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Chitosan Film Functionalized with Grape Seed Oil—Preliminary Evaluation of Antimicrobial Activity

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Abstract: Although the fishing and wine industries undoubtedly contribute significantly to the economy, they also generate large waste streams with considerable repercussions on both economic and environmental levels. Scientific literature has shown products can be extracted from these streams which have properties of interest to the cosmetics, pharmaceutical and food industries. Antimicrobial activity is undoubtedly among the most interesting of these properties, and particularly useful in the production of food packaging to increase the shelf life of food products. In this study, film for food packaging was produced for the first time using chitosan extracted from the exoskeletons of red shrimp (*Aristomorpha foliacea*) and oil obtained from red grape seeds (*Vitis vinifera*). The antimicrobial activity of two films was analyzed: chitosan-only film and chitosan film with the addition of red grape seed oil at two different concentrations (0.5 mL and 1 mL). Our results showed noteworthy antimicrobial activity resulting from functionalized chitosan films; no activity was observed against pathogen and spoilage Gram-positive and Gram-negative bacteria, although the antimicrobial effects observed were species-dependent. The preliminary results of this study could contribute to developing the circular economy, helping to promote the reuse of waste to produce innovative films for food packaging.

Keywords: bioactive molecule; crustacean; marine invertebrate; shelf life

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1. Introduction

The fishing industry is an important economic sector for many countries [1,2]. Despite the importance of this sector in food production, waste creation remains extremely high, often representing a danger to the economy and the environment [3–6]. It is estimated that approximately two-thirds of all catches are not consumed, thereby representing a source of waste [7]. Furthermore, waste production from the fishing industry has increased considerably in recent decades from 27 billion tonnes (in 1970) to 92.1 billion tonnes (in 2017); an increase of 254% [8]. However, waste can be an interesting source of bioactive molecules, such as proteins, lipids and chitin [9], and, in recent years, researchers have studied how to make fish waste a possible valuable resource in the context of the circular economy [7]. A significant proportion of fish waste is constituted by vertebrate species; however, invertebrate species also account for a large part [10]. An interesting product to be extracted from invertebrate waste (for example, many crustacean species) is chitin, one of the most abundant polysaccharides in nature, also found in molluscs, insects and fungi [11,12]. Due to its chemical characteristics, this molecule is insoluble

and poorly biodegradable [13,14]; however, deacetylation produces chitosan [15], a more soluble, biocompatible and biodegradable molecule [16]. Chitosan is the second most abundant polysaccharide found in nature and commonly contained in the exoskeletons of crustaceans [17]. It is biodegradable, nontoxic and has some interesting biological properties, such as antimicrobial [18], anticancer [19], antioxidant [20] and anticoagulant [21] activities. It has also been used as a biomaterial in the artificial regeneration of skin, bone and cartilage [22,23], and for pharmaceutical purposes [24]. It is clear from the above that chitosan is of interest to a series of industrial sectors, including food and nutrition [25], cosmetics [26], wastewater treatment systems [27] and agriculture [28]. Regarding the food sector in particular, this molecule has shown significant potential in the production of new food packaging. Films containing antimicrobial properties are useful when seeking to increase the shelf life of food [29–31]. Recent research has also turned its attention towards the production of chitosan film and its potential to improve the antimicrobial and antibacterial properties [32] with the addition of different types of essential oils [33]. This results in an increase in antimicrobial activity against several bacterial species, such as *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli* [34–37]. In addition to the fishing industry, the wine sector also produces enormous quantities of waste [38]. Almost 39% of production (approx. 20% of which is generated during distribution) [39] consists of pomace, grape seeds, stalks, leaves, wastewater, greenhouse gas emissions and inorganic waste [40]. Like fishing waste, many of these waste streams are a rich resource of polyphenolic compounds with antimicrobial and antioxidant effects [41,42], which could be treated or reused to reduce environmental impacts [43–45]. Scientific literature has shown that grape seeds contain oils which are rich in fatty acids and triacylglycerols with many beneficial [46,47] antimicrobial [48], antioxidant [49], anti-inflammatory [50], cardioprotective [50,51] and antitumoral [52] properties. These properties have been associated with various components, such as flavonoids, carotenoids, phenolic acids, tannins and stilbenes [46,53], catechins, epicatechins, trans-resveratrol and procyanidin B1 [54,55], vitamin E, unsaturated fatty acids (UFA) and phytosterols [56]. The oils are generally extracted using both organic solvents and mechanical techniques [53]; however, they can also be obtained by cold pressing, thus better safeguarding the properties of the substances contained in the oil [57]. From a food preservation perspective, it is known in scientific literature that microorganisms are the primary factor in food degeneration during transport and storage [58]. Examples of these are non-pathogenic Gram-negative bacteria (e.g., *Pseudomonas* spp., *Enterobacteriaceae*, *Acinetobacter* spp., *Staphylococcus aureus*, *Brochothrix* spp., *Escherichia coli*, *Listeria monocytogenes*), which dominate on food-processing surfaces and are implicated in food safety and quality [59–64]. Furthermore, research has revealed a number of pathogenic bacteria which are harmful to human health and are responsible for food contamination, food poisoning and infection [59,60,64,65].

In the literature, several authors have evaluated the antimicrobial activity and properties of chitosan film functionalized with grape seed extract [66–68]. Moreover, other authors who have used chitosan to produce film often used synthetic chitosan [69–72]. For this reason, the novelty of the current study was to analyze the antimicrobial activity of chitosan film extracted for the first time from *A. foliacea* exoskeletons (an economically important species and highly appreciated by consumers) functionalized with grape seed oil (obtained from grape of *V. vinifera* [47]) to evaluate its potential in food packaging. Regarding the grape seed in this study, a new protocol of extraction was performed to obtain oil from grape seed and not powder. This could favor the chitosan functionalization. In particular, the aim of this study was to evaluate the optimal oil concentration to improve the potential antimicrobial capabilities of the films tested, with the future aim of obtaining an exploitable film that can contribute to the circular economy and thus reduce the economic and environmental damage caused by these wastes. In fact, after evaluating which film will have the greatest antimicrobial potential, future studies will aim to test this directly on food and to characterize its chemical–physical properties before being proposed for entry into the market.

2. Materials and Methods

2.1. Chitosan Extraction

Chitosan extractions were carried out using shrimp waste and, in particular, *Aristaeomorpha foliacea* exoskeletons provided by local fishmongers (Palermo, Italy). Firstly, the frozen shrimp waste (−20 °C) was thawed and all organic parts removed; it was then washed in distilled water and dried at 60 °C for two nights. After the drying phase, the sample was ground, weighed and used for the extraction of chitosan. The extraction of chitosan was carried out according to the modified protocol of [73–76]. Extraction involves four basic steps: deproteinization, demineralization, decolorization and deacetylation. Deproteinization was performed using a 3% NaOH (1:10 *w*/*v*), incubating the powder for two hours at 65–70 °C. At the end of incubation time, the solution was cooled, the powder was rinsed 3 times in distilled water and dried overnight at 60 °C. For the demineralization step, the sample was solubilized in a 1N HCl solution for 1 h at 25 °C at a ratio of 1:15 (*w*/*v*). The powder was rinsed three times, re-covered and dried overnight at 60 °C. During the decolorization step, the sample was weighed and solubilized in a 0.315% NaClO solution for 5 min at 25 °C at a ratio of 1:10 (*w*/*v*), rinsed and dried as described above. At the end of this step, chitin was obtained [75]. To obtain chitosan, however, a final deacetylation step needs to be performed. In detail, the chitin was solubilized in 50% NaOH at 90 °C for 5 h (1:15 *w*/*v*). After 5 h, the powder was rinsed three times, recovered and dried overnight at 60 °C. To obtain pure chitosan powder, an additional purification step was performed, effectuating a slight modification to the protocol of [75]. The chitosan sample was solubilized in 0.5 M acetic acid at a ratio of 1:100 (*w*/*v*) overnight at 25 °C. Subsequently, the solution was filtered, neutralized with NaOH until pH 8, and rinsed using distilled water and ethanol–water solution (70:30). At the end, the purified chitosan was recovered and dried.

The IR spectrum was recorded on a Jasco FT/IR 420 spectrometer, in KBr disk sample holders, in the range 4000–300 cm^{-1}. DDA of chitosan was calculated according to the following equation [77].

$$DDA\% = 100 - [(A1655/A3450) \times 115]$$

in which A1655 and A3450 are absorbed at 1655 and 3450 cm^{-1}.

2.2. Grape Seed Oil Extraction

For the extraction of grape seed oil (GSO) from red grapes of *Vitis vinifera*, a methodology based on the absence of chemical solvents was used in this study to achieve better oil extraction yields and to ensure the activity of the oil was not affected when added to the chitosan film. The grape by-products were supplied to us by various Sicilian wine companies. The experimental protocol comprised three main phases: sieving, drying and grape seed oil extraction as described by [47]. In greater detail, wine wastes (grape pomace, grape seeds and woody stalks) were sifted to isolate the grape seeds; the seeds were then dried under a continuous cycle of hot air at 24 °C for four days. At the end of this step, oil from the grape seeds was extracted using a loading hopper (Cgoldenwall CAN-684 apparatus). The oil obtained was filtered and centrifuged at 4200 rpm for 10 min at 25 °C. The properties of grape seed oil were described by [47].

2.3. Films

Three types of films were produced: film with chitosan alone, and film with chitosan functionalized with grape seed oil at two different concentrations (0.5 mL and 1 mL), modifying the protocol of [48,66,68,78]. The chitosan films were obtained dissolving 2% chitosan (*w*/*v*) in 1% acetic acid solution. The solution was placed in an Ultra-Turrax homogenizer at 9500 rpm for 20 min and incubated overnight at 20 °C. Subsequently, the solution was filtered and stirred at 40 °C. Once temperature was reached, glycerol (0.5 mL/g) was added and the solution was stirred for 30 min. The sample was then sonicated for 30 min at

room temperature and 20 mL of solution was placed in Petri dishes (8.5 cm in diameter). To produce chitosan film fortified with grape seed oil, the previous steps were followed; however, after adding glycerol, 0.2% Tween 80 (*v*/*v*) was mixed into the solution for 1 h at 40 °C. Red grape seed oil (*w*/*w*) was added (0.5 mL in one case and 1 mL in other case) to the solution after 30 min, and blended in an Ultra-Turrax homogenizer at 9500 rpm for 5 min. After sonication, the sample was distributed into Petri dishes as in the previous step. All films produced were dried for 48 h at 20 °C.

2.4. Antimicrobial Activity of Films

The experimental plan included an initial qualitative screening to evaluate the antimicrobial activity of films tested on pathogenic and spoilage Gram-positive and Gram-negative bacteria. Several assays were carried out to evaluate the antimicrobial activity of the films by consulting various scientific studies [79,80] and, based on the preliminary results, other methods were applied. In particular, the protocols of [81,82] were modified in the following way: a double layer agar consisting of a support plate count agar layer covered by a soft 0.7% (*w*/*w*) agar layer of the optimal growth medium for each indicator strain was used. Bacteria were inoculated at 10^6 CFU/mL in soft agar and circular discs (10 mm in diameter) of films were placed on top. The plates were incubated at 37 °C for 24 h and the width of the inhibition area was evaluated for quality analysis. Antimicrobial evaluation of the films was investigated in triplicate.

3. Results

3.1. Chitosan Characterization

FT-IR analysis (Figure 1) of chitosan powder showed the characteristic peaks of the chitosan functional groups. In detail, the absorption bands at 1655 cm^{-1} and 3450 cm^{-1} indicated the characteristic peaks of primary amino groups and hydroxyl groups, respectively, of chitosan. Calculated DDA% of the chitosan samples was ca. 30%.

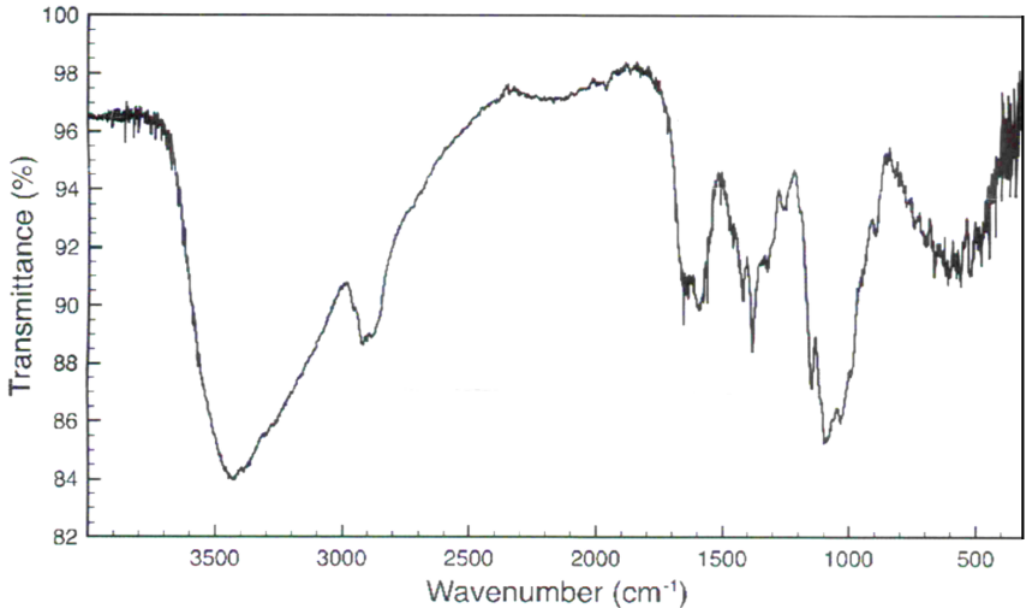

Figure 1. FT-IR spectrum of chitosan.

3.2. Antimicrobial Activity

The antimicrobial activity of the film was evaluated against 21 pathogenic and spoilage bacterial strains, including seven Gram-positive (Table 1) and sixteen Gram-negative (Table 2).

Table 1. Antimicrobial activity against Gram-positive bacteria (− no inhibition; ± low inhibition <1.2 mm; + clear inhibition 1.2–1.5 mm; ++ strong inhibition 1.5–1.8 mm; +++ very strong inhibition >1.8 mm).

Species	Code	Classification	Chitosan Biofilm Inhibition	Chitosan + 0.5 mL GSO Biofilm Inhibition	Chitosan + 1mL GSO Biofilm Inhibition
Listeria monocytogenes	DHPS 11B0	Pathogen	+++	+++	++
Listeria monocytogenes	DHPS 13B0	Pathogen	+++	+++	++
Listeria monocytogenes	ATCC 19114	Pathogen	−	−	−
Bacillus cereus	ICE 170	Pathogen	++	+	±
Staphylococcus Aureus	ATCC 33862	Pathogen	++	++	++
Staphylococcus Epidermidis	ICE 244	Pathogen	−	−	−
Brochothrix Thermosphacta	SP10	Spoilage	+++	+++	+++

Table 2. Antimicrobial activity against Gram-negative bacteria (− no inhibition; ± low inhibition <1.2 mm; + clear inhibition 1.2–1.5 mm; ++ strong inhibition 1.5–1.8 mm; +++ very strong inhibition >1.8mm).

Species	Code	Classification	Chitosan Biofilm Inhibition	Chitosan + 0.5mL GSO Biofilm Inhibition	Chitosan + 1mL GSO Biofilm Inhibition
Escherichia coli	PSL 52	Pathogen	++	++	++
Escherichia coli	ATCC 25922	Pathogen	+	+	±
Stenotrophomonas maltophilia	ICE 272	Pathogen	+	++	+
Acinetobacter guillouiae	ICE 24	Pathogen	+	++	++
Hafnia alvei	4G 44	Pathogen	+	+	+
Hafnia paralvei	4G 53	Pathogen	++	++	+
Salmonella typhimurium	50432	Pathogen	++	++	+
Salmonella enteritidis	ATCC 13076	Pathogen	+	+	±
Enterobacter amnigenus	60 A2	Pathogen	+	+	++
Pseudomonas aeruginosa	PSA 68	Pathogen	+	++	++
Pseudomonas lactis	SP 198	Spoilage	+	+	−
Pseudomonas poae	4G 558	Spoilage	−	−	−
Pseudomonas endophytica	4G 764	Spoilage	−	−	−
Pseudomonas fluorescens	4G 628	Spoilage	+++	−	−

Regarding the inhibition of pathogenic Gram-positive bacteria, the film containing only chitosan showed strong activity against two strains of *Listeria monocytogenes* (DHPS 11B0, DHPS 13B0), medium activity against *Bacillus cereus* and *Staphylococcus aureus*, while no inhibition was registered against *L. monocytogenes* (ATCC 19114) and *Staphylococcus epidermidis* (ICE 244). The addition of 0.5 mL of red grape seed oil did not generally increase the antimicrobial activity of the film. In the case of *B. cereus* (ICE 170), the antimicrobial activity was even reduced. Similar behavior of the film was observed when grape

seed oil was added to the concentration of 1 mL; in particular, the antibacterial activity remained unchanged against *S. aureus*, *S. epidermidis*, *L. monocytogenes* (ATCC 19114) and decreased against *L. monocytogenes* (DHPS11B0 and DHPS 13B0) and *B. cereus*. The only spoilage bacterium used as an indicator, *Brochothrix thermosphacta* (SP10), was strongly inhibited by the chitosan film, but the addition of grape seed oil did not increase inhibition at any concentration.

The inhibitory spectrum of the film was also evaluated against 10 pathogenic and 4 spoilage species with Gram-negative bacteria. The film containing chitosan alone showed strong inhibition against *Escherichia coli* (PSL52), *Hafnia paralvei* and *Salmonella typhimurium*, clear inhibition against *E. coli* (ATCC 25922), *Stenotrophomonas maltophilia*, *Acinetobacter guillouiae*, *Hafnia alvei*, *Salmonella enteritidis*, *Enterobacter amnigenus* and *Pseudomonas aeruginosa*. The addition of grape seed oil at the lower concentration (0.5 mL) increased the antimicrobial activity against *S. maltophilia*, *A. guillouiae* and *P. aeruginosa*, while the activity against *E. coli* (PSL52), *E. coli* (ATCC 25922), *H. alvei*, *H. paralvei*, *S. typhimurium*, *S. enteritidis*, *En. amnigenus* remained constant. The addition of grape seed oil at the higher concentration (1 mL) did not increase activity against *E. coli* (PSL52), *S. maltophilia* and *H. alvei*, decreased the inhibition of *E. coli* (ATCC 25922), *H. paralvei*, *S. typhimurium* and *S. enteriditis* and, surprisingly, increased the inhibition of *En. amnigenus*. Regarding spoilage bacteria, no activity was registered from any film towards *Pseudomonas poae* and *Pseudomonas endophytica*. There was clear inhibition against *Pseudomonas lactis* bacteria of the chitosan-only film and the film with the addition of 0.5 mL grape seed oil, while no inhibition was found with the film combined with 1 mL grape seed oil. Strong antimicrobial activity was only shown by the chitosan-only film against *Pseudomonas fluorescens*.

4. Discussion

4.1. Chitosan Characterization

The analysis carried out for the evaluation of the degree of acetylation of chitosan allowed us to observe that the product obtained and used for the production of the films was of sufficient quality.

4.2. Effects on Gram-Positive Bacteria

Our results showed that the antimicrobial activity of films against Gram-positive bacteria was higher than that registered against Gram-negative strains. This could be due to the different structure of the bacterial cell wall [30]; Gram-positive bacteria have lipoteic acids (LTA) and a higher peptidoglycan content than Gram-negative species, which contain lipopolysaccharides (LPS) in the outer membrane of the cell-wall structure [83]. This differing structure confers distinctive activity to the chitosan. It is known, in fact, that the bactericidal mechanism of chitosan is closely related to its positively charged amino groups (−NH3+). These latter can bind with negatively charged groups of teichoic acids present in the Gram-positive peptidoglycan structure, thus favoring interaction. This is not the case for Gram-negative bacteria, where the LPS in the cell wall can act as a barrier, limiting the penetration of chitosan [84,85]. Our results showed very strong inhibitory activity in the chitosan-only film against two strains of *L. monocytoges* (DHPS 11B0 and DHPS 13B0), as also observed by [79,86]. This could be owing to the biocidal action of chitosan at temperatures above 12 °C [87]; *L. monocytogenes* is a psychotropic bacterium and is adapted to growth at low temperatures thanks to the production of phospholipids with shorter and more branched fatty acids [88]. Another bacterial strain against which the chitosan-only film showed extremely strong antimicrobial activity is *B. thermosphacta*. Although in the literature some authors have demonstrated antibacterial activity against this strain in chitosan combined with other compounds [89], to the best of our knowledge, there are no studies which have analyzed the activity of film with chitosan on its own. Indeed, chitosan activity against *B. thermosphacta* has only been tested by immersion, demonstrating important antimicrobial activities in this compound [90]. The antimicrobial activities observed in our study may be due to the interaction between chitosan and the

hydrolysis products of microbial DNA, which lead to the inhibition of mRNA and protein synthesis [91,92]. Regarding other bacterial strains tested, strong antimicrobial activity was observed against *B. cereus*, most likely due to the fact that chitosan is able to bind rapidly to this species, causing its death [93]. We also found antimicrobial activity also against *Staphylococcus aureus*. However, activity was lower than that registered against *L. monocytoges*, similar to results reported in previous investigations [87]. The pathogenicity of bacteria can be amplified as bacteria are able to form films consisting of a matrix composed mainly of exopolysaccharide (EPS). EPS is an exclusive component of each bacterial strain and allows its colonization [94]. Our results could also be explained by the fact that *S. aureus* has a lower level of film formation, meaning the chitosan is more available to exert its antibacterial activity [95]. The different inhibitory capacities of chitosan against different Gram-positive bacterial strains, and the absence of inhibitory activity exerted by the chitosan film against a strain of *L. monocytogenes* (ATCC 19114) and *Staphylococcus epidermidis*, could be explained by various intrinsic and extrinsic factors affecting the antimicrobial activity of chitosan [31,91,96]. It has been found that the antimicrobial activity of chitosan may depend on the type of microorganism tested, the growth phase of the given bacterium [84,96], its molecular weight, the degree of deacetylation, the positive charge density, the interaction with some materials, the pH and the temperature [31]. Although some authors argue that film activities are reduced because chitosan is unable to diffuse through adjacent agar media and can only inhibit the growth of organisms interacting directly with the active sites [97], our results demonstrated important antimicrobial activities against some bacterial strains even at a concentration of 2%. However, the combination of chitosan with other antimicrobial agents, such as grape seed oil, could favor the chitosan migration process and the final inhibitory activity. After testing the antimicrobial activity of chitosan-only films, we also analyzed the antimicrobial activity of chitosan film with the addition of grape seed oil at two different concentrations to evaluate whether this could improve activity. Antimicrobial activities remained unchanged compared to chitosan-only films against *B. thermosphacta*, *S. epidermidis*, *S. aureus* and two strains of *L. monocytogenes*, demonstrating that grape seed oil does not contribute to increasing activity against Gram-positive bacteria. Indeed, the inhibitory activity, which remained unchanged at the lower concentration of oil compared to the chitosan-only film, decreased as the oil concentration increased. This could be due to a shielding effect of grape seed oil, which may reduce binding between chitosan and the bacterial wall, as observed by other authors who used other essential oils [98].

4.3. Effects on Gram-Negative Bacteria

Regarding the antimicrobial activity of chitosan-only film against Gram-negative bacteria, in agreement with other authors, our study showed lower activity with respect to Gram-positive bacteria [84,99]. This could be due to the to the different structure of the bacterial cell wall, as previously described [30,83]. However, the activity of chitosan against Gram-negative bacteria, albeit lower, may well be due to the interaction between chitosan and the OmpA protein in the outer membrane. This interaction can cause damage to the membrane and lead to death of the bacteria [100]. The most interesting results concerned the strong inhibition of the chitosan-only film against the spoilage bacterium *Pseudomonas fluorescens*. Other authors in the literature have demonstrated that chitosan exerts substantial activity against this bacterial strain, despite it being characterized by a very thick matrix [60]. The particular susceptibility of this species to the action of chitosan could be due to the polyanionic nature of the exopolysaccharides of the *Pseudomonas* matrix [101,102]. However, as regards other bacterial strains belonging to the *Pseudomonas* genus, chitosan-film activities have been shown to be lower or absent (*P. poae* and *P. endophytica*), and this could depend on the ability of bacteria to form films as a survival tool and protection against attack [103,104]. The different response to chitosan could also depend on the nature of the matrix diffused by the film produced by the bacterium, the charge and thickness of the bacterial cell wall, the size, the degree of deacetylation and the concentration of

chitosan [31,60]. Reasonably strong film inhibition exerted by chitosan only was observed against *Hafnia paralvei*, *Salmonella typhimurium* and a strain of *Escherichia coli* (PSL 52). While no data are available in the literature regarding the effects of chitosan on *H. paralvei*, likely due to the fact that this is a relatively new species [105], our results for *S. typhimurium* and *E. coli* are in agreement with those of other authors [106–108]. In particular, the antimicrobial activity against *E. coli* could be due to the ability of chitosan to alter the permeability of the membrane and induce cell death [109]. Furthermore, the chitosan antibacterial action involves not only a reaction of the bacterial wall, but it can also influence the structure of the phospholipid bilayer in the cell membrane, resulting in the release of some internal components [110]. Moreover, [111] suggested that chitosan may be able to form a polymeric membrane around the bacterial cell, preventing it from receiving nutrients. Against all other strains (e.g., *Stenotrophomonas maltophilia*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*), except two strains of *Pseudomonas* (*Pseudomonas poae* and *Pseudomonas endophytica*), clear antimicrobial activity exerted by the chitosan was found, albeit lower than that of other species previously described [60,112–114]. This lower antimicrobial activity could be due to the formation of film by the bacteria [115,116]. Despite this ability to form film, chitosan was able to exert an affect, probably by eliminating the exopolysaccharide, thus reducing bacterial activity [95,117]. Regarding *Acinetobacter guillouiae*, *Hafnia alvei*, *Enterobacter amnigenus* and *Pseudomonas lactis*, our study showed inhibitory effects of chitosan film for the first time. Regarding the addition of grape seed oil at the lowest concentration, the antimicrobial activity of chitosan films increased only against *S. maltophila*, *A. guillouiae* and *P. aeruginosa*. This could be explained by the fact that the use of low pH film, such as that of chitosan, combined with a grape seed extract may increase antimicrobial inhibitory activity against pathogenic bacteria [118]. Moreover, this increase in antimicrobial activity could also be due to the fatty acids contained in grape seed oil. These fatty acids exhibit significant antimicrobial activity at low pH values, thereby acting as anionic surfactants. Their hydrocarbon chains are able to interact with the phospholipids in the phospholipid bilayer of the cell membrane, increasing the membrane's permeability [119]. On the other hand, the oil reduced antimicrobial activity against *P. fluorescens*, most probably due to either the poor migration capacity of chitosan from the film to the surrounding culture, or to the oil-shielding effect between chitosan and the bacteria wall [97,98]. This low inhibitory effect could also be explained by the presence of lipopolysaccharides in the membrane of Gram-negative bacteria. The hydrophobic nature of the membrane may limit the diffusion of lipophilic compounds, such as oils [120,121]. This is in agreement with other authors, who showed that Gram-negative bacteria are generally more resistant to oils compared to Gram-positive bacteria [122]. At higher oil concentrations (compared to chitosan-only films), antimicrobial activity remained unchanged for *E. coli* and *H. alvei* strains, increased against *A. guillouiae*, *E. amnigenus* and *P. aeruginosa*, and decreased against *S. maltofilia*, *H. paralvei*, *S. typhimurium*, *S. enteritidis* and *P. fluorescens*. The differences in chitosan antimicrobial activity observed against different bacterial species could depend on the type of microorganisms tested [31]. Furthermore, the antibacterial efficiency of chitosan may be affected not only by differences between Gram-negative and Gram-positive, but also by the growth phase of the bacteria or by the initial population of microorganisms [84,96].

5. Conclusions

Packaging is an essential element providing protection for the final product and a guarantee of food safety during marketing. The food industry today raises many social concerns and is considered one of the main sources of environmental pollution, primary among which is, undoubtedly, the colossal use of non-degradable plastic packaging materials. The use of biodegradable materials in the production of food packaging is currently considered of primary importance in the bid to alleviate this problem. The use of chitosan as an active packaging material for fresh produce (vegetables, meat and fish) or foods with a short to medium shelf life could help mitigate the environmental problem. In our study, a preliminary evaluation of chitosan-only film has shown significant antimicrobial activity against

bacterial strains typically affecting food preservation. This film, therefore, has interesting potential application in the creation of innovative food packaging. However, the inhibitory capacity was found to be species-dependent and strain-dependent, most likely due to the chemical/physical characteristics of the extracted polysaccharide: molecular weight, degree of deacetylation, solubility, pH of the solution, temperature and viscosity [85]. For this reason, further investigation is needed to see if it is possible to obtain an increase in antimicrobial activity against these bacterial strains by modifying the characteristics of chitosan through changes to the various phases of chemical extraction from the exoskeletons of *A. foliacea* species. The addition of grape seed oil in general did not improve the inhibitory efficiency of the chitosan-only films, except against strains *Stenotrophomonas maltophilia*, *Acinetobacter guillouiae* and *Pseudomonas aeruginosa*. This lower inhibitory effect could be attributed to the poor migration capacity of chitosan from the film to the surrounding culture, or to a screening effect of the oil. Therefore, the antimicrobial activities of films with grape seed oil should be tested in the future by changing the concentration of chitosan and the concentration of the oil. This evaluation is extremely important as grape seed oil provides an opaquer color to the film and may have the potential to protect food from UV degradation. Furthermore, after identifying the most effective concentrations of chitosan and grape seed oil to ensure greater antimicrobial activity, these films should then be tested on food, including an evaluation of any physical characteristics.

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