seven) to 8367 µg/kg (day 37), 1867 to 7600 µg/kg, 700 to 5800 µg/kg, and demoted from 1400 to 267 µg/kg when packed in CE:CH, TA:CH, CH and PA:PE packaging, respectively (Figure 3a).

Higher CPA results measured from cheese could be considered as a consequence of tannin chemistry that allows conjugation with CPA (a tetradic indole acid/N-compound) to form stable linkages between carboxylic groups and amines, similarly to the protein–tannin complex reaction [40]. Additionally, the high temperature is advantageous for tannins chemical reactivity [25] and optimal for lactic acid bacteria (LAB) from cheese to be dominant in the microbial competition [41]. Accumulation in CH film at 25 °C may be attributed to the chemical properties of chitosan, possessing more free hydroxyl radicals [42] to continually interact with (Figure 3b). Undetectable CPA in the iTA:CH films is most likely the result of the higher chemical reactivity of tannic acid, but only until a certain limit (being an unvaried chemical) compared to the iCE:CH film, which contains a source of various, more abundant tannin-rich chestnut extract [43]. Furthermore, chitosan-based materials have been observed to have a strong absorption mechanism for various chemical components [44,45], macromolecules [46,47], and also for mycotoxins [48,49], which is used as a safety precaution to conjugate the unwanted particles. This explanation appears to correlate with the specific film activity of this study in terms of mycotoxin. Rapid CPA forming in cheese samples (at 4 °C) is an irrefutable indication of the film's low activity after packaging, seemingly related to mechanical properties. Cooling storage has a uniforming effect on biopolymers allocation and molecular configuration in materials [50,51]. This is confirmed by the results of inexistent CPA concentration within films on days zero to seven and 37. In time,

the chitosan-based chestnut extract film reaches its moisture equilibrium [34], depending on the food product that it is in contact with.

Furthermore, the films potentially act as an absorber in low-temperature conditions, as on day 14 the iCE:CH and iCH film samples depicted higher CPA presence—most certainly influenced by moisture mobility.

Lately, only a few attempts to quantify CPA in cheese have been reported [40]. Comparatively, in white mold cheese, CPA was reported within a range of 1.83–3610 µg/kg measured by HPLC-MS/MS [50,52], and in inoculated cheddar cheese under modified atmosphere ($CO_2 + O_2$) within range of 4–280 µg/kg measured by HPLC [53]. To our knowledge, there are no reports covering CPA quantification in biofilm matrices that have been in contact with food. Although, theoretical multiple mycotoxin absorption by cross-linked chitosan polymer has been reported to be 5.67 g/kg [48]. The results of inoculated gouda cheese (<8367 µg/kg) and chitosan-based films (<3200 µg/kg 2×2 cm^2) remain in the same range reported by Zhao et al. [48] and therefore report non-toxicity in protective films. This is concluded based on the 50% (LD50) lethal dose in rats by oral ingestion (36,000 µg/kg) [54].

3.3. pH Value

The overall pH deviation between stored Gouda cheese and acidic chitosan-based films was assessed at every time point to determine the flux direction of acidity known to influence microbial growth. In comparison to the CHE PA:PE/iCHE PA:PE samples, a significant conversion of cheese to become more acidic, while the films altered towards a neutral pH, was observed. All the cheese packed in different chitosan films, conditioned at 25 °C, had a pH decrease from 5.6 to 5.2. The pH of cheese from the iCH film started to decrease right away compared to the results of cheese packed in the iCE:CH and iTA:CH films (Figure 4b,c). The incorporation of CE into chitosan-based film showed not to have an additional pH lowering effect on the cheese compared to the TA effect ($p = 0.05$). A delayed change of pH was determined in the latter packaging's until day 14 when it started to stabilize. In regard to the iCHE PA:PE cheese samples, a wave-like pH change was seen with a sharp pH increase from 5.6 to 5.8, down to 5.7 by day 14 and up again to 5.9 on day 37.

The cheese pH changed slightly differently when stored at 4 °C. While packed in the TA:CH/iTA:CH film, the pH values always stayed above the values of other biofilm-packed cheese, and did not go lower than pH 5.3, regardless of the spoilage microbes ($p < 0.05$). The influence of the spoilage microbes on the cheese pH could be observed. To be more exact, without spoilage bacteria, it took 7 days for the pH of cheese in the TA:CH film to drop (Figure 4a). With Gram-negative bacteria, a pH drop started to happen after 7 and 14 days, respectively, with *E. coli* and *P. fluorescens* (Figure 4d,e). *P. commune* influenced pH decrease uniformly in each biopackaging throughout the time points (Figure 4f).The iCHE PA:PE sample results were diverse with a higher temperature, although after a similar pH jump, they were strongest with *P. fluorescens* presence up to pH 6.0, and the iCHE PA:PE samples reached a plateau in pH value from day 14.

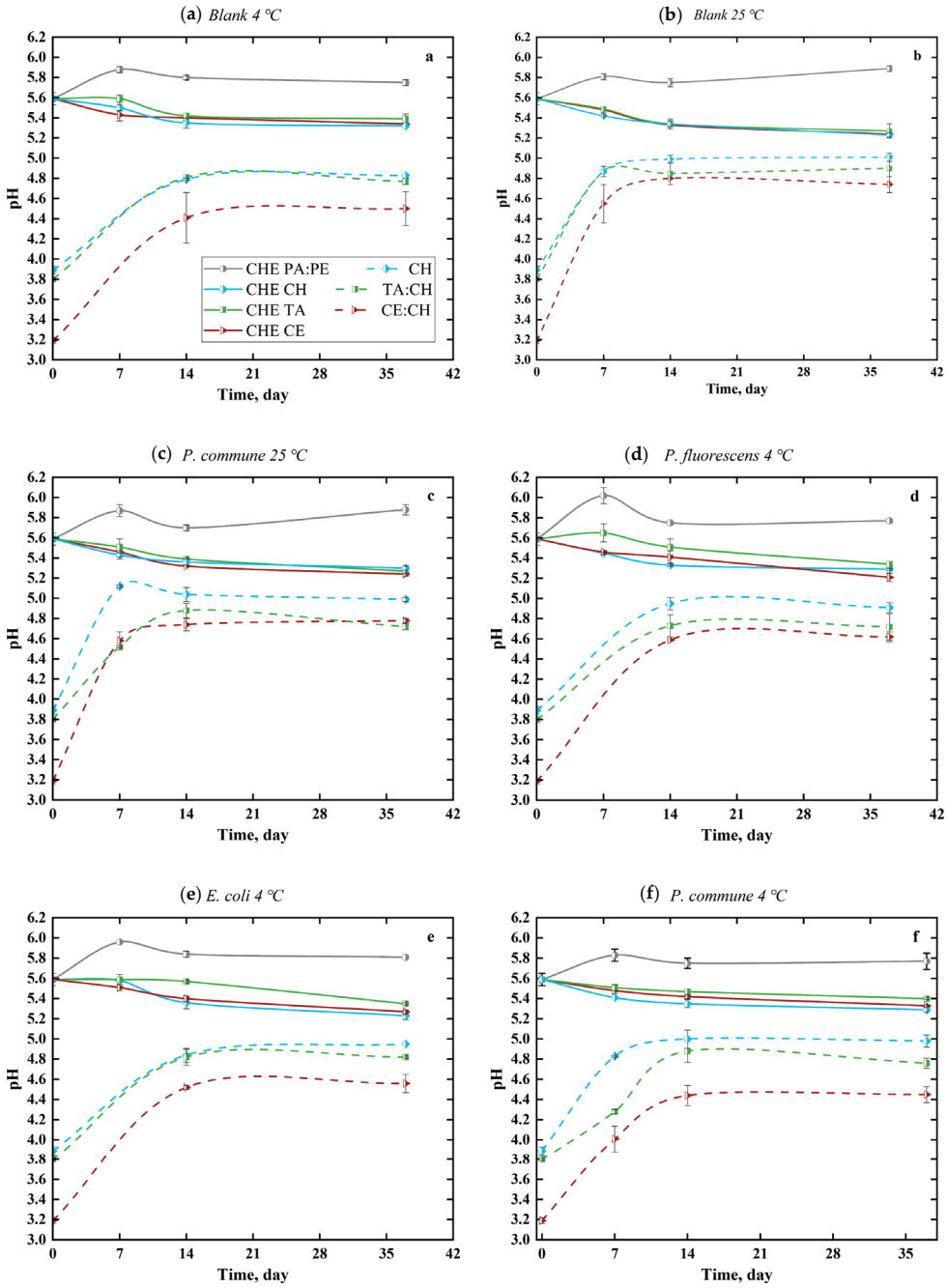


Figure 4. Effect of biopolymer films (CH, TA:CH, CE:CH) on pH in cheese, compared to simultaneous pH change in films (2 × 2 cm) at 4 °C in (**a**) Blank (non-inoculated), (**d**) *P. fluorescens* (**e**) *E. coli*, (**f**) *P. commune* and at 25 °C in (**b**) Blank (non-inoculated), (**c**) *P. commune* sample sets. Dashed lines represent packaging material results. Values are means (n = 2 × 1) with standard errors that are significantly different within columns ($p < 0.05$; Table S3).

The pH trend in films (25 °C) took a similar course in every sample set, with a difference in the films containing tannin-additives, being both lower and identical in values (acidic) ($p < 0.05$) than the CH film. The pH of the CH film rose to 5.0 when it stabilized. In the TA:CH and CE:CH films, the pH reached a plateau at 4.8 on average, which was similar in the presence of P. commune (Figure 4b,c). Simultaneously, lower temperature (4 °C) seemed to prevent pH elevation and kept it at the levels of pH 4.4 (blank, *P. fluorescens*, *E. coli*) and 4.6 (*P. commune*) in the CE:CH films. The preventing effect did not apply to the TA:CH film and the pH was recognized as statistically different from the other tannin-rich films ($p < 0.05$).

Based on the CHE PA:PE/iCHE PA:PE results, moisture mobility influence could be considered, and the samples could experience the so-called buffering effect. This reflects matrix degradation, which leads to free water mobility with a result of elevated pH values. In our previous work, we have seen that active components diffusion in between the food matrix and active CH:CE film reaches an equilibrium and thus explains the plateau after day 14. The observation correlates to the studies of other authors, confirming that the chitosan application has the pH altering effect in a different direction and only to a small extent. For example, when the cheese is packed with gelatin:chitosan film, the pH changes from 4.6 to 4.4 [55]. The pH is reported to increase from 5.3 to 5.9 while incorporated in cheese mixture and packed in alginate coating plus a modified atmosphere environment [56]. Saloio cheese's pH ranged from 4.72 to 5.12 (day 37) when coated with chitosan-based natamycin edible film [57].

The changes in the pH of films are mostly attributed to the electrostatic and hydrophobic interactions between chitosan (cationic) and lipids (anionic) [18,58]. Furthermore, it can be presumed that the warmer environment affects the chitosan network for higher diffusion of active components, while lower temperature keeps the integrity of the material network. Additionally, CE contains fiber, which contributes to the material integrity and thus to a lower diffusion.

3.4. Moisture Mobility

The moisture mobility between chitosan-based films and stored Gouda cheese was evaluated for its possible impact on the growth of pathogenic microbes on cheese. Analysis using an automated moisture analyzer was applied to samples initially and at the end of the experiment. Expectantly, the films acted as moisture absorbers, while cheese yielded moisture. The MC in cheese samples, contaminated with two different Gram-negative bacteria, in an anaerobic surrounding at 4 °C, depicts no distinct species that influence associated differences. Temperature's influence on fungi inoculated cheese, packed in different packaging, stands out with similarity when conditioned at 4 °C and diversity at 25 °C. The CH film without additives performs superior absorbing properties compared to the cheese, while the CE:CH and the TA:CH films, receive results with uniform outcomes, most potentially due to high degradation and intact matrices, respectively (Table 2).

Table 2. The effect of biopolymer films (CH, TA:CH, CE:CH) on cheese's moisture content (MC) compared to moisture accumulation in films (both in % per 0.5 g). Values are means ($n = 2 \times 2$) with standard errors, which within a row are significantly different ($p < 0.05$).

Timepoint	**Day 0**	**Day 37**					
Sample		**Blank 4 °C**	**Blank 25 °C**	***P. fluores.* 4 °C**	***E. coli* 4 °C**	***P. com.* 4 °C**	***P. com.* 25 °C**
Cheese Blank	33.5 ± 1.8	29.5 ± 0.5	31.7 ± 1.7	27.9 ± 1.0	27.9 ± 0.5	30.8 ± 1.2	29.8 ± 2.0
Cheese CH		17.8 ± 3.2	22.0 ± 1.5	19.3 ± 1.6	18.0 ± 1.7	23.5 ± 1.5	19.4 ± 1.5
Cheese TA		24.8 ± 2.1	26.9 ± 2.1	23.1 ± 1.8	24.8 ± 1.0	26.0 ± 1.4	21.9 ± 0.8
Cheese CE		26.0 ± 2.4	24.2 ± 1.6	24.7 ± 1.4	23.0 ± 2.4	28.4 ± 2.1	27.3 ± 1.3
CH	4.0 ± 0.4	62.6 ± 1.7	61.0 ± 1.0	65.0 ± 1.0	61.6 ± 1.6	63.2 ± 3.4	61.2 ± 1.2
TA:CH	2.7 ± 0.4	49.8 ± 1.9	51.8 ± 1.6	52.9 ± 2.3	51.9 ± 2.0	48.7 ± 3.2	51.1 ± 2.1
CE:CH	3.2 ± 0.2	52.0 ± 2.6	52.4 ± 3.1	49.2 ± 3.1	54.2 ± 1.2	50.1 ± 3.3	55.3 ± 2.4

All the samples were packed in additional conventional PA:PE packaging to avoid environmental effects on the MC. However, the CHE PA:PE cheese MC results refer to temperature affected disparities

and diversity aspects of biological material after repacking. A slightly lower MC was measured compared to the starting point MC of 33.5 ± 2.0%. Moisture contents on day 37 were the lowest in the CH/iCH packed cheese samples (20.1 ± 2.2%), with results being on average 24.6 ± 1.8% higher in the TA:CH/iTA:CH packed cheese and even higher in the CE:CH/iCE:CH packed cheese (25.6 ± 2.0%) ($p < 0.05$). Likewise, by the same time, the CH/iCH films gained moisture on average up to 62.4 ± 1.5%, the TA:CH/iTA:CH films 51.0 ± 1.6%, and the CE:CH/iCE:CH films 52.2 ± 2.3% (day 37) showing matrix binding similarities in additive-films. Enhanced moisture absorption is most likely attributed to (i) CH: protein interactions and thus lowering the cheese's protective lipid layer [18], (ii) CE being a starch (good free water binder) incorporator into chitosan-based films [24], and (iii) TA, via its cross-linking mechanism, being able to strengthen the film matrix to be resilient to moisture absorption [59]. Controlling moisture during cheese processing has a technical connotation for final cheese quality [3], and these results show the benefits of CE in regard to moisture absorption, performing absorption to a lower extent and preserving cheese moisture levels time-wise.

3.5. Effect of the Film on a Food Safety

In addition to the mechanical integrity, it is mandatory to determine the safety of a new material when it is applied on food. All the ingredients used in films were of natural origin and declared as food safe [30]. A comprehensive study by Hu and Gänzle [60] shows chitosan's bactericidal effect towards several pathogenic microbes on artificially contaminated intermediate moisture foods, and states that the lethality is limited up to 5 $\log_{10}$ CFU/g. Films incorporated with CE in this study are able to inhibit induced spoilage up to 4 $\log_{10}$ CFU/g, indicating the efficiency to ensure food safety. One of the goals of this study was get more insight into the fungi development and its mycotoxin production. With an interesting outcome and a positive outcome in regard to food safety, the mycotoxin is absorbed into the film's matrix, but does not migrate back to the food surface when the storing temperature is 25 °C (Figure 3). This outcome broadens the storage possibilities surrounding temperature range; however, it should be emphasized that this was not the case at 4 °C and further studies should be conducted for the nethermost temperature. Furthermore, food safety is in balance when several food processes (lipolysis, acidity change) are induced. According to our results of moisture content and pH change that correlates to acidity, the films could be considered as promoting food safety, as the changes were minimal.

4. Conclusions

Based on the results, it can be concluded that chitosan film enriched with chestnut extract reduces extreme bacterial (up to 6 $\log_{10}$ CFU/g) and fungal (up to 4 $\log_{10}$ CFU/g) contamination more actively at 25 °C than at 4 °C. The primary decrease in contaminators happened at 14 days and towards *P. fluorescens* most efficiently. The results indicate that the addition of commercial chestnut extract has the equivalent effect of chemical grade tannic acid and chitosan film solely lacks protection—first because of the high degradation. The chestnut extract enriched chitosan film seems to operate as an absorbent of mycotoxin cyclopiazonic acid at 25 °C while lowering the toxicity level in cheese. Although, even in airtight conditions, the cheese yielded moisture to the chestnut extract chitosan film throughout all the samples, on average up to 8 ± 2% of its initial moisture content. This alters the authentic Gouda cheese color to a dark brown (Figures S4 and S5). Consequently, packing a high lipid food product—Gouda cheese—into chestnut extract enriched chitosan film could be a good strategy as it influences its pH for only 0.2 units and ensures food safety with active compounds, certainly even longer than 37 days. For further studies, the selected antioxidant and antimicrobial biomarkers extracted from the CE can be used to provide cheese with no visual effects. Additionally, the components chosen for in vivo protection of packed food are entirely safe for use in the food.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/11/1645/s1, Supplementary Materials: Supplementary data; Table S1: The effect of biopolymer film (CH, TA:CH, CE:CH) on bacterial counts ($\log_{10}$ CFU/g) on cheese during storage at 4 °C and 25 °C when inoculated with E. coli, P.

fluorescens and P. commune, Table S2: The effect of biopolymer film (CH, TA:CH, CE:CH) on CPA production in cheese (0.5 g), compared with mycotoxin accumulation in films (2 × 2 cm) during storage at 4 °C and 25 °C. Slash-marked spaces depict missing parallels for CH and CH:CE samples and inability to record results for TA:CH and iTA:CH. Calculations in µg/kg are expressed per 1 g of the sample, Table S3: The effect of biopolymer film (CH, TA:CH, CE:CH) on pH in cheese, compared to simultaneous pH change in films (2 × 2 cm) at 4 °C and 25 °C, Figure S1: Mechanical properties of films used for packing Gouda cheese, Figure S2: Total phenolic content of films used for packing Gouda cheese. Gallic acid was used as a standard, and the results were expressed as the mass of gallic acid equivalent (GAE) per mass of the film, Figure S3: UHPLC chromatograms of iTA:CH (film) inoculated with P. commune (■) and 0.025 mg/L CPA standard (■). Spectra depict possible shadowing complex formation between the CPA- and TA- containing samples, Figure S4: Gouda cheese packed in CH, TA:CH and CE:CH biofilms (from left to right), Figure S5: The appearance of the cheese at the beginning (day 0) and the end of the storage (after 37 days, for all the other pictures).

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Supplementary data

Reduction in Spoilage Microbiota and Cyclopiazonic Acid Mycotoxin with Chestnut Extract Enriched Chitosan Packaging: Stability of Inoculated Gouda Cheese

Kristi Kõrge [1,2], Helena Šeme [3], Marijan Bajić [1], Blaž Likozar [1], Uroš Novak [1,*]

[1] Department of Catalysis and Chemical Reaction Engineering, National Institute of Chemistry, Hajdrihova 19, 1000 Ljubljana, Slovenia; kristi.korge@ki.si (K.K.); marijan.bajic@ki.si (M.B.); blaz.likozar@ki.si (B.L.)
[2] Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia
[3] Acies Bio d.o.o., Tehnološki park 21, 1000 Ljubljana, Slovenia; helena.seme@aciesbio.com
* Correspondence: uros.novak@ki.si

1. Mechanical properties of the films

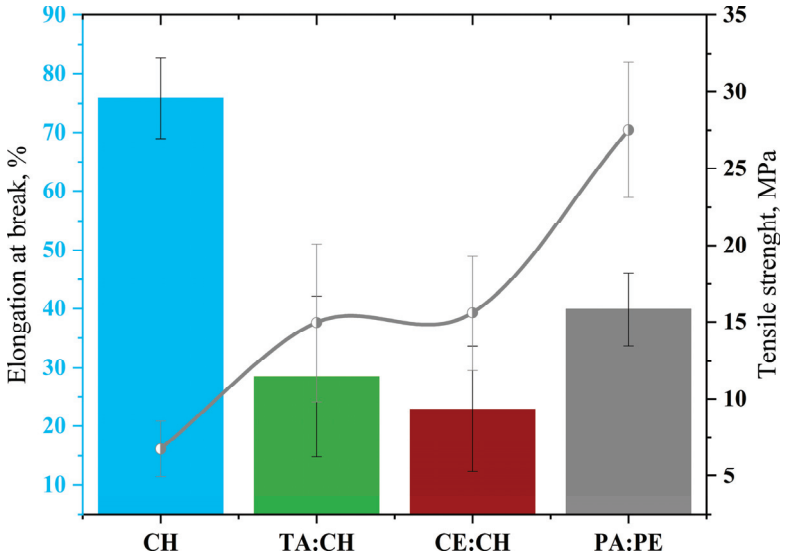

Figure S1. Mechanical properties of films used for packing Gouda cheese. Values are means (n = 4 × 3) with standard errors that are significantly different within columns ($p < 0.05$).

2. Activity of the films

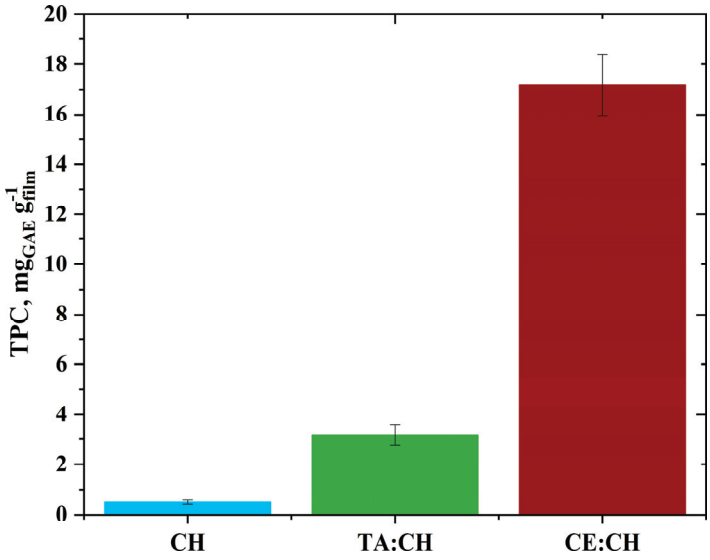

Figure S2. Total phenolic content of films used for packing Gouda cheese. Gallic acid was used as a standard, and the results were expressed as the mass of gallic acid equivalent (GAE) per mass of the film. Values are means (n = 2 × 3) with standard errors that are significantly different within columns ($p < 0.05$).

3. Bacteria and fungi reduction with chitosan-based films

Table S1. The effect of biopolymer film (CH, TA:CH, CE:CH) on CPA production in cheese (0.5 g), compared with mycotoxin accumulation in films (2 × 2 cm) during storage at 4 °C and 25 °C. Slash-marked spaces depict missing parallels for CH and CH:CE samples and unable to record results for TA:CH and iTA:CH. Calculations in μg/kg are expressed per 1 g of the sample. Values are means (n = 2 × 3) with standard errors that are significantly different within rows ($p < 0.05$).

E. coli 4 °C

Sample	0. day	7. day	14. day	37. day
iCHE CH	6.54 ± 0.59	5.94 ± 0.14	4.90 ± 0.23	4.63 ± 0.12
iCHE TA		5.63 ± 0.32	5.58 ± 0.31	5.73 ± 0.21
iCHE CE		5.34 ± 0.13	5.17 ± 0.19	5.55 ± 0.21
iCHE PA:PE		6.74 ± 0.08	6.55 ± 0.20	6.57 ± 0.07

P. fluorescens 4 °C

Sample	0. day	7. day	14. day	37. day
iCHE CH	8.30 ± 0.09	7.30 ± 0.27	6.51 ± 0.19	2.26 ± 0.25
iCHE TA		7.50 ± 0.46	7.00 ± 0.08	4.05 ± 0.19
iCHE CE		7.51 ± 0.34	6.79 ± 0.08	4.33 ± 0.24
iCHE PA:PE		8.13 ± 0.50	7.38 ± 0.99	5.59 ± 0.07

P. commune 4 °C

Sample	0. day	7. day	14. day	37. day
iCHE CH	4.35 ± 0.42	3.65 ± 0.38	3.85 ± 0.06	3.51 ± 0.12
iCHE TA		3.68 ± 0.27	3.35 ± 0.15	3.29 ± 0.13
iCHE CE		3.73 ± 0.27	3.65 ± 0.09	3.61 ± 0.10

iCHE PA:PE		3.86 ± 0.30	3.74 ± 0.11	3.88 ± 0.23

P. commune 25 °C

Sample	0. day	7. day	14. day	37. day
iCHE CH	4.23 ± 0.32	3.68 ± 0.37	3.40 ± 0.18	3.26 ± 0.16
iCHE TA		3.59 ± 0.33	3.47 ± 0.12	2.41 ± 0.27
iCHE CE		3.74 ± 0.29	3.51 ± 0.27	2.56 ± 0.33
iCHE PA:PE		3.83 ± 0.48	3.72 ± 0.07	2.70 ± 0.18

Blank 4 °C and Blank 25 °C

Sample	0. day	7. day	14. day	37. day
iCHE CH	Below detection			
iCHE TA				
I CHE CE				
iPA:PE				

4. Influence of chitosan-based films on mycotoxin CPA from cheese

Table S2. The effect of biopolymer film (CH, TA:CH, CE:CH) on CPA production in cheese (0.5 g), compared with mycotoxin accumulation in films (2 × 2 cm) during storage at 4 °C and 25 °C. Slash marked spaces mark missing parallels for CH and CH:CE samples and unable to record results for TA:CH and iTA:CH. Calculations in μg/kg are expressed per 1 g of the sample. Values are gained by subtraction of spiked noninoculated sample mean from the spiked inoculated sample mean values, that are significantly different within rows ($p < 0.05$).

Time		0. day		7. day		14. day		37. day	
Sample		mg/mL	μg/kg	mg/mL	μg/kg	mg/mL	μg/kg	mg/mL	μg/kg
iCHE PA:PE	4 °C	0.0084 ± 0.0001	33467 ± 503	0.0088 ± 0.0001	35000 ± 283	0.0152 ± 0.0004	60700 ± 1740	0.0220 ± 0.0001	87867 ± 462
CHE PA:PE				0.0084 ± 0.0001	33600 ± 566	0.0145 ± 0.0015	57800 ± 6044	0.0219 ± 0.0001	87600 ± 566
iCHE CH				0.0082 ± 0.0003	32600 ± 1058	0.0151 ± 0.0004	60200 ± 1405	0.0240 ± 0.0005	95800 ± 2104
CHE CH				0.0080 ± 0.0000	31900 ± 200	0.0147 ± 0.0005	58800 ± 2078	0.0225 ± 0.0006	90000 ± 2286
iCHE TA				0.0092 ± 0.0002	36800 ± 924	0.0164 ± 0.0011	65467 ± 4388	0.0298 ± 0.0006	119200 ± 2422
CHE TA				0.0087 ± 0.0002	34933 ± 611	0.0163 ± 0.0002	65200 ± 693	0.0279 ± 0.0012	111600 ± 4607
iCHE CE				0.0119 ± 0.0002	47600 ± 980	0.0199 ± 0.0024	79600 ± 9708	0.0436 ± 0.0035	174267 ± 14110
CHE CE				0.0104 ± 0.0003	41700 ± 1000	0.0194 ± 0.0002	77700 ± 683	0.0415 ± 0.0020	165900 ± 8002
iCHE PA:PE	25 °C	0.0084 ± 0.0001	33467 ± 503	0.0075 ± 0.0001	30133 ± 231	0.0131 ± 0.0001	52200 ± 231	0.0258 ± 0.0023	103200 ± 9304
CHE PA:PE				0.0073 ± 0.0001	29333 ± 462	0.0130 ± 0.0001	52133 ± 231	0.0255 ± 0.0001	101800 ± 283
iCHE CH				0.0076 ± 0.0000	30400 ± 0	0.0130 ± 0.0001	52000 ± 327	0.0277 ± 0.0001	110900 ± 383
CHE CH				0.0076 ± 0.0001	30200 ± 516	0.0130 ± 0.0002	51800 ± 849	0.0277 ± 0.0004	110700 ± 1612
iCHE TA				0.0092 ± 0.0004	36600 ± 1774	0.0153 ± 0.0002	61000 ± 958	0.0304 ± 0.0004	121700 ± 1400
CHE TA				0.0083 ± 0.0003	33000 ± 1200	0.0141 ± 0.0000	56300 ± 200	0.0301 ± 0.0001	120400 ± 566
iCHE CE				0.0127 ± 0.0000	50800 ± 0	0.0188 ± 0.0002	75200 ± 800	0.0367 ± 0.0014	146933 ± 5787
CHE CE				0.0111 ± 0.0009	44400 ± 3464	0.0183 ± 0.0002	73200 ± 800	0.0366 ± 0.0003	146400 ± 1131
iCH	4 °C	0.0100 ± 0.0002	39800 ± 849	0.0092 ± 0.0002	36900 ± 825	0.0125 ± 0.0001	50133 ±231	0.0262 ± 0.0007	104600 ± 2723
CH				/	/	0.0121 ± 0.0001	48400 ± 400	0.0263 ± 0.0002	105200 ± 800

iCH:TA				/	/	/	/	/	/
CH:TA				/	/	/	/	/	/
iCH:CE		0.0107 ± 0.0001	42600 ± 283	0.0153 ± 0.0011	61000 ± 4555	0.0199 ± 0.0002	79400 ± 849	0.0303 ± 0.0020	121000 ± 8017
CH:CE				/	/	0.0191 ± 0.0001	76200 ± 283	0.0244 ± 0.0007	133700 ± 2740
iCH	25 °C	0.0100 ± 0.0002	39800 ± 849	0.0084 ± 0.0002	33700 ± 683	0.0121 ± 0.0002	48533 ± 833	0.0231 ± 0.0001	92500 ± 503
CH				0.0087 ± 0.0002	34933 ± 611	0.0120 ± 0.0002	47867 ± 833	0.0226 ± 0.0041	90300 ± 16390
iCH:TA				/	/	/	/	/	/
CH:TA				/	/	/	/	/	/
iCH:CE		0.0107 ± 0.0001	42600 ± 283	0.0110 ± 0.0002	44100 ± 887	0.0141 ± 0.0003	56400 ± 1131	0.0250 ± 0.0029	100133 ± 11547
CH:CE				0.0109 ± 0.0006	43400 ± 2546	0.0134 ± 0.0005	53467 ± 1973	0.0242 ± 0.0022	96933 ± 8812

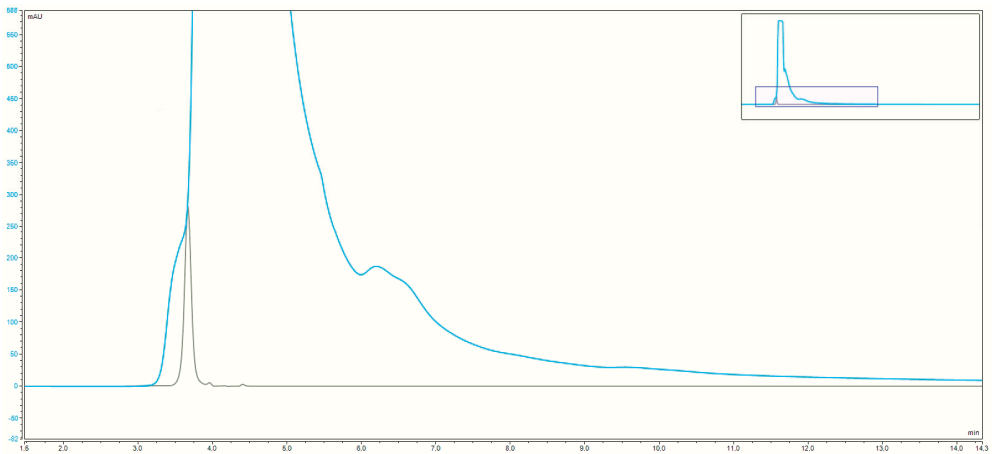

Figure S3. UHPLC chromatograms of iTA:CH (film) inoculated with *P. commune* (■) and 0.025 mg/L CPA standard (■). Spectre depicts possible shadowing complex formation between the CPA- and TA- containing samples.

5. pH value

Table S3. The effect of biopolymer film (CH, TA:CH, CE:CH) on pH in cheese, compared to simultaneous pH change in films (2 × 2 cm) at 4 °C and 25 °C. Values are means (n = 2 × 1) with standard errors that are significantly different within columns ($p < 0.05$).

a	*Blank 4 °C*						
	CHE CH	CHE TA	CHE CE	CHE PA:PE	CH	TA:CH	CE:CH
0. day	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	3.89 ± 0.04	3.80 ± 0.01	3.19 ± 0.03
7. day	5.50 ± 0.03	5.59 ± 0.04	5.43 ± 0.06	5.88 ± 0.02	/	/	/
14. day	5.35 ± 0.05	5.42 ± 0.00	5.40 ± 0.03	5.80 ± 0.02	4.79 ± 0.01	4.81 ± 0.00	4.41 ± 0.25
37. day	5.32 ± 0.00	5.39 ± 0.05	5.34 ± 0.00	5.75 ± 0.03	4.83 ± 0.00	4.77 ± 0.03	4.50 ± 0.17
b	*Blank 25 °C*						
	CHE CH	CHE TA	CHE CE	CHE PA:PE	CH	TA:CH	CE:CH
0. day	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	3.89 ± 0.04	3.80 ± 0.01	3.19 ± 0.03
7. day	5.42 ± 0.01	5.49 ± 0.00	5.48 ± 0.03	5.81 ± 0.03	4.87 ± 0.05	4.87 ± 0.01	4.55 ± 0.19
14. day	5.34 ± 0.03	5.34 ± 0.05	5.33 ± 0.01	5.75 ± 0.04	4.99 ± 0.04	4.85 ± 0.11	4.80 ± 0.00
37. day	5.23 ± 0.02	5.27 ± 0.07	5.24 ± 0.02	5.89 ± 0.03	5.01 ± 0.04	4.90 ± 0.13	4.74 ± 0.08
c	*P. commune 25 °C*						
	CHE CH	CHE TA	CHE CE	CHE PA:PE	CH	TA:CH	CE:CH
0. day	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	3.89 ± 0.04	3.80 ± 0.01	3.19 ± 0.03
7. day	5.43 ± 0.02	5.51 ± 0.01	5.46 ± 0.07	5.87 ± 0.06	5.12 ± 0.01	4.52 ± 0.00	4.58 ± 0.09
14. day	5.36 ± 0.03	5.39 ± 0.01	5.32 ± 0.00	5.70 ± 0.03	5.04 ± 0.07	4.88 ± 0.07	4.74 ± 0.06
37. day	5.30 ± 0.02	5.27 ± 0.02	5.24 ± 0.02	5.88 ± 0.05	4.99 ± 0.01	4.27 ± 0.03	4.78 ± 0.01
d	*P. fluorescens 4 °C*						
	CHE CH	CHE TA	CHE CE	CHE PA:PE	CH	TA:CH	CE:CH
0. day	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	3.89 ± 0.04	3.80 ± 0.01	3.19 ± 0.03
7. day	5.45 ± 0.01	5.65 ± 0.09	5.46 ± 0.02	6.02 ± 0.08	/	/	/

14. day	5.33 ± 0.01	5.51 ± 0.08	5.41 ± 0.06	5.75 ± 0.02	4.95 ± 0.06	4.73 ± 0.11	4.59 ± 0.01
37. day	5.29 ± 0.01	5.34 ± 0.02	5.21 ± 0.04	5.77 ± 0.02	4.91 ± 0.05	4.72 ± 0.13	4.62 ± 0.05

e *E. coli* 4 °C

	CHE CH	CHE TA	CHE CE	CHE PA:PE	CH	TA:CH	CE:CH
0. day	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	3.89 ± 0.04	3.80 ± 0.01	3.19 ± 0.03
7. day	5.58 ± 0.06	5.59 ± 0.00	5.51 ± 0.03	5.96 ± 0.26	/	/	/
14. day	5.36 ± 0.06	5.57 ± 0.01	5.40 ± 0.03	5.84 ± 0.03	4.84 ± 0.07	4.82 ± 0.08	4.52 ± 0.02
37. day	5.23 ± 0.04	5.35 ± 0.01	5.27 ± 0.02	5.81 ± 0.00	4.95 ± 0.00	4.82 ± 0.01	4.56 ± 0.09

f *P. commune* 4 °C

	CHE CH	CHE TA	CHE CE	CHE PA:PE	CH	TA:CH	CE:CH
0. day	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	5.59 ± 0.06	3.89 ± 0.04	3.80 ± 0.01	3.19 ± 0.03
7. day	5.41 ± 0.00	5.51 ± 0.03	5.48 ± 0.03	5.83 ± 0.06	4.83 ± 0.01	4.28 ± 0.02	4.01 ± 0.13
14. day	5.35 ± 0.04	5.47 ± 0.00	5.42 ± 0.02	5.75 ± 0.05	5.00 ± 0.09	4.88 ± 0.11	4.44 ± 0.10
37. day	5.29 ± 0.02	5.40 ± 0.01	5.33 ± 0.01	5.77 ± 0.08	4.98 ± 0.06	4.76 ± 0.05	4.45 ± 0.08

6. Sensorial perception of the cheese packed in chitosan-based films

Visual appearances of the Gouda cheese samples were captured to describe potential sensory perception and quality of the contaminated product when packed in chitosan film packaging (Figure 5). It is possible to see how both the packaging itself and the Gouda cheese looked like in the packaging set at the beginning of the study (Figure S4, S5).

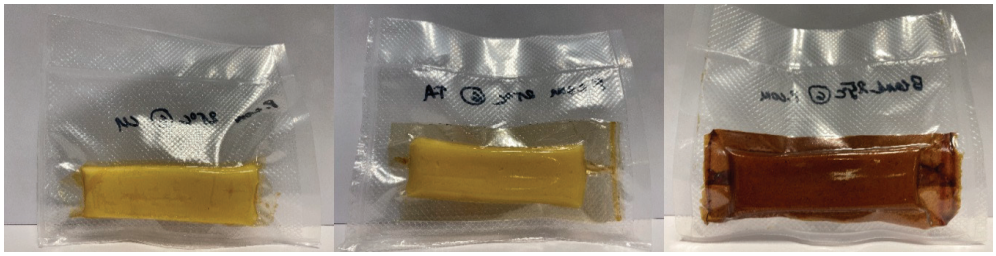

Figure S4. Gouda cheese packed in CH, TA:CH and CE:CH biofilms (from left to right).

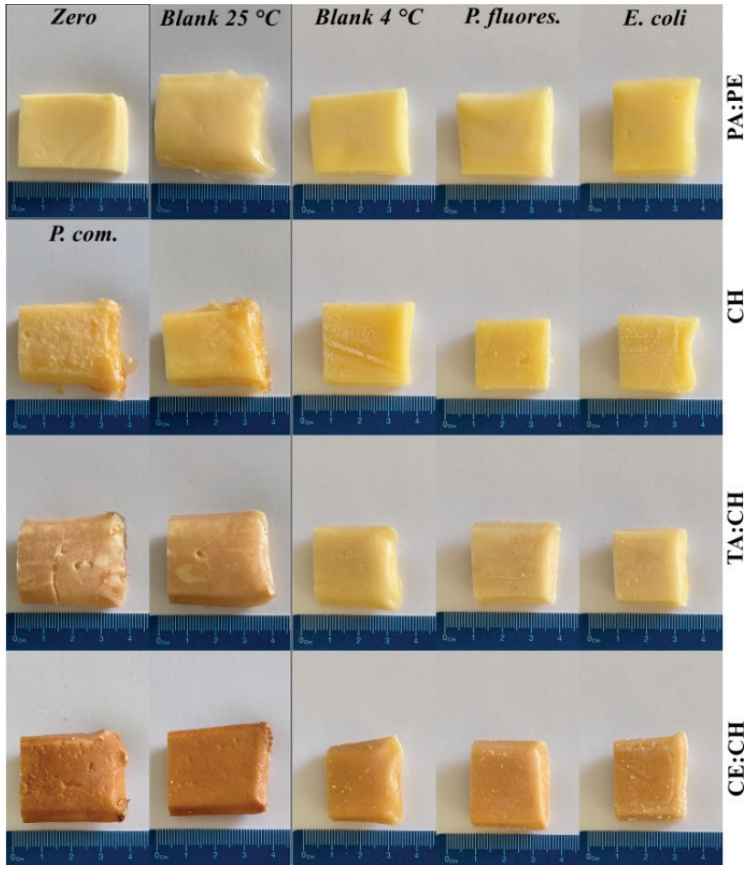

Figure S5. The appearance of the cheese at the beginning (Day 0) and the end of the storage (after 37 days, for all the other pictures).

CURRICULUM VITAE

Personal data

Name:	Kristi Kõrge
Date of birth:	15.04.1988
Place of birth:	Viljandi, Estonia
Citizenship:	Estonian

Contact data

E-mail:	kristikorge@gmail.com

Experience

2019–2020	National Institute of Chemistry (Slovenia), Application of biopolymers as food packaging
2011–2018	Center of Food and Fermentation Technologies, Frozen food quality and stability
2011–2012	Health Board, Laboratory technician

Education

2015–2021	Tallinn University of Technology, School of Engineering, Department of Materials and Environmental Technology, Doctoral degree
2010–2012	Tallinn University of Technology, School of Engineering, Faculty of Chemical and Materials Technology, Master's degree
2007–2010	Tallinn University of Technology, School of Engineering, Faculty of Chemical and Materials Technology, Bachelor's degree
1995–2007	Abja Gymnasium

Language competence

Estonian	Mother tongue
English	C1
Russian	A1

Participation in conferences

2019	Poster presentation, "Interactions of active biopolymer packaging and fresh pasta during shelf-life" conference "Slovenian Chemical Days 2019" (Maribor)
2019	Poster presentation, "Interactions of active biopolymer packaging and fresh pasta during shelf-life" conference "Cutting edge 2019" (Ljubljana)
2013	Oral presentation, "Ice re-crystallization in ice cream using ice structuring proteins from fish" conference "Foodbalt 2013" (Tallinn)
2012	Poster presentation, "Ice re-crystallization in ice cream using ice structuring proteins from fish" conference "Foodbalt 2012" (Kaunas)

Defended dissertations

2012 Kristi Kõrge, Master's Degree, (sup) Katrin Laos; Tiina Klesment, The influence of emulsifiers and ice structuring proteins on structure and crystallization of ice cream pearls, Tallinn University of Technology, Faculty of Chemical and Materials Technology, Department of Food Processing, Chair of Food Technology.

Research projects

EU50205 "Revala Ltd Innovation voucher (15.04.2016−31.07.2016)", Kristi Kõrge, Competence Center of Food and Fermentation Technologies.

EU50179 "Esko Ice creams (1.04.2016−31.07.2016)", Kristi Kõrge, Competence Center of Food and Fermentation Technologies.

3.2.0701.12–0041 "Design and application of novel levansucrase catalysts for the production of functional food ingredients (Functional Food Ingredients) (1.10.2012−31.08.2015)", Kaarel Adamberg, Center of Food and Fermentation Technologies.

EU29994 "Support for Competence Center of Food and Fermentation Technologies (1.01.2009−30.06.2015)", Raivo Vilu, Toomas Paalme, Urmas Sannik, Center of Food and Fermentation Technologies.

ETF7112 "Food Deterioration and Stability (1.01.2007−31.12.2011)", Katrin Laos, Tallinn University of Technology, Faculty of Chemical and Materials Technology.

Supervised dissertations

2015

1. Kadri Ilves, Master's Degree, (sup) Katrin Laos; Kristi Kõrge, The influence of lactose content, source of protein and temperature of preservation the crystallization of lactose in ice cream, Tallinn University of Technology, Faculty of Chemical and Materials Technology, Department of Food Processing.
2. Kaisa Karro, Master's Degree, (sup) Katrin Laos; Kristi Kõrge, Structural and taste characteristics of lactose free ice cream during preservation, Tallinn University of Technology Faculty of Chemical and Materials Technology, Department of Food Processing.
3. Kristi Hüüdma, Master's Degree, (sup) Katrin Laos; Kristi Kõrge, Valgu, Influence of protein, lactose concentration and temperature on lactose crystallization in ice-cream model solutions, Tallinn University of Technology, Faculty of Chemical and Materials Technology, Department of Food Processing.
4. Mirjam Harak, Master's Degree, (sup) Kristi Kõrge; Katrin Laos, Jelly parameters for "oneshot" technology, Tallinn University of Technology, Faculty of Chemical and Materials Technology, Department of Food Processing.

2014

5. Katerina Tšernova, Master's Degree, (sup) Katrin Laos; Kristi Kõrge, The effect of inulin as a sugar and fat substitute on ice cream, Tallinn University of Technology, Faculty of Chemical and Materials Technology, Department of Food Processing.

Publications

2020

1. Kõrge K., Bajić M., Likozar B., Novak U. Active chitosan-chestnut extract films used for packaging and storage of fresh pasta. International Journal of Food Science & Technology. DOI: 10.1111/ijfs.14569.
2. Bajić, M., Oberlintner, A., Kõrge, K., Likozar, B., Novak, U. Formulation of active food packaging by design: Linking composition of the film-forming solution to properties of the chitosan-based film by response surface methodology (RSM) modelling. International Journal of Biological Macromolecules. DOI: 10.1016/j.ijbiomac.2020.05.186.
3. Kõrge, K., Šeme, H., Bajić, M., Likozar, B., Novak, U. Reduction in Spoilage Microbiota and Cyclopiazonic Acid Mycotoxin with Chestnut Extract Enriched Chitosan Packaging: Stability of Inoculated Gouda Cheese. Foods, 9 (11), 1645. DOI: 10.3390/foods9111645.

2019

4. Kõrge, K.; Laos, K. The influence of different packaging materials and atmospheric conditions on the properties of pork rinds. Journal of Applied Packaging Research, 11 (3), 1−11.
5. Novak, U.; Bajić, M.; Kõrge, K.; Oberlintner, A.; Murn, J.; Lokar, K.; Triler, K. V.; Likozar, B. From waste/residual marine biomass to active biopolymer-based packaging film materials for food industry applications – a review. Physical Sciences Reviews. DOI: 10.1515/psr-2019-0099.

2014

6. Klesment, T.; Kõrge, K.; Sannik, U.; Laos, K. Ice re-crystallization in 10% fat ice cream using ice structuring proteins type 1 from fish. Proceedings of the 2014 International Conference on Food Properties: 1st International Conference on Food Properties (ICFP2014), Kuala Lumpur, Malaysia, 24-26 January.

2012

7. Klesment, T.; Kõrge, K.; Laos, K. Ice re-crystallization in ice cream using ice structuring proteins from fish. 7th Baltic Conference on Food Science and Technology FOODBALT-2012, Kaunas, Lithuania, May 17-18, 2012.

ELULOOKIRJELDUS

Isikuandmed

Nimi:	Kristi Kõrge
Sünnikuupäev:	15.04.1988
Sünnikoht:	Viljandi, Estonia
Kodakondsus:	Estonian

Kontaktandmed

E-mail:	kristikorge@gmail.com

Töökohad ja ametid

2019–2020	Rahvuslik Keemiainstituut (Sloveenia), Biopolümeeride rakendamine toidupakendite väljatöötamiseks
2011–2018	AS Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus, Külmutatud toidu kvaliteet ja stabiilsus
2011–2012	Terviseamet, Labori tehnik

Haridustee

2015–2021	Tallinna Tehnikaülikool, Inseneriteaduskond, Materjali- ja keskkonnatehnoloogia teaduskond, doktoriõpe
2010–2012	Tallinna Tehnikaülikool, Inseneriteaduskond, Keemia- ja materjalitehnoloogia teaduskond, Toidutehnika ja tootearendus, magistriõpe
2007–2010	Tallinna Tehnikaülikool, Inseneriteaduskond, Keemia- ja materjalitehnoloogia teaduskond, Toidutehnika ja tootearendus, bakalaureuseõpe
1995–2007	Abja Gümnaasium

Keeleoskus

Eesti keel	Emakeel
Inglise keel	C1
Vene keel	A1

Osalemine konverentsidel

2019	Posterettekanne, “Aktiivse biopolümeerpakendi mõju värske pasta säilivusele” konverentsil “Slovenian Chemical Days 2019” (Maribor).
2019	Posterettekanne, “Aktiivse biopolümeerpakendi mõju värske pasta säilivusele” konverentsil “Cutting edge 2019” (Ljubljana).
2013	Suuline ettekanne, “Jää rekristalliseerumise piduramine kasutades kalast eraldatud jääd struktureerivaid valke” konverentsil “Foodbalt 2013” (Tallinn).
2012	Posterettekanne, “Jää rekristalliseerumise piduramine kasutades kalast eraldatud jääd struktureerivaid valke” konverentsil “Foodbalt 2012” (Kaunas).

Teaduskraadid

2012 Kristi Kõrge, magistrikraad, (juh) Katrin Laos; Tiina Klesment, Emulgaatorite ja jääd struktureerivate valkude mõju jäätise pärlite struktuuri ja kristallisatsiooni vahelistele seostele, Tallinna Tehnikaülikool, Keemia ja materjalitehnoloogia teaduskond, Toiduainete instituut, Toidutehnoloogia õppetool.

Teadusprojektid

EU50205 "Revala OÜ innovatsiooniosak (15.04.2016−31.07.2016)", Kristi Kõrge, AS Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus.

EU50179 "Esko Jäätised (1.04.2016−31.07.2016)", Kristi Kõrge, AS Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus.

3.2.0701.12–0041 "Uudsete levaansukraaskatalüsaatorite disain ja kasutamine funktsionaalsete toidulisandite tootmiseks (FFI) (1.10.2012−31.08.2015)", Kaarel Adamberg, AS Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus.

EU29994 "Support for Competence Center of Food and Fermentation Technologies (1.01.2009−30.06.2015)", Raivo Vilu, Toomas Paalme, Urmas Sannik, AS Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus.

ETF7112 "Toiduainete vananemine ja stabiilsus (1.01.2007−31.12.2011)", Katrin Laos, Tallinna Tehnikaülikool, Keemia ja materjalitehnoloogia teaduskond.

Juhendatud väitekirjad

2015

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