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Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in Biotechnology and Innovation

by

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Abstract

Seafood are highly nutritious and indispensable to a healthy diet. Growing interests in healthy lifestyles result in increasing demands for innovation of seafood products leading to more accessible and less time-consuming confection products. However, seafood is also very perishable, and product degradation is a major cause for the large quantities of product waste, which is a matter of great concert for both the industry and the consumers. This degradation is, in part, consequence of the growth of specific spoilage organisms in the product.

With the objective of increasing shelf-life of a seafood product (squid and shrimp "espetada"), this work focused on the development of natural treatments to inhibit the bacterial proliferation and spoilage throughout 5 days of product storage under refrigeration conditions. To achieve that, an initial microbiological characterization of the product was performed, and the antimicrobial activity of several natural compounds was evaluated against 100 microorganisms previously isolated from the fish samples. Identification of isolates resulted in the detection of several *Enterobacteriaceae* (*Serratia* spp.*, Klebsiella* spp.*, Hafnia* spp.*, Rahnella* spp.*,* etc.), *Pseudomonas* spp. and Lactic Acid Bacteria (*Lactococcus* spp.*, Leuconostoc* spp. and *Weissella* spp.). *Escherichia coli* or *Listeria monocytogenes* were not found in each batch analyzed. Three selected natural compounds, Citrox®, Thyme essential oil and vinegar solutions, were applied through different techniques and their effects in bacteriological growth was analyzed in comparison with untreated samples from the same batch immediately after reception and after 2 and 5 days of refrigerated storage.

Citrox® at 1% v/v (immersion) and 3% v/v (pulverization) as well as Thyme essential oil at 0.2% v/v (immersion) were not effective in retarding the growth of total viable organisms, Lactic Acid Bacteria, *Pseudomonas* spp. and *Enterobacteriaceae*. On the contrary, bacterial inhibition was observed for both immersion and pulverization of vinegar, with significant higher effect ($P < 0.05$) only after immersion for 5 minutes with reductions around 2 log cycles at the end of 5 days of storage.

Although more experimental tests are required, this preliminary study demonstrated that natural compounds such as vinegar solutions could be used to prevent the bacterial growth and to prolong the shelf-life of such perishable squid and shrimp "espetadas".

Keywords: Antimicrobial activity, Natural compounds, Preservation, Vinegar.

Resumo

Produtos de pescado são alimentos altamente nutritivos e indispensáveis para uma dieta saudável. Com o crescimento do interesse por hábitos de vida saudáveis, a procura por produtos de pescado inovadores levou à criação de produtos acessíveis e de fácil confeção. No entanto, os produtos de pescado são alimentos facilmente degradáveis, resultando no desperdício de produto de grande relevância para a indústria e consumidores. A degradação de produtos de pescado é, em parte, resultado da proliferação de microrganismos deteriorantes.

Com o objetivo de estender o período de vida de um produto de pescado (uma "espetada" de lula e camarão), este trabalho focou-se no desenvolvimento de tratamentos à base de produtos naturais, de forma a inibir a proliferação bacteriana e o degradação ao longo de cinco dias de armazenamento do produto. Inicialmente, a caracterização microbiológica do produto foi realizada e a atividade antimicrobiana de vários compostos foi testada contra 100 isolados recolhidos da amostra. A identificação genómica dos isolados resultou na deteção de várias *Enterobacteriaceae* (*Serratia* spp*., Klebsiella* spp*., Hafnia* spp*., Rahnella* spp*.,* etc.)*, Pseudomonas* spp. e Bactérias do Ácido Lático (*Lactococcus* spp*., Leuconostoc* spp. e *Weissella* spp.). *Escherichia coli* ou *Listeria monocytogenes* não foram encontrados em nenhum dos lotes analisados. Três compostos, Citrox®, óleo essencial de tomilho e vinagre de vinho tinto, foram aplicados ao produto através de diferentes técnicas, tendo sido analisado o seu efeito no crescimento bacteriano e comparado com amostras controlo imediatamente após a aplicação do tratamento, assim como após dois e cinco dias de refrigeração. A aplicação de Citrox® a 1% v/v (por imersão) e a 3% v/v (por pulverização) e óleo essencial de tomilho a 0,2% v/v (por imersão), não foram eficazes em retardar o crescimento de microrganismos viáveis, bactérias do ácido lático, *Pseudomonas* spp. e *Enterobacteriaceae* Contrariamente, foi observada inibição bacteriana em amostras imersas e pulverizadas com vinagre 50% v/v, sendo a eficácia do tratamento significativamente maior ($P < 0.05$) apenas após imersão durante 5 minutos. Com exceção das bactérias do ácido lático, neste tratamento foram observadas reduções de 2 ciclos logarítmicos para todos os outros grupos de microrganismos ao fim de 2 dias de armazenamento.

Embora mais testes sejam necessários, este estudo preliminar demonstrou que a aplicação de tratamentos de vinagre pode ser usada para prevenir/inibir o crescimento bacteriano e estender o tempo de vida do produto em questão.

Palavras-chave: Atividade antimicrobiana, Compostos naturais, Conservação, Vinagre.

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It is with great happiness that I deliver this master thesis. More than a product of my work in this last year, it is the result of what I was able to do with the love and friendship of those who walked by my side throughout my whole life. I would not be here if not for any of you.

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I thank God for life and Our Lady for Her protection.

May I be worthy of Your love.

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1. Introduction

1.1 Seafood market and global relevance

Seafood is regarded as a great source of nutrients, indispensable to a healthy diet, ranging from proteins and fatty acids to vitamins and minerals (Wu et al., 2019). Due to this, there is increasing demand for new, fresh, pre-cooked and ready-to-eat seafood products (Møretrø et al., 2016).

With the continued rise of the global population and increasing awareness of the health benefits of a seafood-based diet, demand for seafood products has been growing for the last decades (FIOR Markets, 2019). Seafood represents a wide variety of products produced or fished in water. Fish, mollusks, aquatic plants and microalgae are some of the organisms commonly included in this gastronomic group (FIOR Markets, 2019).

Global seafood market has witnessed a steady growth lately, representing 125 445 million US dollars in 2017 and it is expected to rise to 155 316 million US dollars by 2023. Besides the healthy characteristics of seafood, changes in the lifestyles of western and far-eastern populations, as well as increasing disposable income have been driving the always increasing demand for innovative and more convenient seafood products (Kumar & Deshmuk, 2020).

The adoption of processed food as a convenient alternative to the traditional non-processed products, have made these products more accessible and adaptable to the busy lifestyle common in developed nations. Because of this, the supply of healthy, ready-to-cook seafood products is no longer a luxury but a necessity (FIOR Markets, 2019).

Portugal is the largest consumer of seafood products per capita in Europe, with an average of 55.9 kg in 2015, a considerably higher quantity than the European average of 25,1 kg (European Commission, 2019). While the fishery industry does not represent a large contribution to the national economy, its cultural and social relevance contributes to this industry being one of the most important sectors in Portugal. This culture and social significance are a result of the seafaring traditions of the country and the historical significance of the Atlantic Ocean in Portuguese history. As a consequence of this, fish and other seafood products are common ingredients in Portuguese gastronomy (Almeida et al., 2015). Due to their nutritional characteristics and composition, seafood products are highly perishable foods. Product degradation as well as economic pressures results in

large quantities of product waste (Fidalgo et al., 2018). The loss of large amounts of product culminates in financial losses and quality concerns for both the industry and the consumers (Wu et al., 2019).

Even with increased public awareness and investment from organizations to control the waste of food, over 20% of the annual production of fish products are not consumed (He et al., 2019). A large proportion of waste of seafood occurs, especially in western countries, during the distribution phase and due to consumption at home, resulting in the rising of product price and decreasing revenues for the industry (Gustavsson et al., 2011). This loss of available nutrition precipitates in nutrient losses to the overall population both due to the lack of available product in the market and the associated rise of the product monetary value (Getu & Misganaw, 2015).

In addition, the overfishing of the oceans presents a serious environmental issue. Rising demands from the population, as well as financial pressures, due to wasted product from the industry culminate in the constant increase in seafood production and fishing, destroying several habitats and emptying our oceans in the process. As of now, 30% of fish stocks are overfished and there has been a decrease in marine species of around 39% in the last 40 years (WWF, 2014).

1.2 Degradation and spoilage of seafood products

A product is considered spoiled once the alterations of the product are so representative that it is no longer fit for human consumption. Sensory characteristics such as odor, taste and overall aspect change during spoilage. Spoilage can be induced by a variety of causes, ranging from oxidative spoilage, autolytic enzymatic spoilage and microbial spoilage. The spoilage of food products due to microbial activity occurs as a result of the production of metabolites by the microorganisms that can lead to foul smells and undesirable flavors (Getu & Misganaw, 2015). The production of sulfur compounds, ketones, ammonia, biogenic amines, like putrescine and cadaverine, organic acids, alcohols and others, result in the degradation of the product and the formation of unpleasant smells and flavors (Giarratana et al., 2016). The nutritional value of the product also decreases during spoilage. This spoilage of product is impacted by specific qualities of the product in question, conditions of handling and conditions of storage. High concentration of fat, protein and general moisture in addition to low tissue stability provide bacteria with a near optimum medium for proliferation.

Along with degradation of food products and loss of nutritional value of the product, consumer safety can also be affected by the proliferation of pathogenic organisms capable of either producing harmful metabolites or leading to bacterial infections in humans (Getu & Misganaw, 2015).

The degradation of fish products by microbiological activity of specific spoilage organisms (SSO) is the most concerning cause of spoilage of product faced by the producers (Giarratana et al., 2016). Specific species of spoilage organism can vary depending on the origin of the product and the processing techniques employed during its handling and storage (Boziaris & Parlapani, 2017).

While fish muscle is sterile, gills, skin and the gastrointestinal tract have significant microbial populations. This microbiota varies depending on the environment in which the fish develops and lives. Most of the bacteria found in seafood products are of Gram negative psychrotolerant genera. Of these, there is an over-representation of the phylum of Proteobacteria. Usually, genera found in seafood products of this phylum are the *Pseudomonas*, *Shewanella*, *Acinetobacter*, *Aeromonas* and *Photobacterium*, among others. While Gram negative species, especially those belonging to the phylum of proteobacteria, are the most common bacteria found in seafood, Gram positive bacteria of the genus *Micrococcus* and *Clostridium,* as well as lactic acid bacteria, can also be present (Montet & Ray, 2011). The production of trimethylamine, ammonia, hydrogen sulphide, methylmercaptan and dimethyl-disulphide from the reduction of trimethylamine oxide, the metabolization of urea or deamination of amino acids, and the breakdown of sulphurous compounds are typical consequences of the proliferation of bacteria like the *Shewanella* spp., lactic acid bacteria and *Photobacterium* spp. The production of biogenic amines, such as cadaverine, putrescine and histamine can be induced by the decarboxylation of amino acids by the aforementioned bacteria like *Shewanella putrefaciens* and *Enterobacteriaceae* like *Hafnia alvei* and *Morganella morganii*. Histamine, and its precursor histidine, is a highly regulated compound due to its activity in provoking allergic reactions in the consumer (Montet & Ray, 2011).

Furthermore, besides spoilage-inducing bacteria, the growth of pathogenic microorganisms in food products also demands a great amount of attention. Seafood-associated illnesses have been linked to the proliferation of a variety of viruses, bacteria and parasites (Iwamoto et al., 2010). Due to its over-representation as the major cause of foodborne disease, the presence of pathogenic bacteria will be the primary focus in achieving food safety in this work. The presence of *Vibrio* spp., *Clostridium* spp.,

Salmonella spp., *Shigella* spp. and some strains of *Staphylococcus aureus*, is capable of inducing severe diseases and is of paramount importance when evaluating whether a product is safe for consumption or not. These pathogens are mostly present in food products due to the contamination and pollution of the environments in which the seafood was produced (Iwamoto et al., 2010). Also, pathogens such as *Listeria monocytogenes* and some strains of *Escherichia coli* are commonly found, resulting from cross-contamination and proliferation of microorganisms from the gastrointestinal tract of the product (Costa, 2013; Vongkamjan et al., 2017). Møretrø et al. (2016) noted the presence of *Pseudomonas* spp. and *Shewanella* spp. on equipment and zones of bleeding and short-time storage of several processing plants. The presence of higher levels of bacteria in industrially processed fish compared to those processed under "strict hygienic conditions" alerted to the necessity of scrupulous conditions in the procedure to avoid contaminations (Møretrø et al., 2016). The presence of pathogens in food products, not only lead to loss of nutritional value but their ingestion by humans can also lead to possibly fatal foodborne illnesses (Getu & Misganaw, 2015; FAO, 2020).

1.3 Strategies of preservation of seafood products

To accommodate financial expenses, related to the waste of product and to achieve better ecological balance, there has been calls to the development of new methods of preservation. These new models of preservation and maintenance of quality are matters of great interest to the industry (Wu et al., 2019). Currently, the most common form of fish preservation is refrigeration (He et al., 2019). However, while a high ambient temperature is one of the most important factors for the proliferation of unwanted bacteria (Getu & Misganaw, 2015), refrigeration is not capable of providing long shelf-life periods to fish (He et al., 2019). While somewhat efficient at the preservation of the product and inhibition of degradation agents, for a short period of time, refrigeration has negative impacts on the sensory qualities of fish (He et al., 2019). Because of this, new techniques strive to induce natural and artificial forms of antimicrobial activity to control the spoilage microorganisms (Giarratana et al., 2016). These new techniques include, but are not limited to Superchilling, UV-C radiation, High-pressure processing (HPP), Hyperbaric Storage and Biodegradable films and edible coatings (Fidalgo et al., 2018; Eliasson et al., 2019; He et al., 2019; Bottino et al., 2016; Koutchma et al., 2016; Rode & Hovda, 2016; de Alba et al., 2019; Huang et al., 2013; Moreira et al., 2015; Rezaei & Shahbazi, 2018).

Superchilling, or sub-chilling in industrial terms (Eliasson et al., 2019), is the procedure through which water in food is cooled below freezing point (Eliasson et al., 2019). The freezing temperatures induce the formation of ice crystals inside the product and inhibit the growth of undesirable microbiological cultures, like H_2S-producing bacteria, and the consequential spoilage of the product (Eliasson et al., 2019). The combination of this process with ice-glazing techniques, where the formation of an icy barrier coating the product is observed, was effective in the improvement of the preservative activity of the technique (He et al., 2019).

The use of radiation, specifically UV-C radiation, as a method of food preservation is a new non-thermal technique seen as a viable alternative to the conventional methods of decontamination and preservation of the product (Bottino et al., 2016). It is a non-reactive, non-chemical, cheap and accessible technology (Bottino et al., 2016), being less energy-consuming than traditional thermal methods (Koutchma et al., 2016). By not adding any chemical nor producing any by-products, and not using temperature as a germicidal agent, this process guarantees the preservation of sensory characteristics of the product (Koutchma et al., 2016).

Another non-thermal and non-chemical innovative process of decontamination is the employment of high-pressure processing (HPP) techniques. HPP is an adequate alternative to the more conventional methods of food preservation, as thermal methods (de Alba et al., 2019). It acts by processing the product at high pressures, suppressing bacterial growth and extending shelf-life expectancy (Rode & Hovda, 2016). HPP activity in the inactivation of enzymes and protein denaturation could also prove useful in controlling allergenicity of foods (Huang et al., 2013).

Also, the storage of food products at high pressures allows the storage of these products at room temperature (25-37 ºC) (Fidalgo et al., 2018). Results from different studies using pressures ranging from 50-75 MPa (Fidalgo et al., 2018) to 100-150 MPa (Moreira et al., 2015) showed both equal and superior efficiency in conservative activity when compared to traditional refrigeration. Being this process sufficient to enhance, or at least to match the preservative action of refrigeration, it thrives due to its low energetic cost (Fidalgo et al., 2018). Specifically, in fish products, hyperbaric storage at room temperature demonstrated that it can be used as a method through which extension of shelf-life is achieved while preserving organoleptic and chemical characteristics of the product (Fidalgo et al., 2018).

However, the techniques described above require either expensive equipment, as is the case of UV-C treatment, high-pressure processing and hyperbaric storage, or alter the market characteristics of the product, as is the case of superchilling, since this technique demands the storage of the product in a *quasi*-frozen state. Edible coatings, on the other hand, are relatively cheap and simple processes with proven potential (Rezaei & Shahbazi, 2018).

There are also increasing demands from consumers to introduce natural substitutes to conventional synthetic antimicrobials (Kim et al., 2018). With that in mind, some of these natural food additives can also add value to the product by adding functional properties to the product, like antioxidative activity and support for the delivery of bioactive compounds (Albertos et al., 2019). Both the substitution of synthetic antimicrobials and the addition of product value through nutritional enrichment of food products can be accomplished via the application of edible coatings.

1.4 Edible coatings – compounds and application techniques

The application of non-toxic, edible preservatives as a coating to extend shelf life and inhibit bacterial proliferation has been the focus of several recent studies (Rezaei & Shahbazi, 2018). These coatings have the potential to retard the spoilage of product, retaining the hedonic characteristics of fish, like smell, texture, and flavor (Rezaei & Shahbazi, 2018). The non-synthetic nature of these compounds provides the treated products with an all-natural characteristic which in turn can contribute to a greater appeasement of consumer interests. Natural antimicrobials also present alternatives to the undesirable synthetic antimicrobials (Rezaei & Shahbazi, 2018). Every coating component should be generally regarded as safe (GRAS) or food grade. Thus, no safety issue should arise when using the compound under the recommended conditions. On account of the inherent safety of GRAS and food grade products, natural edible coatings can be applied to most food products to extend their shelf life (García et al., 2016).

Natural coatings not only are inhibitory to the bacterial growth but can also, depending on the compound used in the coating, prevent temporarily the natural degradation of the product, protecting it from damage due to exposure to oxygen and low refrigeration temperatures, among others (Rezaei & Shahbazi, 2018).

The application of natural coatings is an encouraging innovation capable of answering the increasing demand for ready-to-cook products while maintaining a completely natural

essence (Kanatt et al., 2013). As a consequence of its popularity as a promising innovation, numerous components with antimicrobial activity have been already implemented in these edible coatings with positive results (Rezaei & Shahbazi, 2018; Han, Sun & Chen, 2019; Alagawany et al., 2020). An edible coating usually consists of a solution of lipids and polymers, such as proteins and polysaccharides, with antimicrobial activity, like chitosan, or capable of forming a structural matrix operating as a carrier of antimicrobial compounds, like sodium alginate and carboxymethylcellulose (García et al., 2016; Rezaei & Shahbazi, 2018; Pizarro et al., 2016).

The application in food products of several plant-based compounds have been increasingly regarded as a safe alternative to the conventional synthetic compounds used for preservation of food products and extension of their shelf-life (Memar et al., 2017). Due to their antimicrobial activity and functional properties, essential oils, like limonene, thymol, oleuropein and carvacrol, has been the focus of several studies (Han, Sun & Chen, 2019; Marchese et al., 2016; Himour, Yahia, & Belattar, 2017). Through the application of essential oils and its constituents, the formulation of edible coatings with antimicrobial properties can induce great medicinal advantages (Alagawany et al., 2020). For example, thyme essential oil, a safe and natural essential oil extracted from *Thymus vulgaris*, has been regarded as an alternative to the commonly used preservatives and shelf-life extending agents due to its intense antimicrobial activity (Casquete et al., 2016). Due to its high concentrations in thymol, a strong antibacterial component, Thyme essential oils have been the subject of extended studies regarding their application in food products (Alagawany et al, 2020; Ozogul et al., 2020).

Besides their antimicrobial activity and shelf-life extension of food products, essential oils are also known to yield strong smells and flavors due to their rich composition in characteristically volatile compounds (Casquete et al., 2016). For instance, limonene has been lately applied in the food industry due to its aromatic and flavor inducing properties (Giarratana et al., 2016). Limonene is an aromatic compound present in a variety of natural essential oils, specifically those extracted from citrus fruits. It is regarded as a safe compound and its application in food products is regulated in the Union List of Flavorings and Source Materials of the European Union (EU No 872/2012, 2012). With antimicrobial, anti-oxidant and anti-inflammatory properties, limonene application in food products has been the target of several studies (Giarratana et al., 2016; Ibáñez, Sanchez-Ballester & Blázquez, 2020). Similarly to limonene, carvacrol is a monoterpene with proven antimicrobial activity and present in high concentrations in essential oils of

plants of the *Lamiaceae* family (Mauriello, Ferrari & Donsì, 2020). Antimicrobial, anti-oxidant, anti-inflamatory, cardioprotective and neuroprotective properties have been reported as carvacrol characteristics (Memar et al., 2017). Due to its wide spectrum antimicrobial activity and functional properties, carvacrol is a compound of great interest (Marinelli, Di Stefano & Cacciatore, 2018).

Along with plant-based molecules and essential oils, fungal and animal-based compounds, such as propolis and chitosan, could also provide valuable new opportunities in shelf-life extension and food safety (Przybyłek & Karpiński, 2019; Casquete et al., 2016). Propolis is a resinous mixture of beeswax and other resins collected by honeybees, usually of the *Apis melifera* specie. It provides structural and protective action to the honeycomb. Propolis has been regarded as a substance of great interest being commonly used in alternative medicine products due to its reported functional properties (Przybyłek & Karpiński, 2019). While propolis extract composition may vary according to geography and surrounding flora, strong antibacterial activity is commonly found as a characteristic of this compound (Casquete et al., 2016).

Chitosan is another biopolymer which has been recently highlighted as a result of its antimicrobial properties (Sahariah & Másson, 2017; Haghighi et al., 2020). Originating naturally only in fungi, such as those of the *Mucroaceae* family, it can be manufactured through the deacetylation of chitin, one of the most abundant components of insect and crustacean's exoskeletons (Sahariah & Másson, 2017). Its nontoxic, biocompatible, safe to use, biodegradable and antibacterial characteristics suggest chitosan as a strong candidate for application in food products (Sahariah & Másson, 2017; Qu & Luo, 2020). In some cases, its application in edible films has proven to preserve foodstuff with great efficiency (Hu & Gänzle, 2018; Priyadarshi & Rhim, 2020). Besides their antimicrobial activity, chitosan-based solutions are capable of incorporating functional compounds and, in doing so, increase the nutritional and functional value of food products (Ortiz de Elguea-Culebras et al., 2019). Chitosan is, however, only an efficient antimicrobial agent in acidic solutions, due to it being only soluble in low pH mediums. This property reduces considerably the use of this compound as food preservative and value adding agent in non-acidic products (Sahariah & Másson, 2017).

2. Objective

This work aimed to define new strategies to extend the shelf-life of sea food "espetadas" by the application of innovative natural treatments to the product. "Espetada" is a traditional Portuguese ready-to-cook food that consists in the aggregation of diverse food products through the use of wooden or metal skewers. In the present study, "espetada" was composed by squids and shrimps, which had no treatment during processing, resulting in a short shelf-life. The highly perishable characteristic of seafood products presents a necessity to find new strategies to prolong their shelf-life. To achieve shelf-life extension, several spoiling and/or pathogenic microorganisms isolated from this product during and after its shelf-life were identified and screened for their inhibition by different natural compounds. The treatment of samples was tested by immersion and pulverization in water-based solutions.

This work was performed under the scope of ValorMar project, which is focused on the development of technologies, services, products, and other value adding properties to marine resources. Through product innovation, ValorMar project proposes the creation of healthy, eco-friendly, and efficient seafood products. Moreover, this Portuguese project encourages cooperation between seafood producing companies and universities, making use of both industrial and academic research in order to satisfy industrial and consumer demands.

3. Materials and methods

3.1 Sampling

The product analyzed was a traditional Portuguese ready-to-cook product known as "Espetada" composed of raw shrimps (*Parapenaeopsis*, *Penaeus* and *Metapenaeus* genus) and squid (*Loligo duvauceli)*, a common squid in Portuguese gastronomy.
The product was packaged in plastic boxes with two "espetadas" each and, for each experiment, the samples were transported to the laboratory under refrigeration conditions and analyzed immediately. To perceive the bacterial growth during the spoilage of the product, samples were also taken after five days of storage, i.e., after its due-by date. All experiments were performed in duplicate and the samples were stored refrigerated (5 ºC) until the day of the analysis to guarantee the same conditions of a common public market.

3.2 Microbiological analysis

Several pieces of "espetada", randomly selected until a total of 25 g, were added to 225 mL of sterile buffered peptone water (Biokar Diagnostics, Beauvais, France), according to standard microbiological methods, and homogenized in a stomacher (Interscience, Saint Nom la Brèteche, France) for 2 min. Each sample was analyzed in duplicate. Appropriate decimal dilutions were prepared in Ringer's solution (Biokar Diagnostics) for microbial enumeration: colony counts of viable microorganisms at 30 °C on Plate Count Agar (PCA, Biokar Diagnostics; ISO Standard 4833-1:2013); Lactic Acid Bacteria (LAB) at 30 °C on de Man-Rogosa and Sharpe agar (MRSA, Biokar Diagnosis; ISO Standard 15214:1998); *Enterobacteriaceae* at 37 ºC on RAPID′ *Enterobacteriaceae* medium (Bio-Rad, Hercules, CA, USA; ISO Standard 21528-2:2017); *Pseudomonas* spp. counts at 30 ºC on *Pseudomonas* Agar Base (PAB, Biokar Diagnostics) supplemented with Cetrimide, Fusidic acid and Cefaloridin (VWR, Alfragide, Portugal) according to ISO Standard 13720:2010; *Escherichia coli* on Tryptone Bile X-glucuronide Agar (TBX, Bio-Rad) at 44 ºC according to ISO Standard 16649-1:2018 and *Listeria monocytogenes* on Agar *Listeria* Ottavani & Agosti (ALOA, Biomérieux, Marcy l'Etoile, France) at 37 ºC according to ISO Standard 11290-2:2017. For total viable microorganisms, LAB, *Enterobacteriaceae* and *Pseudomonas* spp., also incubation at 11 ºC was applied, in order to analyze bacterial growth at temperatures similar to those in which the product is stored

during its shelf life. Following incubation, appropriate confirmatory tests were performed and colony forming units per gram (cfu/g) were calculated. Samples were analyzed immediately after reception and after 2 and 5 days of refrigeration conditions. Microbial counts were transformed to log cfu/g.

3.3 Selection of isolates

Only presumptively colonies of *Pseudomonas* spp. and LAB were confirmed, since the other microorganisms were grown on chromogenic culture media or on non-selective culture media such as for the counts of total viable microorganisms.
Characteristic colonies in terms of color, type, elevation and opacity were confirmed by Gram staining and oxidase test (*Pseudomonas* spp.) and Gram staining and catalase test (LAB).
Colonies from each culture media/incubation temperature were randomly selected (10%) and cultured on MRSA (only LAB) and TSAYE - Trypticase Soy Agar (Biokar Diagnostics) with 0.6% Yeast Extract (Biokar Diagnostics) and incubated for 24 to 72 h under the same conditions.

3.4 Growth and storage conditions

Each isolate was stored in Trypticase Soy Broth (TSB, Biokar Diagnostics) or MRS broth both containing 30% (v/v) glycerol (Sigma, Steinheim, Germany), at -20 °C. Isolates were sub-cultured twice before being used in the following assays.

3.5 Identification of selected isolates by 16S rRNA sequencing

Each selected isolate was identified by 16S rRNA sequencing. Gram staining was performed for all isolates and their DNA was extracted according to the protocols for Total DNA purification from Gram-positive or -negative bacteria of the GRS genomic DNA Kit (Grisp, Porto, Portugal). The primers used for the amplification of the gene fragments for the 16s rRNA identification were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Vaz-Moreira et al., 2011). For the preparation of the master mix, the following solutions were used: 1 µL of dNTP's (10 mM), 5 µL of Taq buffer KCl (10X), 5 µL of $MgCl_2$ (25

mM), 0.5 µL of primer 27F (100 µM), 0.5 µL of primer 1492R (100 µM) and 1.25 µL of Taq polymerase (1 U/mL) and 2 µL of bacterial DNA. To reach the volume of 50 µL, 34.75 µL of UP H_20 was added to the solution. PCR cycling conditions were performed in a Thermocycler (Bio-Rad) following the above-mentioned protocol: one cycle at 94 ºC for 5.5 minutes, a first phase encompassing both start and denaturation cycles; 30 alternating cycles of annealing and extension for 30 seconds at 55 ºC and 90 seconds at 72 ºC, respectively; and one final cycle at 72 ºC for 90 seconds for the final extension. After the last cycle, the products were cooled to 12 ºC. For each PCR reaction, a negative control (sample without template) and a positive control (sample with known DNA) were included. Each amplified product (10 µL) was combined with 3µL of loading buffer (Bio-Rad) and added in a 1.2% (w/v) agarose gel (Seakem® LE Agarose, Rockland, ME, USA), submersed in 1x TAE Buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8.3; Bio-Rad) containing 1x of Xpert Green DNA Stain (Grisp). On each gel, two positions were filled with a molecular weight marker (NZYDNA ladder VI, 50 to 1500 bp, Nzytech, Lisbon, Portugal) and electrophoresis separation was then performed under 80 V for 45 minutes. Gel images were collected through an UV light transilluminator and the ImageLab program (Bio-Rad).

PCR product purification was performed according to the protocol of the GRS PCR & Gel Purification Kit (Grisp) and used as templates. The resulting DNA sequences were aligned and compared in the Gene Bank through the use of the BLAST program (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997).

3.6 Study of antimicrobial activity of several natural compounds

The antimicrobial activity of twelve natural compounds was tested against each selected isolate and the most effective natural compounds were selected for further experiments.

3.6.1 Preparation of the inoculum

Inoculum of each isolate was performed from each culture grown in TSAYE or MRS (Biokar Diagnosis) at 30 ºC for 24 h, by suspending isolated colonies in Ringer solution in order to obtain a turbidity equivalent to 0.5 McFarland scale.

3.6.2 Natural compounds used in this study and their preparation

3.6.2.1 Ethanolic propolis extracts (EPE)

Two samples of propolis from different geographic locations were used: one sample collected from Maia, Porto, Portugal (EPE1) and the other from Vila Franca, Viana do Castelo, Portugal (EPE2). Both samples were kept in the dark until their use. Stock solutions of 0.1 g/mL ethanolic propolis extracts were prepared in 48 h for each sample according to Casquete et al. (2016). Each propolis sample was chopped into small pieces and extracted with 95% (v/v) ethanol (Sigma) for 24 h with magnetic agitation. Afterwards, the resulting mixture was filtered and the residue was re-extracted in the same conditions. The filtrate solutions were combined and each extract (0.1 g/mL) was stored at room temperature, in the dark.

3.6.2.2 Chitosan solutions

Chitosan of different molecular weights were obtained from Sigma-Aldrich (St. Louis, USA): low molecular weight (LMW; deacetylation degree of 75-85%), medium molecular weight (MMW; deacetylation degree of 75-85%) and high molecular weight (HMW1; >75%). Also, another chitosan of HMW was used (HMW2; Aqua Premier Co., Thailand; deacetylation degree of 90%). Chitosan solutions were prepared in 3% (v/v) solution of glacial acetic acid 99% (Panreac, Barcelona, Spain) to a final concentration of 30 mg/mL, according to Casquete et al. (2016). Afterwards, the solution was stirred overnight for the dissolution of chitosan. Each chitosan solution was stored at 4ºC.

3.6.2.3 Carvacrol and Limonene solutions

Solutions of 0.05% (v/v) Carvacrol (99%, Sigma-Aldrich) and 0.05% (v/v) Limonene (≥95%, Sigma-Aldrich) were prepared by dissolution in polysorbate 80 (Sigma-Aldrich, St. Louis, USA). The solutions were prepared shortly before usage and no special conditions of storage were deemed as necessary.

3.6.2.4 Olive leaf extract solution

A solution of 1.5% (w/v) olive leaf extract (Nutexa, Haylake, UK) was prepared by dissolution of 0.3 g of the extract in 20 mL sterile distilled water. Since the active compound oleuropein is presented in 20% (w/w) of the olive leaf extract, only 0.3% (w/v) of oleuropein was presented in the final solution.

3.6.2.5 Citrox® solution

Citrox® solution was obtained from ProGarda 14 WP (Cirras Ltd, Middlesbrough, England) and used directly or diluted in sterile distilled water. ProGuarda 14WP is a natural, GMO free and food grade processing aid based on Citric acid that inhibits the growth of microorganisms through pH regulation. Dilutions were prepared in sterile water, and a solution of 50% (v/v) was used in the antimicrobial assay.

3.6.2.6 Stabilizer-based solutions

Two food stabilizers into powder form were also tested for its antimicrobial activity and their solubilization in sterile water was performed before testing. Stabilizer 300 (Mendes Gonçalves, Golegã, Portugal) is composed by alcohol vinegar, sodium bicarbonate and maltodextrin and stabilizer 440 (Mendes Gonçalves) by salt (NaCl), antioxidants

(ascorbic acid and sodium ascorbate), and sodium citrates. Both compounds should control bacteriological growth due to their acidic and antioxidant properties.
Preparation of the stabilizer-based solutions was performed at the concentration of 1.5% (w/v) owing this to be the highest recommended concentration by the manufacturer.

3.6.3 Antimicrobial activity screening of each natural compound

The antibacterial activity of the compounds was evaluated by the agar diffusion method (Bauer et al., 1966). Each solution, prepared in section 3.6.2 was combined with the emulsifier Polysorbate 80 (Sigma-Aldrich, St. Louis, USA) in the proportion of 1:10 (polysorbate 80: natural compound solution). Blank disks (Oxoid, Basingstoke, United Kingdom) were immersed in each solution and left submersed for 30 minutes to absorb the solution. Sterile swabs were immersed in each inoculum suspension (prepared as described in 3.6.1) and spread in Mueller-Hinton Agar (MHA, Biokar diagnostics). Disks containing the compounds were added to the top of the culture medium and plates were incubated at 30 ºC during 24 h. Inhibition by each compound was recorded as positive if a translucent halo zone of over 10 mm was observed around the disk. As control, disks adsorbed with distilled water, polysorbate 80, acetic acid and ethanol were used.

3.6.4 Minimum inhibitory and bactericidal concentrations of effective natural compounds

Minimum inhibitory (MIC) and bactericidal (MBC) concentrations were assessed only for Citrox® solution which was the only compound showing antimicrobial activity for the majority of the isolates. Following the protocol for microdilution broth susceptibility test described by Aumeeruddy-Elalfi et al. (2016), a solution of 100 µL (10 µL of polysorbate 80 and 90 µL of MHB) was distributed in 96-well microplates. The Citrox® solution was diluted and 8 different dilutions, ranging from 0.39% to 50% (v/v), were distributed throughout the wells. Then, 50 µL of each inoculum, with a turbidity equivalent to 0.5 McFarland scale, was added to each well and the microplates were incubated at 30 ºC for 24h. After the incubation period, 40 µL of 0.02% (w/v) solution of iodonitrotetrazolium (INT) chloride Biochema dye (PanReac AppliChem ITW Reagents, Barcelona, Spain) was added to each well. The reaction between INT chloride and viable bacterial cells in the suspension induced a change of color, from a transparent mild yellow

to an intense red due to the reduction of INT chloride into INT formazan. The lowest concentration of Citrox® in which there was no change of color was considered the minimum inhibitory concentration.

To determine whether the compound was bactericidal or bacteriostatic, 10 µL of suspension were collected from the wells not experiencing color change in the microplate and inoculated on Mueller-Hinton agar. After incubation for 24 h at 30 ºC, the compound concentration would be considered bactericidal if no growth could be observed, otherwise, the concentration would be defined as bacteriostatic.

3.7 Development and application of solutions for the treatment of samples

In this work, the application of natural solutions to the product was executed through spraying or dipping due to their easy application in industrial circumstances. Both techniques followed standards in order to guarantee the reproducibility of the experiment. Hence, spraying was performed in all surfaces of the product, ensuring that there was a uniform treatment, and dipping was carried out by immersing the product for 5 minutes in the solution. Enumeration at two incubation temperatures of total viable microorganisms, lactic acid bacteria, *Enterobacteriaceae* and *Pseudomonas* spp. was performed as described above (section 3.2) immediately after, after two and after five days of application.

Citrox® and Thyme oil extract were selected based on previous results, but also red wine vinegar was used as antimicrobial compound after a preliminary experiment. In this experiment, three natural solutions were studied: vinegar (Red wine vinegar, 6% acidity, Continente®, Portugal), lemon juice (manually squeezed and immediately used) and Olive Leaf Extract (32 mg/mL, Nutexa, Haylake, UK). Each solution was directly used (100%) or diluted in sterile deionized water in 1:1 (50% v/v) or 1:2 (25% v/v) and their antimicrobial activity was evaluated against three isolates previously isolated and identified (sections 3.2 and 3.5) as *Serratia liquefasciens, Pseudomonas psychrophile* and *Lactococcus gerviae*. Suspensions of approximately 10^8 cfu/mL of each isolate were obtained by adjustments based on initial OD600 readings and added to each test solution. As control, each inoculum was added to a Ringer's solution. Samples were collected immediately after inoculation and after 5, 10, 20, 40 and 60 minutes after inoculation and counted through spread-plating method in TSAYE and MRS agar (for *Lac. gerviae*). After incubation at 30 ºC for 24 h, colonies were counted and the cfu/mL calculated.

3.7.1 Application of a citrox-based solution through pulverization and immersion

Citrox® solutions in sterile deionized water were formulated and applied in the product through pulverization (3% v/v) and immersion (1% v/v). These concentrations were applied according to the maximum recommended concentration of Citrox® in solutions used in the spraying and immersion of foodstuffs. As a negative control of each technique, one sample was sprayed and another was immersed with sterile deionized water, following the same procedure as described above.

3.7.2 Application of Thyme oil extract-based solutions through immersion

The product was immersed in a Thyme essential oil (0.2% v/v) solution with sterile deionized water. As control, one sample was immersed in sterile distilled water and bacterial growth was observed and compared to the treated sample.

3.7.3 Application of a red vinegar-based solution through pulverization and immersion

A 50% (v/v) red vinegar solution, diluted in sterile deionized water, was used to spray all the surfaces of the product or to its immersion during 5 minutes. Three samples were used as control, one pulverized with sterile deionized water, one immersed in sterile deionized water for 5 minutes and one with no treatment or coating.

3.7.3.1 Sensorial evaluation

Sensorial alterations at the day of reception and after 5 days of storage were evaluated for the different types of treatment with 50% (v/v) vinegar. Special attencion was given to the smell, visual aspect as texture, since these characteristics are more easily noticed when the product is uncooked. This process was executed by direct visual comparison at the moment of sampling.

3.8 Statistical analysis

Each experiment was carried out in duplicate. All calculations were carried out using the software IBM SPSS Statistics (version 27.0, IBM Corporation, Armonk, NY, USA). Significant differences in microbial counts of each experiment were analyzed using the non-parametric Kruskal-Wallis test. A significance level of $P < 0.05$ was applied to all statistical procedures.

4. Results and discussion

4.1 Microbiological analysis

Microbiological enumeration was performed for samples of 2 batches immediately after reception (0 days) and after 5 days of storage. Since the recommended storage of the product is at refrigeration temperatures, bacterial counts were performed not only according to each specific ISO standard (30 ºC or 37 ºC in the case of *Enterobacteriaceae*) but also at 11 ºC, due to increase knowledge about the presence and numbers of spoilage bacteria in the product that could be able to grow at low temperatures. The obtained results are presented in table 4.1.1.

Table 4.1.1 – Microbial enumeration of "espetadas"

log cfu/g

	30 ºC		11 ºC	
	Day 0	Day 5	Day 0	Day 5
ALOA	<1.0 ± 0.0	<1.0 ± 0.0	na	na
TBX	<1.0 ± 0.0	<1.0 ± 0.0	na	na
Rapid *Ent	4.7±0.2	8.3±0.2	4.8±0.1	8.2±0.0
PCA	5.7±0.0	8.0±0.0	6.3±0.0	8.2±0.0
MRS	3.9±0.3	6.3±0.0	4.2±0.1	6.5±0.0
PAB	5.3±0.1	8.0±0.0	5.0±0.1	6.3±0.0

Legend: na – not applicable; *incubation at 37 ºC instead of 30 ºC; ALOA- Agar Listeria Ottavani & Agosti; TBX - Tryptone Bile X-glucuronide Agar; Rapid *Ent*. - RAPID′ *Enterobacteriaceae* agar; MRS - De Man-Rogosa Sharpe agar; PCA – Plate Count Agar; PAB - *Pseudomonas* Agar Base.

It is possible to observe that *E. coli* and *L. monocytogenes* were not present in detectable values in the product. *E. coli* is usually considered a consequence of unsanitary practices during processing of seafood, and its presence in food products can represent risks to the consumer (Costa, 2013). No detection of *E. coli* was also reported by Ozogu et al. (2008) in European squid and shrimp after 3 months of storage at 4 ºC. Despite not common, the presence of *L. monocytogenes* in seafood may occur (Das et al., 2013), but the contamination by this pathogen is more associated with contaminations during processing

procedures (Vongkamjan et al., 2017). The absence of *E. coli* and *L. monocytogenes* suggests that safe procedures have been applied during production.

High numbers (~4 to 5 log cfu/g) of *Pseudomonas* spp., *Enterobacteriaceae* and LAB were found at day 0 when incubation was at 30 ºC and 11 ºC. After 5 days of storage, i.e., after the shelf-life of the product, an increase of more than 2 log cfu/g was observed in all these groups of microorganisms, except for *Pseudomonas* spp. at 11 ºC, for which an increase of only 1 log cfu/g of growth was observed.

Also, high numbers of total viable microorganisms (about 6 log cfu/g) at day 0 and an increase of 2 log cfu/g after 5 days of storage were observed with no differences between both temperatures tested. Similar results were reported by Farajzadeh et al. (2016) in shrimps, but with lowest initial counts of total viable microorganisms found (about 2 log cfu/g).

4.2 Identification of selected isolates by 16S rRNA sequencing

After enumeration, about 10% of colonies were collected from the different culture media and identified. Table 4.2.1 shows the several species identified.

Table 4.2.1 - Identification of 100 isolates by 16S rRNA sequencing

Identification	% of isolates	16S rRNA sequencing similarity (%)
Hafnia alvei	6	99.40 - 99.90
Klebsiella oxytoca	2	99.90 - 100.00
Lactobacillus sakei	5	99.90 - 100.00
Lactococcus garvieae	2	99.90 - 100.00
Lactococcus piscium	2	99.40 - 99.90
Leuconostoc gelidum	1	99.40 - 99.90
Leuconostoc mesenteroides	1	100.00
Leuconostoc miyukkimchii	12	99.10 - 99.90
Morganella morganii	3	99.80
Obesumbacterium proteus	1	99.90
Pantoea brenneri	1	98.50
Pseudomonas endophytica	1	99.70
Pseudomonas fragi	1	99.70
Pseudomonas helleri	2	99.80 - 100.00
Pseudomonas helmanticensis	1	99.80
Pseudomonas marginalis	1	100.00
Pseudomonas orientalis	1	99.80
Pseudomonas paralactis	6	99.80 - 100.00
Pseudomonas psychrophila	3	99.70 - 99.90
Pseudomonas trivialis	4	99.90
Pseudomonas weihenstephanensis	2	99.60 - 99.90
Rahnella aquatilis	4	99.60 - 99.90
Rahnella inusitata	10	99.80 - 99.90
Serratia fonticola	2	99.90
Serratia liquefaciens	20	99.40 - 99.90
Serratia nematodiphila	1	100.00
Weissella fabalis	3	97.10 - 98.10
Weissella beninensis	1	97.60

Of the 100 isolates, 49 were identified as members of the *Enterobacteriaceae* family, being these distributed by the *Hafnia*, *Klebsiella*, *Serratia*, *Morganella*, *Rahnella*, *Obesumbacterium* and *Pantoea* genus. This represents a clear dominance of this family of bacteria in the total number of isolates. Bacteria of the *Serratia* genus (*S. liquefaciens*, *S. nematodiphila* and *S. fonticola*), commonly found in the environment, as soil, water and intestinal tract of digestive tracts of various animals, represented over 20% of all

isolates identified in the studied product. The facultative anaerobic bacteria *S. liquefaciens* is, after *Serratia marcescens*, the most common *Serratia* species involved in human infections, being previously connected with infection outbreaks (Mahlen, 2011). More than 15% of the isolates were identified as *Rahnella* spp., another genus that can be found in soil, water and intestinal tracts of herbivores (Liang et al., 2020). *Rahnella aquatilis* is a fish pathogen capable of inducing hemorrhagic septicemias in vulnerable hosts. In humans, *R. aquatilis* is known as an opportunistic pathogen, since it can cause various infections, from wound infection to sepsis (Liang et al., 2020). Two isolates were also identified as *Klebsiella oxytoca*, which is recognized as a pathogen of interest, due to its opportunistic characteristics and resistance to antibiotics (Singh, Cariappa & Kaur, 2016). This bacterium is usually present in water and soil and can be found in the intestine tracts of several animals, including humans (Singh, Cariappa & Kaur, 2016). Its presence in ready-to-eat food products has been previously reported (Nyenje et al., 2012), alerting to unsafe practices that can pose serious threats to consumers. *Hafnia alvei* was also detected in the samples analyzed (6.00% of isolates). Jeyasekaran et al. (2010) also reported the presence of *Hafnia* spp. in *Loligo duvaucelli*, despite the higher numbers found (74% of the total flora of squid tubes).

None of the 22 isolates of *Pseudomonas* spp. found are known to be human pathogens being all the species widespread in various environments (Silby et al., 2011).

Regarding LAB, *Lactococcus* spp. and *Leuconostoc* spp. were found in high numbers. *Leuconostoc* spp. are mesophilic and psychrotolerant bacteria and some species such as *Leuconostoc gelidum* are commonly found in meat and fish products (Endo, Maeno & Liu, 2020). Production of lactic and acetic acids by *Leuconostoc* spp. through the fermentation of carbohydrates is a cause of food spoilage. Formation of slime, discoloration and alteration of smells and flavors are products of *Leuconostoc* activity (Feiner, 2006; Wang et al., 2013). While negative effects to food products can ensue of the proliferation of these bacteria, some *Leuconostoc mesenteroides* and *Leuconostoc lactis* strains have been recorded as potentially probiotic and bacteriocins producers, having antibacterial activity against *L. monocytogenes* (de Paula et al., 2014; Oliveira et al., 2020). *Lactococcus piscium* was previously identified as a fish pathogen in diseased rainbow trout (Williams et al., 1990), however the relation between the presence of this bacteria and the disease has never been established (Saraoui et al., 2016), unlike its relation to the food spoilage. This spoilage is, however, dependent on the strain and the type of foodstuff; *L. piscium* is known to cause the spoilage of meat but seems to have no

impact in the degradation of seafood (Saraoui et al., 2016). *Lactococcus gerviae* has been shown to cause "septicemias, opthalmias and haemorrhages" in marine farmed fish. In humans, *Lac. gerviae* has been known to be the cause of some infections; its proliferation in the human body possibly lead to infective endocarditis (Malek et al., 2019).

Despite some fish pathogens identified, no relevant foodborne pathogens commonly found in seafood were found, such as *L. monocytogenes*, *Vibrio* spp., *Yersinia* spp., *Salmonella* spp. and *Clostridium* spp. (Novoslavskij et al. 2015). The absence of *L. monocytogenes* in the product could be a consequence of good practices of food processing (Vongkamjan et al., 2017) or the presence of high concentrations of LAB, due to their bacteriocins production and the alteration of the environment acidity (Azizoglu & Kathariou, 2016; Pinto et al., 2020).

4.3 Antimicrobial activity screening of several natural compounds

Antimicrobial activity of several natural compounds against the selected 100 isolates was evaluated and the results are presented in Table 4.3.1.

Table 4.3.1 – Screening of antimicrobial activity of 12 natural compounds and respective controls

Compounds	% Resistant	% Sensitive
0.05% (v/v) Carvacrol	**99**	**1**
Polysorbate 80	**94**	**6**
1.5% (w/v) Olive leaf extract	**100**	**0**
0.05% (v/v) Limonene	**94**	**6**
3% (v/v) Chitosan High Weight 1	**31**	**69**
3% (v/v) Chitosan High Weight 2	**32**	**68**
3% (v/v) Chitosan Medium Weight	**31**	**69**
3% (v/v) Chitosan Low Weight	**31**	**69**
3% (v/v) Acetic Acid	**21**	**79**
10% (w/v) EPE 1	**87**	**13**
10% (w/v) EPE 2	**80**	**19**
95% (v/v) Ethanol	**99**	**1**
1.5% (w/v) Stabilizer 1 (300)	**100**	**0**
1.5% (w/v) Stabilizer 2 (440)	**100**	**0**
50% (v/v) Citrox®	**0**	**100**

Legend: EPE – ethanolic propolis extract

Solutions of 0.05% (v/v) carvacrol and limonene showed antimicrobial activity against 1.0% and 6.0% of the isolates, respectively. Isolates that were inhibited by limonene were also inhibited by the emulsifier polysorbate 80, whereby this inhibition was not considered. Contrary to what was found in this study, Bnyan et al. (2014) described minimum inhibitory concentrations under 0.04% (v/v) of carvacrol against *Serratia* spp. Thielmann and Muranyi (2019) also reported minimum inhibitory concentrations of Limonene for *Klebsiella* spp., *Leuconostoc* spp. and *Pseudomonas* spp. of 1.1%, 2.0% and 0.8% (v/v), respectively. This indicates that in order to inhibit the bacterial growth, much higher concentrations of limonene would have to be used.

Both stabilizers 1 (300) and 2 (440) at 1.5% (w/v), and 1.5% (w/v) Olive leaf extract solution did not show antimicrobial activity against any isolate. While Sudjana et al. (2009) demonstrated that Olive leaf extract at this concentration can inhibit bacterial growth of *Helicobacter pylori*, methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*, in this work only concentrations between 25% and 50% (v/v) showed some efficiency in inhibiting growth of *Klebsiella* spp. and *Serratia* spp. .

While all 3% (w/v) chitosan compounds appeared to inhibit over 65% of the isolates, the same isolates were as well inhibited by the 3% (v/v) acetic acid used as control. Because of this, it was assumed that chitosan solutions, independently of molecular weight, were inefficient in inhibiting the growth of all the isolates tested.

Ethanolic propolis extracts at 10% (w/v) inhibited the bacterial growth, but this inhibition varied depending on the propolis used, being EPE2 more efficient than EPE1. Antimicrobial activity of propolis is known to vary accordingly to the environmental characteristics from which it originates (Przybyłek & Karpiński, 2019). The results obtained are supported by the lack of bacterial inhibition by the ethanolic solution used as control, showing that antibacterial activity is a consequence of the propolis properties.

Of all the compounds, only Citrox® was able to inhibit all of the isolates tested. For this reason, only minimum inhibitory concentrations of the Citrox® solution were further determined.

4.4 Minimum inhibitory and bactericidal concentrations of Citrox®

Concentrations ranging from 0.03% to 50% (v/v) Citrox® solution were prepared with sterile deionized water and minimum inhibitory concentrations were determined. The results obtained are shown in Table 4.4.1.

Table 4.4.1 – Inhibitory efficiency of Citrox® solutions at different concentrations

%(v/v) Citrox®	%Sensitive	%Resistant
50.00	**100**	**0**
25.00	**100**	**0**
12.50	**100**	**0**
6.75	**100**	**0**
3.38	**100**	**0**
1.69	**100**	**0**
0.84	**53**	**47**
0.42	**17**	**83**
0.21	**13**	**87**
0.11	**8**	**92**
0.06	**3**	**97**
0.03	**3**	**97**

When diluted to a final concentration of 1.69% (v/v) or higher, Citrox® inhibited all the isolates tested. At our knowledge, no studies about the minimum inhibitory concentrations of Citrox® ProGuarda against the same species identified in this study are available in the literature. However, other formulations of Citrox® are used for the disinfection of different surfaces, as Citrox® BC30 and Citrox® MDC30 (Hooper et al., 2011). Different combinations of bioflavonoids with malic and citric acids cause the variation of its antibacterial activities; 2% (v/v) Citrox® BC30 showed activity against *Clostridium difficile* while 8% (v/v) Citrox® MDC30 was unable to inhibit the same pathogen (Hooper et al., 2011).

All the suspensions where no growth was observed were plated in Mueller-Hinton agar, as described in section 3.6.4. and bacterial growth was observed in 97 out of the 100 isolates tested. This result indicates that Citrox® acts as a bacteriostatic compound, inhibiting bacterial growth but not destroying bacterial cells. Considering that the shelf life of the product is considerably short, and the purpose of the treatment is to extend it, the bacteriostatic characteristics of Citrox® were deemed sufficient to the purpose intended.

4.5 Application of solutions through pulverization and immersion

The application of solutions in food products require direct interaction between the solution and the product. This interaction can be performed through different techniques. Such techniques include but are not restricted to spraying, dipping, panning and fluidized-bed coating. The selection of the best technique depends on the characteristics of the product, such as pH, permeability, and overall constitution. Also, the feasibility of the process in industrial circumstances must be taken into consideration. As a result of being particularly simple process, and it being easily automated, spraying is one of the most common techniques for the application of edible compounds. (Pizarro, Cervera & Rhenals, 2016)

4.5.1 Bacterial growth after application of a Citrox-based solution

Attending to the results observed in the antimicrobial activity assay, solutions with at least 1.69% (v/v) of Citrox® concentrations should be used for the preparation of the potential solution. According to the maximum concentrations recommended by the manufacturer, a solution of 3% (v/v) Citrox® was formulated to be pulverized over the product and a solution of 1% (v/v) Citrox® was applied to the product through immersion. While 1% (v/v) Citrox® is lower than the minimum inhibitory concentration to most of the isolates tested, it is the maximum recommended concentration for Citrox® in solutions destined to the immersion of products.

After the application of the Citrox® through both pulverization and immersion techniques, bacterial growth was analyzed in order to ascertain the effect of Citrox®-based preservative treatment in the product.

Results regarding bacterial counts of the product along time without (control) and after Citrox® application are represented in table 4.5.1.

Table 4.5.1 - Bacterial enumeration during shelf-life of "espetadas" treated by pulverization and immersion in water (control) and Citrox® solution

	Control						Citrox®					
	Pulverization			Immersion			Pulverization			Immersion		
	t0	t2	t5	t0	t2	t5	t0	t2	t5	t0	t2	t5
MRS30ºC	4.2 ±0.2	6.4 ±0.0	6.7 ±0.0	4.2 ±0.0	6.0 ±0.0	6.9 ±0.1	3.9 ±0.0	6.6 ±0.3	6.9 ±0.3	4.1 ±0.1	5.7 ±0.5	7.0 ±0.1
MRS11ºC	6.1 ±0.1	6.5 ±0.1	6.7 ±0.1	5.2 ±0.0	6.9 ±0.1	>7.0 ±0.0	5.5 ±0.0	6.5 ±0.7	7.9 ±0.5	5.1 ±0.2	6.5 ±0.6	>7.0 ±0.0
PCA30ºC	5.3 ±0.0	7.4 ±0.1	8.9 ±0.3	5.6 ±0.1	7.8 ±0.5	8.7 ±0.1	5.1 ±0.0	8.0 ±0.4	9.3 ±0.1	5.9 ±0.2	7.3 ±0.2	8.9 ±0.2
PCA11ºC	6.4 ±0.1	7.3 ±0.0	8.8 ±0.4	5.7 ±0.2	7.9 ±0.5	8.6 ±0.1	5.3 ±0.0	7.9 ±0.5	9.1 ±0.1	5.9 ±0.2	7.6 ±0.0	8.8 ±0.2
RAPID30ºC	2.1 ±0.5	3.9 ±0.1	5.1 ±0.2	4.6 ±0.2	4.8 ±0.1	7.2 ±0.1	2.2 ±0.1	3.9 ±0.5	5.3 ±0.0	4.7 ±0.2	4.8 ±0.1	7.2 ±0.0
RAPID11ºC	3.2 ±0.3	5.4 ±0.1	6.6 ±0.1	4.6 ±0.0	6.3 ±0.1	7.8 ±0.1	3.6 ±0.1	5.2 ±0.6	>6.0 ±0.0	4.6 ±0.0	5.6 ±0.5	7.4 ±0.3
PAB30ºC	4.1 ±0.1	6.4 ±0.1	8.0 ±0.1	6.0 ±0.1	7.3 ±0.1	8.7 ±0.0	4.5 ±0.1	6.6 ±0.3	8.3 ±0.1	6.1 ±0.2	7.5 ±0.2	8.5 ±0.1
PAB11ºC	5.1 ±0.2	6.4 ±0.1	7.9 ±0.0	5.6 ±0.3	7.3 ±0.2	8.8 ±0.1	4.1 ±0.1	6.7 ±0.3	8.0 ±0.1	6.0 ±0.2	7.2 ±0.2	8.6 ±0.0

Legend: MRS - De Man-Rogosa Sharpe agar; PCA – Plate Count Agar; Rapid - RAPID′ *Enterobacteriaceae* agar; PAB - *Pseudomonas* Agar Base.

The values obtained with control samples, treated with sterile water, were used to evaluate the efficiency of the treatments. It was observed a growth of 2 to 4 log cfu/g of the microbiological agents in the control samples analyzed through the period of five days of refrigeration, at both 30 ºC and 11 ºC and for both techniques. The application of Citrox® through pulverization, did not inhibit bacterial growth during the five days of refrigeration. No significant differences ($P > 0.05$) were observed between the bacterial growth on the samples pulverized with water or Citrox® solution. These results allow to conclude that the pulverization of 3% (v/v) Citrox®-based solution, i.e. superior than to its minimum inhibitory concentration, failed in inhibiting the bacterial proliferation in the product, not impacting positively its shelf life. This could be a result of various factors, such as the inability of the technique to interact with non-external parts of the product, the high percentage of water in the product, which could result in the further dilution of the inhibiting-compound and the pH characteristics of the product.

Similar to the samples treated through pulverization, no significant differences ($P > 0.05$) were observed in bacterial growth between the control samples and those immersed in

1% (v/v) Citrox® solution. Therefore, it was concluded that treatment of the tested samples through immersion in a 1% (v/v) Citrox® solution was not efficient in reducing bacterial growth. While this technique provides a more complete and uniform interaction with the product, including the ability to reach more internal zones, the use of the recommended concentration of Citrox® for this technique, i.e lower than the minimum inhibitory concentration previously observed, could be a potential cause for the inefficiency found. At our knowledge, no similar studies with Citrox® are available in the literature, but considering its citric acid composition, Uranga et al. (2018) developed a citric acid- and chitosan-incorporated film and found that only films prepared with 20% wt citric acid was able to reduce the growth of *E. coli*. This concentration is much higher than the concentration used in this work, far exceeding the recommended concentration of Citrox® (3% (v/v) for pulverization and 1% (v/v) for immersion). For this reason and due to their ineffectiveness, no further tests were carried out with this compound.

4.5.2 Bacterial growth after application of a Thyme essential oil-based solution

A solution of thyme essential oil was also applied since its antimicrobial activity against several microorganisms is recognized and has been reported (Carvalho, Albano & Teixeira, 2019; García-Diez et al., 2017). Carvalho, Albano & Teixeira (2019) and García-Diez et al. (2017) described minimum inhibitory concentrations of Thyme essential oil of 0.78% (v/v) and 0.05% (v/v), respectively. Despite the importance of the sensorial quality of these products, it was decided to apply a treatment based on essential Thyme oil at a concentration of 0.2% (v/v) using the immersion technique.

The results obtained in the different culture media, during 5 days of refrigeration, are presented in the table 4.5.2.

Table 4.5.2 – Bacterial enumeration during shelf-life of "espetadas" treated by immersion in water (control) and Thyme essential oil solution

	Control			Thyme Essential oil		
	t0	t2	t5	t0	t2	t5
MRS 30ºC	4.2±0.02	6.0±0.04	6.9±0.09	3.9±0.12	5.4±0.13	>7.0±0.00
MRS 11ºC	5.2±0.01	6.9±0.09	>7.0±0.00	4.8±0.19	6.3±0.28	>7.0±0.00
PCA 30ºC	5.6±0.08	7.8±0.51	8.7±0.09	5.4±0.26	7.1±0.03	8.4±0.20
PCA 11ºC	5.7±0.24	7.9±0.48	8.6±0.05	5.4±0.19	7.1±0.06	8.4±0.14
RAPID 30ºC	4.6±0.21	4.8±0.07	7.2±0.08	2.7±0.19	4.6±0.16	7.7±0.08
RAPID 11ºC	4.6±0.03	6.3±0.05	7.7±0.06	3.7±0.23	5.6±0.26	7.6±0.17
PAB 30ºC	6.0±0.10	7.3±0.14	8.7±0.03	4.7±0.27	7.2±0.08	8.5±0.25
PAB 11ºC	5.6±0.30	7.4±0.20	8.8±0.06	4.7±0.33	7.1±0.27	8.2±0.22

Legend: MRS - De Man-Rogosa Sharpe agar; PCA – Plate Count Agar; Rapid - RAPID′ *Enterobacteriaceae* agar; PAB - *Pseudomonas* Agar Base.

The impact of the immediately contact with the 0.2% (v/v) Thyme essential oil solution varied; no significant differences ($P > 0.05$) were obtained for bacterial counts of total viable microorganisms and LAB, but a decrease of more than 1 log cfu/g in bacterial populations in RAPID'*Enterobacteriaceae* and *Pseudomonas* Agar Base at both 30 and 11 ºC was clearly noted. However, although apparently promising to inhibit microbial loads of the product, Thyme essential oil only had effect immediately after contact, with a sharp increase in bacterial counts in the following days of storage. No significant differences ($P > 0.05$) were found between each control and treated sample after 2 and 5 days of refrigerated storage. However, the initial inhibition conferred by Thyme essential oil solution suggests their potential application as the active constituent of an edible coating but in higher concentrations or in combination with other strategies. Jouki et al. (2014) reported the inhibition of total viable microorganisms, *Enterobacteriaceae*, *Pseudomonas* spp. and LAB in rainbow trout fillets wrapped with films of quince seed mucilage containing 2% (v/v) thyme essential oil during 18 days of refrigerated storage. Also, Kostaki et al. (2009) found better preservative effect with the application of 0.2% (v/w) Thyme essential oil combined with Modified Atmosphere Packaging (60% CO_2/30% N_2/10% O_2) on sea bass fillets instead of the application of Thyme essential oil alone. The authors reported substantially more effective results in extending the microbiological shelf life of the fillets by 12 days at refrigerated storage.

Considering the low solubility of Thyme essential oil in water, the antimicrobial activity of this compound could be possibly improved by substituting water as the solvent agent for some emulsifier as polysorbate or soy lecithin. This substitution could also mitigate the impact of water in product degradation. These solvents have, however, higher viscosity than water, which could lead to difficulties in the application of the solution through pulverization. In response to this, the formulation of micro emulsions could result in the desired antibacterial activity while maintaining the viscosity necessary for the pulverization of the solution.

4.5.3 Bacterial growth after application of a red wine vinegar-based solution

4.5.3.1 Preliminary experiments that led to red vinegar selection

It was concluded that both Citrox® and Thyme essential oil solutions were unsuccessful in inducing extended shelf-life to the product. The excess of water in the product post-treatment could be responsible for the rapid and intense proliferation of bacterial cells owing to the increasing softness of the product when pulverized or immersed in water.
As a consequence, preliminary tests with other natural compounds were carried out. Survival of three isolates – *S. liquefasciens*, *P. psychrophile* and *Lac. gerviae* - as representative bacteria from *Enterobacteriaceae* family, *Pseudomonas* spp. genera and LAB group, respectively, were evaluated for lemon juice, olive leaf extract and red vinegar during 1 h of contact. Bacterial counts in Ringer's solution, and used as control, were 8.37, 8.74 and 8.57 log cfu/mL for *S. liquefasciens*, *P. psychrophile* and *L. gerviae*, respectively. Bacterial counts obtained for the three isolates in the presence of each natural compound are presented in tables 4.5.3.1.1, 4.5.3.1.2 and 4.5.3.1.3.

Table 4.5.3.1.1 - Survival of each isolate in the presence of pure and diluted lemon juice

		log cfu/mL					
		5s	**5'**	**10'**	**20'**	**40'**	**60'**
Serratia liquefasciens	**Lemon Juice (Pure)**	7.2±0.3	6.6±0.7	3.6±0.4	3.0±0.4	<2.0±0.0	<2.0±0.0
	Lemon Juice (50%)	8.3±0.3	7.4±0.5	6.4±0.4	6.2±0.1	6.2±0.2	6.3±0.4
	Lemon Juice (25%)	8.2±0.3	7.6±0.1	7.3±0.0	6.7±0.5	6.6±0.3	6.5±0.4
Pseudomonas psychrophila	**Lemon Juice (Pure)**	7.9±0.1	7.3±0.3	7.1±0.3	6.5±0.4	5.2±0.1	4.3±0.0
	Lemon Juice (50%)	7.5±0.0	7.2±0.1	7.3±0.1	7.4±0.2	7.4±0.3	7.1±0.0
	Lemon Juice (25%)	7.6±0.1	7.3±0.4	7.3±0.0	7.3±0.3	7.2±0.0	7.2±0.1
Lactococcus gerviae	**Lemon Juice (Pure)**	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Lemon Juice (50%)	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Lemon Juice (25%)	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0

Unlike *Lac. gerviae* which was inhibited by all the concentrations of lemon juice tested, only pure lemon juice demonstrated some effectiveness in reducing *P. psychrophila*, inhibiting its growth by approximately 4 log cfu/mL after 60 minutes of exposure. Lower concentrations of lemon juice (50 and 25% v/v) had no impact on *P. psychrophila*, but inhibitions of approximately 2 log cfu/mL was recorded for *S. liquefasciens*. Pure lemon juice also reduced *S. liquefasciens* to levels below the detection limit of the enumeration technique after 40 minutes of contact. The antimicrobial activity of lemon juice against a wide variety of microorganisms has already been reported by others (Hindi & Chabuck, 2013; Oikeh et al., 2016).

Table 4.5.3.1.2 - Survival of each isolate in the presence of pure (32 mg/mL) and diluted olive leaf extract

		log cfu/mL					
		0'	**5'**	**10'**	**20'**	**40'**	**60'**
Serratia liquefasciens	**Olive Leaf Extract**	7.5±0.3	7.3±0.0	7.2±0.3	7.3±0.4	7.1±0.3	7.2±0.1
	Olive Leaf Extract	7.6±0.1	7.3±0.3	7.4±0.2	7.4±0.2	7.3±0.0	7.4±0.1
	Olive Leaf Extract	7.6±0.0	7.5±0.0	7.4±0.0	7.3±0.0	7.4±0.0	7.4±0.0
Pseudomonas psychrophila	**Olive Leaf Extract**	8.1±0.5	7.9±0.4	7.9±0.4	7.7±0.3	7.7±0.0	7.7±0.4
	Olive Leaf Extract	8.1±0.1	7.9±0.2	7.7±0.0	7.6±0.4	7.9±0.0	7.7±0.3
	Olive Leaf Extract	8.0±0.3	7.5±0.3	7.8±0.1	7.9±0.1	7.8±0.0	7.7±0.2
Lactococcus gerviae	**Olive Leaf Extract**	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Olive Leaf Extract	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Olive Leaf Extract	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0

All tested concentrations of Olive leaf extract were able to inhibit *S. liquefasciens* to about 1 log cfu/mL after immediately and until 60 minutes contact.

Although less evident the reduction of *P. psychrophila* immediately after contact, also all concentrations of Olive leaf extract led to its reduction in 1 log cfu/mL after 60 minutes. *Lactococcus gerviae* was reduced to levels below the detection limit of the enumeration technique immediately after contact of the tested compound.

Similar results were reported in the study of Gokmen et al. (2014) where the authors found that Olive leaf extract at 32 mg/mL failed in inhibiting the growth of Pseudomonads and *Enterobacteriaceae*. However, Testa et al. (2019) detected antibacterial activity against Pseudomonads and LAB when using Olive leaf extract at the concentrations of 5 mg/mL and 10 mg/mL, respectively. Being a compound extracted from natural sources, it is presumed that the disparity between results could be a consequence of the extraction method.

Table 4.5.3.1.3 - Survival of each isolate in the presence of pure and diluted red wine vinegar

		log cfu/mL					
		0'	**5'**	**10'**	**20'**	**40'**	**60'**
Serratia liquefasciens	**Vinegar (Pure)**	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Vinegar (50%)	4.6±1.2	4.3±1.4	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Vinegar (25%)	7.3±0.4	6.1±0.3	6.2±0.3	5.4±0.2	5.1±0.1	4.4±0.3
Pseudomonas psychrophila	**Vinegar (Pure)**	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Vinegar (50%)	4.6±0.7	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Vinegar (25%)	6.3±0.3	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
Lactococcus gerviae	**Vinegar (Pure)**	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Vinegar (50%)	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0
	Vinegar (25%)	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0	<2.0±0.0

Pure red wine vinegar (6% acidity) inhibited all the isolates to levels below the detection limit of the enumeration technique immediately after and until 60 minutes contact. Dilution of vinegar by 50% (v/v) resulted in an initial inhibition of about 4 log cfu/mL of *S. liquefasciens* and *P. psychrophila* and their inhibition to levels below the detection limit after 10 and 5 minutes of contact, respectively. Vinegar at 25% (v/v) was also effective to reduce *P. psychrophila* at undetected levels after 5 minutes of contact, but only reductions of about 4 log cfu/mL were observed for *S. liquefasciens* after 60 minutes. All the concentration of vinegar tested also inhibited the growth of *Lac. gerviae*.

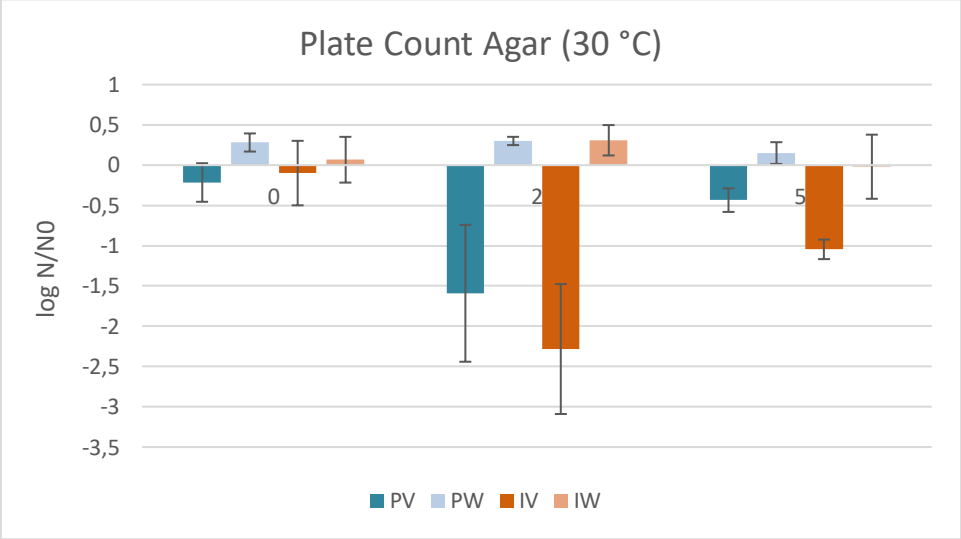
The bacterial growth inhibition by vinegar was somewhat expected due to its known antibacterial activity and antioxidant potential (Bakir *et al.*, 2017). Many studies reporting the antimicrobial activity of vinegar against different bacterial species can be found in the literature (Laranjo et al., 2019; Lingham et al., 2012).

Attending to the strong antibacterial activity observed, a solution of 50% (v/v) of red wine vinegar in sterile water was formulated to be applied through pulverization and immersion in the product.

4.5.3.2 Application of a vinegar-based solution through pulverization and immersion

The effect of the vinegar treatment was tested and microbial counts obtained by vinegar application were transformed to logarithmic reduction using the equation: log (N/N0), where N is the microbial cell count at a particular sampling time and N0 is the microbial cell count after any treatment.

Bacterial growth in each culture media at both incubation temperature is represented in Figures 4.5.3.2.1, 4.5.3.2.2, 4.5.3.2.3 and 4.5.3.2..4



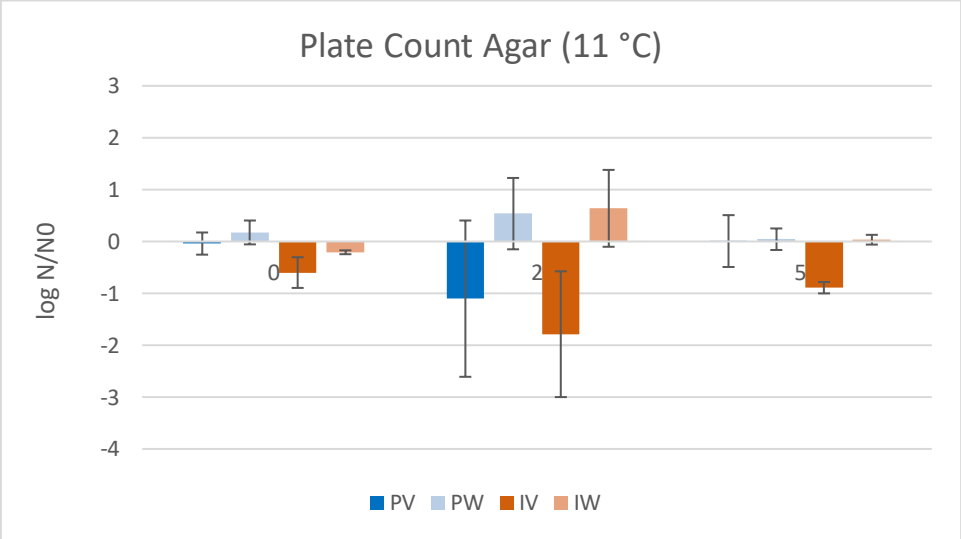


Figure 4.5.3.2.1 – Bacterial counts in Plate count agar at 30 and 11 ºC of "espetadas" treated by pulverization ( ) and immersion ( ) with 50% v/v vinegar solution or by pulverization ( ) and immersion ( ) with water (as control). All the results are represented by the mean ± the standard error of the mean of 2 replicates.

It is possible to observe that at 30 ºC, no significant differences ($P > 0.05$) in bacterial counts can be recognized immediately after treatment. However, greater differences in the reduction of total viable microorganisms were found between the samples treated with vinegar by immersion and the respective control in water after 2 days of storage with reductions of more than 2 log cycles. After 5 days of refrigerated storage, bacterial inhibition was less evident, but bacterial counts for vinegar-treated samples by immersion still demonstrated antibacterial activity, being observed reductions around 1 log cycle in the treated samples compared with the control.

At 11 ºC, slight reductions were detected only for samples immediately after immersion in the vinegar solution. The total viable microorganisms from the same immersed samples were reduced to approximately 1.5 and 1 log cycles after 2 and 5 days of storage, respectively. These results show a clear effect on the reduction of total viable microorganism proliferation during the period of refrigeration in samples immersed in a 50% (v/v) vinegar solution.

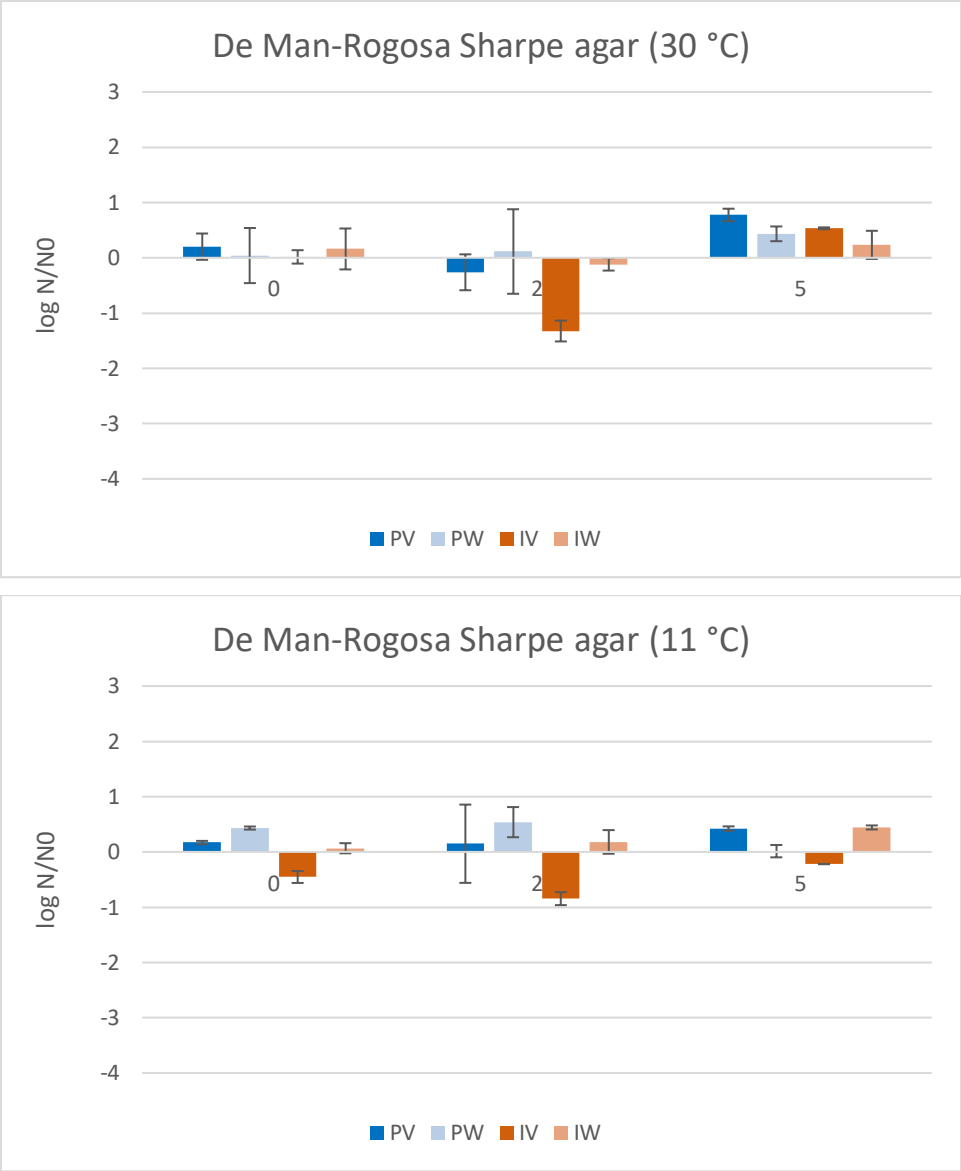


Figure 4.5.3.2.2 - Bacterial counts in De Man-Rogosa Sharpe agar at 30 and 11 ºC of "espetadas" treated by pulverization (■) and immersion (■) with 50% v/v vinegar solution or by pulverization (■) and immersion (■) with water (as control). All the results are represented by the mean ± the standard error of the mean of 2 replicates.

Reductions on Lactic Acid Bacteria at 30 ºC were only detected in vinegar-immersed samples and at the second day of storage. No significant differences ($P < 0.05$) were found between treated samples and respective controls immediately after treatment and after 5 days of storage.

At 11 ºC the observed reductions were similar as those observed at 30 ºC. No significant differences ($P > 0.05$) were found immediately after each treatment. At second day of storage, immersed samples had LAB reductions around 1 log cycle, but no significant

differences ($P > 0.05$) were observed after 5 days of storage between all the treated samples and respective controls.

This result may be due to the acidophilic properties of lactic acid bacteria (Menconi et al., 2014). Considering that tolerance, and the ability to growth in acidic environments, it is comprehensible that the efficiency of acetic acid, present in vinegar, could be inept to inhibit the growth of lactic acid bacteria. Different results were obtained by Zhang et al. (2018) with reported significantly reduction in LAB counts (2 log cycles), during the entire storage period, in raw pork chops treated with a chitosan and bamboo vinegar edible coating.

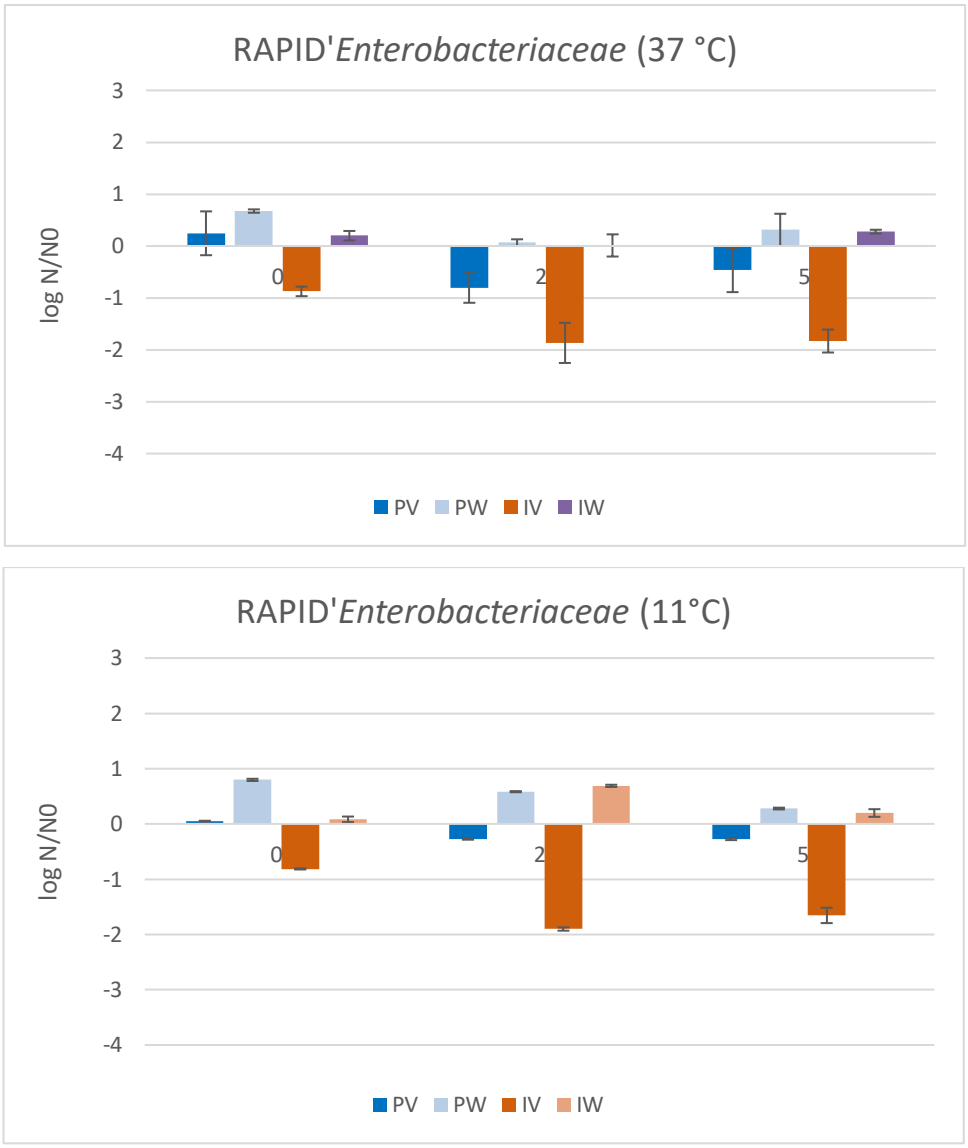


Figure 4.5.3.2.3 - Bacterial counts in Rapid'*Enterobacteriaceae* at 30 and 11 ºC of "espetadas" treated by pulverization (■) and immersion (■) with 50% v/v vinegar solution or by pulverization (■) and immersion (■) with water (as control). All the results are represented by the mean ± the standard error of the mean of 2 replicates.

Significant reductions ($P < 0.05$) of *Enterobacteriaceae* at both 37 and 11 ºC were found in the samples immersed in 50% (v/v) vinegar. After immediately treatment, reductions around 1 log cycle were observed and after 2 and 5 days of storage, reductions were more accentuated (about 2 log cycles). Zhang et al. (2018) also found 2 log cycle reductions of the *Enterobacteriaceae* present in raw pork chops treated with a chitosan and bamboo vinegar edible coating.

At 37 ºC, despite the 1 log cycle reduction at second day of storage for pulverized-samples with 50% (v/v) vinegar, no significant differences ($P > 0.05$) were obtained with this

technique. Curiously, high *Enterobacteriaceae* counts were observed for water-pulverized samples, especially in the culture medium incubated at 11 ºC. Although not able to justify this result, we believe that this increase can be a result of an increase of the water content of the product.

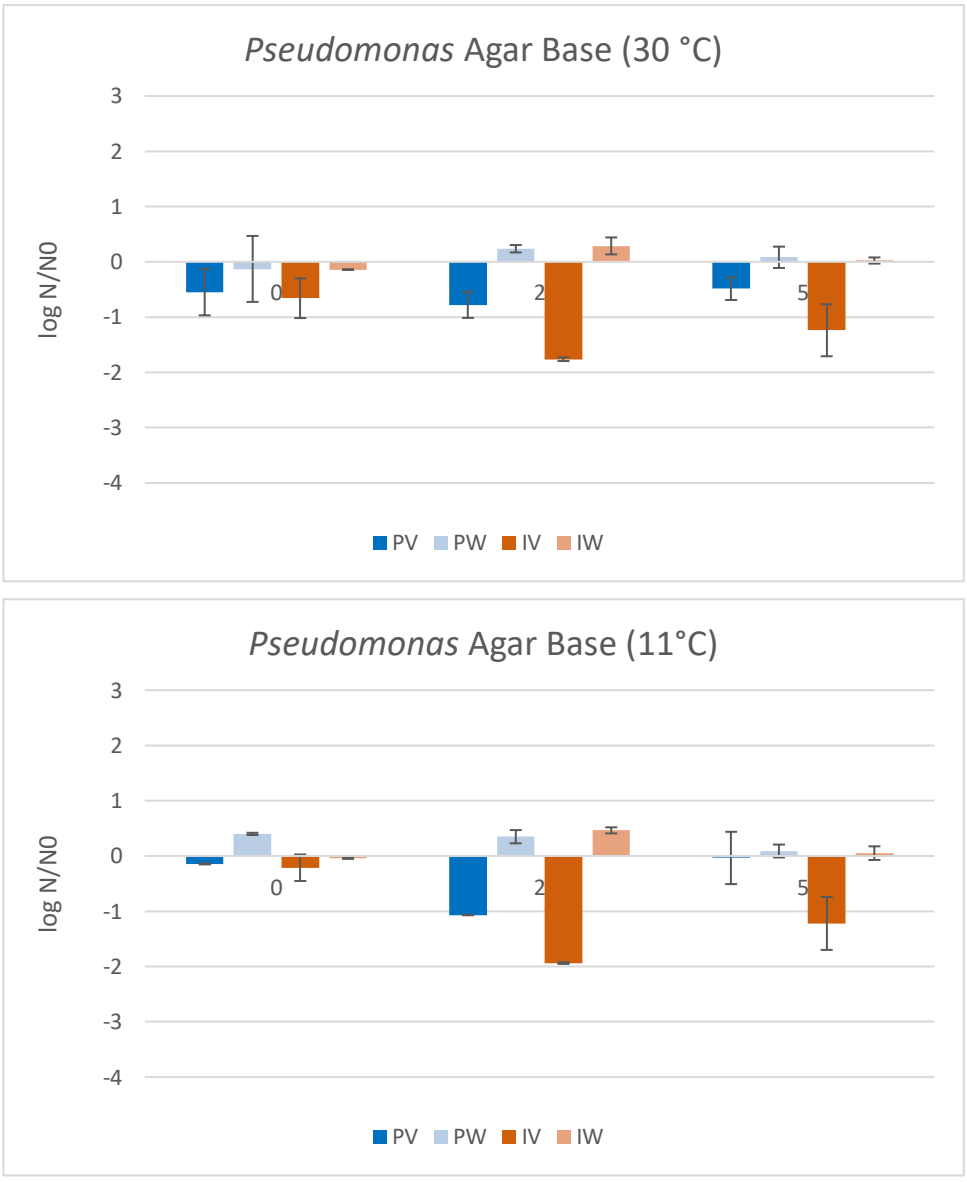


Figure 4.5.3.2.4 - Bacterial counts in *Pseudomonas* agar base at 30 and 11 ºC of "espetadas" treated by pulverization (■) and immersion (■) with 50% v/v vinegar solution or by pulverization (■) and immersion (■) with water (as control). All the results are represented by the mean ± the standard error of the mean of 2 replicates.

Decrease in *Pseudomonas* spp. counts at 30 ºC was observed immediately and during storage after pulverization and immersion of the product with 50% (v/v) vinegar.

However, reductions in vinegar pulverized-samples were not statistically significant ($P > 0.05$). As observed before for the other microorganisms, the higher reduction occurred at the second day of storage, but only for vinegar immersed-samples (around 2 log cycle). After 5 days of storage, no significant differences ($P > 0.05$) were observed in both pulverized- and immersed-samples with vinegar.

In *Pseudomonas* agar base at 11 ºC also no significant reductions ($P > 0.05$) were detected immediately after treatments and after 5 days of storage. At the second day of refrigeration, significant reductions ($P < 0.05$) of 1 and 2 log cycle of *Pseudomonas* spp. were observed in vinegar-pulverized and vinegar-immersed samples, respectively. In sum, it was evident that only the immersed samples with 50% (v/v) vinegar showed reductions in total viable microorganisms, *Enterobacteriaceae* and *Pseudomonas* spp. and that reductions were only significant at the second day of storage. Although the treatment applied did not allow to increase the shelf-life of the "espetadas", the fact that it allowed significant reductions over its shelf life (i.e. 2 days) indicates that vinegar treatments can induce clear advantages to microbiological quality of the product.

4.5.3.3 Sensorial evaluation

Pictures were collected to evaluate and compare product deterioration of control samples, vinegar-pulverized and vinegar-immersed samples and presented in Figure 4.5.3.3.1.

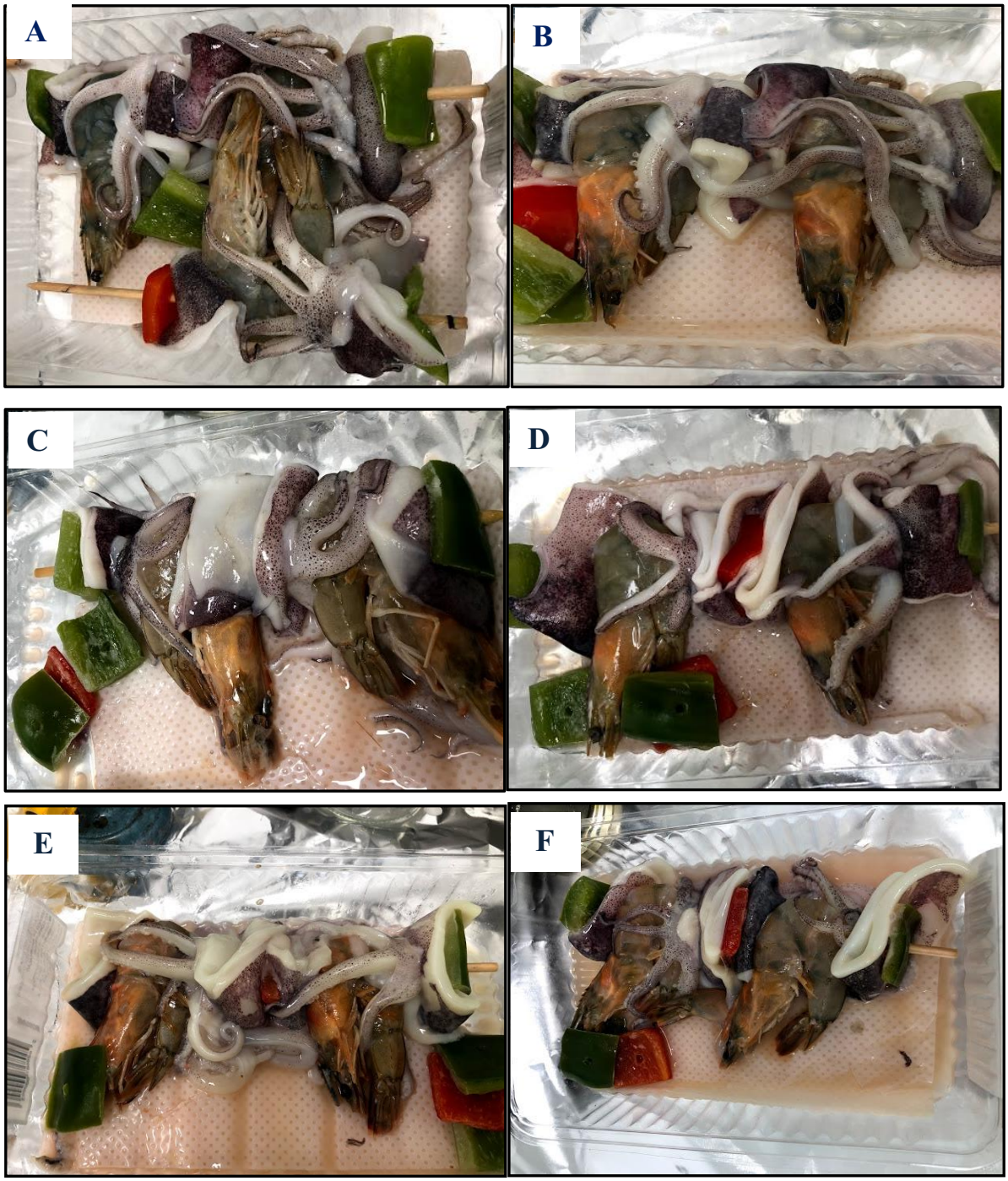


Figure 4.5.3.3.1 – Pictures of control sample at day 0 (A), control sample after 5 days of storage (B), vinegar-pulverized sample after 5 days (C), vinegar-immersed sample after 5 days (D), water- pulverized sample after 5 days (E) and water- immersed sample after 5 days (F).

Evaluation of the product immediately after reception was performed (Figure 4.5.3.3.1 A). The product was hard to cut, clean, colorful and a clear, not unpleasant, smell of seafood was detected at this point.

Immediately after treatment with 50% (v/v) vinegar by both pulverization and immersion techniques, squid flesh color turned to a light rose and a somewhat intense vinegar odor was recognized. Due to the characteristic sensorial properties of red wine vinegar, this result was expected. No additional alterations were detected at the first day of sampling.

After 5 days of refrigerated storage, visual comparison of each treatment effect between samples was performed. Clear signs of spoilage were observed for control samples without any treatment (Figure 4.5.3.3.1 B) and control samples treated with water (Figure 4.5.3.3.1 E and Figure 4.5.3.3.1 F). In these samples, squid flesh developed a yellowish tonality, turned soft and sponge-like and had white slime in its surface. Shrimps darkened considerably and had black spots on their flesh. The intensity of color reduced significantly during storage, having the intensely purple external surface of squid turned to a mild gray. Also, an extremely unpleasant odor was developed during storage, resulting in a completely undesirable product.

When pulverized in 50% (v/v) vinegar, sensorial properties showed deterioration of the product after 5 days of refrigerated storage (Figure 4.5.3.3.1 C). While no slime was observed, changes in squid flesh color and texture were detected, such as the loss of product color and softness of squid flesh. Also, smell was not as pungent as in the control samples. This indicate that pulverization ameliorates the quality of the product after 5 days of storage, but it is not enough to extend its shelf-life (as previously discussed in section 4.5.3.2).

Samples immersed in 50% (v/v) vinegar were noticeably different (Figure 4.5.3.3.1 D). Squid meat maintained its original colors, losing the pink tonality acquired immediately after the treatment. The intense purple coloration of squid exterior surface was maintained. Also, no changes in texture were detected, being observed resistance to cut and consistency in squid. No slime was observed. No dark spots were observed in shrimp and maintenance of lively colors was achieved. Regarding odor, no vinegar scent was noticed, and product smell was similar to what was found in the first day of sampling. Vinegar immersion showed efficiency in controlling the deteriorative progress of the product, maintaining good appearance, texture and smell.

Conclusions

In the present study, "espetadas" of squid and shrimp were characterized microbiologically at the same day of confection and after its shelf-life. No foodborne pathogens *L. monocytogenes* and *E. coli* were detected and high numbers of total viable microorganisms (~6 log cfu/g), *Pseudomonas* spp., *Enterobacteriaceae* and LAB (~4 to 5 log cfu/g) were found after incubation temperatures of 30/37 ºC and 11 ºC. These results indicate that the adequate cooking of the studied "espetadas" would result in a presumably safe product with no serious threat to the consumers. However, an increase of 2 log cycles after 5 days of storage, turns necessary the development of strategies to control the growth of deteriorating bacteria and to increase the shelf-life of these products. The randomly isolation of 100 microorganisms, allow the identification of several different species as *Serratia* spp., *Klebsiella* spp., *Hafnia* spp., *Rahnella* spp., *Pseudomonas* spp., *Lactococcus* spp., *Leuconostoc* spp. and *Weissella* spp.

Antimicrobial activity of several natural compounds was tested against the isolated microorganisms and after several experiments, Citrox®, Thyme essential oil and red wine vinegar were selected to be applied as treatment solutions using pulverization and/or immersion techniques. No significant bacterial reductions ($P > 0.05$) were found in the samples treated with Citrox®, but the fact that the maximum recommended concentration for immersion (1% v/v) is inferior to the minimum inhibitory concentration determined (1.69% v/v), it is assumed that the application techniques for this compound might not be the most appropriate. In addition, the 3% (v/v) Citrox® recommended for pulverization might not be sufficient for the solution penetrate the product and interact with its internal parts. Also, the possible dilution of the solution after contact with the product due to its high humidity was speculated as a possible reason for its ineffectiveness. Similarly, Thyme essential oil did not allow significant bacterial reductions ($P > 0.05$) in the treated samples by pulverization and immersion.

The application of an edible vinegar-based solution applied to the product by immersion resulted in growth inhibition of total viable microorganisms, *Pseudomonas* spp. and *Enterobacteriaceae*, but not of LAB, during two days of storage. Additionally, also the deteriorative progress of the product was delayed, allowing the maintenance of good appearance, texture and smell after five days of storage.

Although the treatment applied with vinegar did not allow to increase the shelf-life of the "espetadas", the fact that it allowed significant reductions over its shelf life (i.e. 2 days of

refrigerated storage), turn the use of a vinegar-based solution, a potential strategy to achieve shelf-life extension to this product. Additional tests such as other concentrations/types of vinegar/techniques used alone or in combinations with other natural compounds are required to guarantee an effective solution for food industry.

Future Work proposals

Due to external pressures, namely the 2020 COVID-19 crisis, some planned experiments were not executed during the period allocated to this study. These are of utmost importance to approve the application of preservative solutions to ”espetadas” of squid and shrimp:

1. Sensorial evaluation of product treated by immersion in 50% (v/v) vinegar after cooking should be performed to determine its sensorial quality. Changes in color, texture, flavor and smell must be analyzed by a trained panel.

2. Metagenomic analysis of the product under different conditions (with and without treatments) should be performed immediately after treatment and during the storage period to increase knowledge about the possible changes in the spoilage microbiota.

Due to the preliminary results obtained in this study, further testing of compounds and techniques could be performed:

3. The application of other vinegars such as white wine or cider vinegar to found out their effectiveness in bacterial reductions and their impact in the product flavor.
4. New formulations of thyme essential oil or other essential oils as edible-coatings should be tested. The formulation of microemulsions with each essential oil is crucial to provide homogenous solutions and its easier application.
5. The study of synergistic activity of different combinations of natural compounds as well as preservative techniques should be evaluated.

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