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DEVELOPING BIO-BASED MATERIALS AND COATINGS FROM WHEY PROTEINS

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Developing bio-based materials and coatings from whey proteins

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To my greatest home "Palestine" . . .

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Table of Content

TABLE OF ABBREVIATIONS

Abbreviations	Description
DPPH	2,2-diphenyl-1-picrylhydrazyl
EB	Elongation at break
EOs	Essential oils
FFSs	Film Forming Solutions
GC-MS	Gas Chromatography-Mass Spectroscopy
GLY	Glycerol
mTG	Microbial transglutaminase
MIC	Minimal Inhibitory Concentration
MHB	Muller Hinton Broth
NPs	Nanoparticles
PNSs	Pecan nut shells
PNSE	Pecan nut shell extract
PHBHHx	Poly-3-hydroxybutyrate-co-hydroxyhexanoate
PHBV	Poly-3-hydroxybutyrate-co-hydroxyvalerate
PHAs	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PLA	Polylactic acid
RH	relative humidity
SEM	Scanning Electron Microscopy
SD	Standard deviation
TS	Tensile strength
TEO	Thymbra essential oil
TEO1	Thymbra from Local shop in Nablus city, Palestine

TEO2	Thymbra from Qabatia city mountain, Palestine
TSB	Tryptic Soy Broth
WV	Water vapor
WP	Whey Protein
YM	Young’s module

TABLE OF FIGURES

LIST OF TABLES

SUMMARY

Plastics are inexpensive, lightweight, strong, durable, corrosion-resistant materials, with high thermal and electrical insulation properties. The diversity of polymers and the versatility of their properties are used to make a vast array of products that bring medical and technological advances, energy savings and numerous other societal benefits. However, the growing production of petroleum-based plastics has incurred in disposal issues raising the concerns of plastic pollution and impact to the environment. These issues have encouraged innovation and research activities in the field of bio-plastics, offering alternatives to conventional plastics. One potential option to pursue would be to explore agri-food wastes and by-products for bio-plastic production. In this scenario the present thesis provides insights into the production and characterization of bio-plastics obtained by using the proteins contained in milk whey, the main by-product derived from cheese production. Even though recycled in different ways, too much whey is wasted in the environment and disposed off illegally. Consequently, there is a significant interest in finding new applications to avoid whey water pollution mostly due to its high content of whey proteins (WPs). In this work we investigated the possibility to use WPs to prepare bio-plastics with improved features. Different experimental conditions, such as *i)* pH modification, *ii)* heating treatment, *iii)* enzymatic treatment, *iv)* plasticizer addition, and *v)* blending with nanoparticles (NPs), were exploited in order to modify the technological features of WP-based bio-plastics prepared by casting method. Furthermore, the films showing the best characteristics were also added with bioactive molecules from Pecan (*Carya illinoinensis*) Nut Shell and Thymbra (*Satureja capitata*) leaf extracts, both to influence film technological properties and to confer antioxidant and antimicrobial activity to the produced bio-plastics. The results reported in the present thesis suggest a possible use of the obtained WP-based films functionalized with active biomolecules as new environmentally friendly candidates for food packaging, by preserving the product integrity against mechanical damage and reducing the microbial spoilage.

RIASSUNTO

Le diverse plastiche di origine petrolifera fanno parte, da anni e con una diffusione sempre maggiore, della nostra quotidianità, poiché rappresentano materiali leggeri, forti, duraturi e resistenti alla corrosione. La loro versatilità ed il loro basso costo hanno portato ad una esagerata produzione annuale che, a livello mondiale, ammonta oggi ad oltre 335 milioni di tonnellate. Si può parlare di "età della plastica" già a partire dalla metà del diciannovesimo secolo, quando fu scoperta la nitrocellulosa, materiale ottenuto mescolando cotone, acido nitrico ed acido solforico, anche se soltanto nella seconda metà del ventesimo secolo iniziò la vera e propria "*plastics revolution*" a seguito della brevettazione del PET (polietilene tereftalato), comunemente utilizzato per la realizzazione delle bottiglie di plastica. I decenni successivi hanno visto uno sviluppo progressivo dei materiali plastici, fino ad arrivare ai cosiddetti tecno-polimeri che, per le loro caratteristiche di resistenza termica e meccanica, sono risultati anche superiori a molti metalli speciali. Sebbene l'uso dei materiali plastici sia aumentato di circa 20 volte negli ultimi 50 anni, meno del 10% della plastica prodotta ogni anno viene riciclato, mentre la restante parte finisce in discariche e nei mari dando origine, nel tempo, ad innumerevoli piccoli petro-polimeri (le cosidette micro- e nano-plastiche) che alla fine vengono ingeriti dagli animali marini e dal zooplancton. All'inquinamento di oceani e suolo sta contribuendo, in maniera sempre crescente, il packaging utilizzato per preservare le proprietà nutrizionali e organolettiche degli alimenti. Spesso, però, il confezionamento alimentare risulta sproporzionato ed ingiustificato. Pertanto, diventa sempre più urgente individuare strategie differenti ed alternative capaci di ridurre il più possibile l'impatto ambientale dovuto al packaging. La ricerca scientifica, multi- e inter-disciplinare, ha tra i propri obiettivi quello di trovare una valida alternativa alle plastiche tradizionali, nonché di limitare l'emissione di CO_2, evitando l'utilizzo di risorse non rinnovabili, come i carbon fossili. Tale obiettivo può essere raggiunto applicando i principi della "bio-economia circolare" che, secondo quanto definito dalla Commissione Europea, è un'economia che promuove il riciclo ed il riutilizzo di prodotti di scarto per ottenere prodotti ad alto valore aggiunto e minimizzare la

quantità di rifiuti prodotti. Lo scopo principale di questo lavoro di tesi è stato quello di produrre nuovi materiali biodegradabili a partire da uno scarto industriale rappresentato dal siero del latte, responsabile di un rilevante inquinamento ambientale a causa sia del cospicuo volume di liquido depositato illegalmente nel suolo e nelle acque che del suo alto contenuto di sostanze organiche. In particolare, le molecole rinnovabili prese in esame sono state le proteine del siero del latte (WPs) ottenute dagli scarti delle industrie casearie. La frazione proteica del siero è una miscela omogenea di diverse proteine tra cui, soprattutto, la β-lattoglobulina e l'α-lattoalbumina. La β-lattoglobulina è la WP più abbondante del siero e, grazie alle sue caratteristiche molecolari, è in grado di influenzare fortemente l'aggregazione delle WPs causata dalla temperatura o dai cambiamenti del pH. Infatti, attraverso trattamenti termici, tale proteina è capace di auto-assemblarsi in una varietà di strutture sovramolecolari (esistenti come ottameri, in un range di pH compreso tra 3.5 e 5.2, e come dimeri tra pH 5.2 e 7.0) mentre, al di sopra di pH 8.0, la β-lattoglobulina è un monomero con peso molecolare di circa 18 kDa. Le WPs sono note da tempo per la loro capacità di produrre film biodegradabili ed edibili, essendo dotati di una buona estensibilità, una buona resistenza alla trazione, e perché sono alquanto insapori ed inodori. Diverse condizioni sperimentali, come i) il cambiamento del pH, ii) il trattamento termico, iii) il trattamento enzimatico, iv) la diversa concentrazione di glicerolo (GLY) usato come plasticizzante e v) il "*blending*" con diverse nanoparticelle (NPs), sono stati utilizzati nel corso del lavoro di questa tesi nel tentativo di migliorare le caratteristiche tecnologiche delle bio-plastiche a base di WPs preparate mediante il metodo del casting. In particolare, i diversi materiali sono stati prodotti preparando soluzioni filmanti (FFSs) di WPs sia a pH 7.0 che a 12.0, in presenza del 30, 40 o 50% (w/w di WPs) di GLY usato come agente plasticizzante. I risultati preliminari ottenuti hanno dimostrato che la concentrazione minima di GLY per ottenere film manipolabili preparati a pH 12.0 è del 30%, anche senza un trattamento termico preliminare (80°C per 25 min) delle proteine, mentre a pH 7.0 era necessario pretrattare termicamente le FFSs contenenti almeno il 40% di GLY. Dopo l'analisi della stabilità delle FFSs mediante misurazioni del potenziale zeta e della dimensione delle particelle, sono state studiate le proprietà meccaniche dei film derivati dalle diverse FFSs. I risultati ottenuti hanno dimostrato che le FFSs trattate a a pH 12.0, non sottoposte a trattamenti termici e

contenenti il 40 o il 50% di GLY portavano alla produzione di materiali più estensibili. Infatti, i film ottenuti hanno mostrato un più alto allungamento a rottura e un modulo di Young più basso, anche molto più alto e più basso, rispettivamente, di quelli osservati con film ottenuti a pH 7.0 derivati da FFSs trattate termicamente. Ulteriori esperimenti sono stati effettuati utilizzando una transglutaminasi di origine microbica (mTG), enzima in grado di catalizzare la formazione di legami isopeptidici tra glutammina e lisina presenti in proteine substrato. L'enzima è stato utilizzato in questo lavoro sperimentale al fine di migliorare ulteriormente le proprietà tecnologiche delle bio-plastiche a base di WP. La reticolazione mediata da mTG, ottenuta nelle diverse condizioni sperimentali e valutata mediante analisi di SDS-PAGE, ha dimostrato che l'enzima migliorava le proprietà tecnologiche dei film aumentando l'allungamento a rottura e riducendo il modulo di Young. Anche le proprietà barriera verso CO_2 e O_2 sono apparse migliorate in seguito ai legami isopeptidici prodotti dalla mTG. Inoltre, è stato esaminata anche la possibilità di migliorare le caratteristiche dei film a base di WPs aggiungendo alla matrice polimerica NPs ottenute da poliidrossialcanoati (PHA), poliesteri biodegradabili termoplastici sintetizzati da vari generi di batteri (*Bacillus, Rhodococcus, Pseudomonas*) attraverso la fermentazione di zuccheri o lipidi. I risultati ottenuti hanno dimostrato che le proprietà tecnologiche dei bio-nanocompositi preparati grazie all'utilizzo di NPs prodotte dai PHA conferivano caratteristiche di maggiore plasticità ai film migliorandone anche le proprietà barriera nei confronti dei gas. Ciò è stato confermato dall'analisi della microstruttura del materiale ottenuto mediante microscopia elettronica a scansione che ha evidenziato una dispersione omogenea delle NPs nella matrice del film. Infine, i film a base di WPs sono stati esaminati come supporto per l'incorporazione di molecole bioattive. In particolare, l'attenzione è stata focalizzata sull'intrappolamento sia di antiossidanti che di agenti antimicrobici derivati, rispettivamente, dalle foglie di Thymbra (*Satureja capitata*, L.) e dal guscio di noce di Pecan (*Carya illinoinensis*, L.). In particolare la Thymbra, una delle più comuni piante selvatiche molto diffuse in Palestina, possiede interessanti proprietà terapeutiche. Infatti, diversi studi hanno dimostrato che possiede proprietà antibatteriche, antifungine ed antivirali, protegge da infezioni respiratorie, diarrea e disturbi digestivi. La noce Pecan, prodotta principalmente negli Stati Uniti e in Messico, ha destato invece particolare interesse perchè il suo guscio rappresenta una

preziosa fonte di composti fenolici antiossidanti, principalmente tannini condensati. Queste molecole naturali sono state in grado di dotare i film a base di WPs di buone proprietà antiossidanti e notevoli proprietà antimicrobiche contro diversi ceppi batterici responsabili del deterioramento degli alimenti. In particolare, dalla Thymbra sono stati ottenuti degli olii essenziali che, in seguito a gas cromatografia accoppiata a spettrometria di massa (GC-MS). Hanno dimostrato contenere molecole quali γ-terpinene, carvacrolo, p-cimene che conferivano alle soluzioni filmanti proprietà antimicrobiche nei confronti di diversi microrganismi sia gram-positivi che gram-negativi (*Salmonella enteriditis, Salmonella enterica e Staphylococcus aureus*). Inoltre, l'estratto acquoso ottenuto dai gusci di noce Pecan ha dimostrato di poter arricchire i film ottenuti di attività antimicrobile verso *Enterococcus faecalis* and *Salmonella enterica,* anch'essi microrganismi responsabili del deterioramente di numerosi alimenti. I film ottenuti con gli estratti di Pecan hanno mostrato di possedere anche notevoli proprietà antiossidanti. Tutte le FFSs ottenute mediante la funzionalizzazione con tali molecole si sono mostrate stabili dal punto di vista del potenziale zeta e i film da esse derivati possedevano ottime caratteristiche tecnologiche. Infatti, i film funzionalizzati con estratti di noce Pecan erano meno flessibili dei film preparati in loro assenza e mostravano una estensibilità inferiore, un valore di modulo di Young più alto ed un aumento significativo della resistenza alla trazione, tutti effetti probabilmente dovuti all'interazione delle molecole fenoliche con le WPs. Nel caso degli oli essenziali di Thymbra, i dati sperimentali hanno evidenziato che essi portavano ad una riduzione della resistenza alla trazione e del modulo di Young dei film ottenuti, mentre non sono stati registrati cambiamenti nel loro allungamento a rottura. In aggiunta, gli additivi presenti negli scarti di Pecan hanno determinato un ulteriore miglioramento delle proprietà barriera ai gas. Infine, esperimenti condotti per indagare la digestione in condizioni fisiologiche dei film variamente funzionalizzati, ha dimostrato che i materiali preparati con estratto di Pecan erano ancora digeribili dall'intestino umano, indicando la possibilità di utilizzare questo materiale per la realizzazione di film non solo edibili ma anche digeribili. In conclusione, i risultati ottenuti, nel loro insieme, rafforzano la convinzione di poter utilizzare film a base di WPs, preparati in presenza di diversi additivi, come materiali alternativi alle plastiche di origine petrolchimica per la conservazione degli alimenti e l'estensione della loro shelf-life. Ulteriori studi sull'applicabilità di

detto materiale per la conservazione di uno specifico prodotto alimentare dovranno essere effettuati per ottimizzare la funzione di questo promettente nuovo *packaging* attivo.

1. INTRODUCTION

1.1. An overview on bio-plastics

The first plastic material, based on nitrocellulose, was produced in 1845 by Christian Schönbein. For making it, Schönbein mixed cotton, nitric acid, and sulfuric acid, and successfully obtained the guncotton, a material showing high flammability and explosive properties[1]. Then, 10 years later, Alexander Parkes was able to improve nitrocellulose properties through the addition of camphor and commercialized the new material, known as celluloid, under the tradename of Parkesine (Figure 1, A)[2].

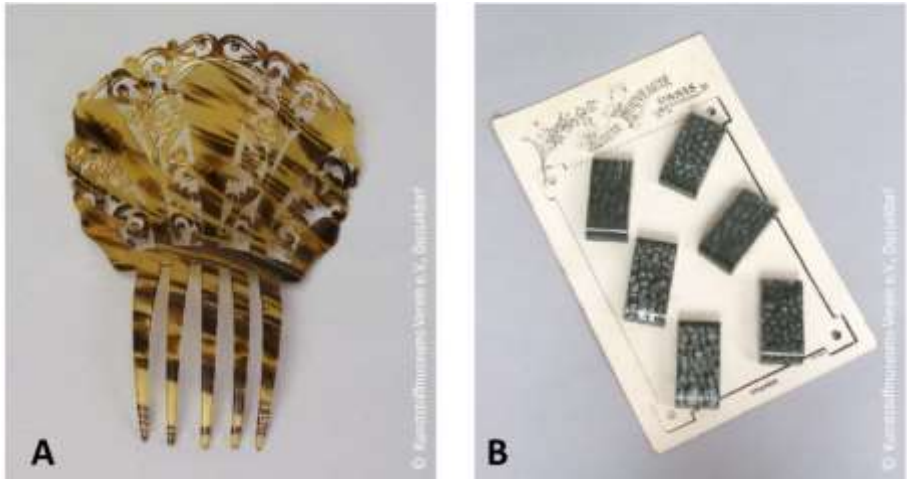


Figure 1: (A) Hair pin made from celluloid used during 1920/1950 **(B)** Buttons made from casein 1920/1940

In addition, caseins, proteins occurring in milk, were also used from the end of 19th century until 1939s to obtain a main raw material to produce galalith (*Greek: milk stone*)[3], which was used for producing buttons (Figure 1, B)[2], personal decorative items, and also for insulation materials in electrical installations.

During the second decade of the 20th century, in the USA, Henry Ford had the first patent in producing new bio-based materials made

from wheat, corn, and soybean. Their main application was in automobile manufacturing, but Ford company was able to find out several further applications of the materials made from soy oil, such as paints and lacquers, a substitute for rubber, and for upholstery fabrics. These early “bio-based plastics” were soon forgotten in the age of petroleum-based polymers converted to “plastics”, considered today as the most pollutant factor of our planet. Plastics have numerous and different applications due to their technical properties such as hardness, formability, rigidity, elasticity, heat and chemical resistance[4]. They are also more economical than many other materials and for all of these reasons they are the most used for different industrial applications: packaging, building, automobile production, electronics, agriculture[1]. However, plastic materials are not easily degradable, being, thus, responsible for huge environmental pollution. Although oil-derived plastics are considered to be one of the greatest innovations in the last century, currently their wide use is regarded as a major threat of damage of the environment. More than 35 million tons of wastes deriving from different plastic items are produced each year in the world, and only 7% of them are recycled, the remaining waste being deposited in the landfills or dispersed in the oceans[5]. Plastics are used in a wide range of food packaging purposes, including containers, bottles, drums, trays, boxes, cups and vending packaging, baby products, and protection coatings. In addition, most plastic-based materials are used in the food industry to protect food products and prolong their shelf-life. Hence, from the beginning of 1980 there has been an increased interest from the food packaging and distribution industry toward alternative materials, such as the “biodegradable plastics”, that become a hot focus of research to reduce plastic production. It is worthy to mention that the transition from fossil-based economy to a bio-based economy is an important EU 2020 strategy target[6].

Among the various “bio-plastics” proposed thus far, hydrocolloid-based materials, made of hydrocolloid macromolecules such as carbohydrates and proteins[7,8], are attracting a particular attention.

Polysaccharides used for edible films or coatings include cellulose, starch derivatives, pectin, seaweed extracts, exudates gums, microbial fermentation gums and chitosan, whereas the most used proteins are represented by soy[9] and bitter vetch proteins[10–12], phaseolin[7,13,14], collagen, gelatin and whey proteins (WPs)[9,15–18].

1.5. Whey proteins

The growing worldwide attention to waste reduction led to focus on the recovery of food by-products. Whey is a by-product of the dairy industry, produced following casein coagulation. The total worldwide milk whey production is estimated to be more than 180 million tons/year, the major amount (approximately 70%) coming from the UE and USA[19]. However, only 50% of this amount is recycled and converted to high added value products[20]. Due to the large volume and the high organic content, the direct disposal of whey in the soil and water is not eco-friendly and it is forbidden in many countries. Therefore, an ecofriendly treatment of whey water, when it is not recycled, is required before its disposal, even because the occurrence of numerous nutrients can be considered as a potential resource for the production of further value-added products. However, large amounts of whey remain generally unutilized, and, thus, whey still deserves attention from researchers to develop innovative processes able to provide maximal benefits from this by-product and to limit its environmental impact**.** In fact, the excellent nutritional value and some properties of WPs originating from the waste stream from the cheese industry (such as solubility in water and ability to act as emulsifiers) have been exploited for the production of transparent, flexible, colorless and odorless edible films with poor moisture barrier, effective aroma barrier and low O_2 permeability. In the EU countries, a total of 170 billion Kg of milk was produced in 2017, 93% of which was commercialized as various dairy products, including cheese (37%), butter (30%), cream (13%), fresh milk (11%), acidified milk (4%), milk powder (2%), and other minor products. Dairy industries produce an average of 2.5 L of

wastewater per L of processed milk, as well as about 9–10 L of cheese whey per kg of cheese produced, resulting in approximately 400 billion L of wastewater per year[21].

Dairy effluents are characterized by a high organic load representing, at the same time, a severe hazard for the environment and a huge opportunity as bioenergy source and for production of different biochemicals. Besides lactose and minerals, milk whey contains functional proteins (WPs) such as β-lactoglobulin (65%), α-lactalbumin (25%) and bovine serum albumin (8%) (Figure 2). Other minor proteins present in the whey are lactoperoxidase, lactoferrin and immunoglobulins[22].

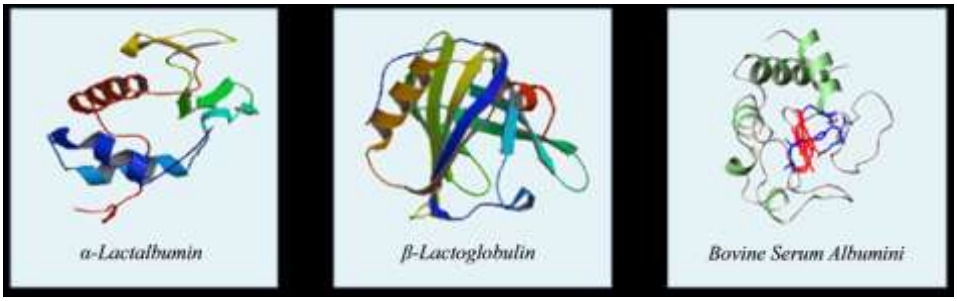


Figure 2: Structure of the main WPs[23]

More in particular, ß-lactoglobulin and α-lactalbumin are endowed with different features (solubility, foaming, and gelling properties)[24] making them of interest for different biotechnological applications. It is known that β-lactoglobulin, upon heating, is capable of self-assembling into a variety of supramolecular structures, existing as an octamer between pH 3.5 to 5.2 and as a dimer between pH 5.2 and 7, whereas above pH 8.0, β-lactoglobulin is a monomer with the molecular weight of 18,277 Da[23]. In addition, β-lactoglobulin is able to resist to the denaturation at acidic pH while, at alkaline pH, two sequential conformational changes occur in its structure (unfolding of helix and exposing sheet domains), similarly to its denaturation occurring in the temperature range between 50 and 90°C, followed by the unfolding of other sheets[25].

WPs have also the ability to give rise to edible bio-plastics endowed with good technological features[23, 26–30]. Edible films and coatings manufactured starting from WP concentrates or isolates have been studied and developed by different methodologies[20,29] and many additives have been used with the aim to improve the characteristics of the obtained WP-based materials. The present thesis describes various experimental attempts performed to further improve the functional properties of WP-derived bio-plastics.

1.5. Microbial transglutaminase

The enzyme transglutaminase of microbial origin (mTG, protein-glutamine γ-glutamyl transferase, E.C. 2.3.2.13) was one tool utilized to reinforce WP films, since this enzyme has been successfully exploited at this aim during the last 15 years[7,9,10,31–35]. mTG, a bio-catalyst able to introduce ε-(γ-glutamyl)-lysine crosslinks into proteins via an acyl transfer reaction, has been proved to modify several food proteins and, among the milk proteins, both caseins and WPs are excellent acyl donor and/or acceptor substrates for mTG[36–38] (Figure 3)[39]. The transamidation reaction occurs when the acyl acceptor is the ε-amino group of an endoprotein lysine residue and the acyl donor is the ɣ-carboxamide group of an endoprotein glutamine residue present either in the same (Figure 3, panel A) or in a different (Figure 3, panel B) polypeptide chain. In the first case, an intra-molecular crosslink is obtained whereas, in the second case, the reaction product is an inter-molecular crosslink. mTG is calcium independent[40], active in a wide range of pH (4-9)[41], resistant between 4 and 60°C[42], commercially available[43], food grade[44] and useful to modify protein structure and biological properties[45].
WP-based films are generally formed in aqueous solutions in the presence of different plasticizers and their properties have been shown to be significantly influenced by the presence of mTG[46]. In particular, the addition of mTG to milk protein-based film forming solution (FFS) was demonstrated to induce an enhancement in film

mechanical resistance and a reduction in its deformability. Even the barrier efficiency toward O_2 was found to be markedly improved in the crosslinked films which showed also a lower permeability to water vapor (WV)[16,17,47].

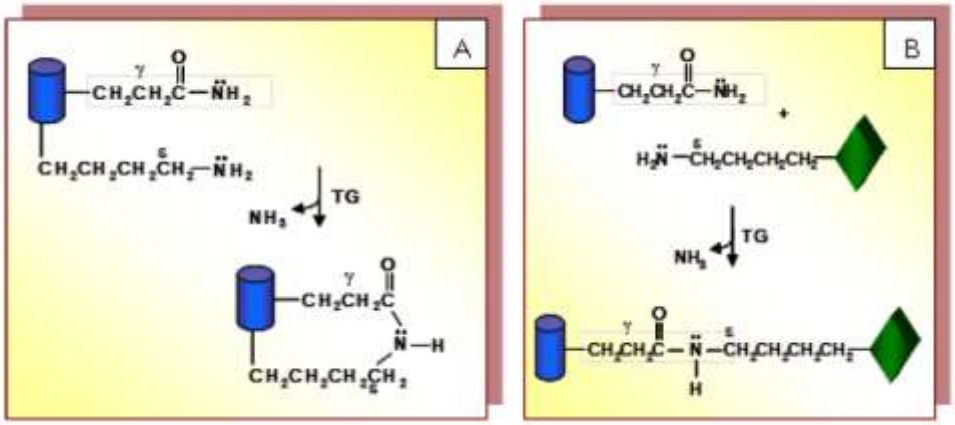


Figure 3: Schematic image representing mTG-catalyzed amide bond formation by intramolecular (A) or intermolecular (B) crosslinking reactions

1.4. Nanoparticles

Besides polysaccharides and proteins, the bio-plastics can be prepared also by using aliphatic polyesters, e.g. polylactic acid (PLA) and polyhydroxyalkanoates (PHAs). In particular, PHAs have attracted research and commercial interests worldwide because they can be used as biodegradable thermoplastics and because they can be produced from renewable resources. Therefore, these polymers possess sustainability and environment-friendly properties[48]. They are a family of bio-polyesters, synthesized as intracellular products by prokaryotic cells, encompassing gram-positive and gram-negative eubacteria[49], oxygen-evolving cyanobacteria[50] and even archaeabacteria, often growing in extreme environments[51]. Different PHAs (polyhydroxybutyrate, PHB; poly-3-hydroxybutyrate-co-hydroxy-valerate, PHBV; poly-3-hydroxybutyrate-cohydroxyhexanoate,

PHBHHx) have been exploited for the preparation of nanoparticles (NPs), able to encapsulate and release various drugs[52–56]. Among the different polymers, PHBHHx displays peculiar elastic properties, thus emerging as promising bio-polymeric source for the designing of bio-nanocomposites with improved flexibility[57–59]. Different NPs have been previously exploited for the matrix reinforcement of bio-plastics, such as the ones prepared from nanocellulose, usually used to improve the properties of different bio-materials[60]. As matter of fact, cellulose nanostructures have been most usually applied as reinforcing agents, but they may also be used as matrices in a variety of materials including films for food packaging applications[61]. As far as WP-based film reinforcement, some efforts have been made by using porous silica (SiO_2) coated titanium (TiO_2) NPs TiO_2@@SiO_2[62], and it was demonstrated that the incorporation of TiO_2@@SiO_2 NPs helps to improve WP-based film mechanical properties. However, the optical properties of the WP films were dramatically modified following incorporation of these NPs, changing from a transparent appearance to an opaque one, even though the embedded NPs contributed effectively to increase the thickness of films and to alter the contact angle of their surface. Therefore, the development of high strength, flexible bio-nanocomposites, tuning of NP properties, as well as their volume fraction and topographic distribution within the scaffold, still remain significant challenges. With the aim to decrease the hydrophilicity of WP-based materials and, consequently, to improve their performance, the possibility to prepare bio-nanocomposites by blending WP-proteins with NPs obtained from PHBHHx has been investigated.

1.5. Thymbra essential oils

Aiming to further improve food packaging materials, many studies have exploited natural additives with antimicrobial and antioxidant activity to produce bio-active food packaging[63–65]. Interestingly, essential oils (EOs) extracted from plants and herbs, could be

interesting natural additives in food packaging applications because of their antimicrobial and antioxidant properties[27,63,66,67]. Among these plants, *Satureja capitata*, an aromatic plant of the *Lamiaceae* family, commonly named “Thymbra” (Figure 4) possesses interesting features. It is also known in Arabic as “za'atar rumi”, “za'atar franji” and “za'atar farsi”[68] (meaning, "Roman hyssop," "European hyssop," and "Persian hyssop," respectively).

Figure 4: Palestinian *Satureja capitata* plant (Thymbra)

In particular, Thymbra has been used for more than 100 years by the Palestinians for tea preparation[69]. Many studies proved that this plant has a powerful anti-bacterial, antifungals, and antiviral activity and can protect from coughing, respiratory infection, diarrhea, and digestive problems[70,71]. In this project Thymbra essential oils (TEOs) have been extracted and used in the attempt to confer antioxidant and/or antimicrobial properties to the produced WP-based bio-plastics.

1.6. Pecan nut shell- water soluble- extract

Bio-active compounds can be extracted not only as EO by hydro distillation method but they can be also extracted via solid liquid extraction method using different solvents (e.g., water, ethanol,

hexane). Pecan nuts (Figure 5)[72] are produced mainly in United States and Mexico in amounts of ca. 420,000 tons per year[73]. The wasted pecan nut shells (PNSs) represent a valuable source of antioxidant compounds, mainly condensed, such as prodelphinidin-type tannins[74-76].

Figure 5: *Carya illinoinensis* (Pecan) nuts

The antioxidant properties of extracts from PNS have been largely explored in the food industry. Water-extractable compounds from PNSs are able to increase the oxidative stability of margarines during storage[77], whereas a PNS hydroalcoholic extract (PNSE) (estimated phenol content: ca. 130 mg gallic acid equivalents/g) has been reported to act as a thermal and photo-oxidative stabilizer of polyethylene and polylactic acid[74,78–80], as well as to confer significant antioxidant and food stabilization properties to these polymers[75]. Moreover, inclusion of PNSE in octenylsuccinate starch films led to improved water resistance and UV-light barrier properties compared to control films[81]. PNS extracts are also characterized by remarkable antimicrobial properties[82], which opens new perspectives toward a full exploitation of the applicability of this material in food field. Indeed, contamination of food samples by microorganisms is a key issue greatly affecting food preservation and shelf-life. It has also to be highlighted that the improper use of conventional antibiotics in food industry is a major issue since it leads to the rapid rise and spread of resistant foodborne pathogens[83,84]. These microorganisms can be easily transmitted to

humans through consumption of fresh and raw foods[85]. Moreover, because of side effects caused by the use of chemical agents[86] and because of the consequences of physical treatments on food organoleptic properties, there is an increasing demand for natural and untreated food products[87]. Aqueous and ethanol PNS extracts have shown antimicrobial activity towards both gram-positive and gram-negative bacteria. Crude extracts were found to be more effective than the single phenolic components, which suggests synergistic effects between the different agents[76,79]. Furthermore, a methanol extract of PNSs showed antifungal activity towards different plant pathogens[88].

1.7. Aim of PhD project

The hypothesis behind this research project resides in that WPs might be used as hydrocolloid source for the production of tailored bio-plastics following their tuning by means of both physico-chemical and biochemical methods. In fact, this thesis is focused on the development of novel WP-based bio-plastics *ad hoc* designed by exploring the effect of protein heating and pH modification. In addition, the influence of GLY used as plasticizer, the WP grafting with polyester-derived NPs, as well as the mTG enzymatic crosslinking action were also evaluated on the features of the derived bio-plastics.
Some of the obtained films were also functionalized by the addition of natural active molecules extracted from Thymbra and Pecan in order to endow the films with biological properties. All the information gained might be useful to assess the application in the next future of the obtained materials for the protection of different kinds of foods (raw or cooked).

2. MATERIALS AND METHODS

2.1. Materials

Commercial WP isolate (~90% dry basis protein) was obtained from BioLine (London, UK), microbial transglutaminase (mTG) from *Streptoverticillium* sp. (Activa WM; specific activity 90 units/g) was supplied by Prodotti Gianni SpA (Milano, Italy). The enzyme solution was prepared by dissolving the commercial preparation in distilled water at a concentration of 100 mg/mL. Clevenger apparatus was used to prepare EOs from palestinian Thymbra; glycerol (GLY) was purchased from Sigma (Steinheim, Germany). All other chemicals and solvents used in this study were analytical grade commercial products.

Different microorganisms were used to evaluate antimicrobial activity. In particular, *Staphylococcus aureus*, *Escherichia coli, klebsiella pneumoniae, Proteus vulgaris, Enterococcus faecium, Pseudomonas aeruginosa, and Candida albicans* were from the American Type Culture Collection with the following ATCC codes: 25923, 25922, 13883, 8427, 700221, 9027 and 90028, respectively. In addition, the clinical pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) was used to evaluate the antimicrobial activity of TEOs. *Salmonella enteriditis* 706 RIVM, *Salmonella enterica* subsp*. enterica* serovar *Typhimurium* (ATCC® 14028), *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were grown in MHB (MHB, Becton Dickinson Difco, Franklin Lakes, NJ) or on Tryptic Soy Agar (TSA) plates (Sigma Aldrich, Milan, Italy).

2.2. Film preparation

2.2.1. Whey protein film preparation

FFSs were prepared by dissolving WP isolate in distilled water (5% WPs, w/v) and adjusting the pH (to 7 or 12) by 0.1 N NaOH addition. FFSs were heated or not at 80°C for 25 min under continuous stirring. After cooling down at room temperature, GLY (30%, 40%, 50%, w/w of WP) was added just before casting 50 mL FFS, containing 500 mg WPs, into 8 cm diameter Petri dishes. The derived films were then obtained by drying in a climatic chamber at 25°C and 45% RH for 24 h and analyzed within 48 h.

2.2.2. Transglutaminase-crosslinked films

Preliminary enzymatic assays were carried out in order to investigate the effect of different concentrations of mTG on WP modification. In this respect, WPs were preliminary treated for 25 min at 80°C and incubated for 1 and 2 hours with different concentrations of the enzyme (0, 8, 16, 24, 32 U/g of WPs) at pH 7.5. The extent of enzymatic crosslinking was carried out by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli[89]. Briefly, 5 µL of sample buffer (15 mM Tris – HCl, pH 6.8, containing 0.5% w/v SDS, 2.5% v/v GLY, 200 mM β-mercaptoethanol and 0.003% w/v bromophenol blue) were added to 20 µL aliquots of each sample and analyzed by 4-20% SDS-PAGE[89]. The electrophoresis was performed at constant voltage (80 V for 2-3 hours) and the proteins were visualized by Coomassie Brilliant Blue R250 staining. Bio-Rad precision protein standards were used as molecular weight markers. SDS-PAGE gel images were acquired using Bio-Rad ChemDoc Imager. The image analysis was carried out using Image Lab software (Bio-Rad, version 5.2.1) as described by Romano et al. (2015)[90].

For the preparation of crosslinked films, the pH of the reaction mixtures, following the incubation with mTG, was adjusted to pH 12. GLY (30 and 40%, w/w of WP), used as plasticizer, was finally added

to prepare the FFSs and films were obtained by casting 500 mg of WPs and FFS drying in a climatic chamber at 25°C and 45% RH for 24 h and analyzed within 48 h.

2.2.3. Poly-3-hydroxybutyrate-co-hydroxyhexanoate nanoparticles grafted films

The preparation and characterization of **PHBHHx-NPs,** described in the attached published article entitled *"Design and characterization of poly (3-hydroxybutyrate-co-hydroxyhexanoate) nanoparticles and their grafting in whey protein-based nanocomposites*", was carried out by Iolanda Corrado attending the same PhD cycle. Hence, the prepared NPs were grafted into WP-based bio-plastics. Briefly, FFSs were prepared by dissolving WPs (500 mg) in distilled water and adjusting the pH to 12 by 0.1 N NaOH addition. The derived solutions were stirred for one hour and, then, aqueous dispersion of PHBHHx-NPs (1, 2, 4 or 8 %, w/w of WPs) was added under continuous stirring at 700 rpm for 15 minutes. GLY (40%, w/w of WPs), used as plasticizer, was finally added, under stirring for 15 min, just before FFS casting. Each FFS (25 mL) was poured into 8 cm diameter polyester Petri dishes and the films were then obtained by drying all the FFSs at 25°C and 45% RH for 24 hours.

2.2.4. Thymbra essential oil-activated films

The essential oils (EOs) were extracted from the leaves of two Palestinian varieties of Thymbra: one (TEO1), coming from a local shop in the city of Nablus, whereas the second one (TEO 2) was straight harvested from Qabatia city. Hence, Thymbra leaves from local shop in Nablus were already dried, on the contrary the ones from Qabatia needed to be dried, procedure that was carried out in an oven at 30°C for 24 h. Then, the TEOs were extracted from the dried leaves by steam-distillation for 3 h using a Clevenger type apparatus according to the method[91] described by Dadalioglu and Evrendilek (2004). $CaCl_2$ salt was used to remove any remaining water, and TEOs, stored in a dark glass bottle at 4°C until use, were

then analyzed by means of gas chromatography-mass spectrometry using Shimadzu QP-5000 GC-MS (Japan). GC apparatus was equipped with a Rtx-5 MS column (30 m long, 0.25 μm thickness and 0.250 mm inner diameter), whereas helium was used as a carrier gas at a flow rate of 1 ml/min, and the injector temperature was 220°C. The oven temperature was programmed from 50°C (1 min hold) to 130°C (5°C/min), then to 250°C (10° C/min) and, finally, kept isothermally for 15 min (transfer line temperature was 290°C). For GC-MS detection, an electron ionization system, with detector volts of 1.7 KV was used. A scan rate of 0.5 sec, and scan speed of 1000 amu/sec was applied, covering a mass range from 38-450 M/Z. The chemical ingredients of the volatile oil were identified by comparing their mass spectra with the reference spectra found in the mass spectrometry data center of the National Institute of Standards and Technology (NIST)[92]. For film preparation in the presence of TEOs, WP solution was prepared by dissolving 5 g proteins in 100 mL distilled water, and adjusting the pH to 12 by 0.1 N NaOH addition then the GLY (50% w/w of WPs) was added. After 1 h stirring, WP solution (10 mL) was used to prepare FFSs containing or not different concentrations of TEO1 (0.1%; 0.4% and 0.8% v/v). Finally, 25 ml FFSs were casted into 8 cm diameter polyester Petri dishes. The derived films were then obtained by FFS drying at 25°C and 45% RH for 24 h and analyzed within 48 h.

2.2.5. Pecan nut shell extract (PNSE)-activated films

PNSE was obtained as previously described[74,75]. Briefly, PNS (1 g) was treated with 20 mL of ethanol/water (3:2 v/v) for 30 min in an ultrasonic bath at room temperature. The mixture was then centrifuged for 20 min at 8000 g, the supernatant was filtered on Whatman paper No.2 (GE Healthcare) and ethanol was removed in a rotary evaporator. The residual solution was then lyophilized to give PNSE as a red powder in 15% w/w yield. For the preparation of PNSE solution, 0.5 g of PNSE were added to 10 mL of water. The suspension was kept in an ultrasonic bath for 30 min and then

centrifuged at 8000 g for 30 min. The supernatant was removed and analyzed at 24 h intervals over 1 week by UV-Vis spectroscopy to check the stability of the components. The pellet was dried by lyophilization and weighted to determine by difference the amount of material gone into solution. PNSE concentration in the supernatant was found to be 30 mg/mL. FFSs were prepared by dissolving 500 mg of WPs in distilled water and by adding subsequently 60% of GLY (w/w of WPs). After PNSE addition (final concentration 9.25 mg/mL), the final pH was brought to pH 12 by using 0.1 M NaOH. Control samples were prepared as above replacing PNSE with distilled water. The films were prepared by pouring FFSs (25 mL) in 9 cm diameter Petri dishes and let them dry at 25°C (45% RH) for 24 h.

2.3 Film physicochemical characterization

2.3.1. Zeta potential and particle size measurements

1.0 mL of each WP-based FFS was analyzed for zeta potential and particle size by using a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK). The device was equipped with a helium-neon laser of 4 mW output power with a fixed wavelength of 633 nm (wavelength of laser red emission). The instrument software programmer calculated the zeta potential through the electrophoretic mobility by applying a voltage of 200 mV and using the Henry equation.

2.3.2. Film forming solution viscosity

FFS viscosity was studied by standard Ostwald capillary viscometer and specific viscosity values were calculated by the following equation:

$$\text{specific viscocity} = \frac{(\text{FFS flow time} - \text{water flow time})}{\text{water flow time}} \qquad (1)$$

2.3.3 Film thickness

Film thickness was measured with a micrometer model HO62 Metrocontrol Srl (Casoria, Naples, Italy) at five random positions over the film area. Values are mean ± standard deviation (SD) of five replicates.

2.3.4. Film mechanical properties

Film tensile strength (TS), elongation at break (EB) and Young's module (YM) were measured by using an Instron Universal Testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). Film samples were cut, using a 30 sharp scissors, into 10 mm wide and 40 mm length strips equilibrated for 48-72 h at 50% ± 5% RH and 23 ± 2°C in an environmental chamber. Five specimens of each film type were tested (1 KN load and 1 mm/5 min speed), as previously reported[93] (ASTM D882-97, 1997).

2.3.5. Film gas and water vapor barrier properties

Film permeability to O_2 (ASTM D3985-05, 2010)[94], CO_2 (ASTM F2476-13, 2013)[95] and WV (ASTM F1249-13, 2013)[96] was determined in triplicate for each film by using a TotalPerm apparatus (Extrasolution s.r.l., Pisa, Italy). Aluminium masks were used to reduce film test area to 5 cm^2, whereas the testing was performed at 25°C under 50% RH.

2.3.6. Film microstructure

A field emission scanning electron microscope (FE-SEM, FEI Nova NanoSEM450) was used to study the morphology of the cast films (cross-section and surface). For cross-section imaging, the films were previously frozen using liquid nitrogen and then cryo-fractured. The images were acquired using an incident electron beam energy between 2 and 5 kV and by collecting secondary electrons with an ETD or TLD detector. All the samples were gold/palladium coated by an automatic sputter coater Denton Vacuum – Desk V, before to be observed.

2.3.7. Film moisture content and moisture uptake

Film samples (2 cm × 2 cm) were weighed and dried at 105°C in an oven for 24 h. Analyses in triplicate of each film were carried out and film moisture content was calculated as:

$$\text{Film moisture content } (\%) = \left(\frac{(W_1 - W_2)}{W_1}\right) \times 100 \quad (2)$$

where W_1 is the initial weight of the film and W_2 is the film weight after drying at 105°C.

Moisture uptake was measured gravimetrically in triplicate following the methodology adopted by Sartori and Menegalli[97] with slight modifications. Samples were cut into 20-mm-sided squares, dried at 105°C for 24 h, conditioned at 23 ± 2°C into a desiccator (50% RH) with a saturated $Mg(NO_3)_2$ solution, and then weighed. The moisture uptake was finally calculated as:

$$Film\ moisture\ uptake\ (\%) = \left(\frac{(W_s - W_d)}{W_s}\right) \times 100 \quad (3)$$

where W_s and W_d are the weight of swollen and dried films, respectively.

2.4. Antimicrobial and antioxidant activity evaluation

2.4.1. Antimicrobial activity of film forming solutions containing Thymbra leaf extract

TEO antimicrobial activity was evaluated by means of the broth micro-dilution method[98] against the following different microorganisms: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Klebsiella*, *Methicillin-Resistant Staphylococcus aureus* (MRSA), and *Proteus vulgaris.* Particularly, two-fold dilutions of the antimicrobial agent were prepared in liquid growth medium and dispensed into a 96-well micro-titration plate. After that, each well was inoculated with microbial cells, which were prepared in the same medium after dilution of the standardized microbial suspension adjusted to 0.5 on the McFarland scale. After mixing, the 96-well micro-titration plates were incubated at 37°C for 24 hours. Minimal inhibitory concentration (MIC) was determined as the lowest antimicrobial

agent concentration able to prevent the visible growth of microorganisms.

To test the antimicrobial activity of the obtained FFSs, microbroth dilution assays, followed by a colony count assay, were carried out. Briefly, 50 µL of bacterial cells (final concentration, 2 x 10^6 CFU/mL) were plated out in 96-well plates. After that, 50 µL of 2-fold serial dilution of the FFSs were added and upon 24 hours of incubation 100 µL of each sample were plated out on TSA (tryptic soy agar) Petri dishes. The plates were incubated at 37°C for 24 hours and, at the end, colony count was performed. FFSs without TEOs were used as controls and bacterial cells alone were used as positive controls of growth. The experiments were carried out in triplicate.

2.4.2 Antimicrobial activity of film forming solutions containing Pecan nut shell extract

Antimicrobial activity of PNSE against *Salmonella enterica* subsp*. entericamser. Typhimurium* (ATCC® 14028) and gram-positive *Enterococcus faecalis* (ATCC® 29212) was analyzed by microbroth dilutions assay[99]. MIC values corresponded to the lowest concentration of extract associated to no detectable bacterial growth**.** Bacterial cells were grown on glass coverslips uncoated (control sample) or coated with FFSs containing or not PNSE in MHB medium, starting from a culture at a concentration of 2 × 10^8 CFU/mL, in static conditions at 37 °C for 16 h. Upon incubation, non-adherent bacteria were removed by gently washing samples with sterile phosphate buffer saline. Viability of cells attached on the surface was determined by sample staining with LIVE/DEAD® BacLight™ Bacterial Viability kit (Molecular Probes ThermoFisher Scientific, Waltham, MA, USA). Staining was performed according to manufacturer's instructions. Images were captured by using a confocal laser scanning microscope (Zeiss LSM 710, Zeiss, Germany) and a 63× objective oil immersion system. The area containing attached bacteria was analyzed by using the Zen Lite 2.3 software package and each experiment was performed in triplicate. Images are 2.5D projections of biofilm structure obtained by

confocal z-stack using Zen Lite 2.3 software. All images were taken under identical conditions and represented the average of at least three different acquisition fields. Scale bar corresponded to 10 μm in all the cases and all images were taken under identical conditions.

2.4.3. Antimicrobial activity of whey protein films containing either Thymbra essential oil or Pecan nut shell extract

In all the experiments bacteria were inoculated and grown overnight in MHB at 37°C. Then, bacteria were transferred to a fresh Tryptic Soy Broth (TSB) tube and grown to mid-logarithmic phase. To evaluate the antimicrobial activity of the WP films in the absence or presence of TEO1 or PNSE as bio-active agents, bacterial cells were diluted in TSB to approximately 2 x 10^7 CFU/mL and inoculated by surface streaking into TSA plates using a swab. A 1.5-cm^2 square of each film was placed into the center of the inoculated plate and pressed to ensure full contact with the agar surface. Plates were incubated at 37 °C for 24 h and the bacterial growth underneath the film was evaluated.

2.4.4. Antioxidant properties of whey protein films

The antioxidant properties were evaluated for the films that were functionalized by PNSE. The experiments were carried out by two different assays:

1) DPPH assay: 1 cm^2 film section (corresponding to 28 mg of material) was added to 10 mL of a 50 or 200 μM ethanol solution of DPPH (2,2-diphenyl-1-picrylhydrazyl), and the absorbance of the solution at 515 nm was periodically analyzed over 4 h. Control experiments were run on films not containing PNSE. Each experiment was run in triplicate.

2) Ferric reducing/antioxidant power (FRAP) assay: 1 cm^2 film section (corresponding to 28 mg of material) was added to 10 mL of a solution of 1.7 mM $FeCl_3$ and 0.83 mM 2,4,6-tris(2-pirydyl)-s-triazine in 0.3 M acetate buffer (pH 3.6). The absorbance of the solution at 593 nm was periodically measured over 4 h. Control experiments were run on films not containing PNSE. Each

experiment was run in triplicate. Results were expressed as Trolox equivalents.
In separate experiments the release of PNSE from the films in the FRAP assay medium was evaluated by adding 1 cm^2 film section (corresponding to 28 mg of material) to 10 mL of 0.3 M acetate buffer (pH 3.6) and periodically recording the UV-Vis spectra of the solution for 4 h. Experiments were run in triplicate.

2.4.5. Film forming solution oral and gastric digestion

FFSs prepared in the absence and presence of PNSE were subjected to two-phase *in vitro* digestion, using an adult model[90,98,100,101] which simulated the physiological conditions of the digestive system at the oral and gastric levels. In particular, 500 µL of each FFS were incubated in 600 µL of simulated salivary fluid (SSF, 150 mM NaCl, 3 mM urea, pH 6.9) for 5 min by stirring at 170 rpm. Afterwards, the samples (75 µL each) were subjected to gastric digestion by adding 100 µL of simulated gastric fluid (SGF, 0.15 M NaCl) the pH of which was adjusted to 2.5 with 6 M HCl,-containing pepsin at protein/enzyme ratio of 20:1 (w/w). Pepsin reaction was stopped at 1, 2, 5, 10, 20, 40, 60 min by adding to each sample 40 µL of 0.5 M ammonium bicarbonate. The samples were then analyzed by SDS-PAGE. Densitometry analysis was performed by calculating the percentage of the average intensity of the 17-kDa β-lactoglobulin normalized to that of control samples.

2.5. Statistical Analysis

Statistical analysis for all the data was performed by means of JMP software 5.0 (SAS Institute, Cary, NC, USA). The data were subjected to analysis of variance, and the means were compared using the Tukey-Kramer HSD test. Differences were considered to be significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

It was considered crucial to study the production of WP-based films under different experimental conditions such as: after protein heat treatment, at different pH, at different concentrations of GLY and, finally, following mTG-mediated crosslinking of the WP matrix. Afterwards, the more appropriate conditions have been pursued as it is possible to infer from the following results.

3.1. Whey protein-based films: influence of pH and glycerol

The pH value has an important role for the protein-based film formation, as well as for the derived material properties[23]. Hence, 1% (w/v) WP solution, heated or not at 80°C for 25 min, has been prepared at pH 2 in order to perform the titration from pH 2 until pH 12 by adding 1.0, 0.5, and 0.1 N NaOH as titrant solutions under constant stirring at 25°C by using a Zetasizer Nano-ZSP (Malvern®,Worcestershire, UK). Zeta potential values, measured at each pH in triplicate demonstrate that under alkaline pHs the WP FFSs were quite stable, regardless the heat treatment (Figure 6). More in particular, they reach zeta potential values lower than -30 mV at pH 12.

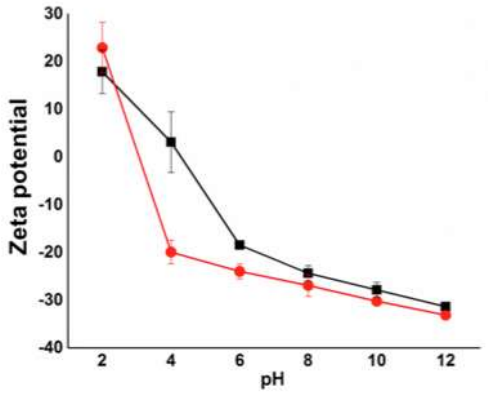
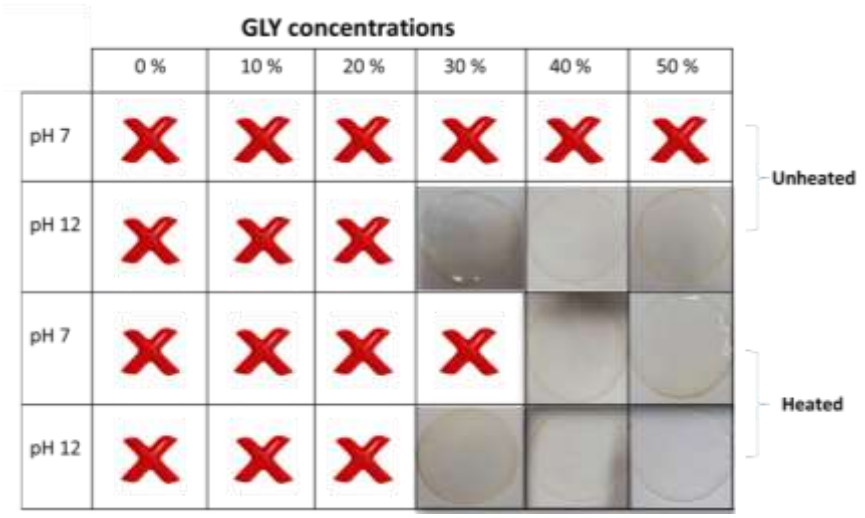


Figure 6: Zeta potential of both unheated (red line) and heated (black line) WPs (1%, w/v) measured at different pH values.

Among the various treatments of WPs (such as chemical modification, alternating electric field, supercritical carbon dioxide or high hydrostatic pressure treatments, etc.) heat treatment and pH change of protein solution are the two methods that could have a broader and easier application on industrial scale. Heat treatment of WPs leads to protein denaturation and, depending on pH value, solution composition and ionic strength, several reactions, such as polymerization or covalent and non-covalent interactions, occur during protein heating[102]. As a result of these interactions, WPs are denatured and the reactions among the exposed free thiol groups lead to protein aggregation depending on the repulsive and attractive forces between particles. Hence, the size and the distribution pattern resulting from WP aggregation depend on pH, heating temperature and protein concentration[102,103]. Herein we investigated the film forming properties of WPs treated or not at 80°C for 25 min by analyzing different properties of the derived films obtained at different pH values and concentrations of GLY, used as plasticizer. Films were prepared by casting, at both pH 7 and 12, WP FFSs containing 30, 40 or 50% (w/w of WPs) of GLY.



(X = *no handleable film formed)*

Figure 7: WP (500 mg)-based films cast either at pH 7 or pH 12 following heat treatment of FFSs containing different concentrations of GLY

The minimum GLY concentration for obtaining handleable films at pH 12 was 30%, also without a preliminary heat-treatment of the proteins, whereas at pH 7 it was necessary to heat-treat the FFSs containing at least 40% GLY (Figure 7). In the following published article an insight about the alkaline treatment on film preparation and on the characterization of the derived materials is reported.

coatings

MDPI

Article

Glycerol-Plasticized Films Obtained from Whey Proteins Denatured at Alkaline pH

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Abstract: Whey represents the major by-product of cheese industry. One possibility to recycle the whey wastes is the use of their globular proteins as a polymer source for the production of biodegradable plastic materials. Whey protein (WP)-based films are usually obtained by protein heat treatment in the presence of glycerol (GLY) as plasticizer at pH 7, a method which would require commercially high costing process. In this work we explored the possibility of producing manageable whey-derived materials without any heat-treatment but under alkaline conditions. The reported results demonstrated that the casting at pH 12 of the unheated WP film forming solutions (FFSs), containing either 40% or 50% GLY, led to produce more resistant and flexible materials than the ones obtained at pH 7. Film opacity was observed significantly increased, being higher in the samples obtained at alkaline pH without WP heating and with higher GLY concentrations. Finally, moisture content decreased with the reduction of GLY content, both in heated and unheated WP-based films, whereas water uptake of the different films prepared at pH 12 did not significantly change.

Keywords: biodegradable materials; whey; protein-based films

1. Introduction

Whey is produced in huge quantities by the dairy industries during the casein coagulation process. Whey can be formed from all types (cow, goat, sheep, camel) of milk and, in particular, bovine whey is the most common whey produced in the western countries, sharing about 85%–95% of the generating milk volume and containing about 55% of the whole milk nutrients [1]. The total worldwide whey production is estimated to be more than 180 million tons/year, the major amount (approximately 70%) coming from EU and USA [1]. Whey is responsible for relevant environmental problems due to both its large volume and high organic content, and thus its disposal into municipal sewers is almost everywhere forbidden. On the other hand, land dumping creates severe pollution concerns for the environment by negatively influencing soil physicochemical characteristics. Therefore, an ecofriendly treatment of whey, when it is not recycled, is required before its disposal, even because the occurrence of numerous nutrients in whey is considered as a potential resource for the production of different value-added products. However, large amounts of whey remain generally unutilized, and thus whey still deserves attention from researchers to develop further innovative processes able to provide maximal benefits from this by-product and to limit its environmental pollution impact.

Among the various whey treatments, heating and pH modification are methods that could have a broader and easier application on industrial scale. Since aggregation depends on the repulsive and attractive forces between the particles occurring in solution, the size and the distribution pattern of whey

proteins (WPs) depend on both pH and heating temperature, as well as on protein concentration [2,3]. β-Lactoglobulin is the major WP of ruminant species, and thus its molecular characteristics strongly influence WP aggregation caused by temperature or pH changes. Upon heating, it is capable of self-assembling into a variety of supramolecular structures, existing as an octamer between pH 3.5 to 5.2 and as a dimer between pH 5.2 and 7, whereas above pH 8.0, β-lactoglobulin is a monomer with a molecular weight of 18,277 Da [4]. β-Lactoglobulin is known to resist denaturation at acidic pH while, at talkaline pH, two sequential conformation changes occur in its structure, i.e., the unfolding of α-helix and of exposed β-sheet domains, similarly to its denaturation occurring in the temperature range 50–90 °C, followed by the unfolding of other β-sheets [5]. Therefore, its tunable structuring capacity makes β-lactoglobulin, and consequently, whey, a possible interesting source for material science. In fact, one possibility to re-use the whey is to turn its protein content into biodegradable/edible packaging films, that can meet consumer demands for safe, convenient, and/or healthy food products with prolonged shelf life as well as sustainability awareness [6]. Edible films, endowed with a low environmental impact, have been progressively improved to effectively protect various food products through tailored mechanical and/or barrier properties, as well as a controlled release of active ingredients [7]. In addition, these materials might replace the petroleum-based plastics, considered to be a major threat of pollution of the environment because they are not easily degradable. In fact, more than 35 million tons of wastes deriving from different plastic items are produced each year in the world and only 7% of them are recycled, the remaining waste being deposited in the landfills or dispersed in the oceans [8]. Therefore, manufacturers are trying to reduce the application of plastic materials, mainly for food packaging, and to develop innovative biodegradable films and coatings [9].

In recent years, edible films obtained from proteins of both plant and animal sources, and particularly WPs, received increasing attention [10–14]. These materials are usually obtained by casting and drying of WP solutions, since the properties of extruded and molded materials derived from WPs are still unsatisfactory [15]. In addition, WP applicability in significant processes of pharmaceutical coating has not yet been really described. In fact, their use has been only proposed in obtaining films targeted as carriers of antimicrobial agents [16] and as a protective barrier to improve food shelf life [17,18], mostly because WP-based films exhibit a poor barrier capacity against water vapor. Therefore, the present paper reports studies specifically addressed to find out new experimental conditions to produce whey-derived films potentially useful for a more extensive application. In this research, the film forming capacity of WPs denatured at high temperatures and/or alkaline pH was systematically studied even in comparison with other proteins. Several properties of the WP-based films obtained at different concentrations of both plasticizer (glycerol, GLY) and structuring (poly-γ-glutamic acid, PGA) agents were also analyzed in the attempt to improve their characteristics.

2. Materials and Methods

2.1. Materials

Commercial WP isolate (~90% dry basis protein) was obtained from BioLine (London, UK), PGA was purchased from Xi'an Fengzu Biological Technology Co., Ltd. (Xi'an, China), and its molecular weight (between 29–30 kDa) determined by capillary viscometry according to Irurzun et al. [19]. GLY and all other reagents were purchased from Sigma (Kawasaki, Japan). Bitter vetch and grass pea seeds were obtained from a local market in Gallichio (Potenza, Italy). Soy protein concentrate was purchased from Laboratori Bio Line s.r.l. (Canaro, Roma, Italy).

2.2. Film Preparation

Film forming solutions (FFSs) were prepared by dissolving WP isolate in distilled water (1% WPs, *w/v*) and adjusting the pH (to 7 or 12) by 0.1 N NaOH addition. FFSs were heated or not at 80 °C for 25 min under continuous stirring. After cooling down at room temperature, GLY (30%, 40%, 50% *w/w* of WPs) and/or PGA (200 mg) were added just before casting 50 mL FFS into 8 cm diameter

polyester Petri dishes. The derived films were then obtained by drying at 25 °C and 45% RH for 48 h and analyzed within 24 h.

Protein concentrates from bitter vetch and grass pea seeds (containing 70%–80% of proteins determined by the Kjeldahl's method) were obtained as previously described [20,21] with some modifications. The respective flours were suspended in distilled water (10%, *w*/*v*), and then the pH was brought to pH 11 with 0.1 N NaOH. After stirring for 1 h, the suspensions were centrifuged for 20 min at 15,000× g at 4 °C and the pH of the resulting supernatants was brought to pH 5.4 with 0.1 N HCl. The obtained precipitates were dried in the climatic chamber at 25 °C and 45% RH. All FPSs were prepared by dissolving the plant protein concentrates (2 g), under stirring for 1 h, in 100 mL of distilled water alkalinized at pH 12 and containing 30% (*w*/*v*) GLY. The pH of some samples was adjusted at pH 7 with 0.1 N HCl. Finally, 50 mL of the differently diluted FPSs, containing 500 mg protein each, were poured in the Petri dishes and allowed to dry in an environmental chamber at 25 °C and 45% RH for 48 h. All the obtained films were analyzed within 24 h.

2.3. Zeta-Potential and Particle Size Measurements

1.0 mL of each WP containing FPS was analyzed for zeta-potential and particle size by using a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK). The device was equipped with a helium-neon laser of 4 mW output power operating at the fixed wavelength of 633 nm (wavelength of laser red emission). The instrument software programmer calculated the zeta-potential through the electrophoretic mobility by applying a voltage of 200 mV and by using the Henry equation [22].

2.4. Film Mechanical Properties

All dried films were cut into 1 cm × 8 cm strips using a sharp scissor and conditioned at 25 °C and 50% RH for 2 h by placing them into a glass chamber over a saturated solution of $Mg(NO_3)_2$ before being tested. Film thickness was measured in six different points with a micrometer (Electronic digital micrometer (Metrocontrol, Srl, Casoria, Italy; sensitivity 0.001 mm) and film tensile strength (TS), elongation at break (EB) and Young's modulus (YM) were determined on five specimens of each sample (5 cm gage length, 1 kN load and 5 mm/min speed) by using an Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA).

2.5. Film Transmittance and Transparency

Each WP film was cut into 1 cm × 4 cm strip and placed in a quartz cuvette and its whole light transmittance and absorbance spectra were obtained by using a Agilent UV-vis spectrophotometer (Santa Clara, CA, USA) in the range of 200–800 nm with a scan rate of 250 nm/min. The transparency analyses were performed as described by Galus and Kadzinska [23] by calculating film opacity as follows:

$$\text{Opacity} = A_{600nm}/X \quad (1)$$

where A_{600nm} was the absorbance at 600 nm and X was the film thickness (mm).

2.6. Film Moisture Content and Uptake

Moisture content test was performed by evaluating the mass loss of the film sample after 24 h at 105 °C as previously described [23]. Analyses in triplicate of each film were made and film moisture content was calculated as:

$$\text{Film moisture content (\%)} = (W_1 - W_2)/W_1 \times 100 \quad (2)$$

where W_1 is the initial weight of the film and W_2 is the film weight after drying.

Film moisture uptake tests were carried out by a gravimetric method described by Manrich et al. [24]. The analysis was performed by determining the mass of film samples after drying at 105 °C

for 24 h and the mass after the samples were put in a conditioning environment at RH 50% (saturated solution of $Mg(NO_3)_2$) for other 24 h. The moisture uptake was finally calculated as:

$$\text{Film moisture uptake (\%)} = (W_s - W_d)/W_s \times 100 \quad (3)$$

where W_s and W_d are the weight of swollen and dried films, respectively.

2.7. Statistical Analysis

All data were analyzed by means of JMP software 5.0 (SAS Institute, Cary, NC, USA), used for all statistical analyses. The data were subjected to analysis of variance, and the means were compared using the Tukey–Kramer HSD test. Differences were considered to be significant at $p < 0.05$.

3. Results and Discussion

3.1. Zeta-Potential and Particle Size

Micro- and nano-particle charge can be quantified by measuring their zeta potential, a quantitative parameter monitoring the particle mobility in an electrical field. It is known that FFS pH value and components may influence the zeta potential of the dissolved or suspended particles. The data reported in Table 1 indicate that both heated and unheated WP FFSs prepared at pH 7 and 12 were quite stable. In particular, zeta-potential values of the FFSs prepared at pH 12 were markedly more negative (around −36 mV) than those detected with FFSs prepared at pH 7 (around −25 mV), with an average particle size between 400 and 600 nm regardless of GLY concentrations and heat-treatment. Therefore, the WP FFSs prepared at pH 12 exhibit a higher stability due to the repulsive forces and, as consequence, should give rise to a more homogeneous distribution of WP nanoparticles during FFS drying and, potentially, to films with improved performance.

Table 1. Zeta-potential and Z-average measurements of whey protein (WP) film forming solutions (FFSs) prepared at pH 7 or 12 and subjected or not to heat treatment.

Whey Protein (WP) Film Forming Solutions (FFSs)	Z-Average (d.nm)		Zeta-Potential (mV)	
	pH 7	pH 12	pH 7	pH 12
+ 30% GLY, heated	147.1 ± 18.4 ^{a,b}	418.9 ± 31.9 ^{a}	−27.0 ± 1.0 ^{a}	−35.3 ± 2.1 ^{a}
+ 30% GLY	1127.0 ± 167.4 ^{c}	610.2 ± 56.5 ^{b}	−21.6 ± 0.4 ^{b}	−38.8 ± 2.6 ^{a}
+ 40% GLY, heated	110.5 ± 21.1 ^{b}	415.4 ± 6.7 ^{a}	−29.1 ± 0.6 ^{a}	−35.4 ± 2.8 ^{a}
+ 40% GLY	522.6 ± 102.5 ^{d}	519.2 ± 30.8 ^{b}	−22.9 ± 0.3 ^{b}	−35.9 ± 2.4 ^{a}
+ 50% GLY, heated	350.1 ± 13.8 ^{d}	403.9 ± 19.7 ^{a}	−27.0 ± 0.1 ^{a}	−36.2 ± 3.3 ^{a}
+ 50% GLY	516.3 ± 23.1 ^{d}	526.1 ± 23.1 ^{b}	−24.0 ± 0.4 ^{b}	−35.6 ± 2.9 ^{a}

Values are mean ±SD; means followed by different letters are significantly different from the values reported in the same column (Tukey–Kramer test, $p < 0.05$).

3.2. Film Mechanical Properties

After the analyses of the stability of all the WP FFSs prepared under different experimental conditions, the mechanical properties only of the handleable films obtained were investigated. In more detail, TS, EB, and YM of the films derived from the FFSs, heated and unheated, prepared at pH 12 in the presence of different amounts of GLY were determined. Conversely, it was possible to evaluate the mechanical properties only of the films derived from the FFSs heated and prepared at pH 7 in the presence of 40% or 50% GLY. In fact, very brittle films were obtained under the same experimental conditions in the presence of 30% GLY, whereas unmanageable sticky materials were obtained with unheated FFS at the same pH and at all GLY concentrations. The results reported in Table 2 indicate that the unheated FFSs, prepared at pH 12 and containing 50% GLY, led to obtain more flexible materials, as demonstrated by the highest EB and the lowest YM detected. In addition, these values were also much higher and lower, respectively, than those observed with counterpart films obtained at pH 7 from heated FFSs in the presence of 50% GLY (Table 3).

Table 2. Mechanical properties of whey protein (WP) films obtained at pH 12.

WP Films	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)	Thickness (μm)
+30% GLY, heated	3.40 ± 0.90 [a]	8.3 ± 4.8 [a]	71.6 ± 14.2 [a]	48 ± 7 [a]
+30% GLY	3.72 ± 0.61 [a]	4.6 ± 0.8 [a]	185.1 ± 16.2 [b]	38 ± 4 [a]
+40% GLY, heated	1.41 ± 0.09 [b]	21.3 ± 5.5 [b]	34.0 ± 7.4 [c]	44 ± 4 [a]
+40% GLY	2.72 ± 0.12 [a]	33.9 ± 8.5 [b]	86.2 ± 3.2 [a]	66 ± 2 [b]
+50% GLY, heated	0.60 ± 0.11 [c]	36.9 ± 10.8 [b]	22.7 ± 5.1 [c]	85 ± 5 [c]
+50% GLY	1.11 ± 0.12 [b]	61.6 ± 8.6 [c]	24.1 ± 4.7 [c]	83 ± 2 [c]

Values are mean ±SD; means followed by different letters are significantly different from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

Table 3. Mechanical properties of whey protein (WP) films obtained at pH 7.

WP Films	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)	Thickness (μm)
+40% GLY, heated	2.81 ± 0.72 [a]	3.6 ± 0.7 [a]	350.5 ± 63.4 [a]	96 ± 6 [a]
+50% GLY, heated	3.20 ± 0.21 [a]	20.8 ± 4.4 [b]	164.7 ± 77.5 [b]	129 ± 35 [a]

Values are mean ±SD; means followed by different letters are significantly different from the values reported in the same column (Tukey—Kramer test, $p < 0.05$).

Among the numerous studies on WP-based films recently reviewed by Zink et al. [13], only few investigations were carried out under experimental conditions similar to those described in the present paper. It has been reported that (i) films obtained from native WP isolates dissolved in water, and plasticized with 30% GLY, were weaker and less extendible than films obtained with heat-denatured WPs, and (ii) the pH (in the range 3–8) of the FFS did not influence the mechanical properties of films made with both native and heat-denatured WPs [25]. The present results, conversely, indicate that WPs denatured at alkaline pH give rise, in the presence of 50% GLY, to films still resistant (TS more than 1 MPa) but more stretchable (EB over 60% and a YM of 24.1 MPa) than those previously obtained at lower pH with heat-denatured WPs and 30% GLY, which exhibited only 8%–18% EB and a YM in the range of 141–472 MPa [25].

Finally, the only way to obtain WP films at pH values lower than 12, without a previous protein denaturation, needed the presence of further additives to the FFS. Table 4 reports the mechanical properties of films prepared in the pH range between 6 and 12 with unheated WPs but in the presence of not only of a plasticizing (GLY) but also of a structuring agent (PGA) [26].

Table 4. Effect of poly-γ-glutamic acid (PGA) on the mechanical properties of whey protein (WP) films prepared at different pHs and containing 50% GLY *.

Film Additive	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)	Thickness (μm)
None, pH 6	ND	ND	ND	ND
PGA, pH 6	0.05 ± 0.01 [a]	1.4 ± 0.3 [a]	149.0 ± 21.4 [a]	45 ± 5 [a]
None, pH 8	ND	ND	ND	ND
PGA, pH 8	1.31 ± 0.60 [b]	2.1 ± 0.4 [a]	155.8 ± 22.7 [a]	70 ± 8 [b]
None, pH 10	ND	ND	ND	ND
PGA, pH 10	0.90 ± 0.20 [b]	1.8 ± 0.6 [a]	124.1 ± 22.8 [a]	73 ± 12 [b]
None, pH 12	1.11 ± 0.10 [b]	61.6 ± 8.6 [b]	24.1 ± 4.7 [b]	83 ± 2 [c,b]
PGA, pH 12	1.11 ± 0.21 [b]	3.1 ± 0.8 [a]	104.9 ± 2.8 [a]	98 ± 11 [c]

* All film forming solutions (FFSs) prepared in the absence of glycerol (GLY) did not give rise to handleable films. Values are mean ±SD; means followed by different letters are significantly different from the values reported in the same column (Tukey–Kramer test, $p < 0.05$); ND, not detectable values because of the unhandleable materials formed.

However, the feature to give rise to plasticized films at pH 12 but not at pH 7, perfectly manipulable, and thus suitable to be studied, seems to be quite specific of WPs. In fact, other protein-based films, such as those derived from bitter vetch, grass pea, and soy seed proteins, were easily obtained at both pH values, in the presence of only 30% GLY, and their mechanical properties also investigated (Table 5).

Table 5. Mechanical properties of glycerol (GLY)-plasticized films obtained at pH 7 and 12 from plant protein sources.

Film Protein Source	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)	Thickness (μm)
Bitter vetch, pH 7	1.73 ± 0.27 ^{a}	68.6 ± 14.7 ^{a}	61.5 ± 15.0 ^{a}	83 ± 8 ^{a}
Bitter vetch, pH 12	2.01 ± 0.28 ^{a}	87.9 ± 19.0 ^{a}	47.9 ± 10.9 ^{a}	99 ± 9 ^{a}
Grass pea, pH 7	8.59 ± 0.41 ^{b}	68.3 ± 30.2 ^{a}	483.0 ± 62.9 ^{b}	110 ± 9 ^{a}
Grass pea, pH 12	4.08 ± 0.39 ^{c}	35.0 ± 12.6 ^{b}	418.0 ± 44.9 ^{b}	117 ± 10 ^{a}
Soy, pH 7	1.59 ± 0.36 ^{a}	105.3 ± 17.7 ^{c}	87.4 ± 19.6 ^{a}	82 ± 7 ^{a}
Soy, pH 12	11.40 ± 1.80 ^{d}	16.8 ± 8.7 ^{b}	574.8 ± 43.3 ^{b}	43 ± 3 ^{b}

Values are mean ±SD; means followed by different letters are significantly different from the values reported in the same column (Tukey–Kramer test, $p < 0.05$).

3.3. Film Transparency

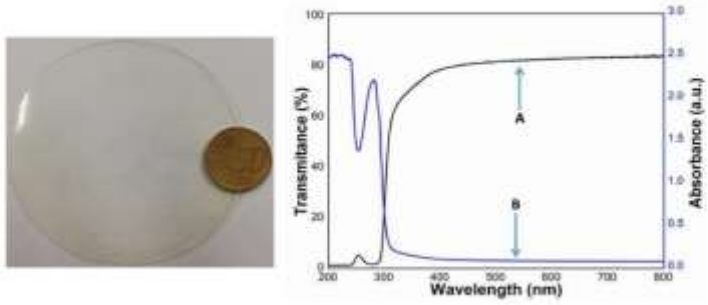
Since the appearance of the coated products plays an important role in consumer acceptability, the opacity of the obtained WP films was evaluated by measuring light transmission through the films at a wavelength of 600 nm [23]. Table 6 clearly indicates that only slight differences were detected between the films prepared at pH 12 and pH 7. Nevertheless, opacity was observed to significantly change, being higher in the films obtained at pH 12 without FFS heating and, when FFS was heated, at higher GLY concentrations. The opacity values detected, which resulted from the same order of magnitude of those of films prepared by Galus and Kandiska at pH 7 from a heated WP isolate [27], were also compared with ones exhibited by traditional commercial plastics such as cellulose triacetate and polypropylene, which resulted more and much less transparent, respectively, than the WP films.

Table 6. Opacity of whey protein (WP)-based films obtained under different experimental conditions.

Whey Protein (WP) Films	Opacity (A_{600}/mm)
+30% GLY, heated, pH 12	1.18 ± 0.64 ^{a}
+30% GLY, pH 12	2.65 ± 0.11 ^{b}
+40% GLY, heated, pH 12	1.23 ± 0.05 ^{a}
+40% GLY, pH 12	2.07 ± 0.25 ^{a,b}
+50% GLY, heated, pH 12	1.57 ± 0.13 ^{a,b}
+50% GLY, pH 12	2.20 ± 0.81 ^{a,b}
+40% GLY, heated, pH 7	1.66 ± 0.01 ^{a,b}
+50% GLY, heated, pH 7	1.27 ± 0.01 ^{a}
Polypropylene *	32.02 ± 3.35
Cellulose triacetate *	0.54 ± 0.09

* Values from Giosafatto et al. [21]. Values are mean ±SD; means followed by different letters are significantly different from the other values (Tukey–Kramer test, $p < 0.05$).

It is worthy to note that no differences were observed among the absorbance and transmittance profiles obtained analyzing the various films prepared at different GLY concentrations and pH values, as well as with heated or unheated WPs. A typical film obtained from an FFS prepared at pH 12 and containing unheated WPs and 50% GLY is shown in Figure 1, together with its whole transmittance and absorbance spectra. Furthermore, the low transmittance of all the films at UV wavelengths should be considered a further interesting feature of the WP-based materials, being potentially able to prevent possible physicochemical alterations of coated/wrapped foods or drugs.



(a) (b)

Figure 1. Film obtained at pH 12 with unheated whey proteins (WPs) in the presence of 50% glycerol (GLY) (a) and its whole transmittance (A) and absorbance (B) spectra (b).

3.4. Film Moisture Content and Moisture Uptake

The prepared WP films were also analyzed for moisture content and moisture uptake, as these features are important for food packaging applications, particularly when the water activity is high or when the film should act as a food protective barrier [28]. In fact, a high moisture content of the coating material considerably limits its use for packaging foods. The results reported in Table 7 showed that the moisture content of the films prepared at pH 12 decreased when the FFS was previously heated, as well as when the amount of GLY was lower in both heated and unheated samples. Conversely, film water uptake did not seem to significantly change in all films cast at pH 12. Finally, the moisture uptake values were found significantly lower when the films were prepared at pH 7.

Table 7. Moisture content and uptake of whey-protein (WP)-based films obtained under different experimental conditions.

Whey Protein (WP) Film	Moisture Content (%)	Moisture Uptake (%)
+30% GLY, heated, pH 12	15.24 ± 1.32^{a}	13.31 ± 1.00^{a}
+30% GLY, pH 12	20.35 ± 1.20^{b}	15.05 ± 0.73^{a}
+40% GLY, heated, pH 12	18.39 ± 1.94^{b}	15.70 ± 0.04^{a}
+40% GLY, pH 12	25.65 ± 0.69^{c}	15.98 ± 2.10^{a}
+50% GLY, heated, pH 12	18.93 ± 3.30^{b}	14.90 ± 0.66^{a}
+50% GLY, pH 12	29.50 ± 2.30^{d}	16.56 ± 0.77^{a}
+40% GLY, heated, pH 7	21.43 ± 0.32^{b}	9.12 ± 0.85^{b}
+50% GLY, heated, pH 7	33.27 ± 0.50^{e}	9.01 ± 0.72^{b}

Values are mean ±SD; means followed by different letters are significantly different from the values reported in the same column (Tukey–Kramer test, $p < 0.05$).

4. Conclusions

Since it is known that denaturation and aggregation of WPs are pH dependent, with strong alkalis producing rod-like microstructures able to form fine-stranded fiber-like matrices, the possibility of obtaining GLY-plasticized materials by using WP isolate treated at pH 12 without heating was investigated and demonstrated. Conversely, at pH 7, it was necessary not only to previously heat at 80 °C for 25 min the WP-containing FFS, but also to increase to at least 40% the GLY concentration to obtain handleable films. The developed experimental conditions allowed the production of hydrocolloid films with higher flexibility with respect to the WP-based films so far obtained at pH 7 following FFS heat treatment, probably because WPs denatured under alkaline conditions form small primary aggregates able to combine into large clusters [29].

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3.2. Preparation and characterization of transglutaminase-crosslinked whey protein films

Further experiments were focused on the use of the enzyme mTG to improve the technological properties of WP-based bioplastics. mTG is an enzyme able to catalyze the formation of intra- and intermolecular isopeptide bonds between endoprotein glutamine (Gln) and lysine (Lys) residues[34]. Several preliminary experiments were carried out to study the best conditions to produce mTG crosslinked WP-based bioplastics. The extent of mTG-mediated crosslinking was assessed by means of SDS-PAGE analysis on the basis of the decrease of α-lactalbumin (Mr ~ 14.2 kDa) and β-lactoglobulin (Mr ~ 17.4 kDa) protein bands and the concomitant increase of both high molecular weight polymer formation and dark smearing. It is worthy to note that the only treatment of WPs, under alkaline conditions (pH 12), leading to protein denaturation, was not sufficient for WPs to act as an effective mTG substrate.

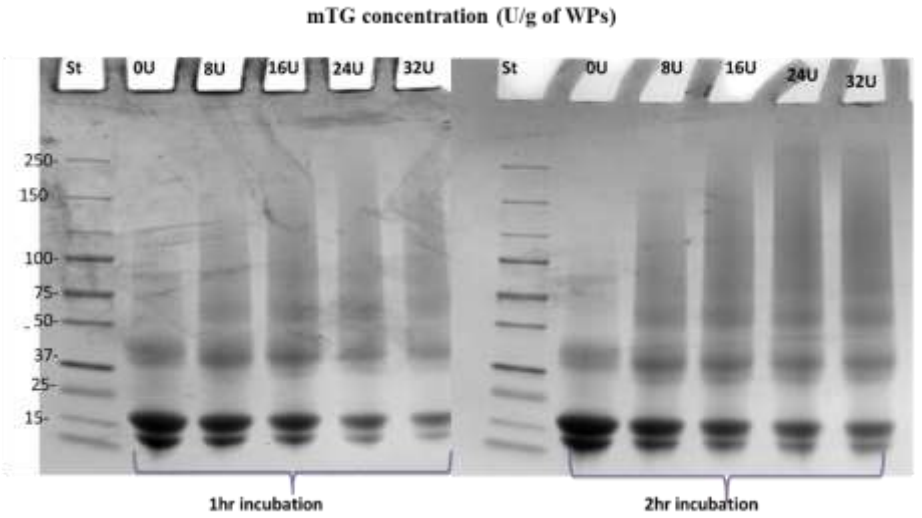

Figure 8: SDS-PAGE of (1%, w/v) WP-containing FFSs, previously treated under alkaline pH with different concentrations of mTG (U/g of WPs)

In fact, the results shown in Figure 8 demonstrated that WPs were barely crosslinked by mTG. Hence, WP heat pre-treatment,

performed for 1 h at 80°C, was crucial as it allowed to expose Gln and/or Lys endoprotein reactive residues to the enzyme. The results obtained under these experimental conditions demonstrated that the incubation of heat-treated WPs at pH 7.0 for 2 h in the presence of mTG was effective in modifying both β-lactoglobulin and α-lactoalbumin (Figure 9).

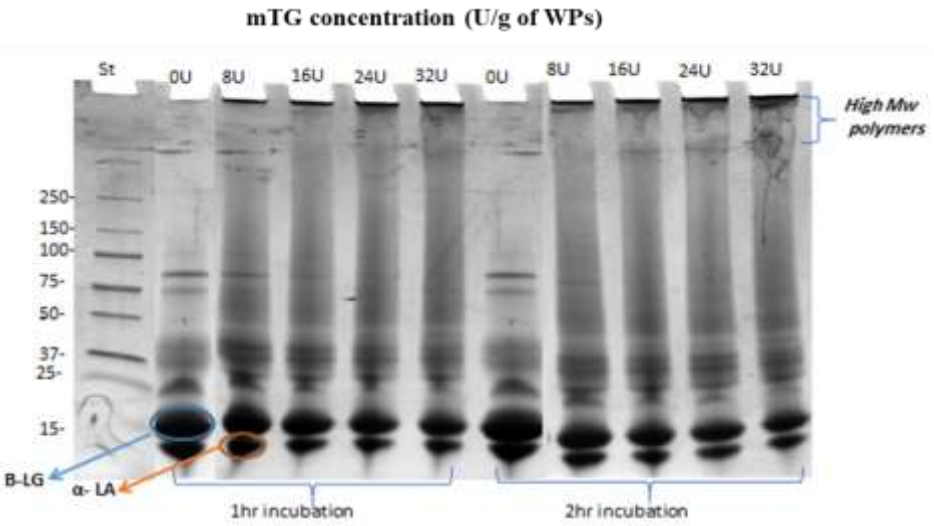

Figure 9: SDS-PAGE of 1% (w/v) heat-treated WPs incubated with different concentrations of mTG (U/g of WPs)

Following mTG modification, the pH was adjusted to pH 12 since, as reported in Abdalrazeq et al.[26], the alkaline pH led to obtain handleable, transparent and more flexible films. GLY (40%, w/w of WP) used as plasticizer was finally added and films were obtained by casting the obtained WP-based FFSs. Preliminary characterization of FFSs was carried out, including the measuring of the FFS stability and particle size (Table 1) and, then, the derived films were prepared and characterized for their mechanical features (Table 2). Zeta potential measurements (Table 1) indicated that the FFSs, either incubated or not with mTG, were quite stable, whereas the mean particle size was affected by the enzyme addition being higher for the samples enzymatically treated, likely as a consequence of the formation of protein isopeptide bonds. Our

results are in agreement with the results reported by Giosafatto et al 2018[104], even though WP-FFSs were more stable than grass pea proteins studied by these authors.

Table 1: Zeta potential and mean particle size of WP (1%, w/w) FFSs containing different concentrations (U/g of WPs) of mTG in the presence of 30% GLY*

WP FFSs	Z- average (d.mn)	ζ-potential (mV)
- mTG	171 ± 7 ^{a}	-33.8 ± 2.5 ^{a}
+ 8 U/g mTG	268 ± 11 ^{b}	-38.8 ± 1.4 ^{b}
+ 16 U/g mTG	263 ± 17 ^{b,c}	-30.5 ± 1.8 ^{a}
+ 24 U/g mTG	182 ± 7 ^{a,c}	-41.8 ± 2.8 ^{b}
+ 32 U/g mTG	233 ± 12 ^{d}	-33.1 ± 2.2 ^{a}

*Values are mean ±SD; the different letters indicate significant differences from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

As far as film characterization, as it is possible to see from Table 2, the mTG containing materials showed an increase in film thickness likely due to an increase of the free volume inside the matrix caused by the isopeptide bond formation catalyzed by the enzyme. Mechanical properties analyses showed that mTG slightly enhanced the EB and reduced the YM, indicating that the enzyme was able to produce more flexible and less stiff films (Table 2).

Table 2: Mechanical properties of films obtained with WPs (500 mg) in the presence of 30% GLY and different concentrations (U/g of WPs) of mTG*

WP films	Tensile strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)	Thickness (μm)
- mTG	2.4 ± 0.6 ^{a}	10.4 ± 4.9 ^{a}	164.7 ± 48.4 ^{a}	112 ± 28 ^{a,b}
+ 8 U/g mTG	1.1 ± 0.2 ^{b}	16.7 ± 5.2 ^{a,b}	33.9 ± 5.0 ^{b}	92 ± 6 ^{b}
+ 16 U/g mTG	0.8 ± 0.2 ^{b}	19.1 ± 6.1 ^{a,b}	25.7 ± 4.5 ^{b}	150 ± 18 ^{a}
+ 24 U/g mTG	0.8 ± 0.2 ^{b}	27.6 ± 8.9 ^{c}	26.6 ± 4.8 ^{b}	122 ± 8 ^{a,b}
+ 32 U/g mTG	0.9 ± 0.1 ^{b}	17.9 ± 3.3 ^{b}	28.6 ± 6.3 ^{b}	156 ± 14 ^{c}

*Values are mean ±SD; the different letters indicate significant differences from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

From the results reported above it was possible to state that 24 U/g was the best concentration of enzyme since a reduction of mechanical properties was observed when a higher amount (32 U/g) was exploited (Table 2). In addition, from a macroscopically point of view, all the films, either untreated or treated with 24 U/g of enzyme, were very similar appearing in both cases transparent and heandleable (Figure 10).

Figure 10: WP (500 mg)-based films prepared in the absence (left) and presence (right) of 24 U/ g mTG

Furthermore, the results of the viscosity test are, showed in Figure 11, indicate that a significantly increased value was obtained with the WP FFS containing 24 U/g mTG. These data confirm the protein polymerization due to the formation of intermolecular isopeptide bonds between endo-protein Gln and Lys residues.

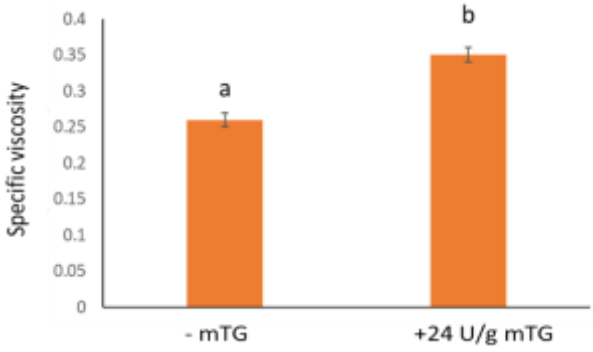

Figure 11: Viscosity of heat-denatured WP (1%, w/w) FFSs prepared either in the absence or presence of 24 U/g of mTG, Values are mean ±SD; the different letters indicate significant differences from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

Further experiments were carried out by using 24 U/g of mTG and by increasing GLY concentration up to 40%. The results reported in Table 3 show that the films prepared under these experimental conditions had an increased resistance and extensibility.

Table 3: Mechanical properties of WP (500 mg)-based films obtained in the presence of 40% GLY after FFS treatment with 24 U/g mTG*

Film	Tensile strength (MPa)	Elongation at break (%)	Young's Modulus (MPa)	Thickness (µm)
- mTG	1.4 ± 0.2 [a]	34.3 ± 3.9 [a]	29.2 ± 4.9 [a]	79 ± 2 [a]
+ mTG (24U/g)	2.2 ± 0.1[b]	49.8 ± 5.3 [b]	50.6 ± 2.6 [b]	77 ± 1 [a]

*Values are mean ±SD; the different letters indicate significant differences from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

It was proved that mTG pretreatment of WPs had a slight but significant effect also on the moisture content and uptake of the WP-based films (Table 4), likely due to the more homogenous and tighter structure created by mTG-catalyzed isopeptide bonds in the WP matrix.

Looking for customers acceptability, film transparency was also investigated. mTG treatment was also shown to significantly improve the transparency of the produced bioplastics, as demonstrated by the reduction of the film opacity (Table 4).

Finally, since CO_2, O_2 and WV permeability properties are important parameters for assessing an industrial application of biodegradable materials, the barrier effects of the prepared films were investigated. The results summarized in Table 4 show that mTG strongly increased CO_2 and O_2 barrier properties of the mTG-crosslinked films, whereas WV permeability was not affected at all by the enzyme treatment. Following studying the above described variables affecting the formation and properties of WP-based films, the developing of additional WP-based bioplastics has been carried out using different strategies and additives in the attempt to further

improve the biological as well as the physico-chemical features of the WP-based biodegradable materials.

Table 4: Functional properties of films obtained with heated WPs (500 mg) cast at pH 12 in the presence of 40% GLY and containing 24 U/g of mTG*

Film	Moisture content (%)	Moisture uptake (%)	Opacity	Permeability		
				CO_2	WV	O_2
				(cm^3 mm m^{-2} day^{-1} kPa^{-1})		
- mTG	22.1 ± 0.9 [a]	12.2 ± 0.9 [a]	1.9 ± 0.6 [a]	0.90 ± 0.06 [a]	0.04 ± 0.01 [a]	0.26 ± 0.02 [a]
+ 24 U/g mTG	18.8 ± 1.0 [b]	10.6 ± 0.5 [b]	1.2 ± 0.1 [a]	0.14 ± 0.02 [b]	0.04 ± 0.01 [a]	0.02 ± 0.01 [b]

*Values are mean ±SD; the different letters indicate significant differences from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

3.3. Whey protein-based film grafted with poly (3-hydroxybutyrate-co-hydroxyhexanoate) nanoparticles

More recently, much attention has been given to the production of biopolymer composites where at least one of their components has nanometric dimensions (1–100 nm)[105]. Such nano-reinforcement is due to NPs of different chemical nature added to a variety of biopolymers with the aim to obtain nanocomposites with improved mechanical and other chemico-physical properties. In this context, we have focused on NPs derived from the aliphatic polyesters PHAs (PHBHHx), since one of the purposes of the present project was to produce WP-based films grafted with PHA NPs (PHBHHx). This experimental part was carried out in collaboration with Dr. Iolanda Corrado, attending the same 33rd Ph.D. cycle, who focused on the preparation and characterization of the PHA-NPs. The results obtained by using PHBHHx demonstrated that this strategy was able to enhance some properties of the WP-based films as it is possible to see in the following published article.

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Design and characterization of poly (3-hydroxybutyrate-co-hydroxyhexanoate) nanoparticles and their grafting in whey protein-based nanocomposites

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ABSTRACT

This work succeeded in the preparation of a nano-biocomposite material based on the use of poly-3-hydroxybutyrate-co-hydroxyhexanoate nanoparticles (PHBHHx-NPs) within a scaffold of whey protein (WP) based films. The experimental conditions for PHBHHx-NPs preparation by solvent-evaporation technique were set up, and the obtained NPs characterized. Dynamic light scattering analyses showed that PHBHHx-NPs are stable, exhibiting a zeta-potential value close to −40 mV and a Z-average size of 80 nm. Morphological characterization by transmission and scanning electron microscopy confirmed nanoparticle average dimensions. The addition of PHBHHx-NPs to WP-based films improved the mechanical properties of the derived bioplastics, producing more extensible materials preserving their mechanical resistance. The grafting of PHBHHx-NPs as material fillers also enhanced the film barrier properties towards O_2, the permeability to both water vapor and CO_2 remaining unaffected.

1. Introduction

The redesigning of plastic materials under a "green perspective" is, nowadays, necessary to address the issue of plastic pollution as well as of shortening of fossil resources. In the bioplastics scenario, materials derived from natural bio-based polymers (i.e. polysaccharides, proteins) or produced by bacteria from renewable resources (*i.e.* polyhydroxyalkanoates, PHA) play a key role, due to their renewable origin and biodegradability features. In particular, the formulation of new biobased composites based on the combination of different classes of biopolymers represents an area of increasing interest for numerous application fields (Li, Yang, & Loh, 2016; Raza, Abid, & Banat, 2018; Zhang, Shishatskaya, Volova, da Silva, & Chen, 2018).

PHAs are a family of polyesters accumulated by various bacteria as carbon and energy storage under stressful conditions. Due to their intrinsic biodegradability and demonstrated biocompatibility (Elmowafy et al., 2019; Koller, 2018), PHA found a range of applications, from food packaging to biomedical sectors. The spectrum of properties displayed by PHA-derived materials is very close to that of conventional petro-plastics, and is dependent on their monomer structure and relative content, which influence the physicochemical parameters of the biopolymers as well as their kinetics of biodegradation (Chanprateep, Buasri, Muangwong, & Utiswannakul, 2010). In the last decade, different PHAs (polyhydroxybutyrate, PHB; poly-3-hydroxybutyrate-co-hydroxyvalerate, PHBV; poly-3-hydroxybutyrate-co-hydroxyhexanoate, PHBHHx) have been exploited for the preparation of nanoparticles (NPs), able to encapsulate and release various drugs (Kalia, 2019; Kiliay et al., 2011; Murueva, Shishatskaya, Kuzmina, Volova, & Sinskey, 2013; Naureen et al., 2015; Sandoval, Rivera, Barrera-Rivera, & Martínez-Richa, 2010; Shrivastav, Kim, & Kim, 2013). Among the different polymers, PHBHHx displays peculiar elastic properties, thus emerging as promising biopolymeric source for the designing of nanocomposites with improved flexibility (Raza et al., 2018; M.; Vastano et al., 2017; Vastano, Corrado, Sannia, Solaiman, & Pezzella, 2019).

However, despite their potential, the effective exploitation of PHA-based materials is still hindered by their high production costs.

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Consequently, sustainable strategies for PHA production are based on the use of waste materials as starting feedstock for microbial fermentation and are considered even more effective if integrated in a biorefinery concept (Kumar & Kim, 2018; Nugroho Prasetyo et al., 2010; Vastano et al., 2019). In this regard, an engineered *Escherichia coli* strain able to produce PHBHHx (40:60 M ratio) was developed (Vastano et al., 2017) and its ability to accumulate the biopolymer through the valorisation of waste materials was recently demonstrated (Vastano et al., 2019).

Another class of renewable biomacromolecules that deserves a special attention is represented by proteins derived from agro-food by-products as they are quite abundant and their recovery might contribute to waste reduction. In particular, it is important to mention proteins present in the whey, a by-product of dairy industries produced in huge amounts following milk casein coagulation. The total worldwide whey production is estimated to be more than 180 million tons/year, the major amount (approximately 70%) coming from the EU and USA (Yadav et al., 2015). Only 50% of whey is recycled and converted into high added value products (Mollea, Marmo, & Bosco, 2013). For both the large volume and the high organic content the direct disposal of whey is not environmental friendly and it is virtually forbidden all over the world. The great majority of whey proteins (WPs) is represented by β-lactoglobulin and α-lactalbumin, endowed with different features such as solubility, foaming, and gelling ability (Norwood, Croguennec, Le Floch-Fouéré, Schuck, & Jeantet, 2018) suitable for several biotechnological applications. In particular, it was recently found out that under alkaline conditions these proteins have the ability to give rise to promising edible bioplastic materials (Abdalrazeq et al., 2019). However, WP-based films, like all the hydrocolloid films, suffer from limitations in barrier and mechanical features, often requiring additives to improve resistance to moisture transfer as well as to enhance their flexibility (Ramos et al., 2012). Many authors investigated the possibility to improve the features of WP-based films by blending them with polysaccharides. Addition of polysaccharides has a significant effect on the physical properties of protein-based edible films (Cieśla, Salmieri, & Lacroix, 2006). In this respect, Di Pierro et al. (2013) (Di Pierro et al., 2013) examined the behaviour of pectin and thermally denatured WPs at various protein/polysaccharide ratios and at various pH values. The same authors also investigated the effect of the addition of chitosan to WP-formulations, in order not only to enhance the film technological properties but also to confer antimicrobial activity to the derived bioplastics thus extending fresh dairy product shelf-life (Di Pierro, Sorrentino, Mariniello, Giosafatto, & Porta, 2011). Moreover, further investigations have been focused on the use of different NPs, such as the ones prepared by nanocellulose, usually used to improve the properties of diverse biomaterials (Sharif Hossain, Uddin, Veettil, & Fawzi, 2018). As matter of fact, cellulose nanostructures have been most usually applied as reinforcing phases, but they may also be used as matrices for a variety of materials including films for food packaging applications (Azeredo, Rosa, & Mattoso, 2017). As far as WP-based films reinforcement some efforts have been made by using porous silica (SiO_2) coated titania (TiO_2) NPs (TiO_2@@SiO_2) (Kadam et al., 2013), and it was demonstrated that the incorporation of TiO_2@@SiO_2 NPs helps to improve WP-based film mechanical properties. However, the optical properties of the WP films were dramatically modified following incorporation of these NPs, changing from a transparent appearance to an opaque one, even though the embedded NPs contributed effectively to increase the thickness of films and to alter the contact angle of their surface. Therefore, the development of high strength, flexible nano-biocomposites controlling nanoparticle properties, their volume fraction and their topographic distribution within the scaffold still remains a significant challenge.

With the aim to decrease the hydrophilicity of WP-based materials and, consequently, to improve their performance, the possibility to prepare nano-biocomposites by blending WP-proteins with NPs obtained from PHBHHx has been studied. In this paper, the optimal formulation of PHBHHx-NPs into the whey protein film matrix was explored and the technological properties of the derived films investigated.

2. Material and methods

2.1. Materials

Commercial WP isolate (−90% dry basis protein) was obtained from BioLine (New Zealand), Glycerol (GLY) and all other reagents were of analytical grade and they were purchased from Sigma (Steinheim, Germany).

2.2. Recombinant production of PHBHHx

For PHBHHx production, recombinant *Escherichia coli* strain, LipoB, was cultured in an Eppendorf NewBrunswick, BioFlo/CelliGen®115 according to (Vastano et al., 2017). LipoB strain was grown in Luria-Bertani (LB) broth at 37 °C, supplemented with ampicillin (100 μg mL^{-1}). After 12 h, the pre-inoculum was transferred in M9 medium at OD_{600nm} of 0.1. The composition of M9 medium is as follows (for 1 L): 100 mL M9 salt [10x]; 0.1 mL of $CaCl_2$ [1 mol L^{-1}]; 5 mL of Glucose [40%]; 2 mL of $MgSO_4$ [1 mol L^{-1}]. Composition of M9 salt [10x] for 1 L [pH 7.4] is as follows: 60 g Na_2HPO_4; 30 g KH_2PO_4; 5 g NaCl; 10 g NH_4Cl. The media were completed after sterilization with the carbon source and other components were prepared in a concentrated solution. A stock solution [20 g L^{-1}] of yeast extract was autoclaved and then added to complete medium at 2 g L^{-1}. M9 was supplemented with 0.01 mM of $FeSO_4$. Sodium octanoate was prepared in a stock solution of 40 [g L^{-1}] and sterilized by filtering. This additional carbon source was added to the cultures (0.1 w/v) as extra-volume. Induction of protein expression was performed after 5 h from the inoculum with 0.5 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside). Cultures were carried out for additional 48 h. At the end of the batch fermentation, cells were recovered by centrifugation and solvent extracted with chloroform by using Soxhlet apparatus (100 mL for gram of lyophilized cells). The dissolved biopolymer was then precipitated by the dropwise addition of cold methanol (10 vol). The polymer was collected by centrifugation (5500 *g*, 30 min at 4 °C), recovered using fresh chloroform and dried under a N_2 flux at 21 °C. The purified polymer was stored at 4 °C until further use.

2.3. Synthesis of PHBHHx-NPs

Synthesis of PHBHHx-NPs was carried out using solvent evaporation protocol. Briefly, polymer was dissolved in chloroform at different concentrations and was then added to 5 mL of aqueous solution of sodium dodecyl sulphate (SDS) (4.44 mg/mL). The mixture was stirred at room temperature for an hour on a magnetic stirring plate, resulting in monodisperse chloroform droplets containing the dissolved polymer, separated from water by the SDS. Sonication (Sonopuls HD 2070; with standard horn) was then used (40% amplitude, 5 s pulse, 8 min) to emulsify the droplets and form the PHBHHx-NPs. Chloroform was removed by evaporation leaving the emulsion at room temperature under stirring for 24 h. Residual chloroform was removed in a rotary evaporator under partial vacuum at RT. NPs were recovered by centrifugation (5500 *g*, 10', 4 °C), resuspended in 10 mL of distilled water and filtrated using sterile filter paper disk (0.45 μm). Different organic: aqueous phase ratios, and SDS to PHBHHx mass ratios were assessed (see Results and discussion). The yield of recovered NPs was calculated as: $Y = M_P{*}100/M_{NP}$, where M_{NP} is the mass of recovered NPs, and M_P the amount of polymer used for their preparation.

2.4. Film preparation

Film forming solutions (FFSs) were prepared by dissolving WP

isolate (500 mg) in 25 mL distilled water and adjusting the pH to 12 by 0.1 N NaOH addition. The derived solutions were stirred for 1 h and, then, aqueous dispersion of PHBHHx-NPs (1, 2, 4 or 8%, w/w of WPs) was added under continued stirring at 700 rpm for 15 min. GLY (40%, w/w of WPs), used as a plasticizer, was finally added, under continued stirring for 15 min, just before casting. Each FFS (25 mL) was poured into 8 cm diameter polyester Petri dishes and the films were then obtained by drying all the FFSs at 25 °C and 45% RH for 24 h.

2.5. Zeta potential and particle size measurements

PHBHHx-NPs dispersion (0.1 mg/mL) in water and 1.0 mL of each WP-based FFSs were analysed for zeta potential and particle size by using a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK). The device was equipped with a helium-neon laser of 4 mW output power with a fixed wavelength of 633 nm (wavelength of laser red emission). The instrument software programmer calculated the zeta potential through the electrophoretic mobility by applying a voltage of 200 mV and using the Henry equation.

2.6. Film mechanical properties

All dried films were cut into 1 cm × 8 cm strips by using a sharp scissor, conditioned at 25 °C and 50% RH for 24 h by placing them into a glass chamber over a saturated solution of $Mg(NO_3)_2$ before being tested. Film thickness was measured in six different points with a micrometer (Electronic digital micrometer, DC-516, sensitivity 0.001 mm) and film tensile strength (TS), elongation at break (EB) and Young's modulus (YM) were determined on five specimens of each sample (5 cm gage length, 1 kN load and 5 mm/min speed) by using an Instron universal testing instrument model no. 5543 A (Instron Engineering Corp., Norwood, MA, USA).

2.7. Film permeability

Gas (CO_2 and O_2) and water vapor permeability (WWP) was determined using a modification of ASTM Standard Method D 3985–8126 with MultiPerm apparatus (ExtraSolutions s.r.l., Pisa, Italy). The samples, duplicates of each film, were conditioned for 2 day at 50% RH before measurement. Aluminum masks were used to reduce film test area to 5 cm^2. The testing was performed at 25 °C under 50% RH.

2.8. Film transparency

The opacity of each sample was investigated as described by Shevkani et al. (2015) (Shevkani & Singh, 2015). This method is based on the measurement of absorbance at 600 nm (spectrophotomer UV/Vis SmartSpec 3000 Bio-Rad, Segrate, Milan, Italy) divided by the thickness (mm). All the samples were cut into pieces of 1 cm × 3 cm and they were let adhere perfectly to the wall of the cuvette. For studying the transmittance, film strips (1 cm × 4 cm) were placed in a quartz cuvette and their whole light transmittance was obtained by using an Agilent UV–vis spectrophotometer (Santa Clara, CA, USA) in the range of 200–800 nm with a scan rate of 250 nm/min.

2.9. Scanning electron microscopy (SEM)

A field emission scanning electron microscope (FE-SEM, FEI Nova NanoSEM450) was used to study the morphology of both films (cross-section and surface) and PHBHHx-NPs. For cross section imaging, the films were previously frozen using liquid nitrogen and then cryo-fractured. To investigate the PHBHHx samples, a droplet of NPs (suspension in water) was deposited on carbon stickers on aluminum stubs and then dried at room temperature. The images were acquired using an incident electron beam energy between 2 and 5 kV and by collecting secondary electrons (SE) with an ETD or TLD detector.

2.10. Transmission electron microscopy (TEM)

Samples for TEM analysis were prepared by placing a drop of a water dispersion of the PHBHHx-NPs on a carbon-coated copper TEM grid and allowing the solvent to evaporate. TEM images were collected using a FEI TECNAI G2 S-twin apparatus operating at 120 kV (LaB_6 source).

2.11. Statistical analysis

The results were analysed statistically using the JMP software 5.0 (SAS Institute, Cary, NC, USA). Arithmetic means and mean square errors were calculated in all cases. Data are referred to experiments carried out in triplicate, both for NPs preparation and film characterization. Significant differences in average values were tested using the Tukey-Kramer HSD test (significance level: $P < 0.05$).

3. Results and discussion

3.1. Characterization of PHBHHx-NPs

PHBHHx-NPs were prepared using the solvent evaporation technique, by dispersing a polymer solution in the aqueous phase containing SDS as surfactant. The effect of different parameters on the Z-average size, polydispersity index (PdI) and zeta potential of PHBHHx-NPs was assessed (Table 1). Taking the SDS concentration invariable, the polymer amount was reduced from 50 to 5 mg mL^{-1}, keeping constant the volume of the organic phase (0.25 mL). This allowed to explore a SDS: PHBHHx mass ratio, ranging from 1.8 to 17.6 mg of surfactant for mg of polymer (Table 1, trials A-D). The highest polymer concentration resulted into the greatest particle size (trial A). The increase of the SDS: PHBHHx mass ratio corresponds to a decrease into particle size down to 82 nm (Trial D), while the PdI is almost comparable (Trials B-D) and indicative of very monodisperse NPs. In all the tested conditions, PHBHHx-NPs revealed a good stability, with zeta potential values < -30 mV. An increase in the NPs recovery efficiency was also observed increasing the SDS: PHBHHx ratio. Another variable explored in PHBHHx-NPs preparation, was the organic: aqueous phase ratio. The effect of this parameter was tested, keeping constant the volume of the aqueous phase and increasing the organic one from 0.5 to 2.5 mL (Table 1, Trials E-G). In these conditions, the same amount of PHBHHx (12.5 mg) was used, leaving constant the SDS: PHBHHx mass ratio. However, particle size was almost unaffected by decreasing the solvent ratios, achieving a diameter of about 110 nm, whilst the PdI slightly increased from trials E to G (remaining always < 0.2), thus indicating a homogeneous particle dispersion. All these conditions produced stable NPs, as revealed by their zeta potential values.

In the preparation of NPs via solvent evaporation, the surfactant plays a crucial role in term of size distribution of the formed NPs. The ionic surfactant forms a repulsive barrier around the droplet in the form of an electrical double layer, thus avoiding coalescence phenomena. Usually, an increase in surfactant concentration makes the particles size to decrease down to a minimum value over which the particles start to increase again (Komaiko & McClements, 2015), probably because of the large increase in viscosity that makes emulsification difficult (Jaouani, Tabka, & Penninckx, 2006; Saberi, Fang, & McClements, 2013; Tebaldi, Maia, Poletto, de Andrade, & Soares, 2019). In trials A to D, a decrease in particle size was observed with increasing SDS: PHBHHx ratio. This result derives from the combination of two effects: *i)* a higher amount of surfactant is available to surround polymer particles; *ii)* a lower polymer concentration reduces the viscosity of the organic phase, thus increasing the stirring efficiency. Similarly, Radu et al. (Radu et al., 2019) have shown that an increase in polyvinyl alcohol to PHBHV ratio causes a progressive reduction into particle size up to an equilibrium, under which smaller particles could not be obtained. The increase in organic/aqueous phase volume ratio was reported to cause a decrease in poly (lactic-*co*-glycolic acid) NP size prepared by emulsification-solvent

Table 1
Effect of SDS/PHBHHx mass ratio and aqueous/organic (A/O) phase volume ratio on Z-average size, polydispersity index (PdI) and zeta potential during PHBHHx-NPs production.
Values are mean ± SD; the different letters indicate significant differences from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

Trial	PHBHHx (mg)	Organic solution (mL)	Polymer concentration (mg mL^{-1})	SDS/PHBHHx mass ratio	A/O phase (mL)	Z-average size (d.nm)	PdI	Zeta potential (mV)	Method efficiency (%)
A	12.5	0.25	50	1.8	20	127^{a}	0.153	−30^{a}	59.8%
B	6.5	0.25	25	3.5	20	99^{b,c}	0.185	−53^{b}	71.3%
C	2.5	0.25	10	8.9	20	90^{c}	0.191	−40^{c}	79.4%
D	1.25	0.25	5	17.6	20	82^{c}	0.195	−44^{c,d}	80.5%
E	1.25	0.5	25	1.8	10	123^{a,b}	0.128	−38^{a,c}	57.6%
F	1.25	1	12.5	1.8	5	117^{a}	0.148	−62^{e}	55.8%
G	1.25	2.5	5	1.8	2	133^{a}	0.184	−50^{b,d}	67.8%

evaporation technique (Mainardes & Evangelista, 2005; Poletto et al., 2008). In this case, the coalescence of droplets is prevented by a greater amount of organic phase available for diffusion in the forming emulsion. The conditions explored in our study (trials E-D) did not lead to the same effect. On the other hand, another study demonstrated that the diameter of poly (lactic-*co*-glycolic acid) nanoparticles prepared with the same method was not influenced by increasing volumes of organic phase (Budhian, Siegel, & Winey, 2007), indicating that the solvent effect is finely dependent on the applied experimental conditions.

Taken together, the results in Table 1 indicate that condition D assures the smallest particle size (in the nano-range) together with a good stability and high recovery efficiency. In addition, these NPs formed a stable colloidal suspension in water which could be stored at RT for at least 7 days without aggregation. The NPs derived from condition D were further characterized by SEM and TEM analyses. Images in Fig. 1 indicate that PHBHHx-NPs derived from condition D were regular and spherical in shape (Fig. 1). Furthermore, their dimensions measured from SEM and TEM images are in accordance with those obtained by Z-average size measurements. In particular, SEM images (Fig. 1A and B) show the presence of aggregated polymeric nanoparticles whereas TEM image, reported in Fig. 1C, highlights the presence of isolated NPs with an average diameter of 60–90 nm.

3.2. Characterization of WP/PHHB-NPs composites

PHBHHx-NPs were tested as nanofillers in the preparation of WP-based films. To this aim, PHBHHx-NPs were added to WP containing FFSs, plasticised with 40% (w/w) glycerol at different concentrations (from 1 to 8%, w/w of WPs), and then mechanically stirred for 15 min. The FFSs prepared either in the absence or presence of different amounts of NPs, were analysed for their zeta potential and particle size, resulting into the formation of stable solutions with zeta potential values between −30.1 and 33.7 mV (Table 2). The addition of PHBHHx-NPs led to a significant reduction in the particle size (Fernandez-Bats, Di Pierro, Villalonga-Santana, Garcia-Almendarez, & Porta, 2018), with a more pronounced effect observed at the highest concentration of NPs tested (Table 2). Then, the FFSs were cast and the derived films characterized. It is worthy to point out that all the films analysed in this study were prepared under alkaline conditions (pH 12.0) without any preliminary WP heat treatment, an experimental condition that allowed to obtain handleable, transparent as well as flexible films (Abdalrazeq et al., 2019).

3.2.1. Film mechanical properties

Mechanical properties of both WP films and WP/PHBHHx-NPs composite films were determined by calculating the stress-strain curves. The data reported in Table 3 show that PHBHHx-NPs have a significant plasticizing effect on the WP-based films, as indicated by the marked decrease of YM and concurrent increase of EB observed by increasing the NP amount into the film matrix. On the other hand, a significant TS reduction was detected only at the highest concentration of NPs tested, thus indicating that PHBHHx-NPs concentrations up to 4% made the films grafted with NPs more extensible without loss of their original resistance. Similar results were obtained by using zinc oxide NPs incorporated into pectin/alginate edible films (Ngo, Dang, Tran, & Rachtanapun, 2018).

Table 2
Effect of different concentrations of PHBHHx-NPs on WP FFS zeta potential and Z-average size.
Values are mean ± SD; the different letters indicate significant differences from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

PHBHHx addition	Z-average size (d.nm)	Zeta potential (mV)
none	844.8 ± 43.3 ^{a}	−32.0 ± 5.9 ^{a}
+ 1%	1241.0 ± 46.4 ^{b}	−32.6 ± 3.0 ^{a}
+ 2%	505.9 ± 9.6 ^{c}	−30.1 ± 4.7 ^{a}
+ 4%	494.7 ± 27.6 ^{c}	−30.1 ± 2.4 ^{a}
+ 8%	382.7 ± 21.1 ^{c}	−33.7 ± 4.6 ^{a}

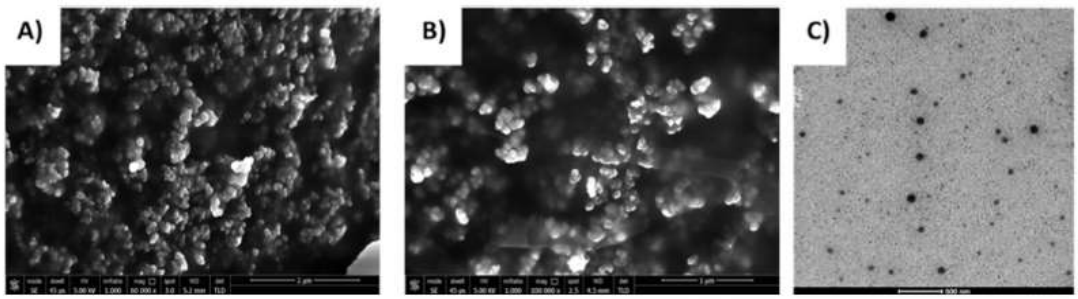

Fig. 1. SEM (A and B) and TEM (C) images of PHBHHx-NPs (2.10^{-1} and 2.10^{-5} mg/mL, respectively).
PHBHHx-NPs were prepared under the experimental condition D described in Table 1; SEM images were obtained at two different magnifications.

Table 3
Effect of different concentrations of PHBHHx-NPs on the thickness and mechanical properties of WP-based films.
(TS, tensile strength; EB, elongation at break; YM, Young's modulus). Values are mean ± SD; the different letters indicate significant differences from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

Film	TS (MPa)	EB (%)	YM (MPa)	Thickness (μm)
WP	3.1 ± 0.8 ^{a}	8.8 ± 1.1 ^{a}	104.1 ± 12.9 ^{a,b}	85 ± 4 ^{a}
WP+1% PHBHHx -NPs	3.3 ± 0.5 ^{a}	7.3 ± 2.5 ^{a}	130.4 ± 13.2 ^{c}	83 ± 3 ^{a}
WP+2% PHBHHx -NPs	3.0 ± 0.2 ^{a}	10.1 ± 2.2 ^{a}	109.1 ± 8.2 ^{a,c}	71 ± 3 ^{b}
WP+4% PHBHHx -NPs	3.2 ± 0.2 ^{a}	16.6 ± 0.8 ^{b}	78.3 ± 3.4 ^{b}	72 ± 1 ^{b}
WP+8% PHBHHx -NPs	2.0 ± 0.2 ^{b}	24.0 ± 3.4 ^{c}	28.5 ± 6.8 ^{d}	67 ± 5 ^{b}

Finally, also the film thickness was observed to decrease significantly in parallel with the increasing amount of NPs inside the matrix, and also this result is in strict agreement with previous data obtained by analysing a different nanocomposite film constituted by a starch-based matrix containing TiO_2 NPs (Goudarzi, Shahabi-Ghahfarrokhi, & Babaei-Ghazvini, 2017).

3.2.2. Film permeability

Water vapor (WV) and O_2 and CO_2 permeabilities are important biomaterial features for their use in various industrial sectors, especially in food packaging. The data reported in Table 4 demonstrate that the presence of PHBHHx-NPs inside the film matrix significantly increased only the O_2 barrier property of the WP-based films. It is well known that the addition of iso-dimensional or elongated nanoparticles in a matrix generally induces a variation of gas (O_2, CO_2) permeability that usually, but not always, decreases with the addition of fillers with high-aspect ratio. However, the final barrier properties are not easy to predict since they are the result of the combination of many variables such as filler/matrix affinity, filler aspect-ratio and the related tortuosity effect, filler orientation and agglomeration (Wolf, Angellier-Coussy, Gontard, Doghieri, & Guillard, 2018). It is worth to highlight that O_2 permeability is one of the most important properties of a packaging material as it is the main factor of organoleptic and nutritional quality degradation of food during storage throughout the oxidation of lipids, proteins, vitamins, pigments and various aroma compounds.

3.2.3. Film transparency

Film transparency is also a crucial parameter specifically influencing the appearance of a product and its acceptance by consumers. Films with low transparency are not generally suitable for food packaging applications especially when they are prepared as a see-through packaging material and used to enhance product visibility. Therefore, analyses of the opacity of the prepared nanocomposite films was carried out by measuring light transmission at a wavelength of 600 nm (Galus & Kadzińska, 2016). As shown in Fig. 2 the obtained WP-based films are quite transparent ($A_{600\ nm}/mm = 1.8 \pm 0.1$), and they did not significantly change this property when they were grafted with PHBHHx-NPs ($A_{600\ nm}/mm = 2.1 \pm 0.3$). Fig. 2 also shows that the presence of NPs inside the WP biopolymer matrix conferred to the film a higher visible and UV barrier property as indicated by the lower light transmission in the range between 200 and 280 nm and 350 and 800 nm, respectively (Leceta, Guerrero, & De La Caba, 2013). It is worthy to highlight that the prevention of lipid oxidation induced by UV is a further requirement in food packaging (Leceta et al., 2013).

Table 4
Barrier properties of WP-based films prepared in the absence or presence of 4% (w/w protein) PHBHHx-NPs.
Values are mean ± SD; the different letters indicate significant differences from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

Film	Permeability ($cm^3\ mm\ m^{-2}\ day^{-1}\ kPa^{-1}$)		
	CO_2	O_2	WV
WP	0.45 ± 0.01^{a}	0.89 ± 0.02^{a}	0.36 ± 0.02^{a}
WP+4% PHBHHx -NPs	0.37 ± 0.03^{a}	0.55 ± 0.01^{b}	0.34 ± 0.01^{a}

3.2.4. Film morphology

SEM micrographs of both surface and cross-section of film samples prepared in the absence and presence of PHBHHx-NPs are shown in Fig. 3. The images show a smooth surface without any fracture in the WP film prepared without NPs (panel A of Fig. 3). In addition, also the film cross-section appears quite smooth (Fig. 3, panel A') despite the presence of some holes, probably due to non-protein colloidal particles, such as fat globule membranes, deriving from the whey (Brooker, 1985). Conversely, as expected, PHBHHx-NPs created rougher structures (white spots) on the surface of the WP film (panel B of Fig. 3), which tend to migrate through the film with a reasonable dispersion (panel B′ of Fig. 3).

4. Conclusions

PHBHH-NPs were produced, characterized and used as nanofillers to obtain WP bio-nanocomposite films under different experimental conditions. The addition of PHBHHx-NPs to WP-based FFSs resulted in a plasticizing effect on the biobased material, producing a resistant but more extensible biomaterial. Moreover, the presence of PHBHHx-NPs inside the matrix enhanced the film barrier property towards O_2, letting to envisage a possible application of this novel material in food packaging, especially suitable for the products requiring preservation from oxidative reactions. This is the first report in which PHA-NPs are successfully used to improve the properties of the protein-based films by achieving the dispersion of a hydrophobic biopolymer into an aqueous FFS. Although this process might open the way to design further smart biomaterials through the incorporation of bio-active molecules into the NPs used, further studies are needed to assess the potential risks of the films grafted with PHBHHx-NPs for both consumers and environment safety.

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.

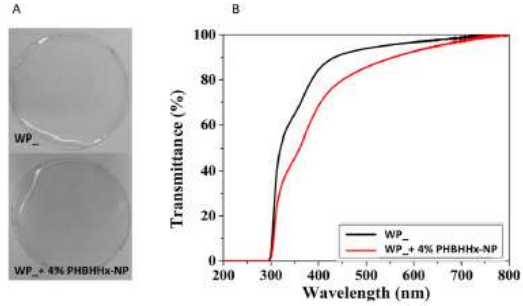

Fig. 2. Images of WP-based films prepared in the absence or presence of 4% PHBHHx-NPs (A) and their whole transmittance spectra (B).

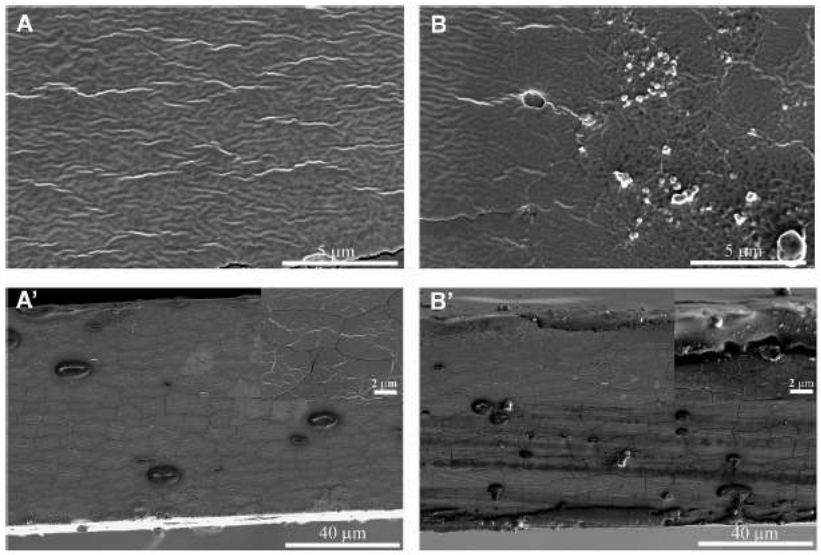

Fig. 3. SEM images of the surface of WP-based films prepared in the absence (A) and presence (B) of 4% PHBHHx-NPs and of the respective cryo-fractured cross-sections (A′ and B′). Insets in A′ and B′ show details at higher magnification.

CRediT authorship contribution statement

Iolanda Corrado: Investigation, Formal analysis. **Manar Abdalrazeq:** Investigation, Formal analysis. **Cinzia Pezzella:** Conceptualization, Writing - original draft. **Rocco Di Girolamo:** Investigation. **Raffaele Porta:** Supervision, Funding acquisition. **Giovanni Sannia:** Supervision, Funding acquisition. **C. Valeria L. Giosafatto:** Conceptualization, Writing - original draft.

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3.4. Bioactive whey protein-based films incorporated with *Thymbra* essential oil

3.4.1. Chemical composition of Thymbra essential oils characterized by means of GC-MS

There are many variables affecting the percentage yield of EO extraction, including: temperature and relative humidity conditions, soil, plant genetics, geographic origin maturity degree and organ, extract distillation time and, finally, pressure and temperature of distillation[91,106,107]. TEO1 percentage yield was 2.36% whereas TEO2 yield was 3.07%.

Following TEO extraction, different components have been identified using GC-MS. As shown in Table 5, the major identified volatiles compounds in TEO1 were γ-terpinene, carvacrol, p-cymene with the following percentages 38.95%, 22.96%, and 19.53%, respectively.

Table 5: Chemical composition of two samples from *TEOs*

Component	TEO 1 (%)	TEO 2 (%)
α-Phellandrene	1.65	3.16
δ-3-Carene	1.48	1.44
Camphene	0.36	0.36
β-Pinene	0.41	0.36
α-Pinene	1.10	-
α- Terpinolene	7.19	0.26
p-Cymene	19.53	-
Ortho-Cymene	-	0.48
γ-Terpinene	38.95	57.81
ψ-Limonene	0.69	-
α-Terpinenol	0.05	4.31
Anisole	0.67	-
Thymol	1.07	0.95
Carvacol	22.96	23.2
Caryophyllene	2.63	4.40
Linalool	-	1.06
Endo-Borneol	-	0.20
α- Humulene	-	0.14
γ – Elemene	-	0.10
Total identified	98.74	98.26
Others	1.26	1.74

As far as TEO2 the Carvacol percentage was in the same range as found in TEO1, whereas γ-terpinene content was higher, being present with a percentage of 57.81%, and no *p*-cymene was detected.

3.4.2. Antimicrobial activity of TEO1 and TEO2

TEO antimicrobial activities were investigated against the following microorganisms: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecium, Klebsiella, Methicillin-Resistant Staphylococcus aureus (MRSA), and Proteus vulgaris.* Table 6 shows MIC values of the two TEOs, revealing powerful antimicrobial activity against both gram positive and gram-negative bacteria. More in details, TEO1 showed stronger inhibition

effect than TEO2 against all the studied microorganisms. It is worthy to note that the investigated Palestinian TEOs showed stronger antimicrobial activity compared with those of the TEOs extracted from Turkish Thymbra previously analyzed by Baydar et al. (2004)[70]. In fact, the Turkish Thymbra EO inhibited the microorganism growth at concentrations of <1/100 (v/v), whereas the Palestinian TEOs showed an antimicrobial effect at concentrations at least <1/800 (TEO1) or <1/400 (TEO2) (v/v).

Table 6: MIC_{100} values showed by TEOs extracted from dried leaves of *Thymbra* against different microorganisms

ATTCC #	ATCC 25923	ATCC 25922	ATCC 13883	ATCC 8427	ATCC 700221	ATCC 9027	Clinical strain	ATCC 90028
Strains name	*Staphylococcus aureus*	*Escherichia coli*	*klebsiella pneumoniae*	*Proteus vulgaris*	*Enterococcus faecium*	*Pseudomonas aeruginosa*	MRSA	Candida albicans
MIC_{100} of TEO 1	1/1600	1/800	1/800	1/1600	1/800	1/1600	1/800	1/800
MIC_{100} of TEO 2	1/800	1/800	1/400	1/800	1/400	1/800	1/400	1/400

3.4.3. Characterization of film forming solution containing TEO1

The particle size and zeta potential values of WP-based FFSs added with TEO1 indicate a significant reduction in the particle size, probably due to formation of emulsions in the presence of TEO1, and that FFS stability decreased by increasing TEO1 concentration (Figure 12).

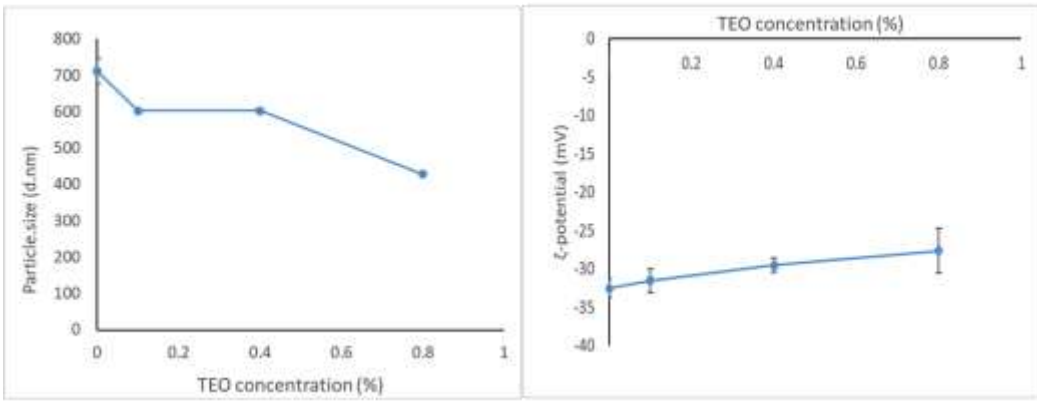

Figure 12: Zeta potential and Z-average measurements of WP-based FFSs prepared in the presence of different concentration of TEO1 (v/v). Values are mean ± SD

Interestingly, FFSs containing WP alone showed a slight antimicrobial activity towards all the strains tested. Surprisingly, the FFSs containing the lowest concentration of TEO1 (0.1 %) was able to completely inhibit bacterial growth of the gram-negative bacteria *Salmonella enteriditis* 706 RIVM, *Salmonella enterica subsp*. *enterica serovar Typhimurium* (ATCC® 14028) and also the gram-positive *Staphylococcus aureus* ATCC 29213 (Figure 13, A, B, C). On the contrary, the FFS containing higher concentrations of TEO1 (0.2 %) was able to completely inhibit the growth of *Enterococcus faecalis* ATCC 29212 (Figure 13, D).

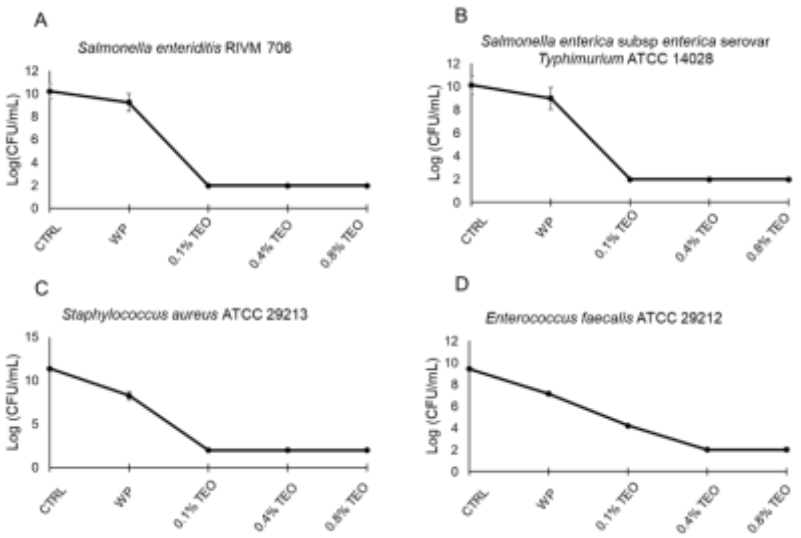

Figure 13: (MIC_{100}) of the TEO1-containing FFS towards *Salmonella enteriditis* 706 RIVM (A), *Salmonella enterica subsp. enterica serovar Typhimurium* (ATCC® 14028) (B), *Staphylococcus aureus* ATCC 29213 (C), *Enterococcus faecalis* ATCC 29212 (D). The graphs report the colony count for each strain. The experiments were carried out in triplicate.

3.4.4. Characterization of whey protein films containing TEO1

3.4.4.1. Opacity, thickness and mechanical properties

The transparency of TEO1 containing films was affected by the oil volume fraction and droplet size distribution in the film forming emulsions, as well as by the droplet rearrangement during drying[108,109]. Moreover, the evaporation of the solvent during the film dryness causes a change in the emulsion structure (i.e. creaming, aggregation and/or coalescence), which have an important role in determining the film optical properties[110]. Figure 14 shows a decreased film transparency by increasing TEO1 concentration.

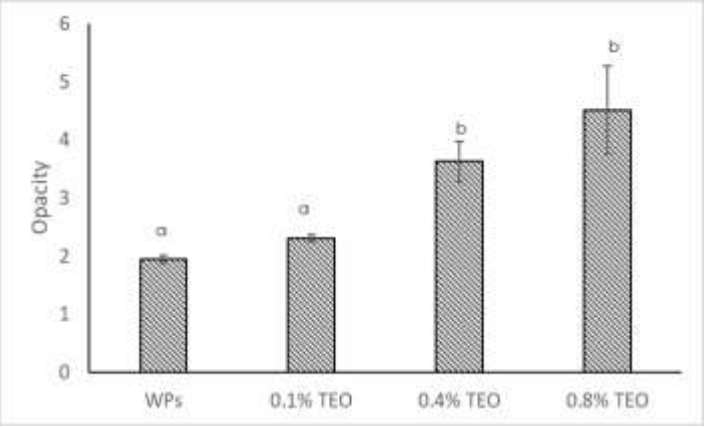

Figure 14: Opacity of WP (500 mg)-based films prepared either in the absence or presence of different concentrations of TEO1 (v/v). Values are mean ± SD; different letters indicate significant differences of the values (Tukey-Kramer test, $p < 0.05$).

These findings are in agreement with the results reported by Galus et al. (2016)[108] who prepared and characterized WP-based films containing rapeseed oil. Figure 15 indicates that no differences were observed among the thickness of the films prepared in the presence of different TEO1 concentrations. Moreover, the mechanical properties of TEO1-containing films showed a progressive decrease in film TS by increasing TEO1 concentrations, whereas a significant increase in the EB was observed only at the highest TEO1 amount present in the FFS. Finally, a marked decrease in the film YM value was also detected at the highest TEO1 concentration used (Figure 15). All these data clearly demonstrate an increased plasticizing effect triggered by TEO1 addition to the WP-based FFSs.

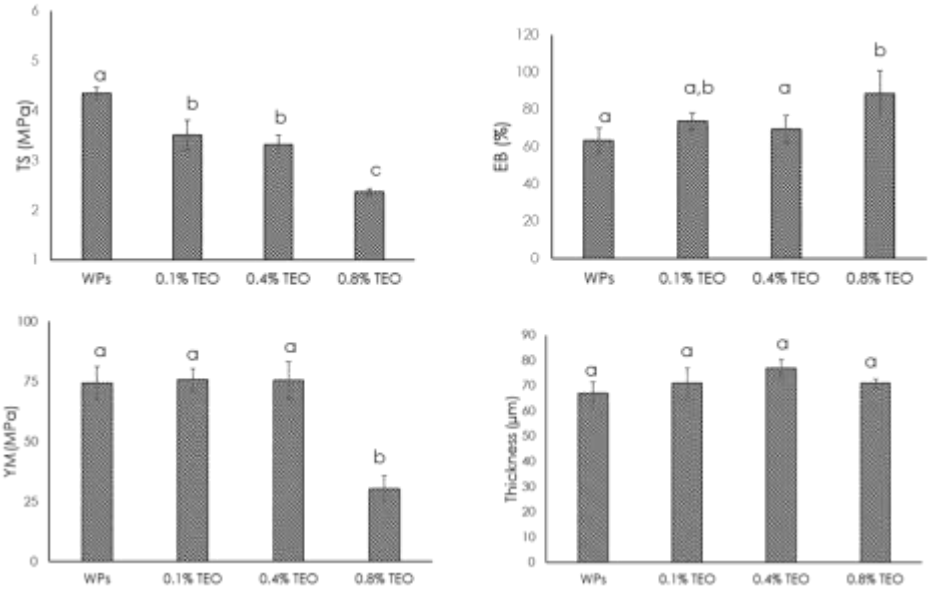

Figure 15: Mechanical properties of WP (500 mg)-based films either incorporated or not with different amounts of TEO1. Values are mean ± SD; means followed by different letters indicate significant differences of the values (Tukey-Kramer test, $p < 0.05$).

3.4.4.2. Moisture content and uptake

Moisture content of WP films progressively decreased following addition of increasing concentrations of TEO1 into the originating FFSs (Table 7). It is worthy to note that even at very low concentrations (0.1%), TEO1 was effective to reduce the film moisture content. These results are in agreement with the previous study reported by Galus et al. (2016)[108] who worked on WP-based materials containing rapeseed oil. On the other hand, slight even though not statistically significant increases of moisture uptake were also observed. Similar results have been reported by Salarbashi et al, 2013[111], who investigated the moisture uptake of soluble soybean polysaccharide films incorporated with Zataria multiflora Boiss (Shirazi thyme).

Table 7: Moisture content and uptake of WP (500 mg)-based films prepared either in the absence or presence of different concentrations of TEO1*.

Film	Moisture content (%)	Moisture uptake (%)
Control sample	22.3 ± 2.6 [a]	11.3 ± 2.9 [a]
+ 0.1% (v/v) TEO1	20.3 ± 1.8 [a,b]	11.7 ± 2.5 [a]
+ 0.4% (v/v) TEO1	18.5 ± 1.4 [a,b]	12.1 ± 1.1 [a]
+ 0.8 % (v/v) TEO1	17.5 ± 0.7 [b]	13.3 ± 0.8 [a]

*Values are mean ± SD; means followed by different letters are significantly different from the values reported in the same column (Tukey-Kramer test, $p < 0.05$).

3.4.4.3. Antimicrobial activity

Finally, the antimicrobial activity of the WP-based films prepared with or without TEO1 was assayed towards *Salmonella enteriditis* 706 RIVM, *Salmonella enterica subsp*. *enterica serovar Typhimurium* (ATCC® 14028), *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 strains by placing small square (1.5 cm x 1.5 cm) from each film into the inoculated plate in full contact with the agar surface[112]. After 24 h at 37°C it was observed that the WP film itself was unable to inhibit the bacterial growth (Figure 16). On the contrary, the films containing TEO1 at the highest concentration tested (0.8%) showed a strong antimicrobial activity towards *Salmonella enteriditis* 706 RIVM, *Salmonella enterica subsp. enterica serovar Typhimurium* (ATCC® 14028) as well as against *Staphylococcus aureus* ATCC 29213. Interestingly, it was possible to observe also a zone of inhibition surrounding the films activated with TEO1, suggesting the ability of TEO1 to diffuse from the film into the agar matrix. Furthermore, no antimicrobial activity of the films was observed against *Enterococcus faecalis* ATCC 29212, even at the highest concentration of the TEO1 tested.

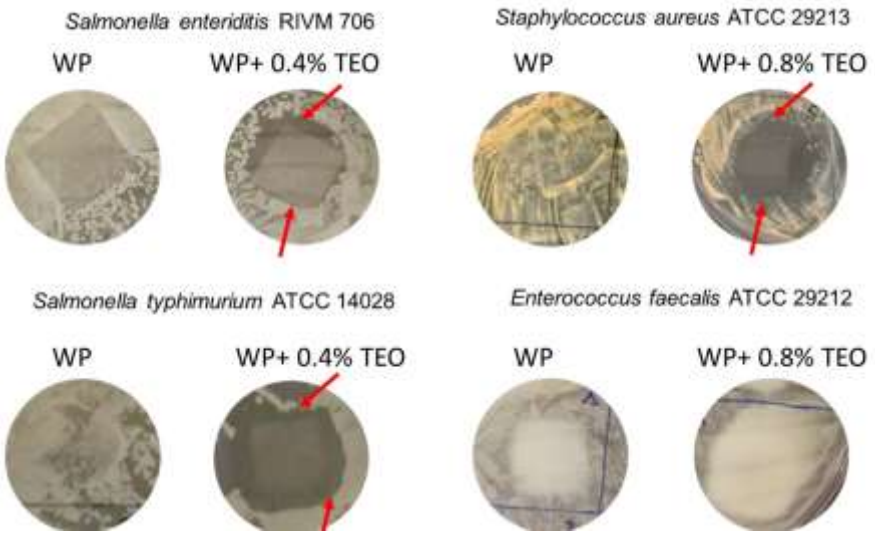

Figure 16: Antimicrobial effect of TEO1 containing films on *Salmonella enteriditis* 706 RIVM, *Salmonella enterica subsp. enterica serovar Typhimurium* (ATCC® 14028), *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 foodborne pathogenic bacteria. Films were tested either alone (left side) or upon bio-activation with different TEO1 amounts (right side). Antimicrobial effects were evaluated by analysing the growth of bacterial cells in direct contact with films. The experiments were carried out in triplicate.

3.5. Bioactive whey protein-based films incorporated with Pecan nut shell extract

The development of an active packaging based on WP-based films functionalized with PNSE was also pursued. To this purpose, FFSs containing WPs, PNSE and GLY were prepared and characterized and corresponding films were produced by casting. Zeta potential measurements revealed that FFSs were stable and the particle size was reduced following addition of PNSE, probably as a result of tannins-WPs interactions. The obtained films were handleable and homogeneous, and PNSE was able to improve their mechanical and barrier properties. Moreover, PNSE-containing films were found to inhibit the growth of the foodborne bacteria *Enterococcus faecalis* and *Salmonella enterica subsp. enterica ser. Typhimurium*. Ferric

reducing/antioxidant power assay clearly highlighted the ability of PNSE to confer antioxidant properties to the films. Finally, simulated digestion experiments of the manufactured films showed a significant reduction of the proteolysis rate in the presence of PNSE, although 40% of the protein was digested after 60 min incubation. Overall, these results put the basis for a possible use of PNSE functionalized WP films as new environmentally friendly candidates for packaging to increase food product shelf-life.

The detailed description of the obtained results is reported in the following manuscript that has been submitted to *Food Packaging and Shelf-Life* journal and it is just accepted.

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Development and characterization of antimicrobial and antioxidant whey protein-based films functionalized with Pecan (*Carya illinoinensis*) nut shell extract

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ABSTRACT

Aim of this study was the development of an active packaging based on whey proteins (WP) functionalized with Pecan nut shell extract (PNSE). To this purpose, aqueous solutions of WP, PNSE and glycerol were mixed and characterized, whereas corresponding films were prepared by casting. Zeta-potential measurement revealed that the film forming solutions were stable, and the particle size was reduced further to the incorporation of PNSE, as a result of tannins-WP interactions. Films were handleable and homogeneous, and PNSE was able to improve their mechanical and barrier properties. PNSE-containing films inhibited the growth of the foodborne bacteria *Enterococcus faecalis* and *Salmonella enterica* subsp. *enterica* ser. Typhimurium. Ferric reducing/antioxidant power assay clearly highlighted the ability of PNSE to impart antioxidant properties to the films. Finally, simulated digestion experiments showed a significant lowering of the proteolysis rate in the presence of PNSE, although 40 % of the protein was still digested after 60 min incubation. Overall, these results put the basis for a possible use of PNSE functionalized WP-based films as new environmentally friendly candidates for increasing the shelf-life of foods.

1. Introduction

The world production of whey is estimated to be around 180 million tons/year, and its disposal represents a problem of considerable environmental impact as it is characterized by a high organic content, which makes it impossible to spill into municipal sewers or in the ground (Yadav et al., 2015; Zhou, Hua, Huang, & Xu, 2019). On the other hand, whey represents not only a source of numerous nutrients, but also a potential resource to produce added value compounds (Ganju & Gogate, 2017). Therefore, in recent years the search for possible applications of this waste has risen a considerable interest. Whey is a proteinaceous material, whose main components are β-lactoglobulin (3.03 g/L) and α-lactoalbumin (1.02 g/L) (Marshall, 2004), which are able, under specific conditions (Abdalrazeq et al., 2019; Corrado et al., 2021; Khwaldia, Perez, Banon, Desobry, & Hardy, 2004) to obtain edible films endowed with good technological properties for food applications. Functionalization of milk whey proteins (WP)-based films with natural and sustainably produced antioxidant and/or antimicrobial additives has also received considerable attention in recent years, prompted also by the increasing need for green approaches to novel functional materials (Xu et al., 2021). In this context, waste products from agri-food industries represent an easily accessible source of phenolic compounds, which apart from their use as food supplements or as additives in functional foods, have become increasingly attractive also from a technological point of view, due to their possible exploitation in materials science, *e.g.* in active packaging (Balasundram, Sundram, & Samman, 2006; Ben-Othman, Joudu, & Bhat, 2020; Moccia, Agustin-Salazar, Agustin-Salazar et al., 2020; Panzella et al., 2020). A noticeable example

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is represented by Pecan nut shell (PNS), which is produced mainly in United States and Mexico in amounts of *ca.* 420,000 tons *per* year (dos Santos, Zabot, Mazutti, & Tres, 2020). PNS represents a valuable source of antioxidant phenolic compounds, mainly condensed, prodelphinidin-type tannins (Agustin-Salazar, Gamez-Meza, Medina-Juárez, Malinconico, & Cerruti, 2017; Moccia, Agustin-Salazar et al., 2020; Pinheiro do Prado et al., 2013). The antioxidant properties of extracts from PNS have been largely explored in the food industry. Water-extractable compounds from PNS are able to increase the oxidative stability of margarines during storage (Ribeiro, de Britto Policarpi, Dal Bo, Barbetta, & Block, 2017), whereas a PNS hydroalcoholic extract (PNSE) (estimated phenol content: *ca.* 130 mg gallic acid equivalents/g) has been reported to act as a thermal and photo-oxidative stabilizer of polyethylene and polylactic acid (Agustin-Salazar et al., 2016, 2017; Alvarez-Chávez et al., 2017; Sánchez-Acosta et al., 2019), as well as to confer significant antioxidant and food stabilization properties to these polymers (Moccia, Agustin-Salazar et al., 2020). Moreover, inclusion of PNSE in octenyl succinate starch films led to improved water resistance and UV-light barrier properties compared to control films (Leon-Bejarano, Durmus, Ovando-Martinez, & Simsek, 2020). PNS extracts are also characterized by remarkable antimicrobial properties (Caxambu et al., 2016), which open new perspectives toward a full exploitation of the applicability of this material in food field. Indeed, contamination of food samples by microorganisms is a key issue greatly affecting food preservation and shelf-life. It has also to be highlighted that the improper use of conventional antibiotics in food industry is a major issue since it leads to the rapid rise and spread of resistant foodborne pathogens (McDermott et al., 2002; White, Zhao, Simjee, Wagner, & McDermott, 2002). These microorganisms can be easily transmitted to humans through consumption of fresh and raw foods (Antunes, Novais, & Peixe, 2020). Moreover, because of side effects caused by the use of chemical agents (Kinderlerer & Hatton, 1990; Sultana et al., 2014) and because of the consequences of physical treatments on food organoleptic properties (John, 2003), there is an increasing demand for natural and untreated food samples (Pisoschi et al., 2018). Aqueous and ethanolic PNS extracts have shown antimicrobial activity towards both Gram-positive and Gram-negative bacteria. Crude extracts were found to be more effective than the single phenolic components, which suggests synergistic effects between the different components (Alvarez-Parrilla, Urrea-López, & de la Rosa, 2010; Pinheiro do Prado et al., 2014). Furthermore, a methanolic extract of PNS showed antifungal activity towards plant pathogenic fungi (Osorio et al., 2010).

Based on all these observations, this work was directed towards the preparation and characterization of WP-based edible films functionalized with PNSE for possible application in food packaging. It has been indeed reported that the addition of crosslinking agents into film forming solutions might improve the mechanical properties of protein-based edible films. Actually, low molecular weight phenolic compounds present in PNSE, such as gallic acid, chlorogenic acid and p-hydroxybenzoic acid, could covalently react further to oxidation with amino or thiol residues in proteins. On the other hand, it is expected that also condensed tannins, which have been reported as the main phenolic constituents of PNSE, could strongly interact with proteins through *e.g.* hydrogen bonds. This would results not only in an enhancement of film technological properties, but also in imparting antioxidant as well as antimicrobial properties to the protein film (Ou, Wang, Tang, Huang, & Jackson, 2005; Pinheiro do Prado et al., 2014). In this paper, the films were characterized for their mechanical as well as gas (CO_2 and O_2) and water vapor barrier properties. In addition, their antimicrobial and antioxidant features together with the gastric digestibility under physiological conditions were evaluated.

2. Materials and methods

2.1. Materials

2,2-Diphenyl-1-picrylhydrazyl (DPPH), iron (III) chloride (97 %), 2,4,6-tris(2-pirydyl)-s-triazine (≥98 %), (+)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (97 %), glycerol and the other reagents were purchased from Sigma-Aldrich, Italy. WP were purchased from Bioline (London UK). PNS was provided by Productora de Nuez S.P.R. de R.I. (Mexico).

2.2. Bacterial strains and growth conditions

Gram-negative *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC® 14028) and Gram-positive *Enterococcus faecalis* (ATCC® 29212) were obtained from ATCC (USA). Bacteria were grown in Muller-Hinton Broth (MHB) at 37 °C over-night. Afterwards, cells were diluted in fresh medium and grown for 3 h at 37 °C until mid-log phase (Bosso et al., 2017).

2.3. Preparation of PNSE and of PNSE solution

PNSE was obtained as previously described (Agustin-Salazar et al., 2017; Moccia, Agustin-Salazar et al., 2020). Briefly, PNS (1 *g*) was treated with 2 × 10 mL of ethanol/water (6:4 v/v) for 30 min in an ultrasonic bath at room temperature. The mixture was then centrifuged for 20 min at 7763 *g*, the supernatant was filtered on Whatman paper No. 2 (GE Healthcare) and ethanol was removed in a rotary evaporator. The residual solution was then lyophilized to give PNSE as a red powder in 15 % w/w yield. For the preparation of PNSE solution, 0.5 *g* of PNSE were added to 10 mL of water. The suspension was taken in an ultrasonic bath for 30 min and then centrifuged at 6793 *g* for 30 min. The supernatant was removed and analyzed at 24 h intervals over 1 week by UV–vis spectroscopy to check solution stability. The precipitate was dried by lyophilization and weighted to determine by difference the amount of material which had gone into solution. PNSE concentration in the supernatant was found to be 30 mg/mL. This solution was then used for incorporation into WP.

2.4. Film forming solution (FFS) preparation and characterisation

FFS was prepared by dissolving 500 mg of WP in 25 mL of distilled water. Subsequently 60 % of glycerol (w/w in respect to proteins) was added. After addition of the PNSE solution (8 mL, containing 240 mg of PNSE), the pH was brought to 12 by using 0.1 M NaOH. A FFS without PNSE was prepared as a blank. 1.0 mL of each FFS was analyzed for Zeta-potential and particle size by using a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK) (Abdalrazeq et al., 2019).

2.5. Film preparation and technological propertycharacterization

The films were prepared by casting method as follows: the FFSs were poured in 9 cm diameter Petri dishes and then allowed to dry at 25 °C (45 % relative humidity) for 24 h.

The obtained films were cut into 1 × 8 cm strips and kept at 25 °C, with relative humidity of 50 %, for 24 h in a glass chamber containing saturated $Mg(NO_3)_2$ before being tested. The thickness of the strips was measured in 6 different points with a micrometer (Electronic digital micrometer - Metrocontrol srl, Casoria Italy - sensitivity 0.001 mm), while the tensile strength (TS), elongation at break (EB) and Young's modulus (YM) were obtained on 5 strips of each sample by means of Instron Universal Testing Instrument model No. 5543A (Instron Engineering Corp., Norwood, MA, USA) using 5 cm gage length, 1 kN load and 5 mm/min speed.

The measurement of film gas permeability towards CO_2, H_2O vapor and O_2 was carried out in triplicate at 25 °C and 50 % relative humidity,

using the MultiPerm permeabilimeter (Extra Solution s.r.l., Pisa, Italy). Aluminium masks were used with the aim of reducing the area of the film tested to 5 cm^2.

2.6. Antioxidant properties of the films

DPPH assay: 1 cm^2 film section (corresponding to 28 mg of material) was added to 10 mL of a 50 or 200 μM ethanolic solution of DPPH, and the absorbance of the solution at 515 nm was periodically analyzed over 4 h. Control experiments were run on films not containing PNSE. Each experiment was run in triplicate.

2.6.1. Ferric reducing/antioxidant power (FRAP) assay

1 cm^2 film section (corresponding to 28 mg of material) was added to 10 mL of a solution of 1.7 mM $FeCl_3$ and 0.03 mM 2,4,6-tris(2-pirydyl)-s-triazine in 0.3 M acetate buffer (pH 3.6). The absorbance of the solution at 593 nm was periodically measured over 4 h. Control experiments were run on films not containing PNSE. Each experiment was run in triplicate. Results were expressed as Trolox equivalents.

In separate experiments the release of PNSE from the films in the FRAP assay medium was evaluated by adding 1 cm^2 film section (corresponding to 28 mg of material) to 10 mL of 0.3 M acetate buffer (pH 3.6) and periodically recording the UV–vis spectra of the solution for 4 h. Experiments were run in triplicate.

2.7. Antimicrobial activity assays

Antimicrobial activity of PNSE was analyzed by the microbroth dilutions assay (Gaglione et al., 2017). In particular, the assay was performed in Difco Nutrient Broth (Becton-Dickenson, Franklin Lakes, NJ, USA) using a bacterial inoculum of 2×10^6 CFU/mL and testing increasing concentrations of PNSE (0–15 mg/mL). Determined minimum inhibitory concentration (MIC_{100}) values correspond to the lowest concentration of extract associated to no detectable bacterial growth.

The antimicrobial activity of the films was tested according to a previously described procedure (Sabbah et al., 2019). Growth inhibition underneath the film in full contact with the agar surface was evaluated. All experiments were run in triplicate.

2.8. Evaluation of the effectiveness of FFS in preventing bacterial cells adhesion by confocal laser scanning microscopy

Bacterial cells were grown on glass coverslips, unfunctionalized (control sample) or functionalized with FFSs containing or not PNSE in 0.5 × MHB medium, starting from a culture at a concentration of 2×10^8 CFU/mL, in static conditions at 37 °C for 16 h. Upon incubation, non-adherent bacteria were removed by gently washing samples with sterile phosphate buffer saline (PBS). Viability of cells attached on the surface was determined by sample staining with LIVE/DEAD® BacLight™ Bacterial Viability kit (Molecular Probes ThermoFisher Scientific, Waltham, MA, USA). Staining was performed according to manufacturer's instructions. Images were captured by using a confocal laser scanning microscope (Zeiss LSM 710, Zeiss, Germany) and a 63× objective oil immersion system. The area containing attached bacteria was analyzed by using the Zen Lite 2.3 software package. Each experiment was performed in triplicate. Images are 2.5D projections of biofilm structure obtained by confocal z-stack using Zen Lite 2.3 software. All images were taken under identical conditions and represent the average of at least three different acquisition fields. Scale bar corresponds to 10 μm in all the cases. All images were taken under identical conditions.

2.9. FFS oral and gastric digestion

FFS prepared in the absence and presence of PNSE were subjected to two-phase *in vitro* digestion, using an adult model (Gionfriddo et al., 2012; Romano, Gionfriddo, Masi, & Mariniello, 2015; Shani-Levi et al., 2017) which simulates the physiological conditions of the digestive system at the oral and gastric level. In particular, 500 μL of each FFS were incubated in 600 μL of simulated salivary fluid (SSF, 150 mM NaCl, 3 mM urea, pH 6.9) for 5 min at 170 rpm. Afterwards, the samples were subjected to gastric digestion in 100 μL of simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5) placed in 1.5 mL microcentrifuge tubes and incubated at 37 °C. Hence, 75 μL of FFS previously incubated with SSF, whose pH was adjusted to 2.5 with 6 M HCl, were added, together with pepsin, to each tube containing SGF to start the gastric digestion reaction. A pepsin to protein ratio of 20:1 w/w was used. At 1, 2, 5, 10, 20, 40, 60 min intervals, 40 μL of 0.5 M ammonium bicarbonate were added to each tube to stop the pepsin reaction. The samples were then analyzed by SDS-PAGE. Briefly, 5 μL of sample buffer (15 mM Tris -HCl, pH 6.8, containing 0.5 % w/v SDS, 2.5 % v/v glycerol, 200 mM β-mercaptoethanol and 0.003 % w/v bromophenol blue) were added to 20 μL aliquots of each sample subjected to oral and gastric digestion and analyzed by 4–20 % SDS-PAGE (Laemmli, 1970). The electrophoresis was performed at constant voltage (80 V for 2–3 hours) and the proteins were visualized by Coomassie Brilliant Blue R250 staining. Bio-Rad precision protein standards were used as molecular weight markers. SDS-PAGE gel images were acquired using Bio-Rad ChemDoc Imager. The image analysis was carried out using Image Lab software (Bio-Rad, version 5.2.1) as described by Romano et al. (2015). Densitometric analyses were performed by calculating the percentage of the average intensity of the 17-kDa β-lactoglobulin normalized to that of control samples.

Table 1
MIC_{100} values of PNSE towards foodborne pathogens.

Strain	MIC_{100} (mg/mL)
Salmonella enterica subsp. *enterica* ser. Typhimurium ATCC® 14028	7.5
Enterococcus faecalis ATCC® 29212	1.85

2.10. Statistical analyses

All data were analyzed by means of JMP software 5.0 (SAS Institute, Cary, NC, USA). Statistical analyses were performed by using ANOVA (film technological property analyses) and Student's *t*-test (antioxidant assays). The data were subjected to analysis of variance, and the means were compared using the Tukey-Kramer HSD test. Differences were considered to be significant at $p < 0.05$.

3. Results and discussion

3.1. Preparation of PNSE solution

To obtain the functionalized WP-based films, an aqueous solution of PNSE was firstly prepared. Given the limited solubility in water of PNSE, the highest achievable concentration was 30 mg/mL. The solution was found to be stable at room temperature at least over 1 week as determined by periodical UV–vis analyses, indicating no variation in its absorption spectra.

3.2. Antimicrobial activity of PNSE

To assess the effectiveness of PNSE in preventing food contamination and spoilage, its antimicrobial activity was tested on the Gram-negative foodborne pathogen *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC® 14028) and on the Gram-positive bacterium *Enterococcus faecalis* (ATCC® 29212). Interestingly, PNSE at a concentration of 7.5 mg/mL was found to be able to completely inhibit the growth of *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC® 14028). The extract was found to be even more active on *Enterococcus faecalis*

Table 2
Average particle size, polydispersion index and Zeta-potential of FFS prepared in the absence and presence of PNSE.

Sample	Average particle size (nm)	Polydispersity index	Zeta-potential (mV)
WP	703.2 ± 46.6 ^{a}	0.7 ± 0.1 ^{a}	−29.9 ± 2.6 ^{a}
WP/PNSE	306.7 ± 3.6 ^{b}	0.5 ± 0.03 ^{b}	−27.3 ± 0.7 ^{a}

Values are mean ± standard deviation. Means followed by the same letters are not significantly different (Tukey-Kramer test, $p < 0.05$).

(ATCC® 29212), being its MIC_{100} value as low as 1.85 mg/mL (Table 1). Determined MIC_{100} values were found to be comparable to those previously reported in the literature (Yemmireddy, Cason, Moreira, & Adhikari, 2020).

3.3. WP/PNSE-based FFS preparation and characterisation of their physicochemical properties

The possibility of using PNSE to prepare WP-based active bioplastics was then tested. To this aim, FPS plasticized with glycerol in the presence or absence of PNSE (30 % w/w) were prepared under alkaline conditions as previously reported (Abdulrazeq et al., 2019; Corrado

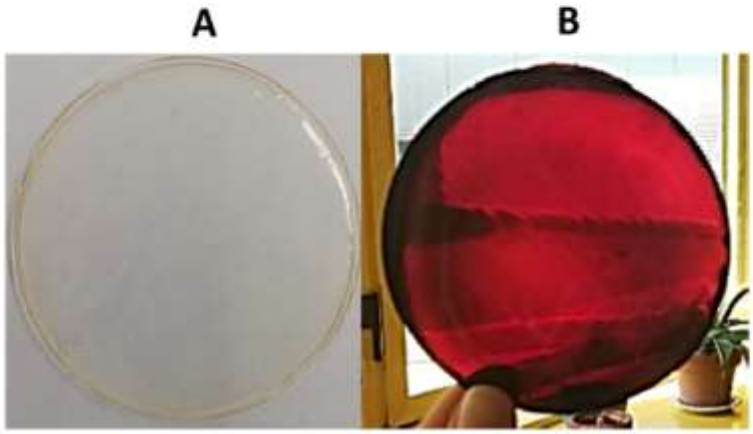

Fig. 1. WP-based films prepared in the absence (A) and in the presence (B) of PNSE.

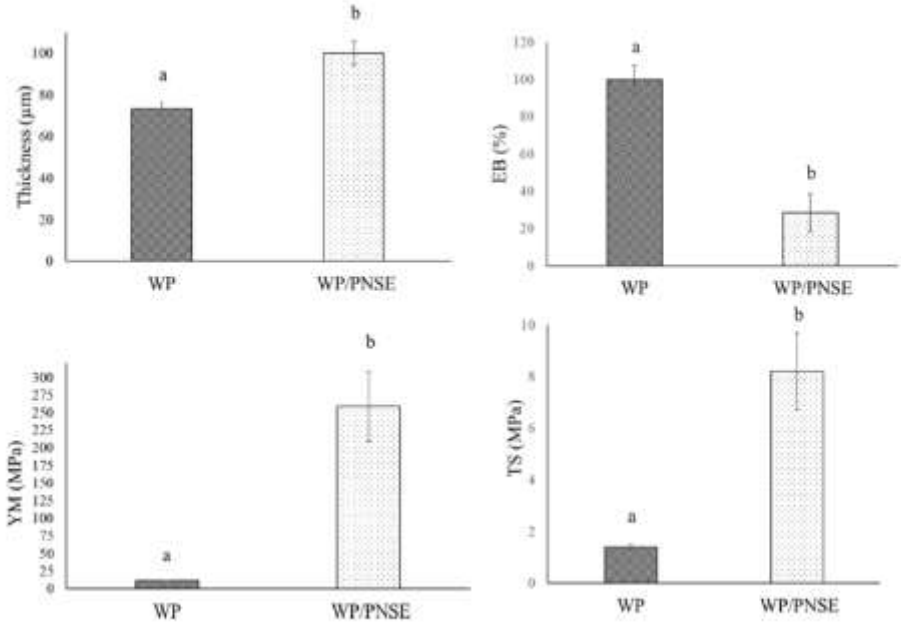

Fig. 2. Effect of PNSE on the thickness and mechanical properties of WP-based films (EB, elongation at break; YM, Young's modulus; TS, tensile strength). Values are mean ± SD; different letters indicate significant differences between the values reported in the same plot (Tukey-Kramer test, $p < 0.05$).

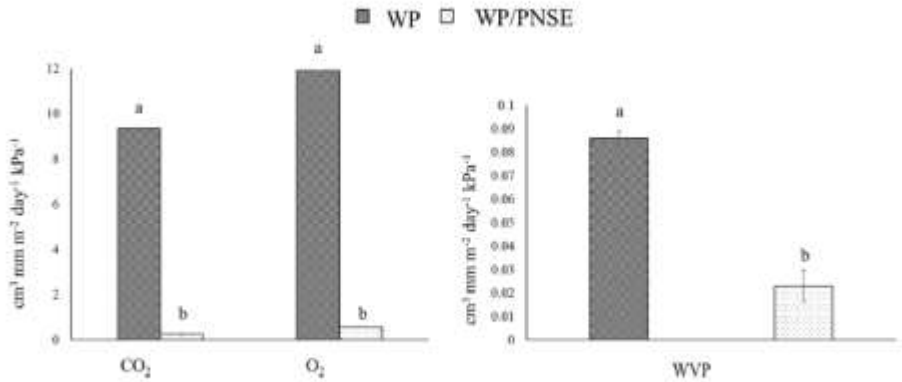

Fig. 3. Barrier properties of WP-based films prepared in the absence or presence of PNSE. Values are mean ± SD; different letters indicate significant differences between the values reported in the same plot (Tukey-Kramer test, $p < 0.05$).

et al., 2021) andanalyzed for the Zeta-potential and particle size to verify the solution stability. In particular, Zeta-potential is a quantitative parameter related to the mobility of particles in an electric field. A good stability is given by the presence of electrostatic repulsions between the particles, which avoids uncontrolled aggregations of colloids in solution (Sabbah et al., 2017). As reported in Table 2, the Zeta-potential value of the PNSE-functionalized FFS, though slightly lower than that of the blank sample, still falls within the stability range (about −27 mV). As far as particle size is concerned, it is worthy to note that it was halved for the FFS prepared with PNSE. This is likely due to the fact that the polyphenol containing extract might reduce the inter-particle interactions of WP, in agreement with previously reported data indicating that *i)* the formation of polyphenol–protein conjugates may reduce the extent of aggregation (Czubinski & Dwiecki, 2017) and that *ii)* the size of the particles varies with the protein: polyphenol ratio (Siebert & Lynn, 2000). The polydispersion index was quite low for both samples. This index, which was approximately 0.7 in the control sample, decreased to 0.5 in the sample prepared in the presence of PNSE, indicating that the particle size in the functionalized FFS was more uniform.

3.4. Film preparation

WP-based films were produced using the solvent casting technique, according to the protocol described in Abdalrazeq et al. (2019). The selected percentage of glycerol (60 % w/w with respect to the protein content) was adequate to obtain films capable to be handled and flexible. The films prepared in the presence of PNSE showed an intense purple red color (Fig. 1).

3.5. Film characterization

3.5.1. Mechanical properties

The mechanical properties determined for the obtained films are reported in Fig. 2. Film thickness increased significantly ($p < 0.05$) in the presence of the extract, in agreement with previous observations. For example, Peng, Wu, and Li (2013) showed that chitosan films incorporated with tea polyphenols had higher thickness probably because in the presence of polyphenols the inter-molecular interactions (including hydrogen bonding and hydrophobic force) increased (Zhang, Yang, Tang, Hu, & Zou, 2000). In particular, polyphenols may act as cross-linkers of protein making the film structure more compact, resulting macroscopically in higher thickness. In particular, condensed tannins, which are the main phenolic components in PNSE (Moccia, Agustin-Salazar et al., 2020), are known to strongly interact with proteins (Cano, Andres, Chiralt, & González-Martínez, 2020; Girard, Teferra, & Awika, 2019; Moccia, Piscitelli et al., 2020; Zeller, Reinhardt, Robe, Sullivan, & Panke-Buisse, 2020). On the other hand, the films functionalized with PNSE were less flexible than the control ones, showing a lower EB and a higher YM value. However, a significant increase in TS was observed as a result of PNSE incorporation. These observations were in agreement with those reported by Hu, Yuan, Han, Li, and Song (2019) who evidenced that in gelatin-based edible films the

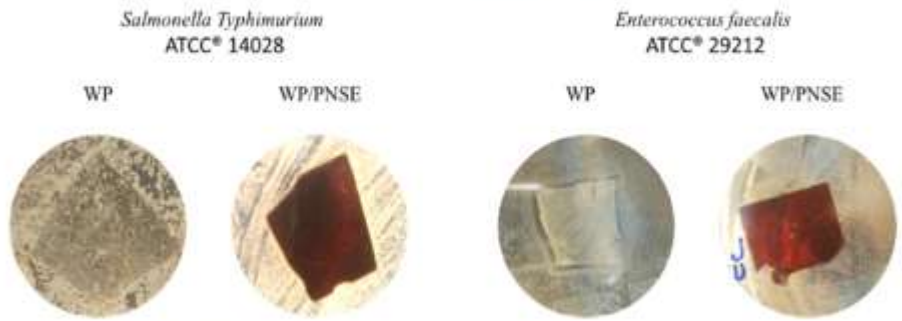

Fig. 4. Antimicrobial activity of WP films functionalized with PNSE. The antimicrobial activity of the films prepared in the absence or in the presence of PNSE was tested by evaluating the growth of both *Salmonella enterica* subsp. *enterica* ser. Typhimurium ATCC® 14028 and *Enterococcus faecalis* ATCC® 29212 under the films in direct contact with agar surface.

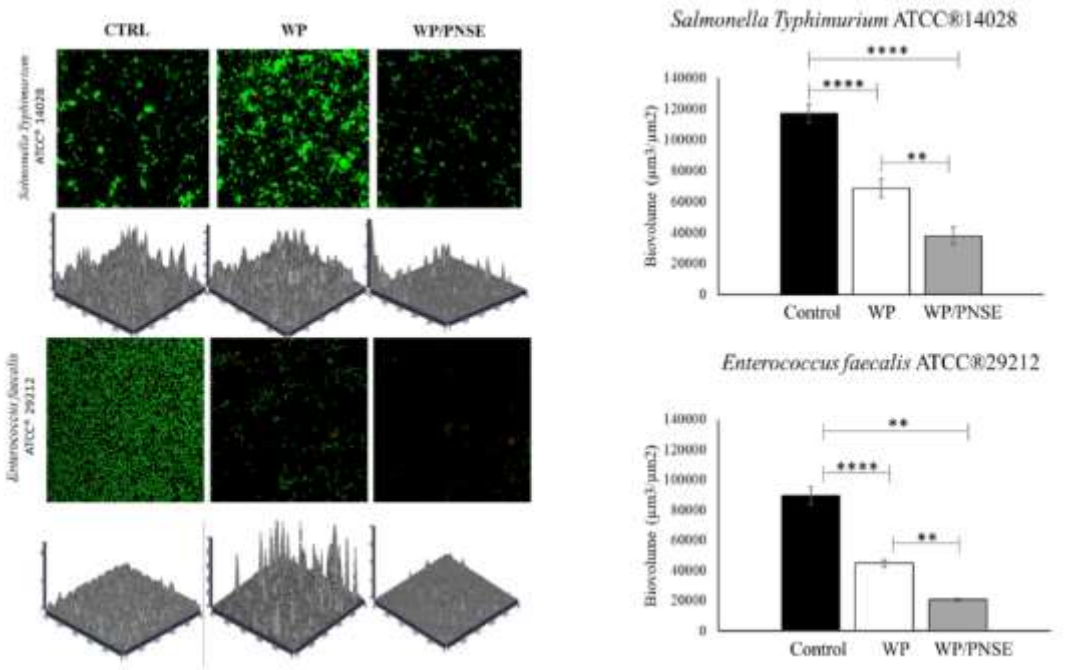

Fig. 5. Confocal laser scanning microscopy analyses of bacterial cells adhesion on functionalized coverslips. The effects of FFS were evaluated on the attachment of *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC® 14028) (upper panel) and *Enterococcus faecalis* (ATCC® 29212) (lower panel) bacterial cells. Images were taken under identical conditions in at least three independent experiments. Scale bar 10 µm. $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$ were obtained for control versus treated samples and WP vs WP/PNSE sample.

crosslinking between the amino group of the proteins and the phenolic components of *Ginkgo biloba* extract enhanced film stiffness, by increasing the TS and lowering the EB. Similar results were also obtained in the studies carried out by Hoque, Benjakul, and Prodpran (2011) in which polyphenols affected the EB values of protein films incorporated with different plant extracts.

3.5.2. Water vapor and gas barrier properties

The barrier properties of the WP-based films towards water vapor, CO_2 and O_2 were also determined. As shown in Fig. 3, the permeability to water vapor and to the two types of gases of the film functionalized with PNSE was found to be significantly lower ($p < 0.05$) compared to the unfunctionalized one. These results are important for a potential use of these materials in the food sector, since, to keep food fresh, the water vapor permeability (WVP) value should be maintained as low as possible (Naidu & John, 2020). In addition, a higher O_2 barrier property is strongly advisable as oxygen is responsible, for example, for the rancidity of fatty acids (Peelman et al., 2014). In addition, the reduced CO_2 permeability can make these active bioplastics suitable as candidates to protect some specific foods from decarbonation (Dong (2016); Idumah, Hassan, & Ihuoma, 2019). The decrease of water vapor and gas permeabilities, as reported by other authors for films containing phenolic extracts (Sun et al., 2019), may be attributed to the increased film thickness.

3.5.3. Antimicrobial activity of WP/PNSE films

The antimicrobial effects of WP-based films were tested on *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC® 14028) and on *Enterococcus faecalis* (ATCC® 29212), as previously described (Sabbah et al., 2019). After 24 h at 37 °C, bacterial cells growth was found to be inhibited only in the presence of PNSE. Indeed, a strong inhibition of bacterial growth of both bacterial strains was observed in the area in direct contact with the film containing PNSE (Fig. 4). Almost no colonies are detected under the film in the presence of PNSE, whereas numerous bacterial colonies are present under the film in the case of the control (Fig. 4). Observed results might be explained by assuming that antimicrobial substances are not released by the film and do not diffuse into the medium, but they act only on bacterial cells at direct contact with the film by inhibiting their growth. Only a slight inhibition of bacterial growth was, instead, observed in the case of unfunctionalized films (Fig. 4). Based on observations reported in the literature, we can hypothesize that PNSE antimicrobial activity might be mediated by oxidation of microbial cell membranes, complexation with essential metal ions, or inhibition of extracellular enzymes (Scalbert, 1991; Serrano, Puupponen-Pimiä, Dauer, Aura, & SauraCalixto, 2009).

3.5.4. Evaluation of the effectiveness of WP/PNSE FFS in preventing bacterial cells adhesion

In order to further investigate the antimicrobial activity of the films functionalized with PNSE, confocal laser scanning microscopy analyses were carried out. To this purpose, both *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC® 14028) and *Enterococcus faecalis* (ATCC® 29212) were grown on glass coverslips coated with FFS prepared in the absence or in the presence of PNSE. The presence of PNSE enhanced FFSs antibacterial activity in the case of both bacterial strains (Fig. 5). Indeed, it should be noticed that the presence of PNSE determines a reduction of biofilm biovolume in the case of both strains under test. Being both bacterial strains able to form biofilm, obtained data suggest that WP/PNSE FFS are able to prevent biofilm attachment by inhibiting cell adhesion. A similar behaviour has been described in the literature for chitosan films functionalized with antibiotics (Smith, Baumgardner, Courtney, Smeltzer, & Haggard, 2010). Interestingly, this

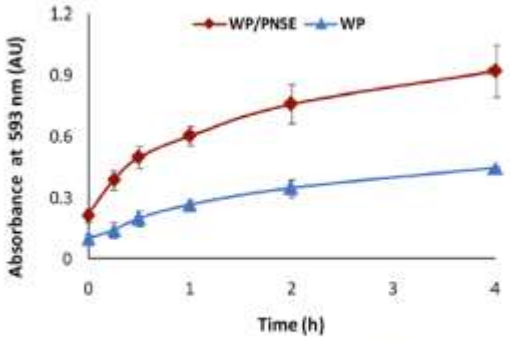

Fig. 6. Fe^{3+} reducing activity of the WP and WP/PNSE films. Mean ± SD values of three experiments are reported.

activity might be associated to specific active ingredients from PNSE, such as tannins, since it has been reported that condensed tannins from the Brazilian medicinal plant *Pityrocarpa moniliformis* are able to act as anti-adhesive substances, both in solution and as immobilized on a surface, against a range of Gram-positive bacteria (Trentin et al., 2015). These results indicate that FFSs containing PNSE might be suitable to be applied in the control and prevention of bacterial contaminations (Smith et al., 2010).

3.5.5. Antioxidant properties of WP/PNSE films

The antioxidant properties of the films were initially evaluated by the DPPH assay, which did not indicate any reducing properties for the PNSE-functionalized films. On the contrary, encouraging results were obtained in the FRAP assay (Fig. 6), which clearly highlighted the ability of PNSE to impart significant antioxidant properties to the film, exhibiting a Trolox equivalent value 2.4 times higher than that of the control film at 4 h (0.6 ± 0.1 μg/mg compared to 0.27 ± 0.02 μg/mg). The antioxidant activity observed for the control film in this assay can be attributed to some WP amino acids able to act as electron donors (Saiz-galo, Lopez-Caballero, Gomez-Guillen, Mauri, & Montero, 2012). The marked differences between the results obtained in the DPPH and FRAP assays can be interpreted on the basis of the higher affinity of the protein-based film for the aqueous medium used in the second assay compared to the organic solvent in which the DPPH assay was performed. This probably allowed a higher permeability and, hence, a more effective interaction of PNSE with the solution, thus resulting in more efficient reducing properties. The ability of phenolic compounds, such as caffeic acid and epigallocatechin gallate (de Morais et al., 2020) as well as proanthocyanidins (Chen et al., 2021), to impart efficient reducing properties to WP in the FRAP assays has been recently reported, whereas less has been done on WP films. Actually, some studies described the efficient reducing properties of functionalized WP films in the DPPH assay (Andrade et al., 2021; Carvalho et al., 2019; Chollakup et al., 2020), whereas only a small number of papers reported relevant results from the FRAP assay (Serrano-Cruz, Villanueva-Carvajal,

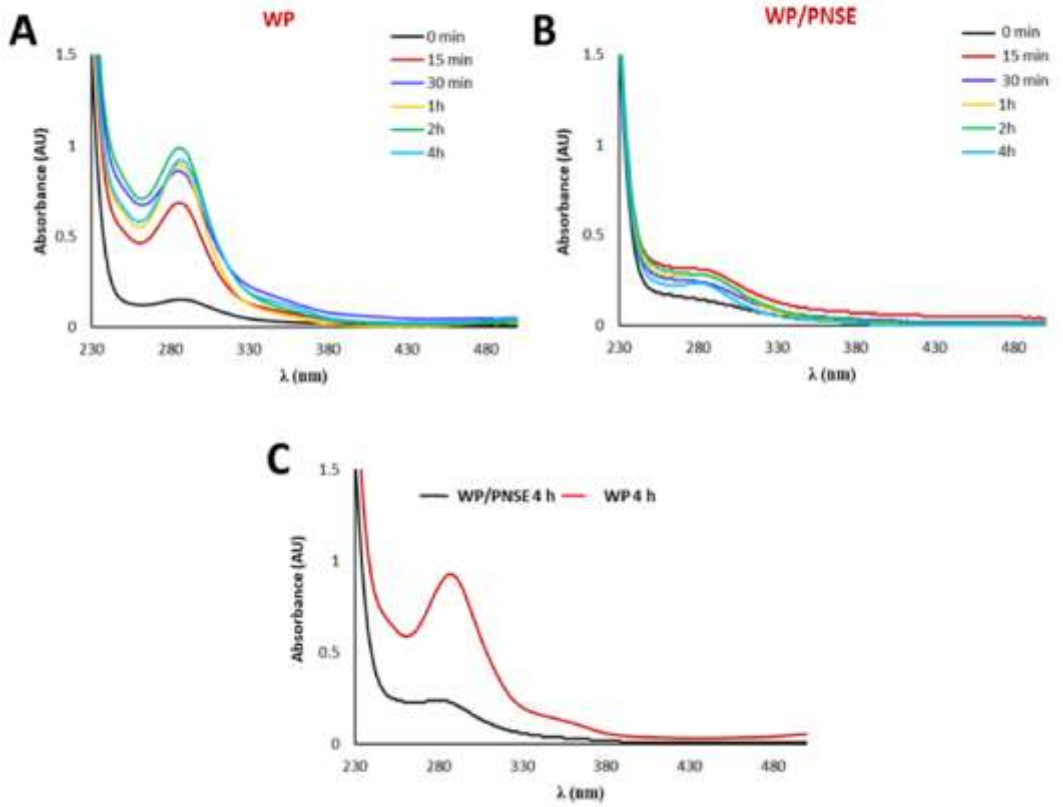

Fig. 7. UV–vis spectra of the solutions of the WP films in 0.3 M acetate buffer (pH 3.6) recorded at different times. (A) WP film, (B) WP/PNSE film, (C) Comparison of the spectra of the WP and WP/PNSE film solutions at 4 h.

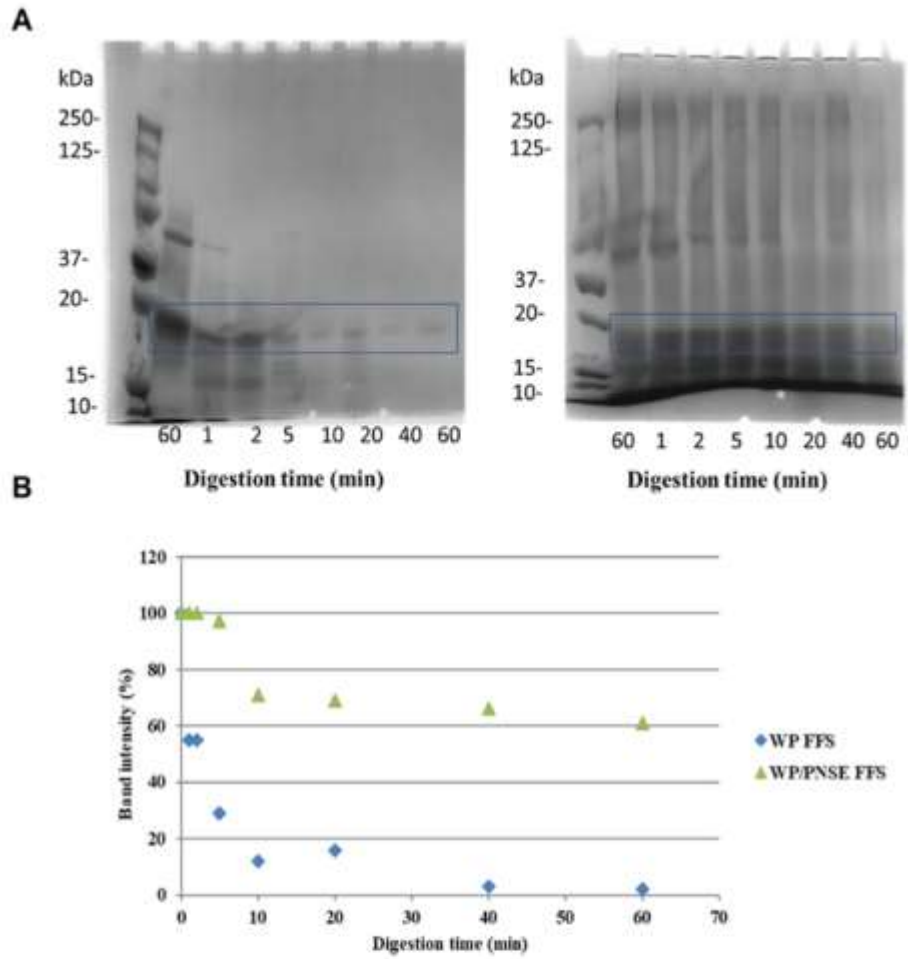

Fig. 8. Panel A: SDS-PAGE (4-20 %) of oral (60 min) and gastric digestion (1-60 min) of FFS based on either WP alone or on WP/PNSE. Panel B: Densitometric analysis of β-lactoglobulin performed by using the software Image lab.

Morales-Rosales, Ramírez-Dávila, & Domínguez-López, 2013; Zuilhuma, Faroze, & Hasiba, 2010). In any case, an efficient interaction between the solvent and the polymer matrix appears to be crucial for the films to exhibit efficient antioxidant properties (Chollakup et al., 2020).

To verify whether the antioxidant properties of the functionalized film are associated to a release of PNSE, further experiments were performed by immersing the film in 0.3 M acetate buffer (pH 3.6) under the same conditions used in the FRAP assay. The solution was periodically analyzed by UV–vis spectroscopy in comparison with a solution of the control film. The results shown in Fig. 7A indicated a significant release of WP (λ_{max} 286 nm) from the control film, reaching a plateau after 2 h. Notably, a remarkable lower absorption was detected in the case of the PNSE-functionalized film (Fig. 7B), suggesting a kind of waterproofing action of PNSE preventing protein leaching, in agreement with the results of the water permeability measurement. The strong similarity between the UV–vis absorption spectra of WP and PNSE (Moccia, Agustin-Salazar et al., 2020) did not allow, however, to attribute the absorbance at around 280 nm to one or another species in the case of the functionalized film. In any case, the amount of released material from this latter reached a maximum after 15 min (Fig. 7B), likely as a consequence of a rapid solubilization of the material localized on the film surface, whereas a progressive increase in the amount of reduced Fe^{3+} was observed over 4 h (Fig. 6), thus ruling out the possibility that the antioxidant properties exhibited by the functionalized films are mainly due to migration of PNSE in the FRAP solution. In any case, assuming that the absorption at 280 nm is due to WP, the lower absorption observed in the case of WP/PNSE compared to WP (0.24 *vs* 0.92, respectively) (Fig. 7C) would indicate a lower release of WP in the solution, thus confirming that the tannin components in PNSE contribute to reinforce the WP structure (Fig. 2). The strong interaction of condensed tannins with WP has been also recently reported in the literature (Freire et al., 2018).

3.5.6. *Oral and gastric digestibility*

In order to verify whether functionalized FFS were digested by the human body in view of a possible application for the preparation of edible coatings, experiments mimicking oral and gastric digestion of FFS under physiological conditions (2012, Gionfatto et al., 2014; Shani-Levi et al., 2017) were performed. The *in vitro* digestion methods have the advantage of being less expensive, less laborious, and free from ethical

restrictions (Shani-Levi et al., 2017). It is worth to point out that only few papers addressed the digestion under physiological conditions of films based on WP. For example, some authors (Sarkar, Zhang, Murray, Russell, & Boxal, 2017) described emulsions with an oil-water interface consisting of a composite layer of WP isolate and cellulose nanocrystals and demonstrated that the presence of the nanocrystals decreased the proteolysis of the proteins.

FFS with or without PNSE were first incubated with a simulated salivary liquid to mimic the oral digestion. Afterwards, the samples were subjected to gastric digestion by bringing the pH to 2.5, and by addition of both pepsin and a simulated gastric liquid prepared under physiological conditions. The samples were then incubated for different times and analyzed by SDS-PAGE (4–20 %) (Fig. 8). Interestingly, alkaline treatment of WPs (Abdalrazeq et al. (2019) seems to improve digestion (Fig. 8) in comparison to WPs not previously treated (Mackie & Macierzanka, 2010). The loss of the tightly packed structure of β-lactoglobulin and α-lactoalbumin at alkaline pH that makes the hydrophobic residues more exposed to the medium and available for digestion with pepsin may likely account for this observation (Ozwulata, Isobe, Tomasula, & Cooke, 2006). Data in Fig. 8 show that the FFS incorporating with PNSE were digested to a lower extent with respect to the control sample. This result is in agreement with the one reported by He et al. (2020) showing a reduced digestibility of WP isolate in the presence of curcumin extract. Densitometric analyses of the protein band relative to β-lactoglobulin characterized by a molecular mass of 17.4 kDa showed that the protein is digested faster in the system prepared in the absence of PNSE. This might be due to the formation of complexes with phenolic compounds that affects the secondary structure of the protein, thus influencing susceptibility to pepsin (Czubinski & Dwiecki, 2017; Girard & Awika, 2020). Another interpretation for the decrease of the digestibility of the extract-containing FFS would invoke the inhibitory activity of the proteases by proanthocyanidins present in the pecan nuts (Vazquez-Flores et al., 2010). In any case, after about 60 min incubation, 40 % of the proteins present in the PNSE-based FFS was digested (Fig. 8B), indicating that films functionalized with PNSE may be considered for developing of novel edible packaging systems.

4. Conclusions

The potential of WP-based FFS as sustainable material for food packaging has been recently increasingly appreciated. In this work a further valorization of this material was explored by incorporation into the FFS and the derived films of an extract obtained from an abundant waste derived from pecan nut production (PNSE). The functionalized films were stiff (YM value equal to 250 MPa) and mechanically resistant (TS value equal to 8 MPa), exhibiting an increased thickness (90 μm) and a decreased EB (30 %), as a result of a substantial interaction of the PNSE condensed tannins with the protein component. Also the barrier properties towards water vapor and CO_2 and O_2 gases were improved by inclusion of PNSE. The mechanical features together with the water vapor and gas barrier properties are of paramount importance to identify the capacity of the bio-based films as packaging materials for protecting different foodstuffs. Notably, PNSE imparted the WP based films good antioxidant (0.6 μg of Trolox/mg of sample in the FRAP assay) and remarkable antimicrobial properties against *Enterococcus faecalis* and the food pathogen bacterial strain *Salmonella enterica* subsp. *enterica* ser. Typhimurium. Finally, although the interaction between proteins and phenolic compounds reduced the rate of digestion, the WP/PNSE-based FFS was still digestible by the human gut, and hence the possibility to use this material for the implementation of edible films remains a viable option. All these observations confirm the possibility to exploit WP/PNSE-based films as an advantageous and eco-friendly alternative for food preservation and shelf-life extension. Of course, further studies on the applicability of WP functionalized with PNSE in preserving a specific food product should be carried out to optimize the function of this active packaging.

CRediT authorship contribution statement

Angela Arciello: Conceptualization, Supervision, Writing - original draft, Writing - review & editing. **Lucia Panzella**: Conceptualization, Data curation, Methodology, Supervision, Writing - original draft, Writing - review & editing. **Eliana Dell'Olmo**: Data curation, Investigation, Methodology. **Manar Abdalrazeq**: Methodology, Supervision. **Federica Moccia**: Investigation, Writing - review & editing. **Rosa Gaglione**: Data curation. **Sarai Agustin-Salazar**: Investigation, Methodology, Writing - original draft, Writing - review & editing. **Alessandra Napolitano**: Conceptualization, Supervision, Writing - review & editing. **Loredana Mariniello**: Supervision, Writing - review & editing. **C. Valeria L. Giosafatto**: Conceptualization, Supervision, Writing - original draft.

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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4. CONCLUSIONS

In the present thesis heating tat 80°C for 25 min and alkaline pH treatment were employed in order to assess the effect of these experimental conditions on the derived materials. Hence, films were prepared by casting WP FFSs, at both pH 7 and 12, containing 30, 40 or 50% (w/w of WPs) GLY. The minimum GLY concentration for obtaining handleable films at pH 12 was 30%, also without a preliminary heat-treatment of the proteins, whereas at pH 7 it was necessary to heat-treat the FFS containing at least 40% GLY. After analyses of the stability of FFSs by zeta-potential and particle size measurements, the mechanical properties of the derived films were investigated. The obtained results demonstrated that the films obtained at pH 12 from FFSs unheated and containing either 40 or 50% GLY led to obtain more flexible bioplastics. In fact, these films exhibited the highest EB and the lowest YM, also much higher and lower, respectively, than those observed with counterpart films obtained at pH 7 from heated FFSs. Further experiments were focused on the use of mTG, a microbial enzyme able to catalyze the formation of isopeptide bonds between endoprotein glutamine and lysine residues. The enzyme was exploited in order to further improve the technological properties of WP-based bioplastics. The extent of mTG-mediated crosslinking, assessed by SDS-PAGE analysis, has proved to improve the technological properties of the films by enhancing the EB and reducing the YM. Also, the barrier properties towards CO_2 and O_2 resulted increased following the mTG-catalysed crosslinking. In addition, in this study for the first time WPs were blended with NPs obtained from PHAs. The technological properties of the derived bio-nanocomposites demonstrated that PHA-NPs conferred plasticity features to the films by also improving the film gas barrier properties. This was confirmed by the material microstructure that evidenced a homogeneous dispersion of NPs into the film matrix. WP-based films were finally used as support for

the incorporation of bioactive molecules. In particular, attention was focused on the entrapment into selected films of both antioxidants and antimicrobial agents from leaves of Thymbra and from nut shell of Pecan, respectively. These natural molecules were able to endow WP based films with good antioxidant and remarkable antimicrobial properties against different food spoilage bacterial strains. Besides the natural additives were also able to influence film technological properties, being the materials prepared in the presence of Pecan extract less extensible and more mechanical resistant, whereas the EOs from Thymbra reduced film mechanical resistance without changing the extensibility. In addition, experiments carried out to investigate the digestion under physiological conditions of such functionalized films, demonstrated that the films prepared with Pecan extract were still digestible by the human gut, and, hence, the possibility to use this material for the implementation of edible films remains a viable option. All these observations confirm the possibility to exploit WP-based films, prepared in the presence of different additives, as an advantageous and eco-friendly alternative to the traditional plastic polymers for food preservation and shelf-life extension.

The scale up of these new materials on industrial scale might open new horizons to produce tailored one time or short-term use items suitable for food packaging. For example, they could be useful to protect and extend the shelf-life of different kind of foods (both raw and cooked) to reduce moisture adsorption. For example, the transparent mTG-crosslinked can be used to protect some milk-based foods in which is important to see through the package. On the other hand, a possible protection from photooxidation of photosensitive compounds contained in various food products might be obtained by using the PNSE-based purple red color. More in general, these findings encourage further investigations since WPs seem a potential renewable bio-source capable to partially substitute the highly pollutant petroleum-derived polymers, the production of which is continuing to exponentially increase. In fact,

from an environmental and economic point of view, it is worthy to note that petroleum is becoming significantly expensive and a progressively limited resource expected to decline over the next few decades. Therefore, an early transition to renewable sources, such as that represented by WPs, might be a valuable milestone for some specific sectors of food packaging industry.

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APPENDIX

➢ Experience in foreign laboratories

1. **Faculty of Medicine and Health Sciences,** New Campus**,** AN-Najah National University**,** P.O. Box 7 Nablus- Palestine
   Supervisor: Prof. Nidal Amin Jaradat
   Topic: Extraction and component identification of Thymbra essential oils, studying their antimicrobial activities.
   Date: July, August 2019
2. **Department Materials Development**, Fraunhofer Institute for Process engineering and packaging IVV, Giggenhauser Str. 35, 85354 Freising
   Supervisor: Dr. Siegfried Fürtauer
   Topic: Coatings experiments of bio-based materials (Whey proteins, Nanocellulose) on different polymer substrate, and analysis their effect on the technological properties of the used substrates.
   Date: March, April, May 2021

➢ Courses

1. Protein structure visualization, analysis and molding (Prof. Eugenio Notomista),2018
2. Bioactive molecules from natural sources: purification and application (Dr. Daria Monti), 2018
3. Bioplastics (Dr. C. Valeria L. Giosafatto) 2018
4. Enzymes as additives or processing aids (Dr. C. Valeria L. Giosafatto) 2018
5. Enzymatic and microbial applications in biotransformations and nanotechnology (Prof. Rachele Isticato), 2018

6. Advanced in mass spectroscopy (Prof. Piero Pucci), 2018
7. First step in writing and publishing a scientific manuscript (Prof. Daria Monti), 2019
8. Carbohydrate active enzymes for biorefineries (Prof. Marco Moracci), 2019
9. Uses and abuse of Chemicals (Prof. Fabio Temussi), 2019
10. The techniques of solid-liquid extraction for industrial uses (Prof. Daniele Naviglio), 2020
11. Enzymes as additives or processing aids (Prof. Giosafatto Valeria), 2020
12. Microscopic methodologies (Prof. Angela Arciello and Dr. Rocco Di Girolamo), 2020
13. Safety Training Course (Prof. Liliana Lista), 2021

- **Seminars**

1. Prof. Henk Haasgmann. “Immunomosulatory antimicrobial peptides: biology and applications”, 15/02/2018
2. Prof. N J Clayden. “A life of magical spinning (Solid state NMR spectroscopy in chemistry)”, 20/02/2018
3. Prof. N J Clayden. “Muons useful in chemistry”, 22/02/2018
4. Prof. Charlotte Blom. “Enzyme discovery at novozymes”, 13/03/2018
5. Prof. Michael M.Cox. “On the Nature of Science”, 13/04/2018
6. Dr. Thierry Tron “En route to synthetic biology”, 22/06/2018.

7. Prof. Carlos Regalado Gonzales “Design and characterization of an edible film based on starch and chitosan”, 29/06/2018.

8. Prof. Dargana Mitic “Inorganic drugs to treat cancer”, 5/07/2018

9. Prof. Carmen Galen “Novel synthetic glycol- tools for biology research”, 26/09/2018

10. Dr. Arianna Mazzoli “Search of new therapeutic targets of obesity and obesity-related diseases”, 21/11/2018

11. Prof.M.J.Collins “Biology of the book and the bee”, 19/02/2019

12. Prof.Mauro Di Fenza “Industrial biotechnology innovation and synthetic biology accelerator”, 26/03/2019

13. Prof. Anna Maria Masdeu Bulto.” Utilization of CO2 from solvent to reactant”, 9/04/2019

14. Prof. Serecko Kirin “Artificial secondary structures in the design of new catalysts and new material”, 07/06/2019

15. Prof. Jesus Jimenez-Barbero “Breaking the limits in glycan recognition by NMR”, 23/09/2019

➢ List of Publications

1- **M. Abdalrazeq**, C.V.L. Giosafatto, M. Esposito, M. Fenderico, P. Di Pierro, R. Porta. Glycerol-Plasticized Films Obtained from Whey Proteins Denatured at Alkaline pH. Coatings,9, 322-331, https://doi.org/10.3390/coatings9050322

2- **M. Abdalrazeq**, C.V.L. Giosafatto, R. Porta. Properties of bio-materials obtained from milk whey proteins at different pH values and plasticizer concentrations. 1st Coatings and

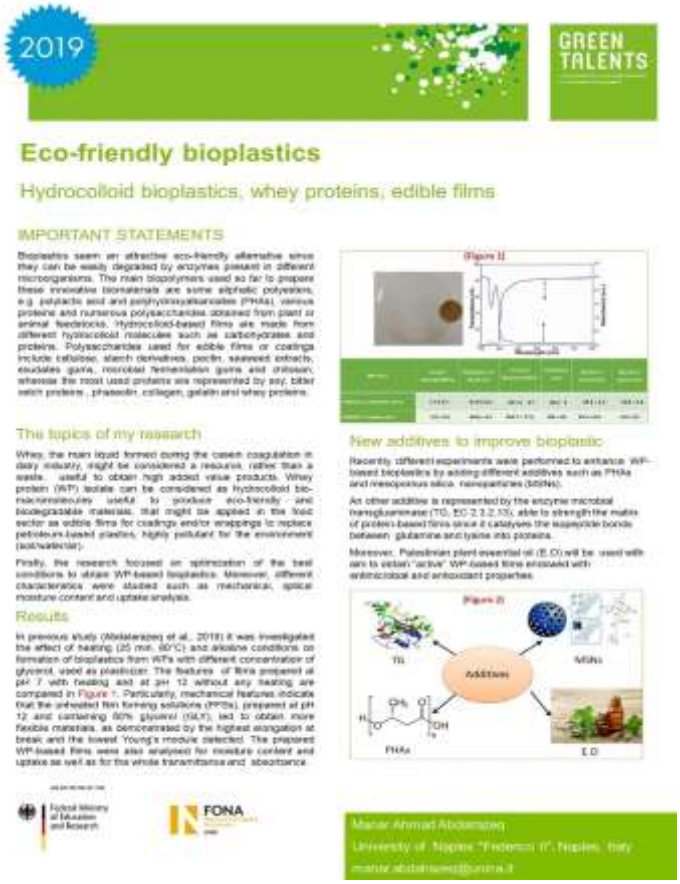
Interfaces Web Conference, https://doi.org/10.3390/ciwc2019-06150

3- **I.Corrado and M. Abdalrazeq** , C. Pezzella, R. Di Girolamo, R. Porta,G. Sannia, C.V.L. Giosafatto. Design and characterization of poly (3-hydroxybutyrate-co-hydroxyhexanoate) nanoparticles and their grafting in whey protein-based nanocomposites. Food Hydrocolloids, 2020, https://doi.org/10.1016/j.foodhyd.2020.106167

4- A.Arciello, L. Panzella, E. Dell'Olmo, **M. Abdalrazeq**, F. Moccia, R.Gaglione, S. Agustin-Salazar, A. Napolitano, L. Mariniello, C. V. L. Giosafatto. Development and characterization of antimicrobial and antioxidant whey protein-based films functionalized with Pecan (Carya illinoinensis) nut shell extract. Food Packaging and Shelf Life, 2021, https://doi.org/10.1016/j.fpsl.2021.100710

➢ List of Communications

1. **M. Abdalrazeq**, Eco-friendly bioplastics, Green Talents – International Forum for High Potentials in Sustainable Development, Fedral Minstry of Education and research, Berlin, Germany. 23th – 25th October 2019.



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2. I. Corrado, G. Sannia, C. Pezzella, R. Di Girolamo, C.V.L. Giosafatto, C. Regalado Gonzàlez, R. Porta and **M. Abdalrazeq**, WHEY PROTEIN/POLYHYDROXYALKANOATE BIONANOCOMPOSITES, SIB 2019_60th CONGRESS, Lecce, Italy. 18th – 20th September 2019.

WHEY PROTEIN/POLYHYDROXYALKANOATE BIONANOCOMPOSITES

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Whey, the liquid obtained from the acid or proteolytic milk clotting, is responsible for relevant environmental issues due to its both large volume and high organic content. Bovine whey, the most common one produced in the western countries, shares about 85–95% of the originating milk volume and contains about 55% of the whole milk nutrients. The use of whey proteins (WPs) to prepare biodegradable packaging materials is an attractive recycling possibility of such by-product of the dairy industries. More recently, much attention has been given to the production of biopolymer composites where nanoparticles (NPs) of different chemical nature were shown to improve the properties of biodegradable materials. In this work the attention was focused on NPs derived from polyhydroxyalkanoates (PHAs), a class of biopolymers produced by bacteria from renewable sources (1). The experimental conditions for PHA-NPs preparation were set up and the obtained NPs were characterized by Transmission Electron Microscopy and Scanning Electron Microscopy. Dynamic light scattering analyses show that PHA-NPs are stable, exhibiting a ζ-potential value of about -40 mV and a Z-average size of 80 nm. Also the WP/PHA film forming solutions, prepared at pH 12 to obtain handleable, transparent and more flexible bionanocomposite films (2), resulted stable. The mechanical properties of the derived blended bioplastics indicate that the addition of PHA-NPs to WPs enhances film elongation at break and decreases its Young's modulus, producing more extensible materials.

1. Raza *et al.* (2018). Polyhydroxyalkanoates: properties and chemical modification approaches for their functionalization. *Biotechnol Prog. 34*:29-41.
2. Abdalrazeq *et al.* (2019). Glycerol-plasticized films obtained from whey proteins denatured at alkaline pH. *Coatings 9*: 322-330.

3. **M.Abdalrazeq**, C.V.L Giosafatto, C. Regalado Gonzales, P. Di Pierro, and R. Porta. Cross-linked milk whey protein-based bioplastics obtained in the presence of microbial transglutaminase. 9th Edition of the Shelf Life International Meeting (SLIM 2019), Congress Center Federico II, Napoli, 17th– 20th June 2019.

PNM30-Cross-linked milk whey protein-based bioplastics obtained in the presence of microbial transglutaminase

Manar Abdalrazeq[1], *Concetta Valeria L. Giosafatto*[1*], *Carlos Regalado-Gonzales*[2], *Prospero Di Pierro*[1] *and Raffaele Porta*[1]

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Abstract

Milk whey (MW), the liquid obtained from the acid or proteolytic milk clotting, is responsible for relevant environmental problems due to its both large volume and high organic content. Hence, the use of MW proteins (MWPs) as biopolymer source to prepare biodegradable/edible packaging materials is an attractive MW recycling possibility. In this work we investigated the film forming properties of MWPs, thermally denatured at 80°C for 25 min at pH 7, following treatment with microbial transglutaminase (mTG, E.C. 2.3.2.13), an enzyme able to catalyze the formation of isopeptide bonds between endoprotein Gln and Lys. After incubation with different amounts of enzyme (8, 16 and 24 U/gr of MWPs), the pH of the film forming solutions (FFS) was adjusted to 12 as we found out that at this pH handleable and transparent films were obtained. Finally, 40% glycerol (*w/w* protein), used as plasticizer, was added to FFSs and films were prepared by casting 1% of MWPs. Under these experimental conditions, it was proved by SDSPAGE that MWPs acted as mTG substrates and, through ζ potential measurements, it was also assessed the physico-chemical stability of the FFSs. Mechanical properties analyses indicated that the microbial enzyme enhanced the elongation at break and reduced the Young's modulus, showing that mTG was able to produce more flexible and less rigid films. Additional experiments, based on blending with different nanoparticles, aliphatic polyesters and anionic polymers are being carried out to further improve the performances of the cross-linked MWP-based bioplastics reported.

Keywords: milk whey proteins, biodegradable materials, microbial transglutaminase, mechanical properties.

Acknowledgement *This work was supported by the Italian Ministry of Foreign Affairs and International Cooperation through the "Fourth Executive Program of Scientific and Technological Cooperation between Italy and the United States of Mexico", 2018-2020.*

4. **M. Abdalrazeq,** C.V.L Giosafatto,R. Porta. A novel bioplastic obtained by whey protein recycling. 2° Workshop BIO/10, Aulua Magna del Complesso delle Biotecnologi, Napoli, 17 May 2019.

5. **M. Abdalrazeq**, C.V.L Giosafatto*,R. Porta. Effect of alkaline pH and heat treatment on the formation of bioplastics from whey proteins in the presence of different concentrations of glycerol. 2° Workshop BIO/10, Aulua Magna del Complesso delle Biotecnologi, Napoli, 17 May 2019.

BIOTEC.3

EFFECT OF ALKALINE PH AND HEAT TREATMENT ON THE FORMATION OF BIOPLASTICS FROM MILK WHEY PROTEINS IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF GLYCEROL

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Milk whey (MW) represents the major by-product of cheese industry. One possibility to recycle the MW wastes is the use of their globular proteins (MWPs) as a polymer source for the production of biodegradable plastic materials. MWP-based films are usually obtained by protein heat treatment in the presence of glycerol (GLY) as plasticizer at pH 7 [1], a method which would require commercially high costing process. Since it is known that denaturation and aggregation of MWPs are pH dependent, with strong alkalis producing rod-like microstructures able to forms fine-stranded fiber-like matrices, it was exploited the possibility to produce manageable MW-derived materials without any heat-treatment but under alkaline conditions. Our results demonstrated that the casting at pH 12 of the unheated MWP film forming solutions, containing either 40 or 50% GLY, led to produce more resistant and flexible films than the ones obtained at pH 7. Film opacity was observed significantly increased, being higher in the samples obtained at alkaline pH without MWP heating and with higher GLY concentrations. The developed experimental conditions allowed to produce hydrocolloid films with improved properties probably because MWPs denatured under alkaline conditions form small primary aggregates able to combine into large clusters [2]. Finally, also moisture content was observed to decrease with the reduction of GLY content, both in heated and unheated MWP-based films, whereas water uptake of the different films prepared at pH 12 did not significantly change.

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2. Bryant, C.M., McClements, D.J. Optimizing preparation conditions for heat-denatured whey protein solutions to be used as cold-gelling ingredients. *Afr. J. Food Sci.* **2000**, *65*, 259–263, Doi: 10.1111/j.1365-2621.2000.tb15990.x.

6. C.V.L Giosafatto , A. Romano, A. Al-Asmar, M. Abdalrazeq, L. Mariniello. Microbial transglutaminase as effective tool to modify functional properties and in vitro digestibility of grass pea (lATHYRUS sATIVUS l.) flour. 6 International Conference on food Digestion Granada conferance center, Spain, 2ed- 4th April 2019.

378/82. MICROBIAL TRANSGLUTAMINASE AS EFFECTIVE TOOL TO MODIFY FUNCTIONAL PROPERTIES AND IN VITRO DIGESTIBILITY OF GRASS PEA (LATHYRUS SATIVUS L.) FLOUR

(1)Giosafatto, C., (1)Romano, A., (2)Al-Asmara, A., (1)Abdalrazeqa, M., (1)Mariniello, L.

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Introduction

Grass pea is a very popular crop in many Asian and African countries where it is grown either for stockfeed or human consumption. It is characterized by a lot of advantageous biological as well as agronomic features such as resistance to drought, high grain-yielding capacity and high protein content of its seeds. Thus, nowadays, this legume is rightly considered as one of the most promising sources of starch and proteins.

Objective

Our aim was to characterize grass pea flour following treatment with microbial transglutaminase (TG), an enzyme catalyzing intra and/or intermoleoular isopeptide bonds between glutamines and lysines into proteins.

Methodology

The impact of the TG modification on microstructure by means of Scanning Electron Microscopy was explored. Protein digestibility was assessed by carring out in vitro digestion experiments following physiological conditions. Finally starch digestibility as well as expected glycemic index (eGI) of grass pea flour was evaluated by using an enzymatic assay kit and by taking into account the rapidly digestible starch and slowly digestible starch, important parameters used also for calculating the eGI.

Main findings

Results demonstrated that grass pea flour proteins act as effective substrate of TG. Microstructural results showed that the addition of TG produced a more compact structure likely due to TG-catalysed heteropolymers. Nutritional properties such as slowly digestible starch and expected glycemic index values followed the order: grass pea flour incubated in the absence of TG> grass pea flour incubated in the presence of TG > raw flour. The TG catalyzed heteropolymers were easily digested as demonstrated by in vitro oral and gastric digestion.

Conclusion

TG-modified grass pea flour can be considered as a new source of starch and proteins, as it possesses nutritional properties that make this legume an inexpensive food source suitable for for feeding a large spectrum of population.

Key words

Estimated glycemic index, food structure, grass pea flour, in vitro digestion, transglutaminase.

7. R. Porta, **M. Abdalrazeq**, P. Di Pierro, C. Regalado Gonzales, and C.V.L Giosafatto. Milk whey protein-based biomaterials with improved properties. XV FISV Congress Sapienza University of Rome, Italy, 18-21 September 2018.

P17.4 - Milk whey protein-based biomaterials with improved properties

Raffaele Porta[1], M. Abdalrazeq[1], C.V.L Giosafatto[1], P. Di Pierro[1], C. Regalado Gonzales[2], C.V.L Giosafatto[1]

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The dairy industry gives rise to considerable quantities of milk whey (MW) that, because of its high organic content, cannot be discharged directly and, thus, should be treated with additional costs for the manufacturing companies. One possible MW recycle is the use of its protein content (~65% β-lactoglobulin, ~25% α-lactalbumin and ~8% bovine serum albumin) as biopolymer source for production of biodegradable/edible films, coatings or wrappings. In the present study MW proteins (MWPs), following heat denaturation and solubilization at different pHs, were exploited to prepare film forming solutions containing different concentrations of uncharged and/or ionic plasticizers. These new formulations gave rise to stable systems, as demonstrated by ζ-potential measurements and, by casting, to manipulable, resistant and flexible films with features comparable to the ones of various commercial biomaterials. Further experiments, based on enzymatic MWP crosslinking and/or blending with different nanoparticles, are being carried out to further improve the performances of the obtained MWP-based edible films. *(Supp. by Ital. Min. For. Aff. & Int. Coop.; IV Progr. Quadro Coop. Italia/Messico).*

➢ Memberships

01/02/2018 – Present

Junior Member in Italian Society of Biochemistry and Molecular Biology

➢ Honours and Awards

12/10/2019- 26/10/2019

- Awards – Green Talent 2019- German Federal Ministry of Education and Research (BMBF): Every year, the German Federal Ministry of Education and Research (BMBF) hosts the prestigious Green Talents – International Forum for High Potentials in Sustainable Development to promote the international exchange of innovative green ideas from various fields of research. I was one of the 25 winners among 837 applicants. During two weeks I visited many universities and research institutes including:

1. Research Center for Artificial Intelligence (DFKI), Saarbrücken.
2. The United Nations University (UNU), Bonn.
3. RWTH Aachen University, Aachen.
4. Wuppertal Institute for Climate, Environment and Energy, Wuppertal.
5. Germanwatch, Bonn.
6. Covestro, Düsseldorf.
7. The IfBB – Institute for Bioplastics and Biocomposites, Hannover.
8. Fraunhofer Institute for Process Engineering and Packaging, Giggenhauser Str. 35, 85354 Freising,
9. Federal Ministry of Education and Research (BMBF), Berlin.

03/12/2019

- Grant from Italian Society of Biological Chemistry (SIB) to participate in the Enzyme Discovery and Engineering for Biotechnological, Monte Sant 'Angelo, University of Naples Federico II, Napoli, Italy.

18/09/2018

- Grant to participate in the (FISV) conference 2018 – Italian Society of Biological Chemistry (SIB), Roma, Italy

13/09/2019 – 13/12/2019

- Scholarship for research activity – Italian Ministry of Foreign Affairs and International Cooperation Entitled. “Nano reinforced edible films to be used in food packaging". University of Naples “Federico II”

01/10/2020 – 1/12/2020

- Scholarship for research activity – Italian Ministry of Foreign Affairs and International Cooperation Entitled. "Preparation and characterization of edible materials containing nanoparticles activated with essential oils. University of Naples “Federico II”

➢ Conferences

- November 5th 2020, Online forum and partnering on biopolymers 2020, Bayern Innovativ GmbH, Germany
- 1st - 2ed October 2020, IFIB 2020 International Online Forum on Industrial Biotechnology and Bioeconomy, Italy
- 18th- 20th, September 2019, SIB 2019_60th Congress (Lecce, Italy)
- 17th- 20th June 2019, 9th Edition of the Shelf-Life International Meeting (SLIM 2019), in "Federico II University " (Naples, Italy)
- 2nd- 4th of April 2019: 6th International Conference on food Digestion, Granada conference center, (Granada, Spain)

- 15th- 29th March 2019: 1st Coatings and Interfaces Web Conference (CIWC 2019)
- 18th- 21st September 2018: XV Congress of the Italian Federation of Life Sciences (FISV), in "Sapienza University" (Rome, Italy)

➢ Workshops

- II Industrial Biotechnology: BioID&A Biotechnology Identity and Application, 28 October 2019. Sala Azzurra, Monte Sant ' Angelo, University of Naples Federico II, Napoli, Italy.
- Scientific communication, 25 October 2019, Federal ministry of education and research, Berlin (Germany)
- 2° Workshop BIO/10 for SIB society , 17 May 2019, Aula Magna del Complesso delle Biotecnologie, Napoli (Italy)
- NanoBioMedicin, 22 March 2019, Conference Room CNR, Napoli (Italy)
- After your PhD what?, 18-21 February 2019, Sala Azzuro, University of Naples Federico II, Napoli, Italy.
- 7th February 2019 Speaker in "Migrant women in university and research",
  Link: https://agenda.infn.it/event/18142/contributions/84943/

➢ Schools

- Enzyme Discovery and Engineering for Biotechnological Applications, 3-5 December 2019, Monte Sant ' Angelo, University of Naples Federico II, Napoli, Italy.
  https://astrobio2020.wixsite.com/edenbiotech2019
- 14th -15th May 2018: Transferable Skills Spring School, University of Naples Federico II, Napoli, Italy
- 25th October 2019, scientific communication workshop, Berlin, Germany

Manar Abdalrazeq

2018 – 2021

93