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運用辛酸/幾丁聚醣/明膠之可食膜
提升肉品安全性與架儲期

Application of chitosan-gelatin-caprylic acid edible coating on fresh meat for enhancing the microbiological safety and shelf life

俞沛恩

Matthew K. Heras

指導教授：盧冠宏 博士

Advisor: Kuan-Hung Lu, Ph.D.

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the microbiological safety and shelf life

本論文係　俞沛恩　君（R08851009）在國立臺灣大學食品安全與健康研究所完成之碩士學位論文，於民國 112 年 1 月 31 日承下列考試委員審查通過及口試及格，特此證明。

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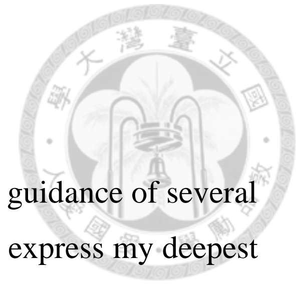

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Institute of Food Safety and Health
National Taiwan University
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Graphical abstract

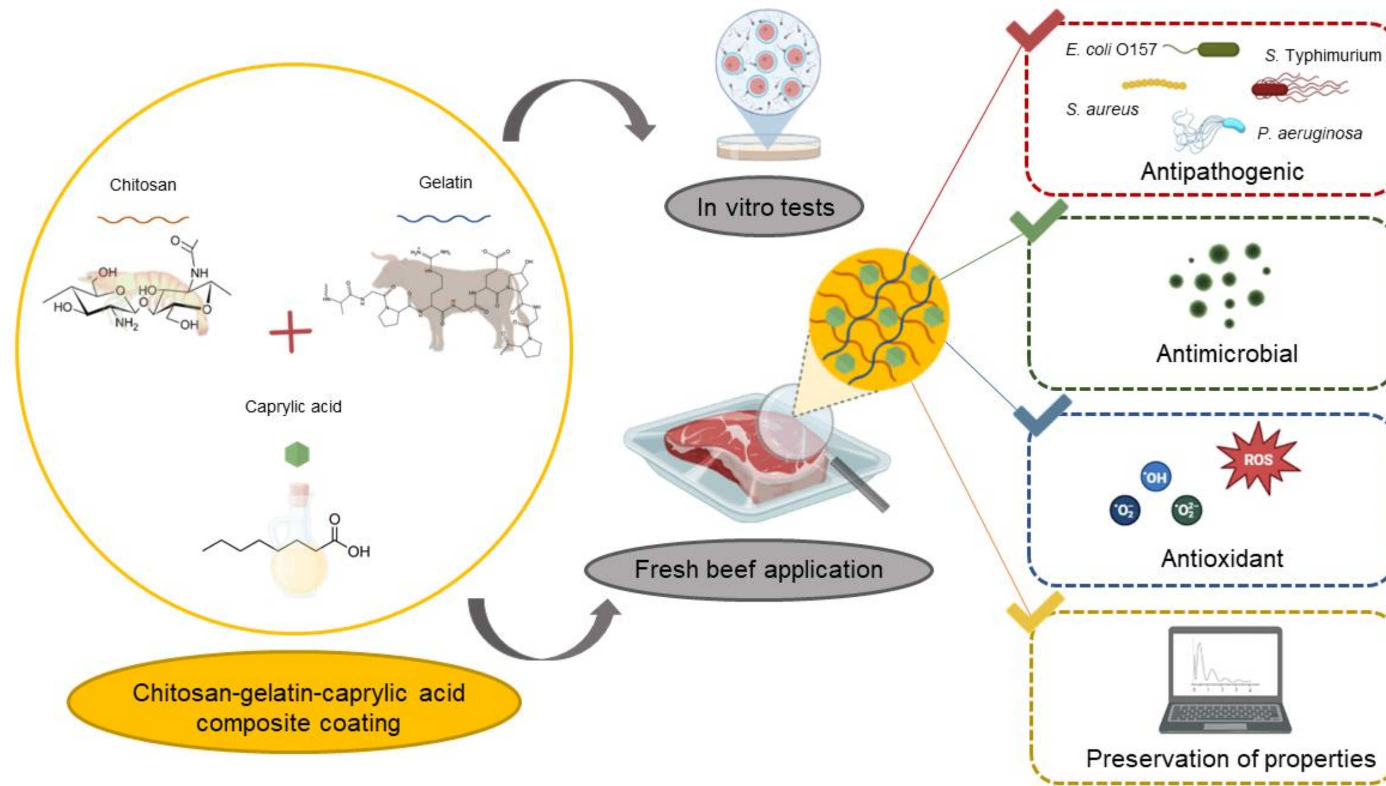

Figure 1. Graphical abstract of this research study.

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中文摘要

長久以來，肉類產品一直是人們動物性蛋白質的主要來源，是飲食中不可或缺的食物之一。然而肉品在屠宰及儲藏過程中，可能因為品質下降 （如：脂質氧化、腐敗）和病原菌交叉汙染等問題，影響架儲期和安全性，對於消費者和零售商而言是一大隱憂。根據我國食藥署的食品中毒事件統計資料，肉類在各類食品中排名第三，且近年來肉品的人均攝食量逐年攀升，亦可能導致肉品引起的食源性疾病風險與發生率隨之上升，因此相關業者必須尋求更完善且有效的方法，以滿足大眾對肉品安全和架儲期的需求，亦是保障公共衛生與健康的重要課題。本研究旨在開發以幾丁聚糖 (CHI)、明膠 (GEL) 和辛酸 (CA) 為基礎之可食用複合塗膜，並測試其在 LB 培養基 和新鮮牛肉基質 (有氧包裝) 之抑菌、抗氧化和抗病原菌功效。此複合塗膜的保存特性測試，是將具有塗膜之新鮮牛肉儲存在冷藏溫度 (4 ℃) 下 20 天，評估其對於抑制鮮肉之微生物腐敗、脂質氧化和品質流失上的影響。而複合塗膜的抗病原菌性能，則是將新鮮牛肉接種病原菌之後再塗膜，然後將肉品置於冷藏 (4 ℃) 和 10 ℃ 低溫下儲存 7 天，藉以評估塗膜對於病原菌生長之影響。在研究初始階段，先以培養基測定了 CA 對常見肉類病原菌 (即金黃色葡萄球菌、大腸桿菌 O157:H7、鼠傷寒沙門氏桿菌和綠膿桿菌) 的最小抑菌濃度和最小殺菌濃度，以篩選最佳 CA 濃度加入幾丁聚糖-明膠 (CHI-GEL) 之複合膜中。培養基實驗表明 1% CHI - 3% GEL 複合膜對於所有病原菌的生長抑制超過 3 log CFU/mL。若進一步添加 0.25% 或 0.5% CA 能夠大幅提升抑菌能力到 4 至 9 log CFU/mL。在新鮮牛肉的塗膜試驗中也觀察到類似的結果，其中 CHI-GEL 複合膜對所有病原菌抑制功效隨著 CA 的添加而加强，達到超過 3 log CFU/g。研究結果顯示出 CA 和 CHI-GEL 膜之間具有協同的抗菌活性。此外，CHI-GEL-CA 複合膜在維持肉品品

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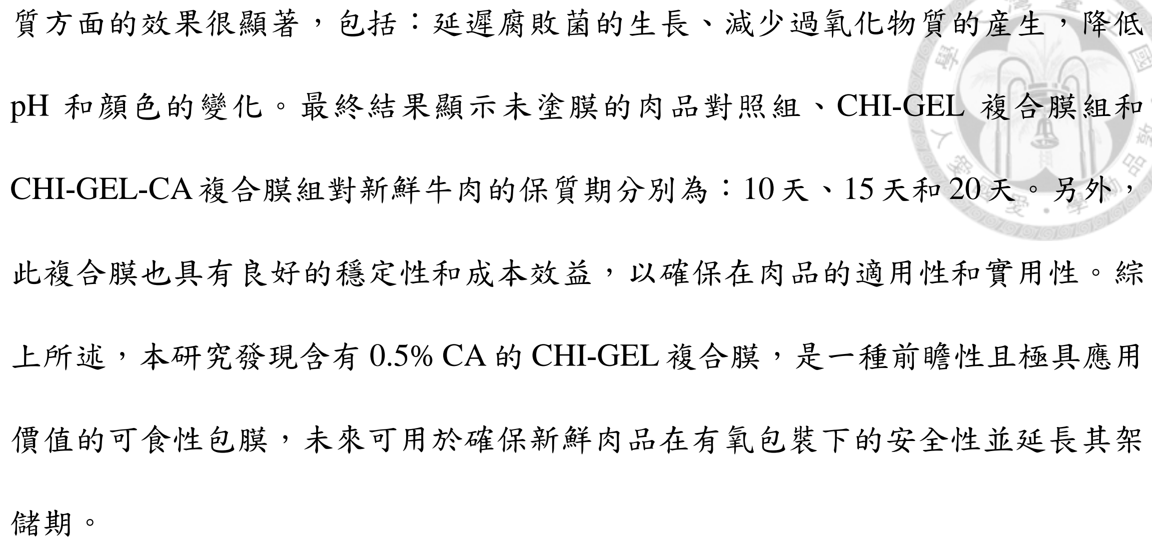

質方面的效果很顯著，包括：延遲腐敗菌的生長、減少過氧化物質的產生，降低 pH 和顏色的變化。最終結果顯示未塗膜的肉品對照組、CHI-GEL 複合膜組和 CHI-GEL-CA 複合膜組對新鮮牛肉的保質期分別為：10 天、15 天和 20 天。另外，此複合膜也具有良好的穩定性和成本效益，以確保在肉品的適用性和實用性。綜上所述，本研究發現含有 0.5% CA 的 CHI-GEL 複合膜，是一種前瞻性且極具應用價值的可食性包膜，未來可用於確保新鮮肉品在有氧包裝下的安全性並延長其架儲期。

關鍵字： 辛酸、幾丁聚糖、明膠、可食用複合塗膜、鮮肉、抗病原菌功效、保存特性

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Abstract

Meat products have long been a staple food in people's diets as the primary source of animal protein. However, during processing and storage, the problems related to quality degradations (i.e., lipid oxidation, spoilage) and pathogen cross-contamination creates shelf life and safety concern for consumers and retailers alike. According to the Taiwan Food and Drug Administration, foodborne outbreaks related to meat products rank 3rd among all food types. Moreover, recently, there has been a continual increase in the average meat intake of the people in Taiwan, which can lead to higher rates of foodborne outbreaks. Therefore, finding effective treatments are important matters for food safety practitioners, to meet the public demand for safe and long shelf life meat products. The objective of this study was to determine the antimicrobial, antioxidant, and antipathogenic activities of a newly developed edible composite coating comprised of chitosan (CHI), gelatin (CHI-GEL), and caprylic acid (CA), under growth medium (Luria-Bertani broth) and aerobically packaged meat (fresh beef) matrix. The preservation properties of the coatings were evaluated based on the extent of microbial spoilage, lipid oxidation, and quality loss in fresh meat stored at refrigerated temperature (4 °C) for 20 days. The inhibitory properties of coatings were assessed based on pathogenic bacterial growth in inoculated meat stored at refrigerated (4 °C) or abusive temperature (10 °C) for seven days. In the preliminary phase of the study, the minimum inhibitory concentration and minimum bactericidal concentration CA against common meat pathogens (i.e., *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Pseudomonas aeruginosa*) were determined to select the ideal CA concentration to be incorporated in CHI-GEL composite coating. The growth medium experiments showed that 1% CHI-3% GEL composite coating effectively inhibited all pathogenic growth by > 3 log CFU/mL. Further addition of 0.25% or 0.5% CA was able to enhance the

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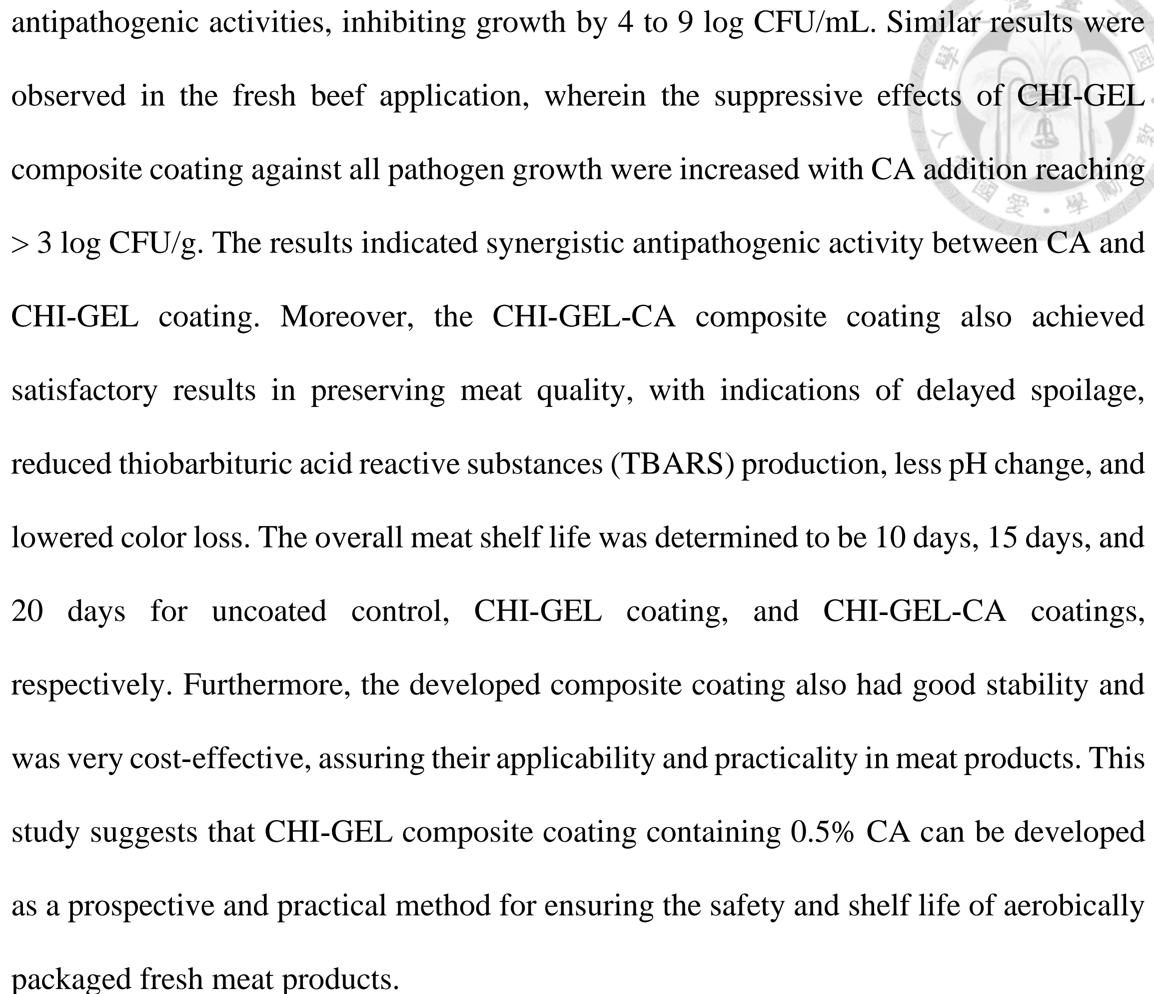

antipathogenic activities, inhibiting growth by 4 to 9 log CFU/mL. Similar results were observed in the fresh beef application, wherein the suppressive effects of CHI-GEL composite coating against all pathogen growth were increased with CA addition reaching > 3 log CFU/g. The results indicated synergistic antipathogenic activity between CA and CHI-GEL coating. Moreover, the CHI-GEL-CA composite coating also achieved satisfactory results in preserving meat quality, with indications of delayed spoilage, reduced thiobarbituric acid reactive substances (TBARS) production, less pH change, and lowered color loss. The overall meat shelf life was determined to be 10 days, 15 days, and 20 days for uncoated control, CHI-GEL coating, and CHI-GEL-CA coatings, respectively. Furthermore, the developed composite coating also had good stability and was very cost-effective, assuring their applicability and practicality in meat products. This study suggests that CHI-GEL composite coating containing 0.5% CA can be developed as a prospective and practical method for ensuring the safety and shelf life of aerobically packaged fresh meat products.

Keywords: Caprylic acid, Chitosan, Gelatin, Edible composite coating, Fresh meat, Antipathogenic activity, Preservation properties

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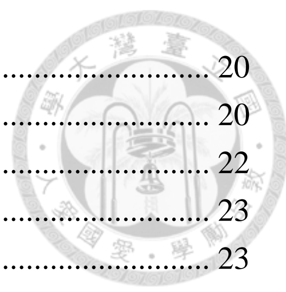

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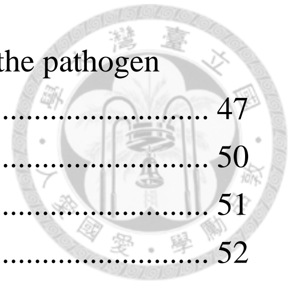

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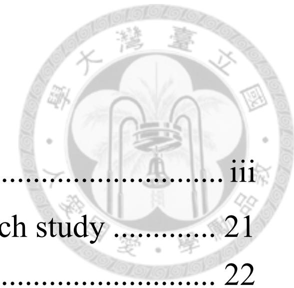

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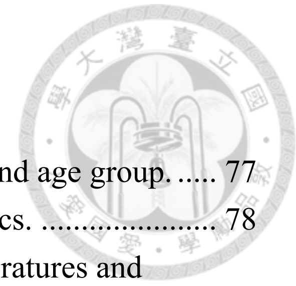

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Chapter 1. Introduction

Food safety and quality assurance are essential aspects that have gotten more attention in the past years. As meats are among the prevalently consumed food products worldwide, keeping their safety and quality during all stages, from production to storage, is an important matter. Microbial contamination (e.g., *Lactobacillus* spp., *Pseudomonas* spp.) has long been the major cause of shelf life, quality, and sensory deterioration during fresh meat storage (Hoa et al., 2022a). Raw meats are also deemed to be one of the major food products containing pathogenic bacteria (e.g., *Salmonella* spp., *Staphylococcus* spp.), as contamination from environmental factors can easily occur. Moreover, improper storage or undercooking can cause these pathogens to grow and potentially induce foodborne diseases (Ortega et al., 2014). Therefore, due to these public health concerns, the importance of developing an efficient and effective method for improving meat safety, quality, and shelf life has long been tackled by researchers. Studies have pointed out that in retail shops and supermarkets, overwrap packaging is the predominantly used packaging for fresh meats, owing to its cheaper cost and better appearance (McMillin, 2008). However, this causes concern as overwrap packaging usually has a shorter shelf life in comparison to other packaging techniques (e.g., vacuum packaging, modified atmospheric packaging) because of the rapid oxidation rate and microbial spoilage (Lorenzo & Gomez, 2012). Consequently, finding ways to overcome the lack of overwrap packaging are necessary and important. As a promising emerging solution, antimicrobial coatings, especially chitosan-based, are being widely studied for meat product applications (H. Y. Zhang et al., 2019). Chitosan-based coatings have proven to be very effective in inhibiting microbial and even pathogenic growth (Guo et al., 2019). However, due to poor moisture barriers of chitosan, studies regarding chitosan composite coatings (i.e., chitosan-gelatin) are rising. Recently, the addition of biopreservatives have also been

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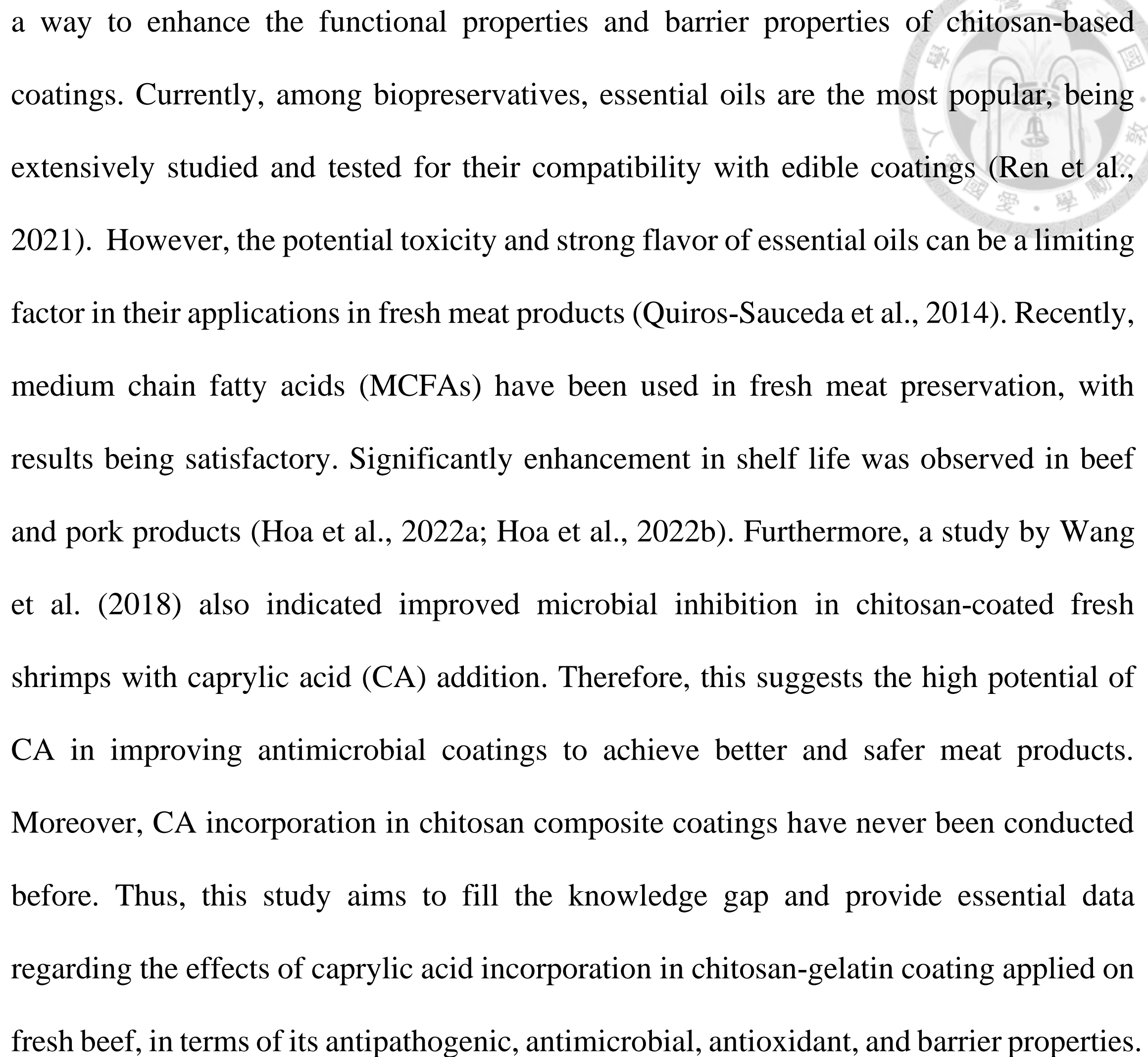

a way to enhance the functional properties and barrier properties of chitosan-based coatings. Currently, among biopreservatives, essential oils are the most popular, being extensively studied and tested for their compatibility with edible coatings (Ren et al., 2021). However, the potential toxicity and strong flavor of essential oils can be a limiting factor in their applications in fresh meat products (Quiros-Sauceda et al., 2014). Recently, medium chain fatty acids (MCFAs) have been used in fresh meat preservation, with results being satisfactory. Significantly enhancement in shelf life was observed in beef and pork products (Hoa et al., 2022a; Hoa et al., 2022b). Furthermore, a study by Wang et al. (2018) also indicated improved microbial inhibition in chitosan-coated fresh shrimps with caprylic acid (CA) addition. Therefore, this suggests the high potential of CA in improving antimicrobial coatings to achieve better and safer meat products. Moreover, CA incorporation in chitosan composite coatings have never been conducted before. Thus, this study aims to fill the knowledge gap and provide essential data regarding the effects of caprylic acid incorporation in chitosan-gelatin coating applied on fresh beef, in terms of its antipathogenic, antimicrobial, antioxidant, and barrier properties.

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Chapter 2. Literature review

2.1 Meat

Meat is generally defined as the flesh of an animal, which is suitable for human consumption. Meat has long been a significant part in the diet of many people due to containing rich nutrients. Meat is generally classified into white (e.g., turkey, chicken, duck), red (e.g., pork, beef, lamb), and processed (e.g., smoked, cured products) meats.

2.1.1 Meat consumption

Based on the study of McAfee et al. (2010), among the meat types, red meat is consumed the most in accordance with the daily diet of people in several countries. Furthermore, findings from the Global Dietary Database have indicated that both Southeast Asia and East Asia regions have a mean daily red meat intake of 87 g/day, which is relatively higher than the world average intake of 51 g/day (Miller et al., 2022). Moreover, data provided by Lien et al. (2020) estimated that the average consumption of beef in Taiwan was 74.53 g/day and 53.08 g/day for male and female adults, respectively, as seen in **Table 1**. These studies reflect red meat (i.e., beef) as an essential food commodity in our country.

2.1.2 Factors influencing meat quality and safety

There are many postmortem factors directly impacting the quality of meat, with pH and temperature being the most crucial ones. The meat pH is determined by the glycogen amount present in meat muscles, with values ranging from 5.4 to 5.7 for freshly slaughtered animals. As the pH lowers, the meat will have decreased water-holding capacity, lighter color, and poorer texture quality. Furthermore, to maintain the postmortem meat quality, meat must be properly stored at a low temperature (refrigerated

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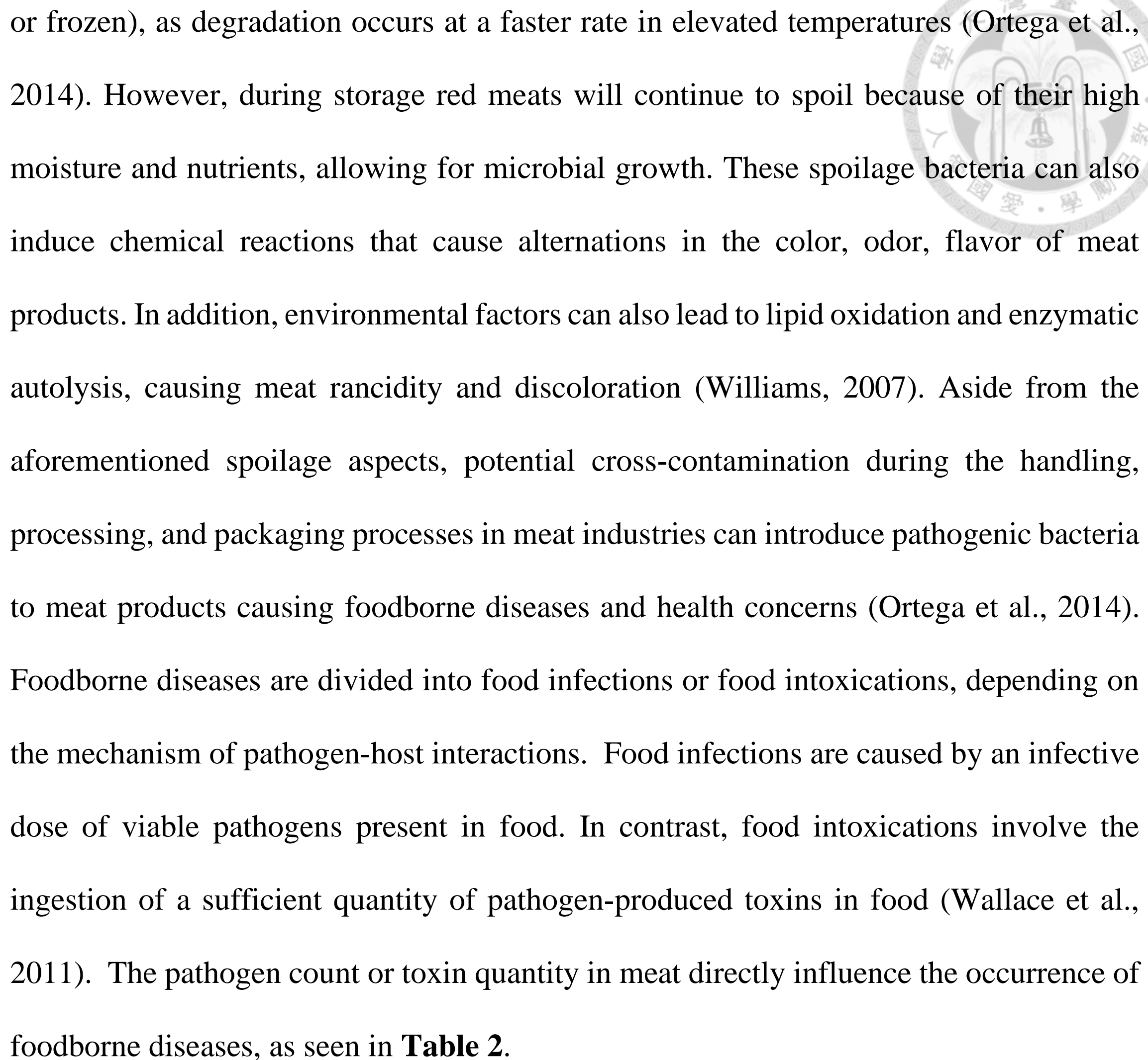

or frozen), as degradation occurs at a faster rate in elevated temperatures (Ortega et al., 2014). However, during storage red meats will continue to spoil because of their high moisture and nutrients, allowing for microbial growth. These spoilage bacteria can also induce chemical reactions that cause alternations in the color, odor, flavor of meat products. In addition, environmental factors can also lead to lipid oxidation and enzymatic autolysis, causing meat rancidity and discoloration (Williams, 2007). Aside from the aforementioned spoilage aspects, potential cross-contamination during the handling, processing, and packaging processes in meat industries can introduce pathogenic bacteria to meat products causing foodborne diseases and health concerns (Ortega et al., 2014). Foodborne diseases are divided into food infections or food intoxications, depending on the mechanism of pathogen-host interactions. Food infections are caused by an infective dose of viable pathogens present in food. In contrast, food intoxications involve the ingestion of a sufficient quantity of pathogen-produced toxins in food (Wallace et al., 2011). The pathogen count or toxin quantity in meat directly influence the occurrence of foodborne diseases, as seen in **Table 2**.

2.1.3 Meat shelf life

The length in time under specific temperature storage where meat starts to deteriorate to an unacceptable level (e.g., microbial growth, lipid oxidation, color change, odor development) is defined as its shelf life. The judgement of shelf life is determined by retailers, wherein a defined minimum acceptable quality is made for the products. This will indicate the length of time the meat products are sold in shops (Potter & Hotchkiss, 1999). **Table 3** shows the shelf life of various fresh meats under different packaging conditions. The shelf life of similar meat products may differ greatly based on initial microbial concentrations, storage environment, and applied packaging. Based on the meat

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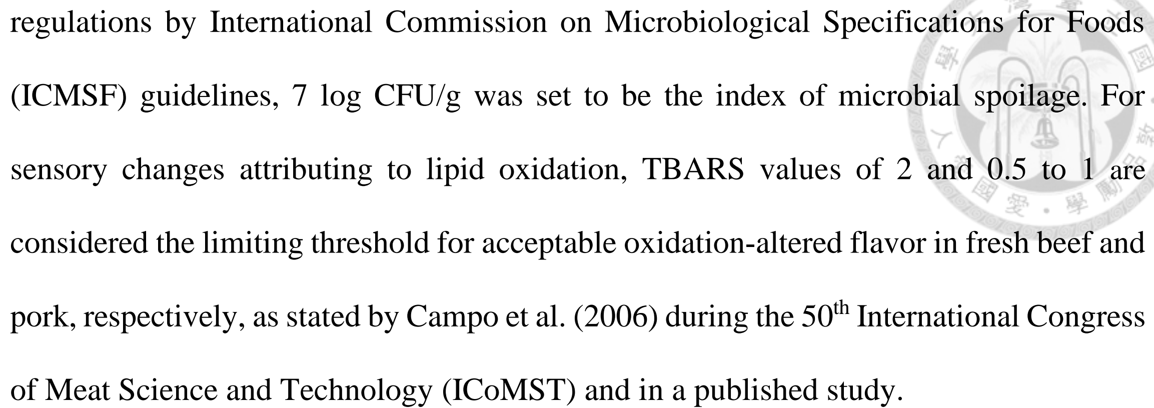

regulations by International Commission on Microbiological Specifications for Foods (ICMSF) guidelines, 7 log CFU/g was set to be the index of microbial spoilage. For sensory changes attributing to lipid oxidation, TBARS values of 2 and 0.5 to 1 are considered the limiting threshold for acceptable oxidation-altered flavor in fresh beef and pork, respectively, as stated by Campo et al. (2006) during the 50th International Congress of Meat Science and Technology (ICoMST) and in a published study.

2.2 Foodborne pathogens associated with fresh meat contamination

Foodborne pathogens are biological agents (e.g., bacteria, viruses, and parasites) that are present in a variety of contaminated foods, causing hazardous health effects and diseases when ingested. Among these, bacterial pathogens have been an ongoing concern in food products worldwide. Based on **Figures 4** and **5**, Taiwan Food and Drug Administration (2021) revealed that the foodborne disease outbreaks related to meat ranked 3rd among all food types from years 1981 to 2021. A total of 3,528 foodborne disease outbreaks were identified as caused by pathogenic bacteria, with *Staphylococcus aureus*, Enteropathogenic *Escherichia coli,* and *Salmonella* spp. having relatively high cases and being pathogens associated with meat. Moreover, the high prevalence rate of these pathogens can also be traced in other countries and regions (Bantawa et al., 2018; Omer et al., 2018; Wu et al., 2018), which will be discussed in the subsections.

2.2.1 *Staphylococcus aureus*

S. aureus is a gram-positive facultative anaerobic bacterium which grows at a temperature between 7 °C to 48 °C. This pathogen is mainly present in the skin, hair, and even nostrils of animals, with an average of 30% to 50% humans being probable carriers of this pathogen (Le Loir et al., 2003). At high concentrations and suitable temperatures, *S.*

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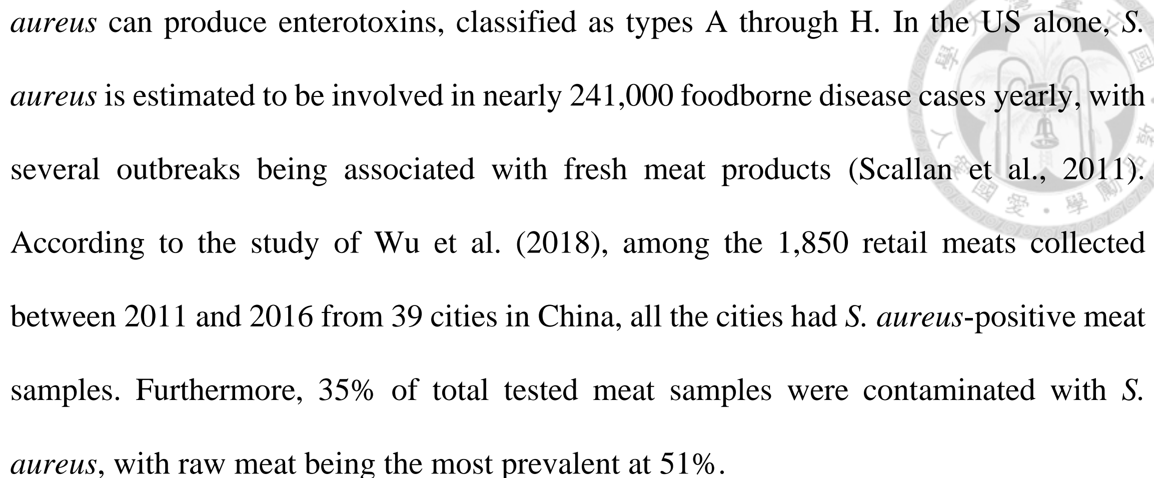

aureus can produce enterotoxins, classified as types A through H. In the US alone, *S. aureus* is estimated to be involved in nearly 241,000 foodborne disease cases yearly, with several outbreaks being associated with fresh meat products (Scallan et al., 2011). According to the study of Wu et al. (2018), among the 1,850 retail meats collected between 2011 and 2016 from 39 cities in China, all the cities had *S. aureus*-positive meat samples. Furthermore, 35% of total tested meat samples were contaminated with *S. aureus*, with raw meat being the most prevalent at 51%.

2.2.2 *Escherichia coli* O157:H7

E. coli is a gram-negative facultative anaerobic bacterium which grows at temperatures between 8 °C to 43 °C. There are six major *E. coli* pathotypes, each containing different virulence factors. Among these pathotypes, Shiga toxin-producing *E. coli* O157:H7 is a continual food safety concern, as it causes hemolytic uremic syndrome with high mortality rate. Infections and outbreaks concerning these pathogens are primarily associated with contaminated cattle products such as undercooked beef (Cody et al., 1999). Based on Scallan et al. (2011), approximately 63,153 foodborne illness cases occur annually in the USA, having a hospitalization rate of 46.2%. A study carried out in Nepal also resulted in over 50% of total tested meat samples (consisting of pork, buffalo, goat, and chicken) being contaminated with *E. coli* spp., including the O157:H7 variant (Bantawa et al., 2018).

2.2.3 *Salmonella* Typhimurium

Salmonella Typhimurium is a gram-negative facultative anaerobic bacterium which grows at a temperature between 6 °C to 46 °C. *Salmonella* spp. has been one of the major causes of gastroenteritis in humans, and data have shown that the majority of

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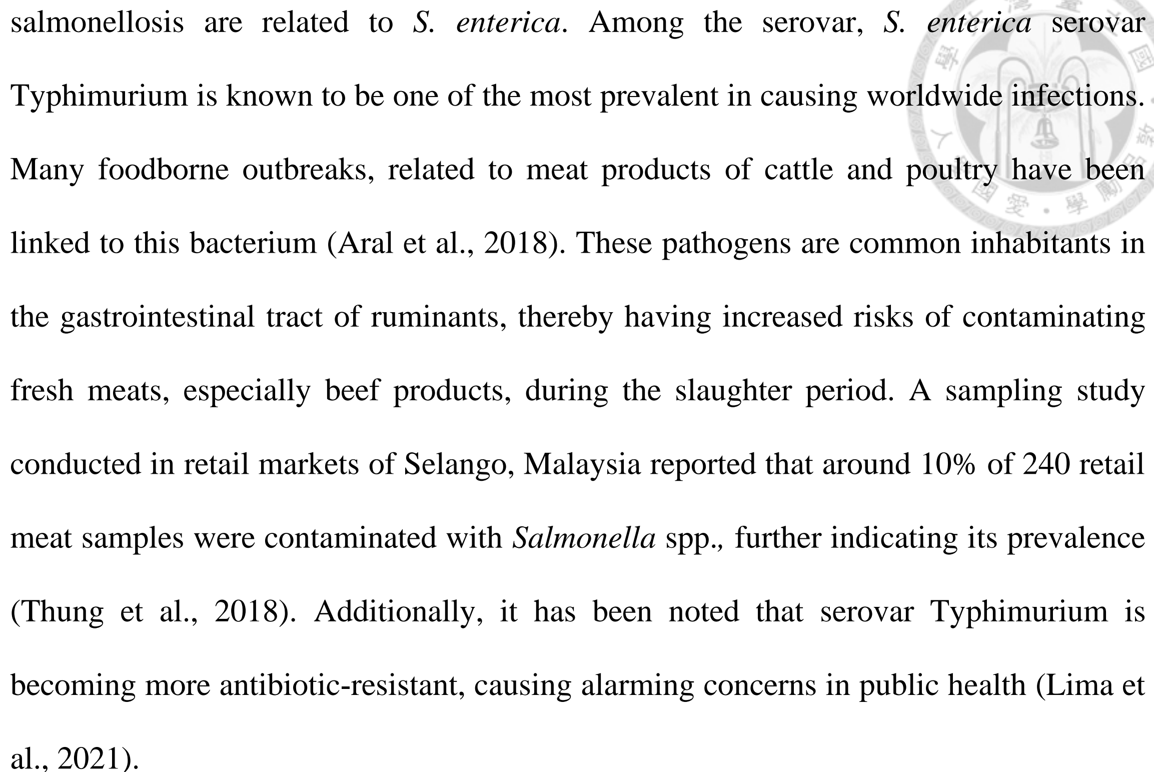

salmonellosis are related to *S. enterica*. Among the serovar, *S. enterica* serovar Typhimurium is known to be one of the most prevalent in causing worldwide infections. Many foodborne outbreaks, related to meat products of cattle and poultry have been linked to this bacterium (Aral et al., 2018). These pathogens are common inhabitants in the gastrointestinal tract of ruminants, thereby having increased risks of contaminating fresh meats, especially beef products, during the slaughter period. A sampling study conducted in retail markets of Selango, Malaysia reported that around 10% of 240 retail meat samples were contaminated with *Salmonella* spp.*,* further indicating its prevalence (Thung et al., 2018). Additionally, it has been noted that serovar Typhimurium is becoming more antibiotic-resistant, causing alarming concerns in public health (Lima et al., 2021).

2.2.4 *Pseudomonas aeruginosa*

P. aeruginosa is a gram-negative aerobic bacterium which grows at a temperature between 4 °C to 42 °C. *P. aeruginosa* is an opportunistic multidrug resistant pathogen, that is generally present in the environment and which can possibly contaminate fresh meats causing serious concerns (Gu et al., 2016). A study by Bantawa et al. (2018) indicated *P. aeruginosa* has a high prevalence rate of 40% in the tested meat samples in Nepal. Additionally, 122 strains of *P. aeruginosa* were detected from 230 bovine meat samples analyzed in Côte d'Ivoire, West Africa, having a prevalence rate at 47.8% for multidrug-resistant types. This matter is concerning as it has been stated that despite therapeutical advancement, the mortality rate of *P. aeruginosa* infection remains high at approximately 30% (Benie et al., 2017).

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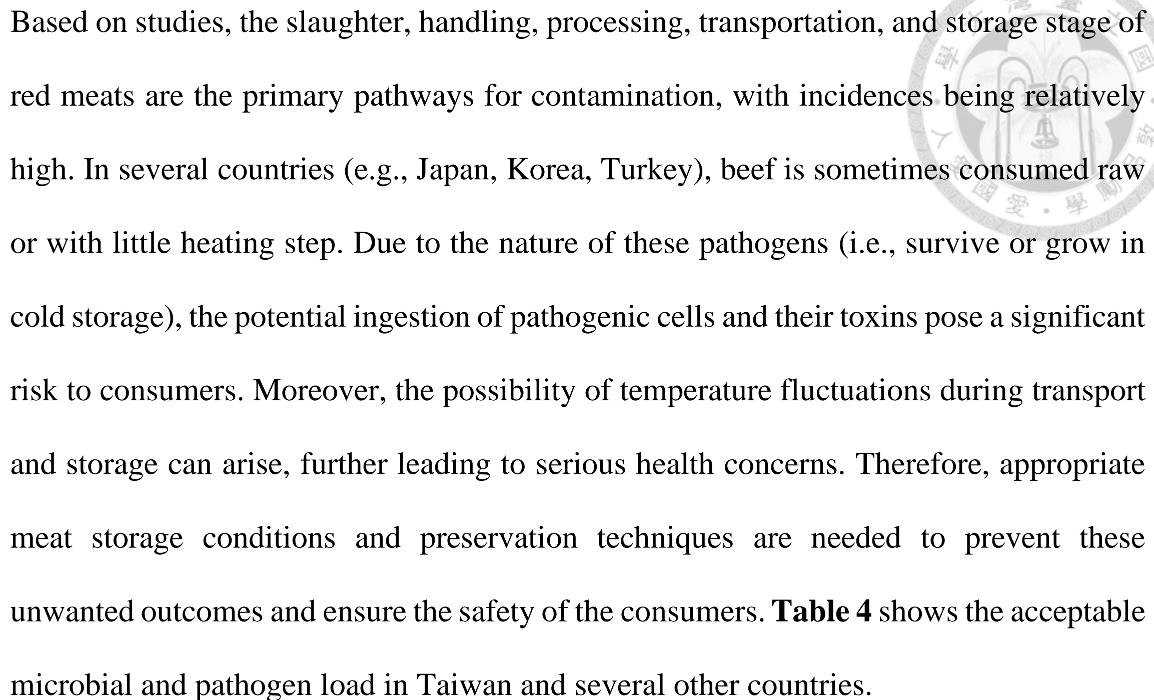

Based on studies, the slaughter, handling, processing, transportation, and storage stage of red meats are the primary pathways for contamination, with incidences being relatively high. In several countries (e.g., Japan, Korea, Turkey), beef is sometimes consumed raw or with little heating step. Due to the nature of these pathogens (i.e., survive or grow in cold storage), the potential ingestion of pathogenic cells and their toxins pose a significant risk to consumers. Moreover, the possibility of temperature fluctuations during transport and storage can arise, further leading to serious health concerns. Therefore, appropriate meat storage conditions and preservation techniques are needed to prevent these unwanted outcomes and ensure the safety of the consumers. **Table 4** shows the acceptable microbial and pathogen load in Taiwan and several other countries.

2.3. Preservation techniques for fresh meats

Due to the continual increase in the human population, there is a large and rising need for meat consumption. Based on Gustavsson et al. (2011), approximately 20% of global meat production, as estimated by the Food and Agriculture Organization of the United Nations (FAO), are wasted yearly due to improper handling or storage. Moreover, recent studies had similar results wherein insufficient preservation methods and technologies resulted in meat loss and waste occurrence during production and storage stages, especially in developing regions (Karwowska et al., 2021). Therefore, achieving effective preservation methods and sanitation practices is necessary to minimize meat spoilage and maximize meat safety.

2.3.1 Cold storage

Cold storage is the common method for preserving unprocessed fresh meats and is mainly divided into conventional chilling (4 °C) and conventional freezing (-18 °C). To ensure

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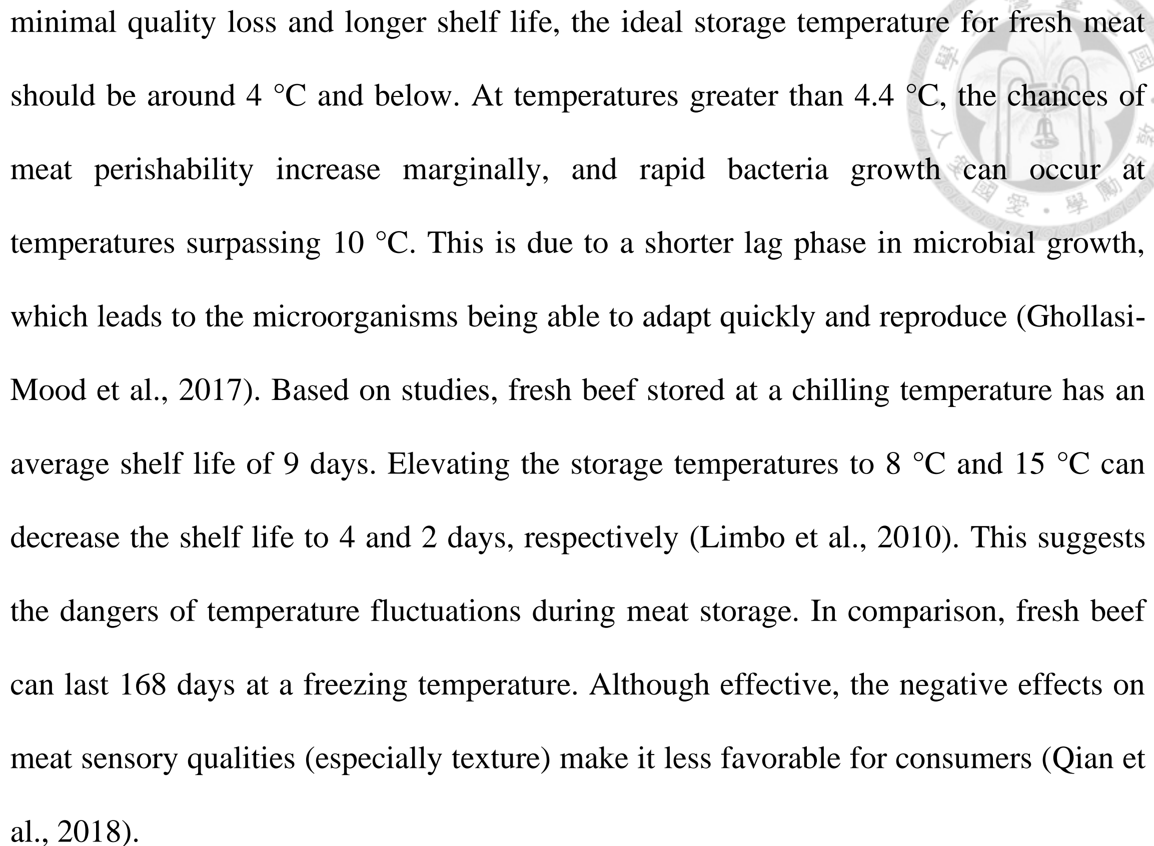

minimal quality loss and longer shelf life, the ideal storage temperature for fresh meat should be around 4 °C and below. At temperatures greater than 4.4 °C, the chances of meat perishability increase marginally, and rapid bacteria growth can occur at temperatures surpassing 10 °C. This is due to a shorter lag phase in microbial growth, which leads to the microorganisms being able to adapt quickly and reproduce (Ghollasi-Mood et al., 2017). Based on studies, fresh beef stored at a chilling temperature has an average shelf life of 9 days. Elevating the storage temperatures to 8 °C and 15 °C can decrease the shelf life to 4 and 2 days, respectively (Limbo et al., 2010). This suggests the dangers of temperature fluctuations during meat storage. In comparison, fresh beef can last 168 days at a freezing temperature. Although effective, the negative effects on meat sensory qualities (especially texture) make it less favorable for consumers (Qian et al., 2018).

2.3.2 Other conventional methods

With the rise in technology, new effective methods have been developed for fresh meat preservation. These include the utilization of irradiation, high-pressure treatment, vacuum packaging, and modified atmospheric packaging. However, the majority of techniques may still possess technological or economic constrains, which limit their widespread application in meat industries (Sampels, 2015). Although synthetic preservatives (e.g., sodium benzoate, potassium sorbate) are cost-effective in preserving fresh meats, recent reports of their toxicity (e.g., carcinogenic, teratogenic) and potential adverse health effects (e.g., gastrointestinal disease, allergic reactions) are alarming and concerning (Ren et al., 2021; Zhang et al., 2020).

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2.4 Edible coatings

To address the aforementioned situation, the use of edible coating appears to be a potential alternative. The edible coating is a thin layer of edible polymer formed as a coating on the food product, which serves as a barrier between the food and the environment. Studies have shown that its application on fresh meats minimizes microbial spoilage, quality loss, oxidation, and discoloration (Kumar et al., 2022). Conventional ways of applying edible coatings in food products are by spraying, spreading, or dipping process. Spraying is done by either using the classical method at a droplet size of 20 μm or by the electro-spraying method at a droplet size of 100 nm. Meanwhile, brushing is the primary method of spreading and applying the coating on the food surface, ensuring full coverage. In fresh meat, dipping is generally used in order to create thicker coatings on the meat surface, giving better physicochemical attributes (Dhumal & Sarkar, 2018).

2.4.1 Classifications

Edible coatings are classified into polysaccharide-based, protein-based, and lipid-based coatings, having different functional properties, each with its own advantages and limitations. Polysaccharides-based coatings (e.g., chitosan, cellulose, starch, pectin, gums) provide selective permeability to gases such as O_2 and CO_2 and have relatively good resistance to fats and oils. Polysaccharides differ in their molecular weight, hydrophobicity, and electrical charge, thus leading to difference in their forming capability and physicochemical properties (Vargas et al., 2008). When polysaccharide coatings are cooked under high heat, they can dissolve and integrate into the meat surface, achieving better texture and reduced moisture loss (Cutter, 2006). Lipid-based edible coatings (e.g., animal oil, vegetable oil, fats, waxes) are excellent moisture barriers due to their hydrophobicity, cohesiveness, and flexibility. The number of carbons they possess

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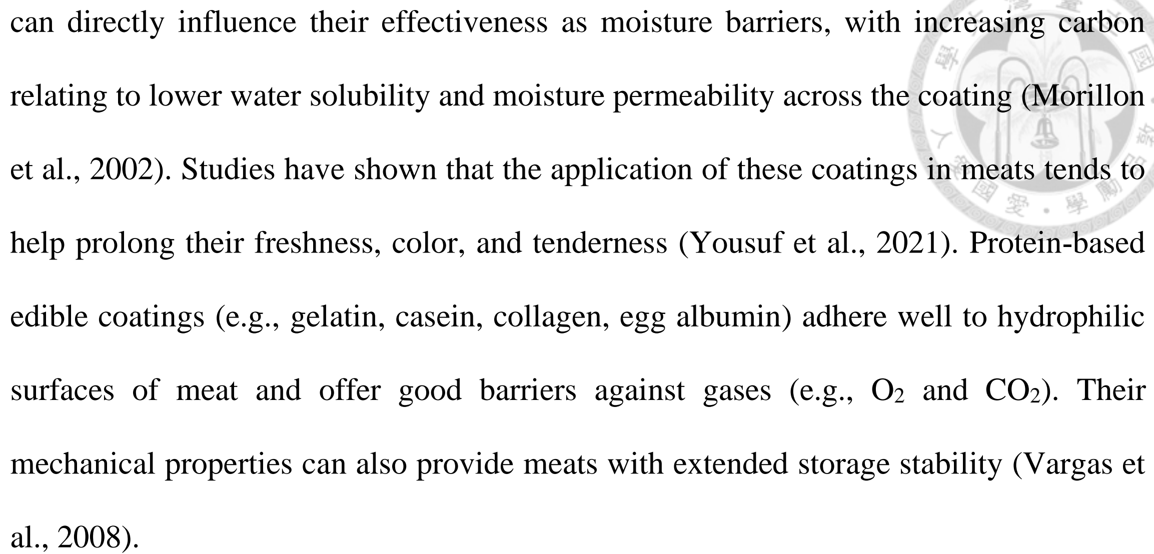

can directly influence their effectiveness as moisture barriers, with increasing carbon relating to lower water solubility and moisture permeability across the coating (Morillon et al., 2002). Studies have shown that the application of these coatings in meats tends to help prolong their freshness, color, and tenderness (Yousuf et al., 2021). Protein-based edible coatings (e.g., gelatin, casein, collagen, egg albumin) adhere well to hydrophilic surfaces of meat and offer good barriers against gases (e.g., O_2 and CO_2). Their mechanical properties can also provide meats with extended storage stability (Vargas et al., 2008).

2.4.2 Composite coatings

Additionally, individual polymer coatings can be combined to form composites, allowing for improved functional and barrier attributes (e.g., moisture, gas, mechanical barrier) and reduced limitations (Yousuf et al., 2021). The development of composite coating can make by the deposition of several coating layers (i.e., multilayered) or a single homogenized coating layer (i.e., monolayered). In general, multilayered coatings can be more effective because of the absence of non-homogenous distribution in lipids. However, monolayered coatings are relatively easier and faster to produce due to only having one drying step. Moreover, the monolayered coating can possess both hydrophilic and lipophilic properties, which can provide more utilization in meat applications (Gallo et al., 2000; Vargas et al., 2008)

2.5 Chitosan-based coatings

Chitosan is a cationic polysaccharide produced from the deacetylation of chitin in alkali. Chitin is one of the most abundant polysaccharides present in nature. It is primarily found in either the exoskeletons of crustaceans or the cell walls of fungi (Alishahi & Aider,

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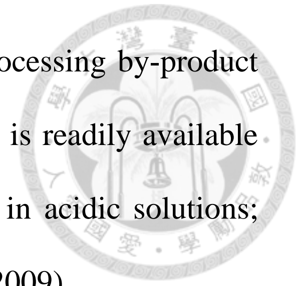

2012). Due to the abundance of crab and shrimp shells as food processing by-product wastes, commercial chitosan is usually made from crustaceans and is readily available (Aranaz et al., 2009). Unlike chitin, chitosan is relatively soluble in acidic solutions; therefore, enabling for its film/coating-forming ability (Pillai et al., 2009).

2.5.1 Toxicity

Based on the United States Food and Drug Administration (US FDA), chitosan is a non-toxic polymer and generally recognized as safe (GRAS). *In vivo* chronic toxicity study of chitosan showed no adverse effects in the organs of mice at daily oral ingestion of 30 mg/kg bw (Mukhopadhyay et al., 2015). Additionally, consumption at a high concentration of 4500 mg for 12 days resulted in negative toxic effects in humans, further indicating its safety (Gades & Stern, 2003).

2.5.2 Antimicrobial properties

Chitosan is reported to show antimicrobial properties against many foodborne pathogens, fungi, and yeasts (Dutta et al., 2009). Several mechanisms of chitosan have been proposed by Raafat and Sahl (2009): (1) interaction of positively charged amino groups of chitosan with the negatively charged cell membrane of bacteria causing the alternation of cell membrane, leakage of intracellular constituents and eventually cell death; (2) action of chitosan as a chelating agent, causing selective binding with metal ions, which disrupts the metabolic enzymes in the microbial cell membranes, ultimately affecting their growth; (3) penetration of chitosan into the cell membrane of bacteria and binding with the DNA, causing interference of mRNA synthesis. Based on collective studies, the antimicrobial activity of chitosan largely relies on the degree of deacetylation (DDA), with higher levels of DDA providing better inhibitory properties (Tsaih & Chen, 2003). The molecular

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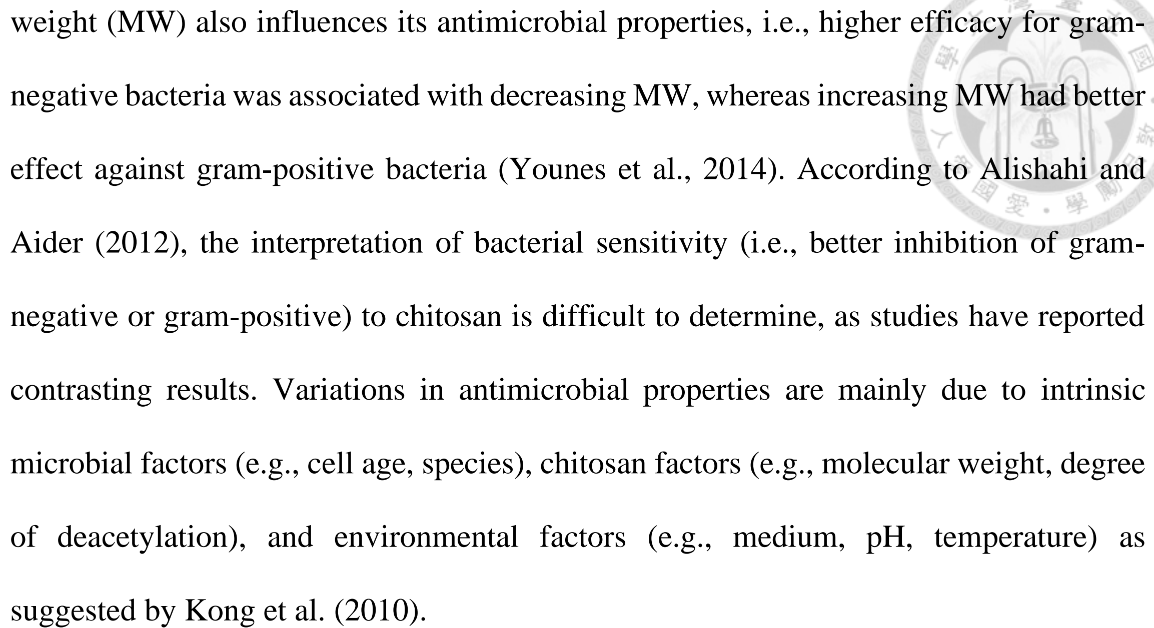

weight (MW) also influences its antimicrobial properties, i.e., higher efficacy for gram-negative bacteria was associated with decreasing MW, whereas increasing MW had better effect against gram-positive bacteria (Younes et al., 2014). According to Alishahi and Aider (2012), the interpretation of bacterial sensitivity (i.e., better inhibition of gram-negative or gram-positive) to chitosan is difficult to determine, as studies have reported contrasting results. Variations in antimicrobial properties are mainly due to intrinsic microbial factors (e.g., cell age, species), chitosan factors (e.g., molecular weight, degree of deacetylation), and environmental factors (e.g., medium, pH, temperature) as suggested by Kong et al. (2010).

2.5.3 Antioxidant properties

Chitosan is also documented to possess excellent antioxidant activities, preserving both the quality and sensory characteristics of beef products (Georgantelis et al., 2007; Kanatt et al., 2008). Chitosan retard lipid oxidation by chelating ferrous ions or by its free radical scavenging ability (Kamil, 2002). Currently, it has been proven that the antioxidant activities of chitosan are largely affected by its DDA and MW (Yuan et al., 2016). Chitosan with higher MW also showed to be more stable in thermal changes, having slower degradation during storage as a result of their less porous molecular structure and lower water-uptake ability (Mucha & Pawlak, 2002).

2.5.4 Combination with other polymers

Although chitosan-based coating has numerous advantages in meat application, its relatively high-water permeability can potentially limit its uses. Therefore, chitosan in combination with other biopolymers is being studied to improve its functionality. Based on several studies, chitosan coatings are commonly combined with protein biopolymers,

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particularly gelatin, due to their high affinity and compatibility.

Gelatin is primarily derived from the hydrolysis of collagen in marine and bovine origin. It is used primarily as an edible coating to lessen both oxygen and moisture migration (Herring et al., 2010). Gelatin coating is effective against oxidation in foods owing to its excellent mechanical and chemical barriers. Recently, chitosan-gelatin coating, a combination of polysaccharide and protein biopolymers, have been applied in various meat products, including seafoods (Farajzadeh et al., 2016; Xiong et al., 2020; Zhang et al., 2020). In addition to enhanced antimicrobial properties, the combination of chitosan and gelatin also results in a polyelectrolyte complex, which forms a compact structure enhancing the moisture barrier, stability, and flexibility of coatings (Benbettaieb et al., 2014; Pereda et al., 2011).

Chitosan-gelatin, based on their combined ratio, yields varying properties. A previous study by Jridi et al. (2014) has indicated that 25:75 chitosan-gelatin is the optimal proportion, giving a good balance of mechanical, barrier properties and antimicrobial effectiveness in comparison to 50:50 and 75:25 ratio. Moreover, the concentrations of chitosan-gelatin are also an important factor in their preservation efficacy. In a comparison study, 18 different chitosan-gelatin-glycerol coatings were tested in preserving fresh beef at 4 °C of storage. Results have determined that gelatin at 3-6% combined with chitosan at 0.5-1% exhibited the best results, having significantly reduced weight loss, lipid oxidation, and discoloration (Cardoso et al., 2016). Similar results were also observed in fresh shrimp preservation at 4 °C, wherein chitosan (1%) and gelatin (3%) were regarded as the optimum concentration for the coating (Farajzadeh et al., 2016).

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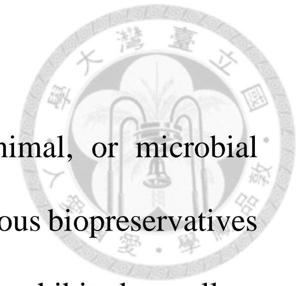

2.6 Biopreservatives for enhancing coating properties

Biopreservatives are natural compounds obtained from plant, animal, or microbial sources that possess antimicrobial or antioxidant characteristics. Various biopreservatives (e.g., essential oil, fatty acids, bacteriocins, plant extracts) have exhibited excellent properties inhibiting the growth of spoilage microorganisms or foodborne pathogens (Ren et al., 2021). There is a current trend of incorporating biopreservative in edible coatings due to their synergy, as coatings allow for the entrapping of biopreservatives, resulting in an active release in meat products (Ortega et al., 2014). **Table 5** shows the common biopreservatives added in chitosan-based coatings for meat preservation.

2.6.1 Essential oils

Essential oils (EOs) are concentrated, aromatic, and volatile liquids acquired from plant parts (e.g., flowers, seeds). In the past years, EOs and their derivatives have been broadly applied in several meat products as antimicrobial or antioxidant additives. Common ones include oregano, carvacrol, thymol, clove, and cinnamon. Their antimicrobial and antioxidant activities are dependent on chemical composition, with phenol-containing ones (i.e., thymol, carvacrol) demonstrating the highest antimicrobial activity (Bassole & Juliani, 2012). EOs generally penetrate through the bacterial membrane, damaging and altering the fatty acid profile, and causing cell destruction (Rao et al., 2019). A recent study showed that 0.4% cinnamon EO addition to chitosan-gelatin composite film significantly reduced *S. aureus* and *E. coli* growth up to 98% (Guo et al., 2019). In meat applications, the addition of 0.25% oregano EO successfully reduced surface microorganisms, which prolonged the product shelf life (Petrou et al., 2012). Moreover, chitosan film containing 0.5% oregano EO and thyme EO was also reported to reduce pathogens significantly: *E. coli*, *S. aureus*, and *Salmonella* levels in fresh ground beef

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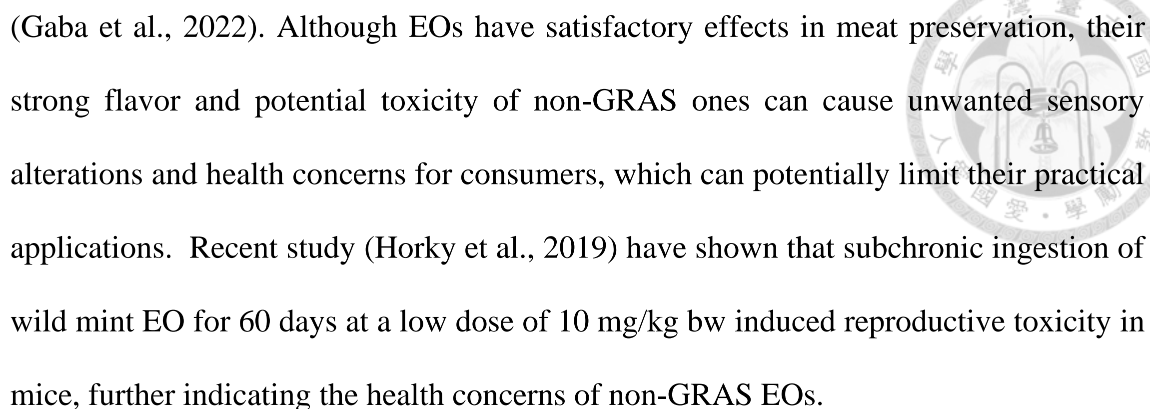

(Gaba et al., 2022). Although EOs have satisfactory effects in meat preservation, their strong flavor and potential toxicity of non-GRAS ones can cause unwanted sensory alterations and health concerns for consumers, which can potentially limit their practical applications. Recent study (Horky et al., 2019) have shown that subchronic ingestion of wild mint EO for 60 days at a low dose of 10 mg/kg bw induced reproductive toxicity in mice, further indicating the health concerns of non-GRAS EOs.

2.6.2 Bacteriocins

Bacteriocins (e.g., nisin, enterocin) are antibacterial peptides produced by probiotic bacteria *Lactococcus lactis* and *Enterococcus faecalis,* respectively. These peptides can effectively suppress the growth of bacteria, especially towards related strains. Amongst the bacteriocins, the majority of the studies in meat preservation are regarding nisin. This is due to nisin possessing good stability under thermal stress conditions. Additionally, they do not cause sensory alterations as additives due to their odorless and tasteless characteristics (Perez et al., 2014). Studies have shown that nisin mainly interferes with the synthesis property of bacterial cell membrane, causing the formation of pores, which leaks internal cellular components leading to cell death. In meat applications, nisin addition in chitosan coatings can effectively inhibit or reduce the growth of spoilage microorganisms and *E. coli* O157:H7, extending the shelf life and safety (Azizian et al., 2019; Cao et al., 2019). However, there have been some instances where antimicrobial activity was not enhanced. Xiong et al. (2020) stated that the incorporation of 0.1% nisin in chitosan-gelatin coating did not yield any improvement in the antimicrobial activity or prolonged shelf life of fresh meat. This may be an indication of nisin not being suitable for incorporation in composite coating matrix as its functional properties may be hindered.

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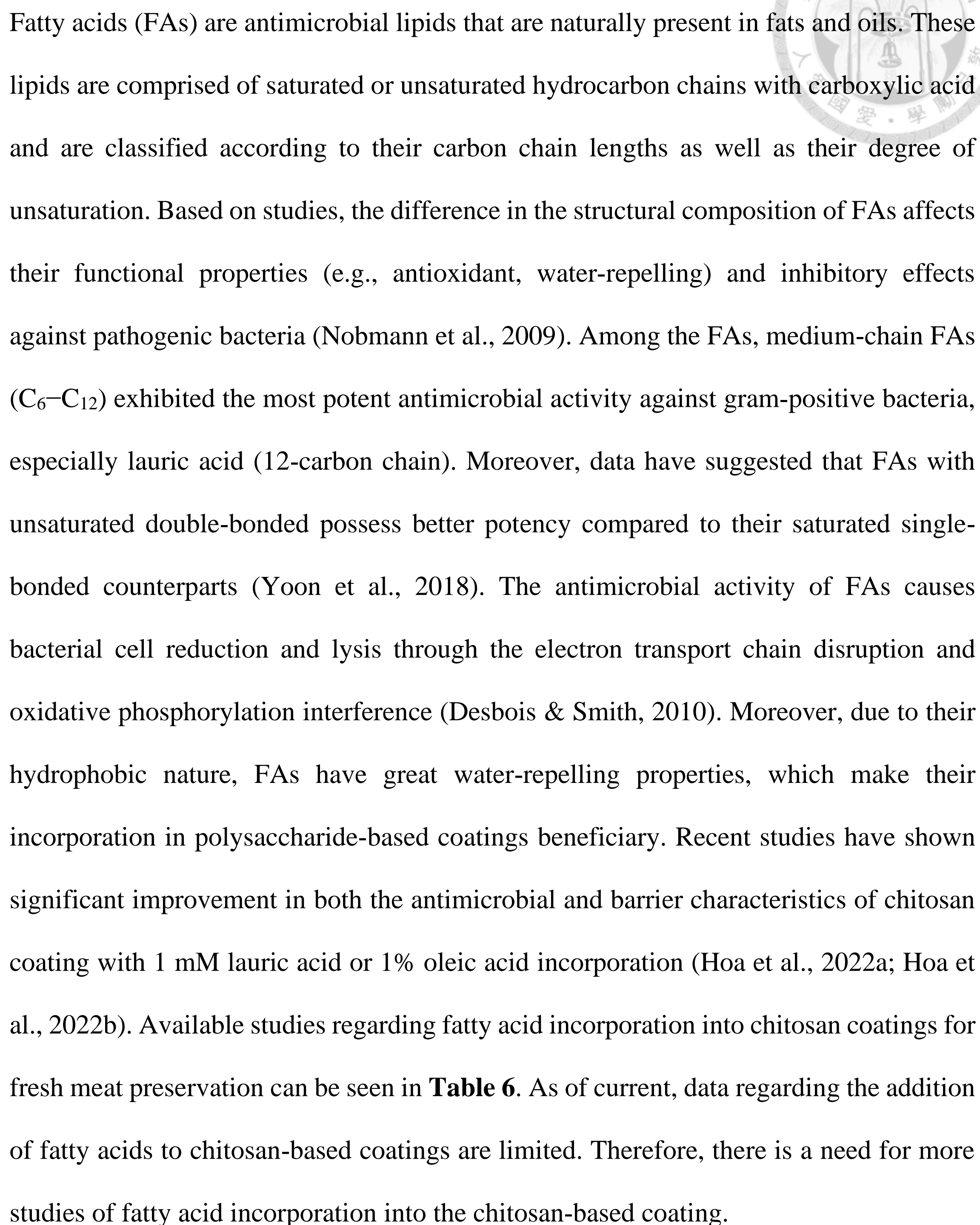

2.6.3 Fatty acids

Fatty acids (FAs) are antimicrobial lipids that are naturally present in fats and oils. These lipids are comprised of saturated or unsaturated hydrocarbon chains with carboxylic acid and are classified according to their carbon chain lengths as well as their degree of unsaturation. Based on studies, the difference in the structural composition of FAs affects their functional properties (e.g., antioxidant, water-repelling) and inhibitory effects against pathogenic bacteria (Nobmann et al., 2009). Among the FAs, medium-chain FAs (C_6–C_{12}) exhibited the most potent antimicrobial activity against gram-positive bacteria, especially lauric acid (12-carbon chain). Moreover, data have suggested that FAs with unsaturated double-bonded possess better potency compared to their saturated single-bonded counterparts (Yoon et al., 2018). The antimicrobial activity of FAs causes bacterial cell reduction and lysis through the electron transport chain disruption and oxidative phosphorylation interference (Desbois & Smith, 2010). Moreover, due to their hydrophobic nature, FAs have great water-repelling properties, which make their incorporation in polysaccharide-based coatings beneficiary. Recent studies have shown significant improvement in both the antimicrobial and barrier characteristics of chitosan coating with 1 mM lauric acid or 1% oleic acid incorporation (Hoa et al., 2022a; Hoa et al., 2022b). Available studies regarding fatty acid incorporation into chitosan coatings for fresh meat preservation can be seen in **Table 6**. As of current, data regarding the addition of fatty acids to chitosan-based coatings are limited. Therefore, there is a need for more studies of fatty acid incorporation into the chitosan-based coating.

2.7 Caprylic acid

Caprylic acid (CA), also known as octanoic acid, is a saturated medium-chain fatty acid (8-carbon chain) that is naturally found in coconut oil and bovine milk (Marina et al.,

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2009). CA has exhibited great antimicrobial activity against a wide spectrum of foodborne pathogens, particularly *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Typhimurium (Boyen et al., 2008; Nair et al., 2005; Nair et al., 2004; Skrivanova et al., 2006). Moreover, as seen in **Table 7,** CA at low concentrations can inhibit the growth of these foodborne pathogens, which makes it a fitting compound to combine with chitosan-based coatings and to examine their potential synergistic antimicrobial activities.

2.7.1 Toxicity

CA has been approved by the US FDA as a GRAS food additive, allowing for its application in ready-to-eat (RTE) and fresh meat products. Based on FDA regulations, the maximum acceptable levels of CA are up to 50 mg/kg bw. Health concerning toxicity levels were determined to be 10080 mg/kg bw based on the oral LD_{50} in rat studies (Jenner et al., 1964). Furthermore, the European Food Safety Authority (EFSA) has reaffirmed the safety of CA, and consumption at doses up to 10% in diet (9000 mg/kg bw/d) is relatively safe, with no expected adverse health effects (Mortensen et al., 2017).

2.7.2 Enhancing biological properties of coatings

CA incorporation in chitosan coating resulted in decreased microbial growth, reduced oxidative rancidity, and prolonged shelf life of fresh shrimps (Wang et al., 2018). **Figure 6** shows the potential mode of action of CA in chitosan-gelatin coating. Moreover, the addition of a CA derivative, sodium octanoate, in gelatin film also effectively lowered both microbial and pathogenic *E. coli* counts in vacuum-packed beef striploin, significantly extending shelf life from 27 to 42 days at 4 °C of storage (Clarke et al., 2017; Reid et al., 2017). A recent study has also reported that CA enhances the mechanical

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properties of basil seed gum films, allowing for better moisture and vapor barrier (Gahruie et al., 2020). Although CA has great antimicrobial and barrier properties, there are concerns regarding its aroma potentially altering the sensory characteristics of meat products. Based on Burnett et al. (2007), CA addition had no significant impact on the organoleptic qualities of RTE and poultry meats, thus supporting its application in fresh meats.

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Chapter 3. Research Motivation

3.1 Research hypothesis

To the best of our knowledge, research on CA coupled with edible coatings is very limited. In addition, coatings with CA have never been applied to red meat products. With CA-incorporated coatings potentially improving the safety and quality of red meat products, there are issues that need to be addressed and explored, as indicated in **Figure 2**. We hypothesized that CA has the potential to enhance the antipathogenic, antibacterial, antioxidant, and barrier effects of chitosan-gelatin composite coatings in fresh beef product preservation. **Figure 2**. also lists the aims and goals developed for this research study. We expect that chitosan-gelatin-CA coating can effectively reduce or eliminate potential pathogenic bacteria present in fresh beef, assuring the safety of consumers. Furthermore, this developed coating is also anticipated to effectively prolong the shelf life of fresh beef by reducing the rate of microbial spoilage, oxidative rancidity, pH change, and color loss.

3.2 Research objectives

The study will be focused on developing the best possible chitosan-gelatin-CA coating ratio, with consideration of the microbial, chemical, and color impacts in fresh meat preservation at 4 °C for 20 days. Moreover, the inhibitory effects on pathogenic growth at 4 °C and 10 °C for 7 days will also be studied. It is expected that the experiment results can provide essential data that was limited or unavailable before, which can have academic contributions in the field of food safety. In practice, this research can be of use in meat slaughterhouses, manufacturing plants, and processing industries. From the view of public health, applications of CA in chitosan gelatin coating in beef products can lead

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to safer and longer shelf life, which lessens spoilage concerns and potential foodborne infection incidence from occurring.

Questions

- Reaffirmation of CA inhibitory effects on foodborne bacterial pathogens **(efficacy)**
- Antipathogenic activities of CA incorporated edible coatings **(data)**
- Interaction between CA and chitosan-gelatin coating properties **(synergy and stability)**
- Effects of CA application on microbial, chemical, and color changes of fresh beef **(quality)**
- Shelf-life prolongation of chitosan-gelatin-CA coating on fresh beef **(duration)**
- Reduction of pathogenic load to safe levels during fresh beef contamination **(consumer safety)**

Hypothesis

- CA are GRAS biopreservatives, which has great potential to enhance antipathogenic, antimicrobial and antioxidant effects of chitosan-gelatin coating
- Incorporation of CA in chitosan-gelatin coating can enhance its barrier properties, thus helps in prolonging meat shelf-life during cold storage

Goals and aims

- Screening of the effective CA concentration against common foodborne bacterial pathogens
- Bactericidal effects of chitosan-gelatin-CA, in relation to the concentration and time needed
- Antipathogenic effects of chitosan-gelatin-CA coating in *in vitro* and meat matrix at 4 °C and 10 °C
- Investigation of beef quality and shelf-life prolongation under chitosan-gelatin-CA coating application
- Evaluating the potential of developed antimicrobial coating in improving safety and quality of fresh beef

Expected Results

- CA application in chitosan-gelatin coating can significantly improve antipathogenic, antimicrobial, antioxidant and barrier properties thus allowing for safer, better quality and longer shelf-life fresh beef
- Potential to commercialize the developed coating to be used in fresh beef preservation and assist meat manufactures, processing plants and retailers in lessening the risks of meat spoilage and potential food poisoning incidence
- The developed coating can help reduce public health concerns in case of accidental consumption of undercooked or contaminated fresh beef products

Figure 2.The significance, goals, and contributions of current research study

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Chapter 4. Study design

Part 1: Growth Medium
Caprylic acid screening
512 mM
to
2 mM
Pathogenic analysis
MBC
MIC
Coating development
CHI
GEL
CHI-GEL
CA incorporation
Log reduction assay
Time-kill curve
Selection of ideal coatings
Stability test

Part 2: Meat Matrix
Meat application
Storage analysis study
Pathogen inoculation
4°C
Non inoculated meat
Inoculated meat
4°C and 10°C
Color
pH
Oxidation
Microbial
Pathogen
S. aureus
E. coli
S. Typhimurium
P. aeruginosa

Figure 3. Experimental design.

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Chapter 5. Materials and methods

5.1 Materials

The purchased reagents were of analytical grade unless specified as food grade (FG).

5.1.1 Reagents

1. Cetrimide agar: Himedia (Maharashtra, India)
2. Chitosan powder, FG: Charming & Beauty (Taipei, Taiwan)
3. Ethanol: Yeong Jyi Chemical Apparatus (New Taipei, Taiwan)
4. Gelatin powder, FG: HydroBio. (New Taipei, Taiwan)
5. Luria-Bertani broth (LB): Bioman Scientific (New Taipei, Taiwan)

Reagents indicated below are purchased from Sigma-Aldrich (St. Louis, MO, U.S.A)

1. Acetic acid (AA)
2. Butylated hydroxytoluene (BHT)
3. Caprylic acid (CA), FG
4. Glycerol
5. Mueller Hinton broth (MHB)
6. Sodium bicarbonate
7. Tetraethoxyproprane (TEP)
8. Thiobarbituric acid (TBA)
9. Trichloroacetic acid (TCA)

Reagents indicated below are purchased from Becton Dickinson (Sparks, MD, U.S.A)

1. Buffered peptone water (BPW)
2. MacConkey agar II with Sorbitol (SMAC)
3. Mannitol salt agar (MSA)
4. Xylose lysine deoxycholate agar (XLD)

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5. Tryptic soy agar (TSA)

5.1.2. Pathogenic bacteria

1. *Escherichia coli* O157:H7, BCRC 15374 (Hsinchu, Taiwan)
2. *Pseudomonas aeruginosa,* ATCC 27853 (Virginia, U.S.A)
3. *Salmonella* Typhimurium, BCRC 12947 (Hsinchu, Taiwan)
4. *Staphylococcus aureus,* ATCC 25923, (Virginia, U.S.A)

5.1.3 General equipment

1. Analytical balance: PA 224, Ohaus (New Jersey, USA)
2. Autoclave: ES-315, Tomy (Tokyo, Japan)
3. Biosafety cabinet: Forma II Type A2, Thermo Scientific (Massachusetts, USA)
4. Centrifuge: Centrifuge 5810R, Eppendorf (Hamburg, Germany)
5. Colorimeter: NH310, 3nh (Shenzhen, China)
6. Drying oven: DK-500DT, Yihder (New Taipei, Taiwan)
7. Electric sterilizer: Dragon 320, Rocker Scientific (Kaohsiung, Taiwan)
8. Electronic balance: D9AD-MK00600, Dogger Scientific (New Taipei, Taiwan)
9. Hot plate stirrer: SP88857100, Themo Scientific (Massachusetts, USA)
10. Low-temperature incubator: LTI-603, TKS (Keelung, Taiwan)
11. Microplate spectrophotometer: Multiskan Go, Thermo Scientific (Massachusetts, USA)
12. pH meter: PH500, Clean L'eau Instruments (Taoyuan, Taiwan)
13. Refrigerator: NR-B659TV, Panasonic (Osaka, Japan)
14. Shaker incubator: LM-570RD, Yihder (New Taipei, Taiwan)
15. Stomacher: BagMixer 400, Interscience (St Nom, France)

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16. Ultralow temperature freezer: CyroCube F740h, Eppendorf (Hamburg, Germany)
17. UV-vis spectrophotometer: UV mini-1240, Shimadzu (Kyoto, Japan)
18. Vortex: SI-0236 (G560), Scientific Industries (New York, USA)
19. Water activity meter: LabTouch aw, Novasina (Lachen, Switzerland)
20. Water bath: B206, Firstek (New Taipei, Taiwan)

5.1.4 Graphical and analytical software

1. BioRender (Toronto, Canada)
2. Excel 2016: Microsoft (Albuquerque, New Mexico, USA)
3. SigmaPlot 14.0: Systat Software, Inc. (Chicago, USA)
4. SPSS 29.0 software: IBM Corp. (New York, USA)

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5.2 Methodology

5.2.1 Preparation of bacteria

Reactivation

Staphylococcus aureus ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* O157:H7, BCRC 15374 and *Salmonella* Typhimurium, BCRC 12947 stored at -20 °C were reactivated and inoculated into Tryptic Soy Agar (TSA), then incubated at 37 °C for 24 h. The forming colonies were then inoculated into a new TSA and cultivated under the same condition. The procedure was repeated until the third generation of bacteria was obtained.

Culture and Wash

For bacteria cultures, one or two colonies from the third-generation bacteria were inoculated into a 50 mL Luria-Bertani (LB) broth. Incubation and shaking conditions for all pathogens were set at 37 °C with 125 rpm shaking for 14 h. For the washing of *S. aureus* cells, the culture medium was centrifuged at 1000xg for 5 mins at 4 °C, and the cells were washed twice with sterile deionized water (DW). For *P. aeruginosa*, *E. coli* O157:H7, *S.* Typhimurium, the above-mentioned procedures were performed with modifications on centrifugation conditions with 3000xg for 5 min at 4 °C, 4000xg for 5 min at 4 °C, and 4000xg for 10 min at 4 °C, respectively.

5.2.2 Pathogenic analysis

Minimum inhibitory concentration test and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) of CA against the pathogenic bacteria was determined using M07-A10 broth microdilution method, as explained by the Clinical and Laboratory Standards Institute (CLSI), with minor modifications. The pathogens in the

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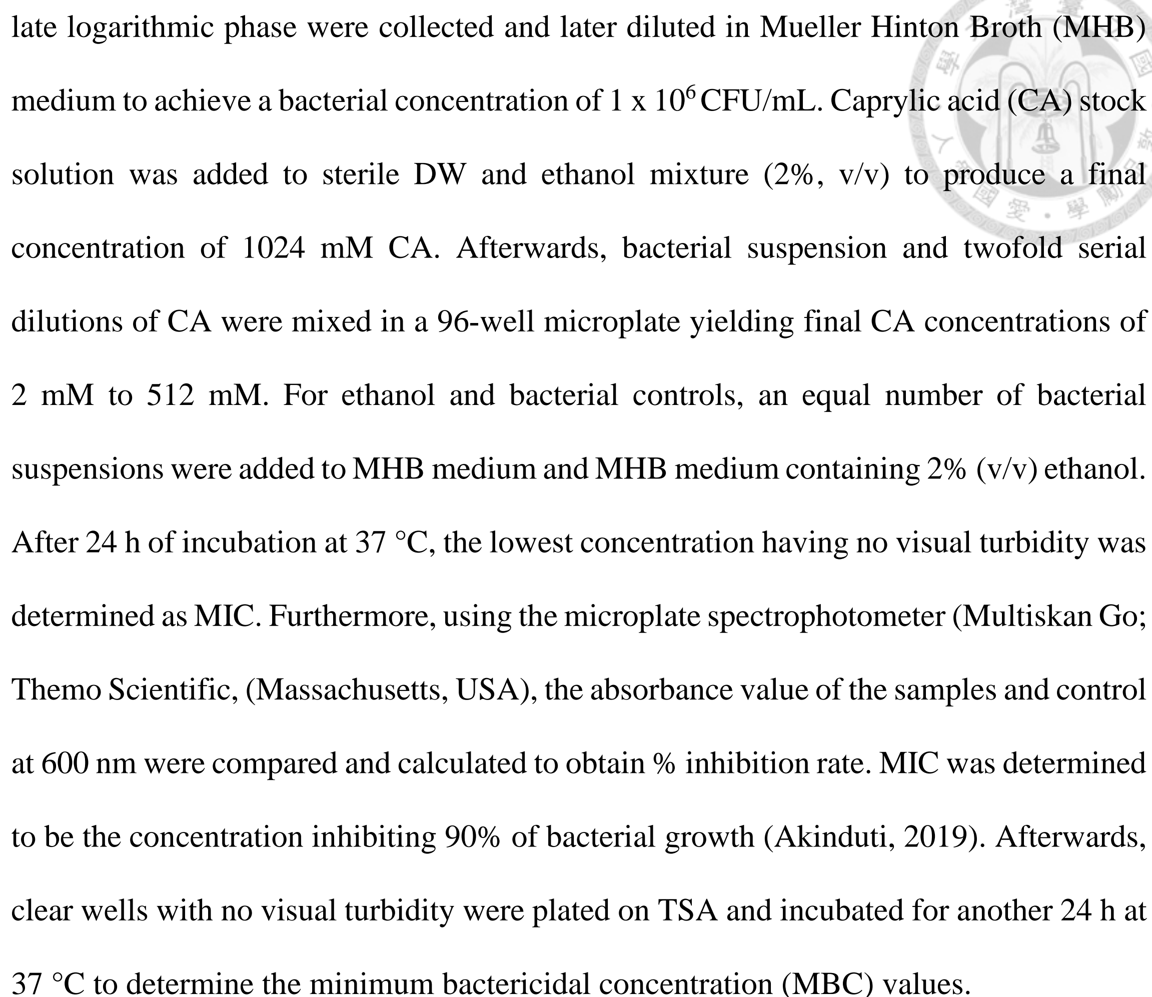

late logarithmic phase were collected and later diluted in Mueller Hinton Broth (MHB) medium to achieve a bacterial concentration of 1 x 10^6 CFU/mL. Caprylic acid (CA) stock solution was added to sterile DW and ethanol mixture (2%, v/v) to produce a final concentration of 1024 mM CA. Afterwards, bacterial suspension and twofold serial dilutions of CA were mixed in a 96-well microplate yielding final CA concentrations of 2 mM to 512 mM. For ethanol and bacterial controls, an equal number of bacterial suspensions were added to MHB medium and MHB medium containing 2% (v/v) ethanol. After 24 h of incubation at 37 °C, the lowest concentration having no visual turbidity was determined as MIC. Furthermore, using the microplate spectrophotometer (Multiskan Go; Themo Scientific, (Massachusetts, USA), the absorbance value of the samples and control at 600 nm were compared and calculated to obtain % inhibition rate. MIC was determined to be the concentration inhibiting 90% of bacterial growth (Akinduti, 2019). Afterwards, clear wells with no visual turbidity were plated on TSA and incubated for another 24 h at 37 °C to determine the minimum bactericidal concentration (MBC) values.

5.2.3 Preparation of antimicrobial coating solutions

The edible coating solutions of chitosan (CHI), gelatin (GEL), and chitosan-gelatin (CHI-GEL) coating were prepared based on Farajzadeh et al. (2016) with minor modifications. For the preparation of 1% (w/v) CHI solution, chitosan powder was dissolved in 1.5% (v/v) acetic acid using a magnetic stirrer at 40 °C, until complete dissolution. The 3% (w/v) GEL solution was obtained by dissolving gelatin powder in distilled water with constant stirring at 35 °C for 15 min. The solution was swelled for 15 min at 7 °C and then heated under 55 °C for complete dissolution. The composite coating of 1% CHI- 3% GEL was prepared by mixing the 2% CHI and 6% GEL solutions for 30 min at 60 °C with constant agitation. Finally, the CHI-GEL-CA coating were obtained by mixing CA

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thoroughly in the cooled CHI-GEL solution, achieving concentrations ranging from 1 mM to 64 mM, which were applied in future tests. The pH of all coating solutions was adjusted to pH 5.5 ± 0.1 using sodium bicarbonate.

5.2.4 Log reduction assay

For the determination of the bactericidal effects of chitosan-gelatin-CA coatings, a time-kill test was conducted to obtain information on the time-dependent or concentration-dependent antibacterial effect. The protocol was based on CLSI M26-A with some modifications. Pathogenic bacteria were suspended in MHB with concentrations of 5 x 10^7 CFU/mL. The tested CA concentrations in chitosan-gelatin coatings were 0.25 x MIC, 0.50 x MIC, and 1 x MIC. A growth control (no coating) was also used to determine the normal bacterial growth. A ratio of 1:1 of MHB bacterial suspension and coating was mixed, achieving a final concentration of 2.5 x 10^7 CFU/mL Incubation for each bacterium was done under 37 °C for varied time intervals of 0, 0.5, 2, 4, 8, 24 h. The percentage of dead bacterial cells were calculated and compared to the growth control for the determination of the number of living cells. The results were expressed as log CFU/mL.

5.2.5 Time-kill curve

The bactericidal activity of different individual antimicrobial treatments (2% ethanol, CA MIC, CA MBC), individual coating solutions (3% GEL, 1% CHI, 1% CHI + 3% GEL) and combined treatments of chitosan coating with incorporated CA (1 x MIC, 1/2 x MIC, 1/4 x MIC, and 1/8 x MIC) were determined using the protocol of Tantala et al. (2021) with some modifications. Pathogenic bacteria were suspended in MHB with concentrations of 5 x 10^7 CFU/mL. A ratio of 1:1 of MHB bacterial suspension and

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treatment was mixed, achieving a final concentration of 2.5 x 10^7 CFU/mL. Samples were taken after 24 h of incubation and plated in TSA. Incubation conditions for all pathogens were 37 °C for 24h. The inhibitory results were expressed as log CFU/mL. Calculations were based on the difference in log initial bacterial population and surviving bacterial population at each time point.

5.2.6 Coating stability assay

The ideal formulated coatings were selected and subjected to stability testing, determining the changes in coating characteristics during long periods of storage. The determination of keeping properties was based on the method of Jagannath et al. (2006), with minor modifications. Approximately 20 mL of coating solution was placed in a centrifuge tube, and the presence of aqueous phase separation was measured and recorded at the start and end of 30-day storage at 25 °C. The separation extent will be graded quantitatively to determine the stability of the coatings. Coating stability was rated as follows: 5 for excellent stability (without separation); 4 for good stability (0.25 mL separation); 3 for slightly stable (1 mL separation); 2 for quite stable (1.5 mL separation); 1 for moderately unstable (2 mL separation); 0 for extremely unstable. Microbial analysis and pH analysis of the coatings were also done on day 0 and day 30 to ascertain their stability. Microbial analysis was conducted by spreading 0.1 mL of coating solution on the TSA plate, following incubation at 37 °C for 24 h. For pH analysis, each coating solution will be directly measured using a digital pH meter (PH500; Clean L'eau Instruments, Taoyuan, Taiwan) calibrated at pH 4 and 7.

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5.2.7 Preparation of beef samples and coating applications

Approximate 4 kg of fresh beef round steak samples were purchased from traditional market OneBeef Wholesale (Taipei, Taiwan) at 24 h post-mortem. With the help of the vendor, the visible fats and skins in the beef samples were removed. The samples were also cut into two different meat sizes (width x length x thickness) based on experiment need: 1) 8 x 5 x 2 cm for non-inoculated meat samples; 2) 2 x 2 x 2 cm for inoculated meat samples using a sanitized measuring ruler. Samples were divided into four treatment groups randomly to ensure equality, with a total of five sampling days. For each treatment and sampling day, three meat pieces (n = 3) were measured.

Apart from the non-coated control group (CON), beef samples will be individually immersed for 30 sec in coating solutions based on **Table 9**. Samples were then taken from the solution using tongs and air dried for 30 min at 4 °C. Storage conditions of fresh beef samples were based on overwrap packaging conditions in retail stores and households. Each fresh beef will be put in 14 x 10 cm plastic tray with an absorbent pad underneath the sample and sealed with oxygen-permeable polyethylene films. All packed samples were stored in a standard fridge at 4 °C. Samples were measured on days 0, 5, 10, 15, and 20 for microbial growth, pH change, color loss, and lipid oxidation.

5.2.8 Screening of bacterial contamination and UV irradiation effects in beef samples from traditional market

Six beef samples were collected from the 2 x 2 x 2 meat batch. Prior to analysis, half of the samples were first subjected to UV irradiation for 30 min (15 min per top and bottom portion). From the collected samples, approximately 5 g of sample was taken and transferred into a stomacher bag containing 45 mL of 0.1% peptone water, which was then homogenized for 1 min using a stomacher (BagMixer 400; Interscience, St Nom,

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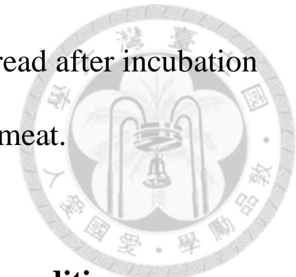
France). Afterward, 0.1 mL of the sample was spread on plates and read after incubation conditions. The results were calculated and expressed as log CFU/g meat.



The bacteria, medium, colony determination, and incubation conditions were as follows:

1. *Escherichia coli*, SMAC, red colonies, 37 °C for 24 h
2. *Escherichia coli* O157, SMAC, colorless colonies, 37 °C for 24 h
3. *Pseudomonas aeruginosa*, Centrimide, yellow green colonies, 37 °C for 24-48 h
4. *Salmonella* Typhimurium, XLD, red with black center colonies, 37 °C for 24 h
5. *Staphylococcus aureus*, MSA, yellow colonies, 37 °C for 48 h
6. *Staphylococcus epidermis*, MSA, red colonies, 37 °C for 48 h
7. Total viable count, TSA, all growing colonies, 37 °C for 24 h

5.2.9 Preparation of inoculated beef samples and coating applications

The preparation of meat samples was from the protocol (in Chapter 5.2.7.). For bacterial inoculation, initial concentrations of 2.5 x 10^8 CFU/mL were obtained and serially diluted with final concentrations of 6 log CFU/g in the beef samples. The inoculation procedure will be based on Stopforth et al. (2006b) with minor modifications, where 500 µL of bacteria cells were inoculated on the top portion of the meat sample and allowed for 15 min attachment at 4 °C. Similar procedure was performed on the bottom portion of the sample. Coating solution immersion and air drying were based on the protocol (in Chapter 5.2.7). Samples were then packed in ziplock bags and stored in a standard fridge at 4 °C and 10 °C storage to mimic temperature conditions at household refrigerators and during potential fluctuations, respectively. Samples were measured on days 0, 1, 4, and 7 for pathogenic bacterial growth.

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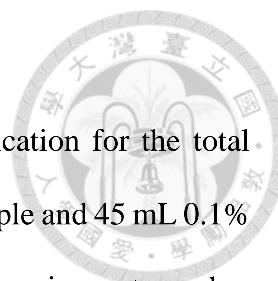

5.2.10 Microbial analysis of beef samples: total viable count

The total viable count (TVC) in beef samples was used as an indication for the total growth of microorganisms during cold storage. About 5 g of beef sample and 45 mL 0.1% peptone water were homogenized in a sterile stomacher bag for 1 min using a stomacher (BagMixer 400; Interscience, St Nom, France). The obtained liquid mixture was diluted in ten-fold serial dilutions using 0.1% peptone water. Afterward, 0.1 mL of the solutions were spread on TSA and incubated at conditions of 37 °C for 48h, with results expressed as log CFU/g meat.

5.2.11 Chemical analysis of beef samples: pH

Ten grams of homogenized sample was mixed with 40 mL of distilled water in a sterile stomacher bag and homogenized for 30 sec using a stomacher (BagMixer 400; Interscience, St Nom, France). Each beef sample will be measured in duplicate reading using a digital pH meter (PH500; Clean L'eau Instruments, Taoyuan, Taiwan) calibrated at pH 4 and 7.

5.2.12 Chemical analysis of beef samples: color

The color of beef sample was determined using a portable colorimeter having a 2° observer and D65 illuminant (NH310; 3nh, Shenzhen, China). The device was calibrated using standard white and black tile prior to testing. Three different locations (upper air-expose surface) of the beef sample was measured for all samples, with the wrapping film not removed. The CIE L*a*b* values, which represents lightness, redness and yellowness were measured. Moreover, the total loss of redness was also calculated and expressed as the percentage loss from initial (day 0) a* values.

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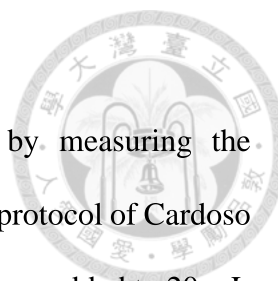

5.2.13 Chemical analysis of beef samples: oxidation

The extent of lipid oxidation in beef sample was determined by measuring the thiobarbituric acid reactive substances (TBARS) values based on the protocol of Cardoso et al. (2019) with some modifications. Approximately 5 g of sample was added to 20 mL of 5% TCA solution and 0.5 mL of 0.15% BHT (in ethanol), then blended for 1 min using vortex (SI-0236 (G560); Scientific Industries, New York, USA). The meat slurry was centrifugated at 3000 xg for 5 min using a centrifuge (Centrifuge 5810R; Eppendorf Hamburg, Germany). The supernatant was recovered and adjusted to 25 mL with 5% TCA. Thereafter, a mixture of 2 mL sample and 2 mL 0.02 M TBA was heated in a water bath for 30 min at 95 °C. After cooling the sample in ice, the absorbance of red pigment formed was measured at 532 nm using a UV-vis spectrophotometer (UV mini-1240; Shimadzu, Kyoto, Japan). The standard curve of TEP was used to calculate the amount of malondialdehyde (MDA) present in the samples. TBARS values were expressed as mg of MDA/kg of beef.

5.2.14 Pathogenic analysis of beef samples

To determine the pathogenic bacterial growth under 4 °C storage, about 5 g of beef sample and 45 mL 0.1% peptone water were blended in sterile test tubes for 1 min using vortex mixer (SI-0236 (G560); Scientific Industries, New York, U.S.A). The obtained liquid mixture was diluted in ten-fold serial dilutions using 0.1% peptone water. Afterward, 0.1 mL solutions were spread in respective agars and incubated at different time periods for bacterial growth enumeration. The incubation conditions were according to the supplier, Becton Dickinson Co. *S. aureus* was spread on the MSA plate and incubated at 37 °C for 48 h. *E. coli* O157:H7 was spread on the SMAC plate and incubated at 37 °C for 24 h. *S.* Typhimurium was spread on the XLD agar plate and incubated at 37 °C for 24 h. *P.*

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aeruginosa was spread on CA plate and incubated at 37 °C for 24-48 h. All the results were calculated and expressed as log CFU/g meat.

5.2.15 Statistical analysis

Statistical analysis was performed using SPSS 29.0 software (IBM Corp., New York, USA). SigmaPlot 14.0 software (Systat Software Inc., Chicago, USA) was used for scientific graphing and data analysis. The main and interaction effects (coating treatments, storage days, temperatures) were assessed as follows. The growth medium experiments were evaluated using the one-way analysis of variance (ANOVA). For the meat matrix experiments, a two-way ANOVA was used for TVC, pH, color parameters, and TBARS tests, while a three-way ANOVA was used for pathogen tests. The statistical significant difference among samples were analyzed by using the Tukey's test at $P < 0.05$. The experiments were conducted in triplicate, and all data were expressed as mean ± standard error.

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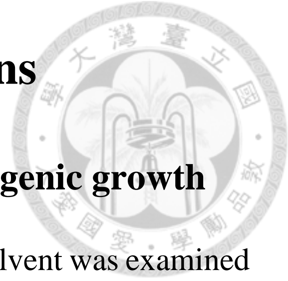

Chapter 6. Results and discussions

6.1 Preliminary testing of ethanol interference on pathogenic growth

Prior to the screening of CA antimicrobial properties, ethanol as its solvent was examined for the inhibitory effect on the growth of *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Pseudomonas aeruginosa*. Cultures were grown in MHB containing ethanol concentrations ranging from 0 to 8% as seen in **Table 10.** After incubation at 37 °C and measuring at 600 nm using a spectrophotometer, the growth rate of each pathogen was calculated. Based on the results, 1% and 2% ethanol concentrations did not exhibit any significant suppressive effect on the growth of all tested pathogens. At 4% ethanol concentration, a significant difference in growth was observed for all pathogens besides *S.* Typhimurium. This can be attributed to variations in their growth characteristic and capability in the presence of ethanol. At 8% ethanol concentration, approximately 5 to 10% reductions in growth were observed in comparison to the growth of control. The results are similar to the observations of Tango et al. (2018), wherein adding ethanol concentrations greater than 3.5% in the growth medium caused significant reductions in the growth rate and maximum population density of *S. aureus*.

In general, ethanol exhibited the highest inhibitory effect on *S. aureus* growth as compared with other tested pathogens. The reason can link to others being gram-negative bacteria, which have more tolerance to membrane disruption due to their impermeable cell membrane. As specific ethanol concentrations can influence the membrane function, synthesis process, or transport systems of bacteria, its interaction as a solvent with antimicrobial compounds can potentially have synergistic effects (Patterson & Ricke, 2015). In order to avoid the interference of added ethanol in CA antimicrobial activities,

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the concentration (2% ethanol) at which no significant inhibitory effects were selected as a solvent for future assays.

6.2 Antipathogenic activity of caprylic acid

The minimum inhibitory concentration (MIC) of CA against the tested pathogens was determined using the visual turbidity and spectrophotometric assay method. Two different endpoint determinations were done to ensure that the obtained MIC results were accurate and precise for future incorporation in the chitosan-gelating coating. The spectrophotometric endpoint was determined to be the concentration at which growth inhibition reached 90%. When comparing the results from visual turbidity in **Figure 7** and spectrophotometric assay in **Figure 8**, the CA MIC values for all pathogenic bacteria were practically the same, indicating a high precision of the experiments.

As shown in both figures, the 2% ethanol control group did not exhibit any independent inhibitory effect on the bacterial growth of all tested pathogens. This further provides evidence that the ethanol as solvent did not contribute to additional antibacterial activities. Therefore, the inhibitory effects can be attributed to the antimicrobial properties of CA alone. Based on the results, the CA MIC against *S. aureus* was the lowest, at 8 mM, as compared to *E. coli* O157:H7, *S. Typhimurium*, *P. aeruginosa,* which were at 16 mM, 64 mM, and 64 mM, respectively. This can be due to the difference in cell membrane characteristics of gram-positive and gram-negative bacteria, which causes CA to have greater inhibitory effects. This is in accordance with existing literature wherein medium-chain FAs typically show greater efficacy against gram-positive bacteria as compared to gram-negative bacteria (Yoon et al., 2018). The obtained MIC results were similar to MIC

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values from previous studies, as seen in **Table 7**. Values were within 10 to 32 mM CA for *S. aureus*, 10 to 20 mM CA for *E. coli* O157:H7, and above 20 mM CA for *S. Typhimurium*.

The minimum bactericidal concentration (MBC) of CA was determined to be the concentration at which there was no occurrence of pathogen growth as seen in **Figure 9**. The CA MBC against *S. aureus* and *E. coli* O157:H7 were both at 32 mM, while *S. Typhimurium* and *P. aeruginosa,* were both at 128 mM, respectively. The bactericidal concentration was approximately two to three times the bacteriostatic concentration for all tested pathogens.

6.3 Antipathogenic activity of coating solution

6.3.1 Log reduction of pathogenic bacteria

The effectiveness of individual treatments: ethanol and CA, together with coating solutions: gelatin (GEL), chitosan (CHI), and chitosan-gelatin (CHI-GEL), as well as combined treatments: chitosan-gelatin with CA at different concentrations were assessed in MHB against several beef related pathogens (*S. aureus* in **Figure 10**, *E. coli* O157:H7 in **Figure 11**, *S. Typhimurium* in **Figure 12**, and *P. aeruginosa* in **Figure 13**) with an initial concentration of 7 log CFU/mL. The number of viable cells in relation to inhibited growth was reported as log reduction in bacterial cell number. Across all tested pathogens, ethanol, and gelatin had similar antibacterial effects ($P > 0.05$), which were both < 1 log in growth reduction, indicating little inhibitory effects. The finding is similar to previous results in chapters 6.1 and 6.2, wherein ethanol at 2% did not induce noticeable effects.

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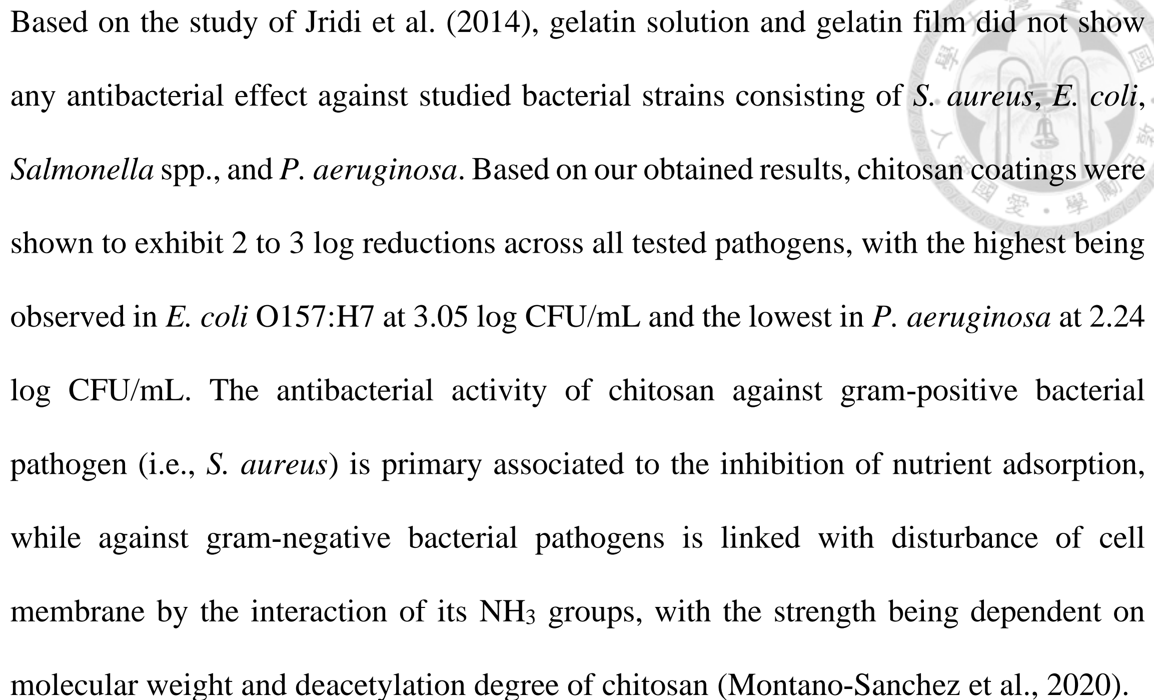

Based on the study of Jridi et al. (2014), gelatin solution and gelatin film did not show any antibacterial effect against studied bacterial strains consisting of *S. aureus*, *E. coli*, *Salmonella* spp., and *P. aeruginosa*. Based on our obtained results, chitosan coatings were shown to exhibit 2 to 3 log reductions across all tested pathogens, with the highest being observed in *E. coli* O157:H7 at 3.05 log CFU/mL and the lowest in *P. aeruginosa* at 2.24 log CFU/mL. The antibacterial activity of chitosan against gram-positive bacterial pathogen (i.e., *S. aureus*) is primary associated to the inhibition of nutrient adsorption, while against gram-negative bacterial pathogens is linked with disturbance of cell membrane by the interaction of its NH_3 groups, with the strength being dependent on molecular weight and deacetylation degree of chitosan (Montano-Sanchez et al., 2020).

The combination of chitosan and gelatin (CHI-GEL) achieved greater than 3 log reductions for all pathogens and had a significant increase in antibacterial effects in comparison to chitosan alone. Combining chitosan and gelatin can induce the formation of a polyelectrolyte complex, allowing for better stability and interaction against bacterial pathogens. However, this was not the case for the *E. coli* pathogen, as there was no improvement with gelatin addition, and a slight decrease in antibacterial activity was observed. A similar result was also seen in Pereda et al. (2011), wherein gelatin addition to chitosan solutions did not improve growth inhibition against *E. coli*, while having a significant increase in growth inhibition against other bacteria.

The addition of CA to CHI-GEL solution showed a significant enhancement in antibacterial properties. Bactericidal effects (9 log CFU/ mL) were observed for all pathogens with the incorporation of CA at 1x and 1/2x MIC. This was a significant leap compared to other CA concentrations. CHI-GEL coatings with CA at 1/2x MIC obtained

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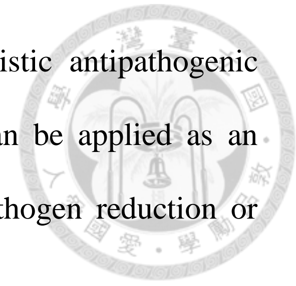
similar effects to CA at MBC concentration, suggesting synergistic antipathogenic activity. In this regard, the formulated CHI-GEL-CA coating can be applied as an effective and promising antibacterial coating with the goal of pathogen reduction or elimination.



6.3.2 Time-kill curve of pathogenic bacteria

For assessing the concentration-dependent and time-dependent antibacterial effects of CA incorporation in CHI-GEL coatings, time-killing experiments were conducted against bacterial pathogens (*S. aureus* in **Figure 14**, *E. coli* O157:H7 in **Figure 15**, *S. Typhimurium* in **Figure 16**, and *P. aeruginosa* in **Figure 17**). From an initial concentration of 7 log CFU/mL, the control group of all tested bacteria reached 9 log CFU/mL after 24 hr of incubation at 37 °C. The concentration-dependent and time-dependent effects of CA were observed in all tested pathogens. Incorporating CA at 1x MIC reduced bacterial growth to below 3 log CFU/mL and below 1 log CFU/mL after 0.5 hr and 2 hr of contact, respectively. Moreover, bactericidal effects were achieved after 4 hr of contact at 1x MIC. As the added CA concentration was reduced in half (based on individual pathogen MIC), the time needed to achieve bactericidal effects was increased to 8 hr for *S. aureus* and 24 hr for *P. aeruginosa*. As the CA concentration was at 1/4x MIC, complete elimination of the pathogens was not achieved even after 24 hr of contact, having a pathogenic count of less than 5 log CFU/mL.

In conclusion, to achieve bactericidal effects on pathogens after 24 hr of contact, the needed CA concentrations in CHI-GEL coatings were 4 mM for *S. aureus*, 8 mM for *E. coli* O157:H7 and 32 mM for both *S.* Typhimurium and *P. aeruginosa*. The differences in needed concentration may be attributed to bacterial cell structure, as the gram-positive

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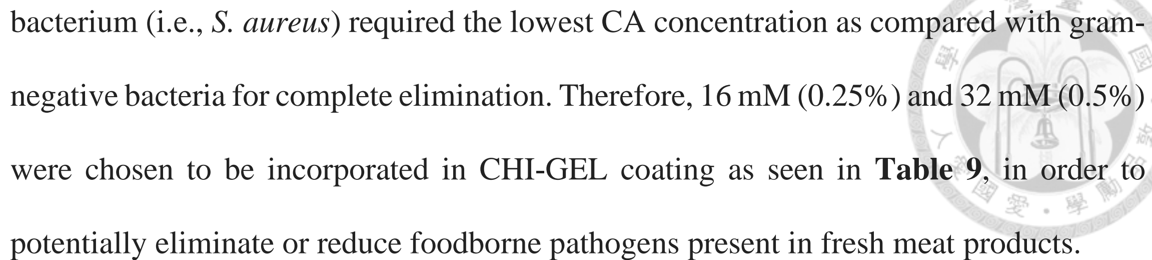

bacterium (i.e., *S. aureus*) required the lowest CA concentration as compared with gram-negative bacteria for complete elimination. Therefore, 16 mM (0.25%) and 32 mM (0.5%) were chosen to be incorporated in CHI-GEL coating as seen in **Table 9**, in order to potentially eliminate or reduce foodborne pathogens present in fresh meat products.

6.4 Characteristic evaluation of coating solutions

The stability of CHI-GEL, CHI-GEL-CA 16 mM (0.25%), and CHI-GEL-CA 32 mM (0.50%) were measured to determine changes in their properties after 30 days of storage, as seen in **Table 11**. For storage stability, all coatings did not exhibit any visible separation at 25 °C. However, when the storage temperature was increased to 35 °C, CA containing 0.5% had 0.30 mL separation, which can still be categorized as having good stability. In terms of microbial presence, no detected growth was observed for all tested coatings. This indicates that aerobic microorganisms cannot grow or survive in the coating matrix, even when coatings are aerobically exposed to air. Moreover, this can also suggest that their antimicrobial activities have not degraded even after a long duration of storage. For pH changes, CHI-GEL coating remained constant for the entire storage duration. CA-incorporated coatings have a slight increase in their pH value. The results provide evidence that the formulated coatings can remain stable at the storage of 25 °C and 35 °C.

6.5 Initial bacterial load in beef samples and the effects of UV irradiation

The bacterial contamination in beef samples can be seen in **Table 12**. Total viable count (TVC) can be used to estimate the total number of microorganisms present, including molds and yeasts, and is used as an important index in determining the safety and quality

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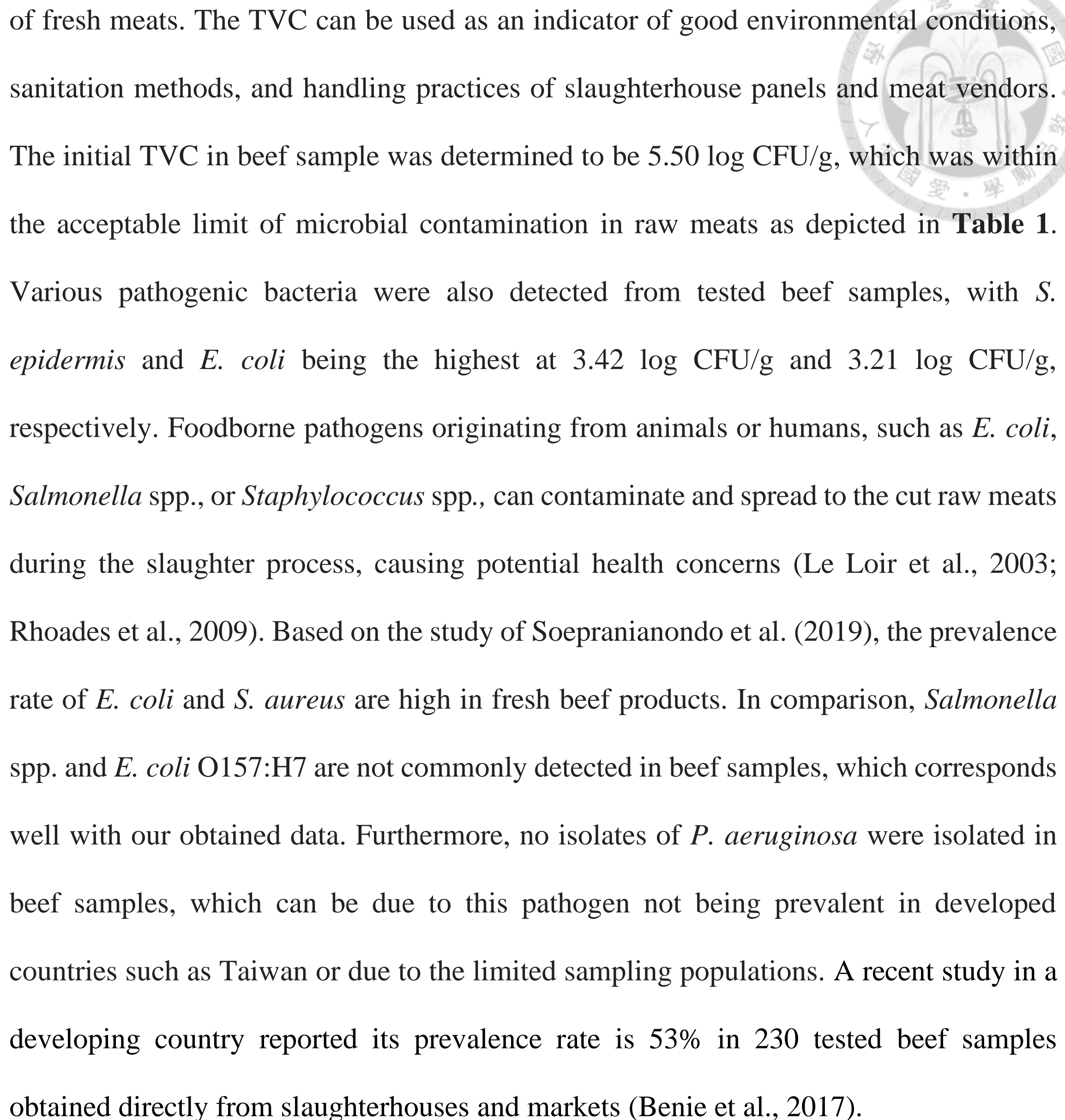

of fresh meats. The TVC can be used as an indicator of good environmental conditions, sanitation methods, and handling practices of slaughterhouse panels and meat vendors. The initial TVC in beef sample was determined to be 5.50 log CFU/g, which was within the acceptable limit of microbial contamination in raw meats as depicted in **Table 1**. Various pathogenic bacteria were also detected from tested beef samples, with *S. epidermis* and *E. coli* being the highest at 3.42 log CFU/g and 3.21 log CFU/g, respectively. Foodborne pathogens originating from animals or humans, such as *E. coli*, *Salmonella* spp., or *Staphylococcus* spp., can contaminate and spread to the cut raw meats during the slaughter process, causing potential health concerns (Le Loir et al., 2003; Rhoades et al., 2009). Based on the study of Soepranianondo et al. (2019), the prevalence rate of *E. coli* and *S. aureus* are high in fresh beef products. In comparison, *Salmonella* spp. and *E. coli* O157:H7 are not commonly detected in beef samples, which corresponds well with our obtained data. Furthermore, no isolates of *P. aeruginosa* were isolated in beef samples, which can be due to this pathogen not being prevalent in developed countries such as Taiwan or due to the limited sampling populations. A recent study in a developing country reported its prevalence rate is 53% in 230 tested beef samples obtained directly from slaughterhouses and markets (Benie et al., 2017).

Due to initial counts of *S. aureus* and potential *E. coli* O157:H7 in purchased beef samples being relatively high at approximately 2 log CFU/g, UV irradiation was applied in the samples to lower the bacterial loads. As seen in **Table 12**, the UV treatment effectively reduced the initial bacterial load of all pathogens, with the elimination of the four major pathogens to be inoculated in beef samples. The reduction and elimination are essential to avoid the potential interference of background bacteria when conducting the storage studies.

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6.6 Effect of chitosan-gelatin-caprylic acid coating in beef preservation

6.6.1 Antimicrobial activity

The effects of coatings with chitosan-gelatin (CHI-GEL) and CA-incorporated chitosan-gelatin on the TVC in fresh beef are shown in **Figure 18**. At day 0, fresh beef had similar initial TVC values at 4.29-4.65 log CFU/g for the non-coated (CON) and coated samples. The TVC values of all beef samples increased during 20 days of storage, among which, CON increased the fastest and was significantly higher than other samples starting at day 5. The rise in TVC may be due to the growth of *Pseudomonas* spp. or lactic acid bacteria (LAB), which are common psychrotrophic bacteria associated with aerobically chilled beef (Pennacchia et al., 2011). Based on ICMSF guidelines, a TVC of 7 log CFU/g is the critical limit in fresh beef, with a higher value indicating spoilage. At day 10, the growth in CON (7.07 log CFU/g) surpassed the limit, which coincides with the reported shelf life of aerobically stored chilled beef. Upon reaching day 15, visible white whiskers in beef surfaces were observed in CON (8.04 log CFU/g). At day 20, the presence of surface slimes was seen in CON (8.98 CFU/g). Based on studies, as the number of microorganisms reaches 8 to 9 log CFU/g, slimes will appear on meat surfaces, with the possible cause being the growth of *Pseudomonas* spp. (Wickramasinghe et al., 2019). Visual observations of beef products can be seen in **Figure 20**.

The results showed that the TVC of CON continuously increased with increased storage time ($P < 0.05$). In comparison, CHI-GEL and CHI-GEL-CA coated samples only increased ($P < 0.05$) after day 5 and day 10 of storage, respectively. There is a significant interaction between coating treatment and storage. After 20 days of storage, the microbial growth in CON was significantly higher, with almost twice and thrice the amount compared to CHI-GEL and CA-incorporated coatings, respectively. Based on the results,

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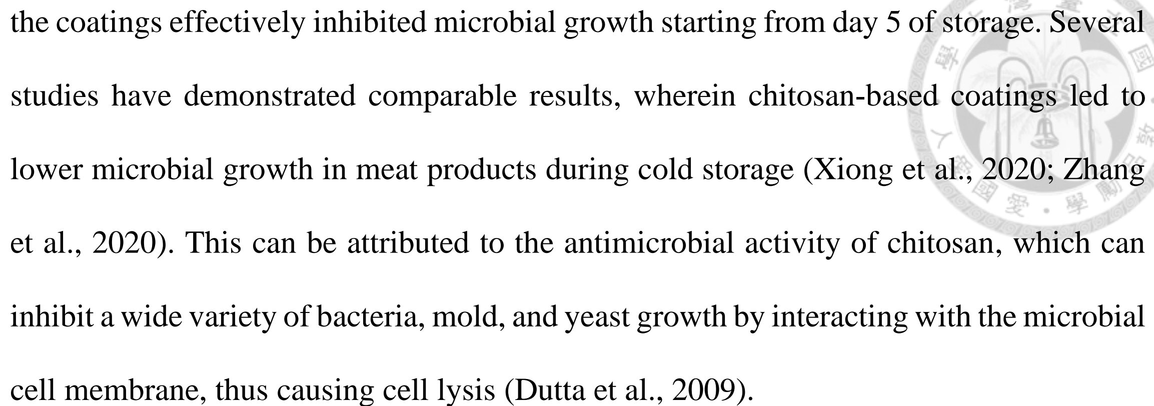

the coatings effectively inhibited microbial growth starting from day 5 of storage. Several studies have demonstrated comparable results, wherein chitosan-based coatings led to lower microbial growth in meat products during cold storage (Xiong et al., 2020; Zhang et al., 2020). This can be attributed to the antimicrobial activity of chitosan, which can inhibit a wide variety of bacteria, mold, and yeast growth by interacting with the microbial cell membrane, thus causing cell lysis (Dutta et al., 2009).

When comparing the coating samples, after 20 days of storage, only CHI-GEL coatings containing CA at 0.5% (5.52 log CFU/g) and at 0.25% (5.69 log CFU/g) concentrations were within the acceptable TVC limit. Both coatings were also significantly lower compared to CHI-GEL coating (7.24 log CFU/g). The result is expected as CA is known to exhibit antimicrobial activity, especially against gram-positive bacteria such as meat spoiling LAB (Yoon et al., 2018). Additionally, this can mean synergistic potentials between CHI-GEL and CA. Similar conclusion was also reached in the study of Hoa et al. (2022a), where a synergistic effect between lauric acid (LA) and chitosan coating was observed. This can suggest the suitability of medium-chain fatty acid (e.g., CA and LA) incorporation in chitosan-based coatings. Furthermore, it can also be concluded that incorporating CA at 0.25% and 0.5% to CHI-GEL coatings did not have significantly varying antimicrobial effects. This indicates that chitosan-gelatin coating with 0.25% CA is effective for keeping aerobically packed fresh beef stored at 4 °C for 20 days under safe microbial levels.

6.6.2 pH change

The pH value is an essential index in determining meat quality, especially concerning its water-holding capacity, color, tenderness, and shelf life (Jankowiak et al., 2021). The pH

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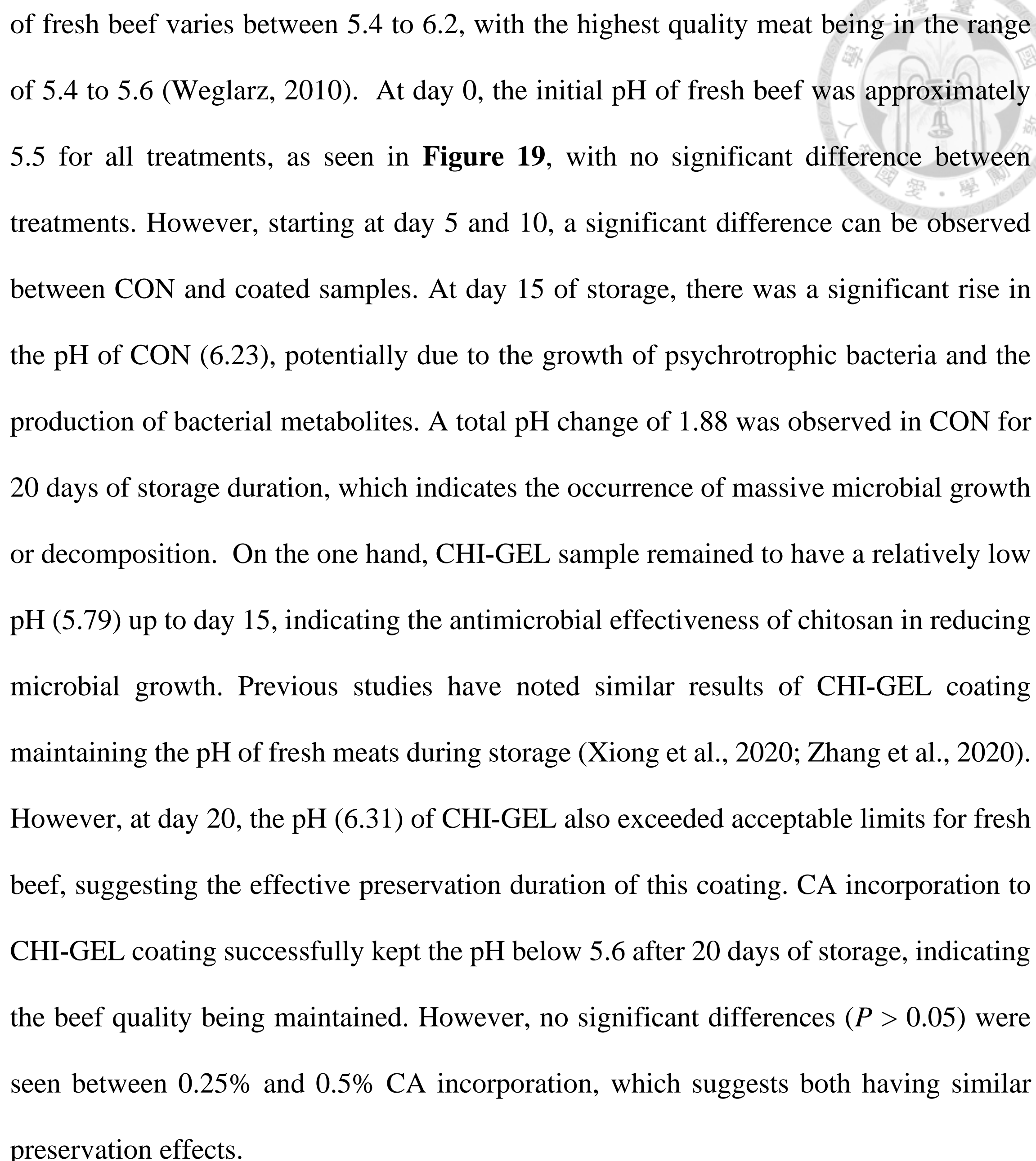

of fresh beef varies between 5.4 to 6.2, with the highest quality meat being in the range of 5.4 to 5.6 (Weglarz, 2010). At day 0, the initial pH of fresh beef was approximately 5.5 for all treatments, as seen in **Figure 19**, with no significant difference between treatments. However, starting at day 5 and 10, a significant difference can be observed between CON and coated samples. At day 15 of storage, there was a significant rise in the pH of CON (6.23), potentially due to the growth of psychrotrophic bacteria and the production of bacterial metabolites. A total pH change of 1.88 was observed in CON for 20 days of storage duration, which indicates the occurrence of massive microbial growth or decomposition. On the one hand, CHI-GEL sample remained to have a relatively low pH (5.79) up to day 15, indicating the antimicrobial effectiveness of chitosan in reducing microbial growth. Previous studies have noted similar results of CHI-GEL coating maintaining the pH of fresh meats during storage (Xiong et al., 2020; Zhang et al., 2020). However, at day 20, the pH (6.31) of CHI-GEL also exceeded acceptable limits for fresh beef, suggesting the effective preservation duration of this coating. CA incorporation to CHI-GEL coating successfully kept the pH below 5.6 after 20 days of storage, indicating the beef quality being maintained. However, no significant differences ($P > 0.05$) were seen between 0.25% and 0.5% CA incorporation, which suggests both having similar preservation effects.

6.6.3 Color loss

Meat color is an important quality characteristic and a key determinant of consumer acceptability. It can also reflect changes during storage in relation to oxidation (Bekhit, 2019). Effects of coating application in color traits: L*(lightness), a* (redness), and b* (yellowness) of fresh beef are listed in **Table 13**. In terms of L*, no significant difference was observed in all beef samples for the first 10 days of storage. At day 20, the significant

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difference was very noticeable, as CON became darker in color (36.60), in contrast to the lighter appearance of coated samples, as seen visually in **Figure 20**. Amongst the coated samples, CHI-GEL coating had the highest L* value (46.01), indicating a paler appearance of fresh beef compared to CA-incorporated ones; however, no significant difference was observed statistically.

According to AMSA (2012), a* is an indicator of meat freshness, as fresher meat generally has high redness value. At day 0, all fresh beef samples have similar initial a* values. However, starting at day 5, a significant difference was observed between CON and coated samples, with chitosan-gelatin also having significantly more redness loss in comparison to CA-incorporated coatings. This trend continued until day 20, where CON had a total redness loss of 59.85%. Hoa et al. (2022a) stated that the coating structure might lessen oxygen permeability to fresh beef, which delays the rate of oxymyoglobin oxidation to metmyoglobin, thus having better redness retention. Moreover, chitosan has always been known for having good antioxidant activities, which also helps in slowing down possible meat discolorations caused by lipid oxidation. Incorporating 0.25% or 0.5% CA into CHI-GEL coating helped in reducing discoloration from occurring, which is possibly due to the additional antioxidant properties of CA (Sengupta et al., 2014). Based on results of a* and % loss of redness, CA incorporated coatings significantly delayed color changes in the initial 5 days of storage as compared to other treatments.

According to Cardoso et al. (2016), b* values are usually neglected in studies of fresh meat color due to the absence of significant difference among the samples during storage. However, a significant difference was observed on day 10 and day 20, wherein CON and CHI-GEL became more bluish as compared to little changes in CA-incorporated CHI-

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GEL coatings, which may be attributed to CA antioxidant effects. Amongst the color parameters, only in b* values were there no significant interactions between coating treatments and storage time.

6.6.4 Antioxidant activity

TBARS is a standard marker for evaluating lipid oxidation in meat, with higher oxidation levels showing a higher TBARS value. The effects of coating application in reducing lipid oxidation during storage are shown in **Figure 21.** The initial TBARs of all beef samples were below 0.30 mg MDA/kg, indicating meat used was relatively fresh. Starting from day 5, a significant difference in TBAR production rate was observed between CON and coated samples. During the 20 days of storage, CON showed the fastest and highest increase among the samples, achieving 2.54 mg MDA/kg beef on the final day. The rapid increase can be linked to the interaction of oxygen with the meat surface due to being aerobically packaged. Based on the studies of Campo et al. (2006) and Suman et al. (2010), TBARS values of 2 mg MDA/kg beef are considered rancid and the limiting threshold for acceptable oxidized beef. Therefore, uncoated beef can only last for 15 days, while coated beef is sensory acceptable even after 20 days.

In comparison to the CON, all coated meat samples had a significantly slower oxidation rate. This is an indication that the formulated coatings can effectively act as barriers and inhibit certain amounts of oxygen exposure to the aerobically packaged meat samples. Multiple studies have suggested chitosan able to delay lipid oxidation due to its metal ion chelation property and reaction with generated volatile aldehydes (i.e., MDA) from degraded fats (Alishahi & Aider, 2012). Moreover, gelatin addition in chitosan coating also helps in lowering the access of oxygen permeability through the coating, reducing

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oxidation reactions from occurring (Cardoso et al., 2016). The application of chitosan-gelatin coatings has also been studied in the preservation of fresh pork (Zhang et al., 2020) and fresh shrimp (Farajzadeh et al., 2016), having evident and satisfactory antioxidant activity results.

The addition of CA in CHI-GEL coating enhanced antioxidant properties significantly, controlling TBARS below 1 mg MDA/kg until day 15. The delayed and retarded lipid oxidation can be associated with the improved metal chelation activity and radical scavenging activity of CA. Sengupta et al. (2014) reported that medium-chain fatty acids such as CA, capric and lauric possess excellent antioxidant properties, with CA having the best effect in reducing oxidation. Similarly, Wang et al. (2018) also reported that CA incorporation improved the antioxidant activities of chitosan-based coating, extending the shelf life of fresh shrimp. Based on obtained results at day 20, no significant difference in antioxidant activities was observed between the added CA concentrations of 0.25% and 0.5%. However, based on the statistical difference in storage days, 0.5% CA significantly delayed the oxidation rate until day 10 as compared to day 5 with 0.25% CA. This suggests that a 0.5% CA addition in CHI-GEL coating can be the optimal formula.

6.7 Inhibition activity of chitosan-gelatin-caprylic acid coating on the pathogen growth in beef

Based on the results of the time-kill curve assays, the coating treatments: CHI-GEL, CHI-GEL-CA 0.25%, and CHI-GEL-CA 0.5%, were assessed for their long-term antimicrobial action against common beef pathogens: *S. aureus, E. coli* O157:H7, *S.* Typhimurium and *P. aeruginosa,* in the meat matrix under refrigerated (4 °C) and abusive (10 °C) temperatures. For CON, the inoculated bacterial populations (approximately 6

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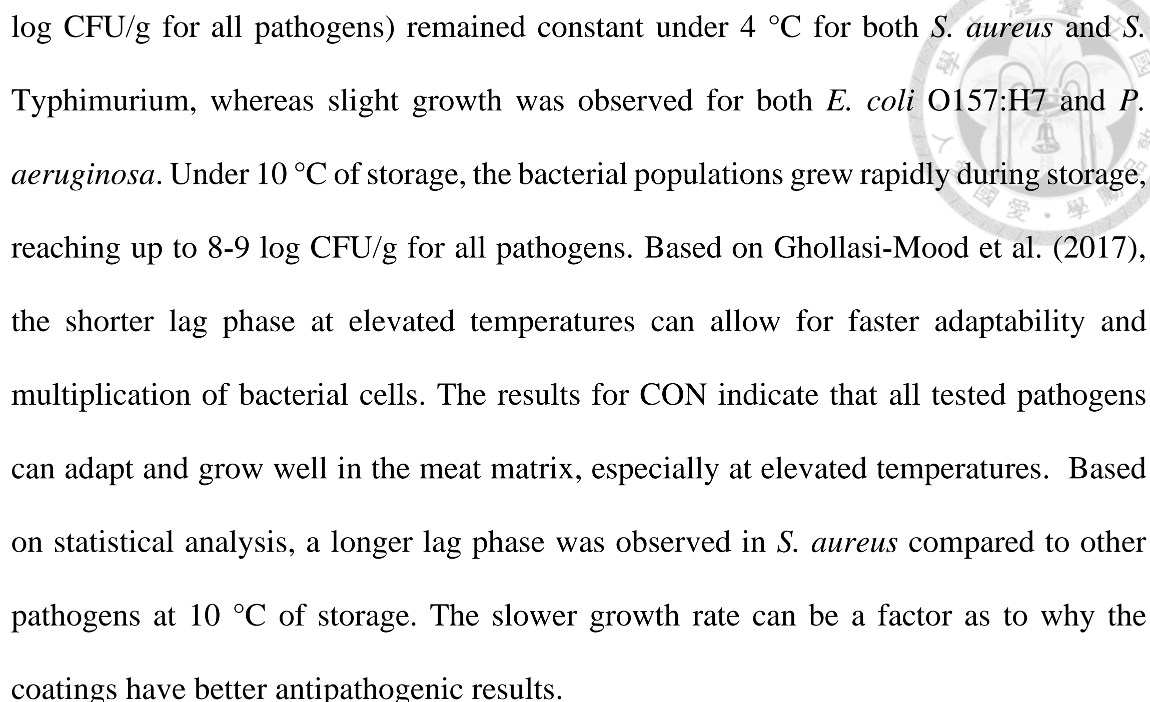

log CFU/g for all pathogens) remained constant under 4 °C for both *S. aureus* and *S.* Typhimurium, whereas slight growth was observed for both *E. coli* O157:H7 and *P. aeruginosa*. Under 10 °C of storage, the bacterial populations grew rapidly during storage, reaching up to 8-9 log CFU/g for all pathogens. Based on Ghollasi-Mood et al. (2017), the shorter lag phase at elevated temperatures can allow for faster adaptability and multiplication of bacterial cells. The results for CON indicate that all tested pathogens can adapt and grow well in the meat matrix, especially at elevated temperatures. Based on statistical analysis, a longer lag phase was observed in *S. aureus* compared to other pathogens at 10 °C of storage. The slower growth rate can be a factor as to why the coatings have better antipathogenic results.

At day 0 (0.5 hr contact time), it can be observed that coating treatments demonstrated a decrease of approximately 1-2 log CFU/g for all tested pathogens. This initial reduction can be attributed to the attached pathogens being washed away during coating application and/or the interactions of antimicrobial components with the pathogens. In a study by Morsy et al. (2014), between day 0 to day 1, the application of edible film treatments in inoculated meat (6 log CFU/cm^2) induced a reduction of approximately 2-4 log CFU/cm^2 for *S. aureus*, *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes,* with that initial reduction being the highest within the 14 days of storage. This can indicate that application of edible coatings and films in contaminated meat can cause a great reduction in pathogen count within a short period of time.

Based on **Figures 22 to 25**, among the pathogens, *S. aureus* was determined as the most sensitive to the formulated coating solutions, especially to CHI-GEL-CA 0.5%. High reductions of 3.93 log CFU/g and 4.93 log CFU/g were observed in inoculated meat

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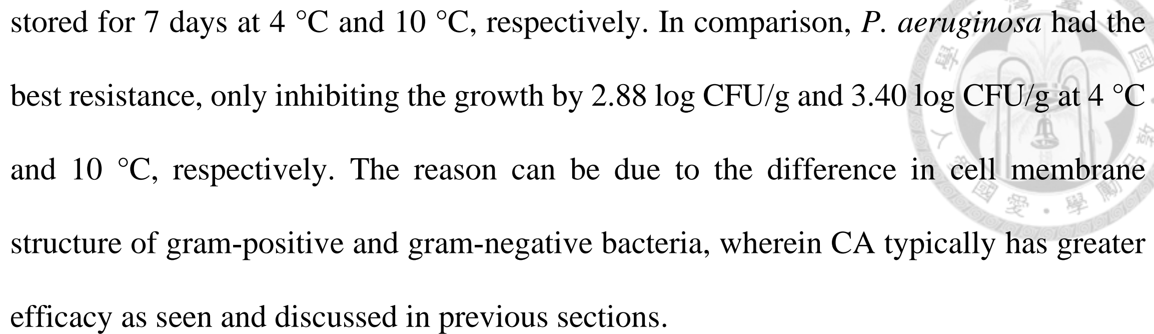

stored for 7 days at 4 °C and 10 °C, respectively. In comparison, *P. aeruginosa* had the best resistance, only inhibiting the growth by 2.88 log CFU/g and 3.40 log CFU/g at 4 °C and 10 °C, respectively. The reason can be due to the difference in cell membrane structure of gram-positive and gram-negative bacteria, wherein CA typically has greater efficacy as seen and discussed in previous sections.

When comparing storage at 4 °C and 10 °C, CHI-GEL coating alone was ineffective in controlling or reducing gram-negative bacteria count at 10 °C storage, as viable organisms appeared to recover and grow at later days of storage. For CA-incorporated coatings, a trend can be observed, wherein the elevated temperature significantly enhanced the antimicrobial activity, causing greater reductions in all populations aside from *E. coli* O157:H7. H. C. Zhang et al. (2019) has stated that higher temperature can contribute to better CA antimicrobial effect due to the increase of fluidization of cell membranes, which allows for greater CA-acting ability on cells and limits its growth.

Overall, there is a statistically significant interaction between time, treatment, and temperature. The effects of time and treatment depend on the storage temperature, which can be observed with the results of CHI-GEL and CA-incorporated coatings as stated above. Among the independent variables, coating treatments seemed to have the largest impact in influencing pathogen growth. All treatments had a significant difference in their antipathogenic activity, with 0.5% CA incorporated CHI-GEL coating exhibiting the strongest activity.

As temperature abuse and fluctuations can occur during storage or transport, chitosan-gelatin coating with 0.5% CA can cause high levels (> 3 log CFU/g) of reduction from

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potentially contaminated meats, reducing the risk of foodborne diseases and keeping consumers safe.

6.8 Cost and applicability of developed coating

In our experiment, the price of food-grade caprylic acid, chitosan, and gelatin was NTD 2150/kg, NTD 1600/kg, and NTD 450/kg, respectively. The cost of the formulated coating containing 0.5% CA, 1% chitosan, and 3% gelatin was approximately NTD 40.25/L of solution. Based on the conducted preliminary study, a meat sample weighing 25 g can absorb 0.5 to 1 mL of the coating solution. One litter of the solution was able to coat roughly 25 to 50 kg of fresh beef. Therefore, the coating cost for 1 kg of beef was calculated to be NTD 1.61 to 0.805. This is very cost-effective, as the shelf life of the beef was extended to 20 days, compared to the 10 days of control. Furthermore, this coating also allows for added protection against bacterial pathogens. The developed coating has great potential to be commercially used in meat slaughterhouses, processing plants, and retail stores as a treatment method for safer, better quality, and longer shelf life of fresh beef products.

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Chapter 7. Conclusion

For the first time, the effect of chitosan-gelatin-caprylic acid composite edible coatings were assessed under growth medium and meat matrix. Synergistic antipathogenic activity was observed between caprylic acid and chitosan-gelatin coating under log reduction and time-kill assays. Moreover, the developed coatings had good stability under room and elevated temperature storage conditions for 30 days. The application of chitosan-gelatin coating in aerobically packaged fresh beef samples achieved inhibitory effects against spoilage microorganisms and pathogenic bacteria growth during 4 °C storage. The coating effectively reduced pH change, TBARS production, and color loss for the whole 20-day storage. The incorporation of CA significantly improved both antimicrobial and antioxidant activities of the CHI-GEL coating. The antipathogenic activity against *S. aureus*, *E. coli* O157:H7, *S.* Typhimurium, and *P. aeruginosa* were also very evident even at abusive 10 °C storage. At 0.5% CA, the coating demonstrated the best results in terms of overall color retention and pathogenic growth inhibition, keeping the population of all inoculated pathogens at low concentrations. The findings suggest that CHI-GEL-CA 0.5% coating has satisfactory antipathogenic, antimicrobial, antioxidant, and barrier properties, suitable for use as a cost-effective preservation method for fresh beef during storage. The proposed method has great potential to help reduce spoilage problems for meat retailers and foodborne disease concerns for consumers.

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Chapter 8. Research limitation and recommendation

Due to time, financial and technological reasons, the following are the confronted limitations and future recommendations of this study.

Experimental aspect:

1. **Using bacterial cocktail for better bacterial species representation**

In the conducted study, only one strain for each pathogen was used for analysis. The obtained results may not accurately represent the effectiveness of developed coating against these bacterial species. Therefore, it is recommended that future studies can use cocktail consisting of at least three strains of bacterial species.

2. **Evaluating the properties of developed coating**

Morphological properties of developed coatings (i.e., chitosan-gelatin coating and chitosan-gelatin containing 0.25% and 0.5% caprylic acid) were not analyzed in the study. In order to ensure uniformity of coating thickness in meat samples, the use of scanning electron microscopy (SEM) is recommended after coating formation on meat. Tests regarding the permeability of moisture and food ingredients (e.g., oils or flavors) were also not conducted in the study. It is recommended that for moisture barrier testing, the water activity of coating treated meats can be compared with uncoated meat under various storage days to evaluate the degree of moisture permeability.

3. **Assessing the influence of developed coating on meat sensory properties**

The current study was not able to determine the acceptability of the coated meat. It is recommended to conduct a sensory evaluation on people of all ages to assess the

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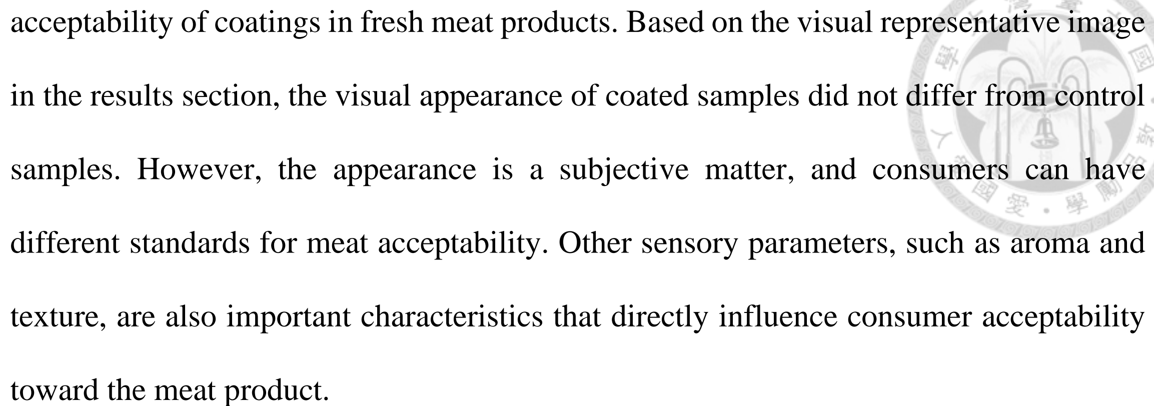

acceptability of coatings in fresh meat products. Based on the visual representative image in the results section, the visual appearance of coated samples did not differ from control samples. However, the appearance is a subjective matter, and consumers can have different standards for meat acceptability. Other sensory parameters, such as aroma and texture, are also important characteristics that directly influence consumer acceptability toward the meat product.

Commercialization aspect:

1. **Conducting a survey of consumer acceptance for coated meat products**

Although the coatings used in coated meats are edible, there remains the question of consumer acceptance. Therefore, knowing the consumers' perception of edible coatings are necessary to improve the commercialization success.

2. **Investigating the effects of cooking on developed coating**

As the final product is cooked meat, the effects of cooking in relation to the coating and the meat product should be further tested. Currently, the influence of heat on caprylic acid, chitosan, or gelatin remains uncertain. There are some discussions regarding the effect of cooking on lipid, polysaccharide, and protein-based coatings, wherein they would be degraded upon high heat and may even give a textural improvement in cooked meats as discussed in the literature review. However, for the developed chitosan-gelatin-caprylic coating, it is unknown whether it will provide similar results.

3. **Determining the produced volatile flavor compound during cooking**

As flavor is considered an important aspect of quality for cooked meat, the evaluation of volatile flavor compounds is recommended for future studies involving coated meat.

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Through the assessment, aside from evaluating flavor characteristics of cooked meat from the Maillard reaction, volatile compounds that are markers of meat spoilage can also be determined in the process.

4. Combining nanotechnology with the developed coating

With the rise of nanotechnology in recent years, it may also be advisable to conduct studies on chitosan-based coatings with nanoencapsulated fatty acids, especially caprylic acid due to its great antibacterial and antioxidant properties. There is a high probability that nanoencapsulated biopreservatives will become the trend in edible coating and film application for fresh meat products, as improved functional properties have been observed in recent studies. However, since these nanomaterials can easily penetrate the human body, their toxicological effects and safety must be clarified. Furthermore, the preparation time and production cost for these types of coating must also be evaluated in terms of industrial-scale production.

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Figures

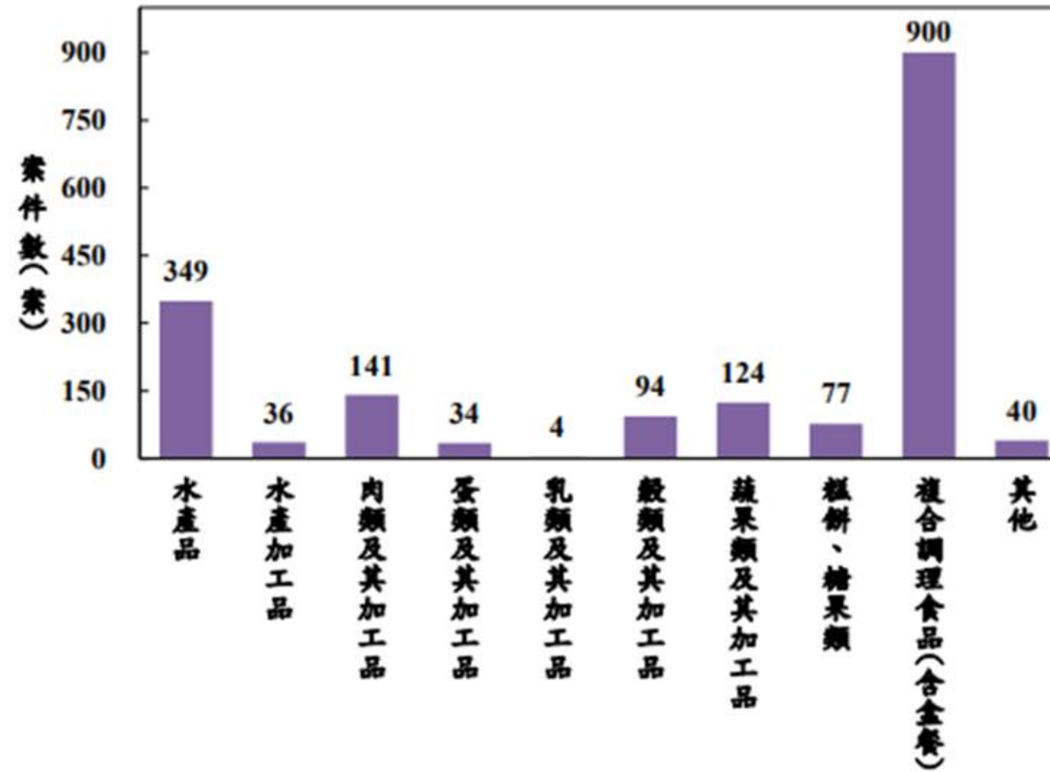

Figure 4. The food origin of collective foodborne disease outbreaks from years 1981 to 2021 in Taiwan.

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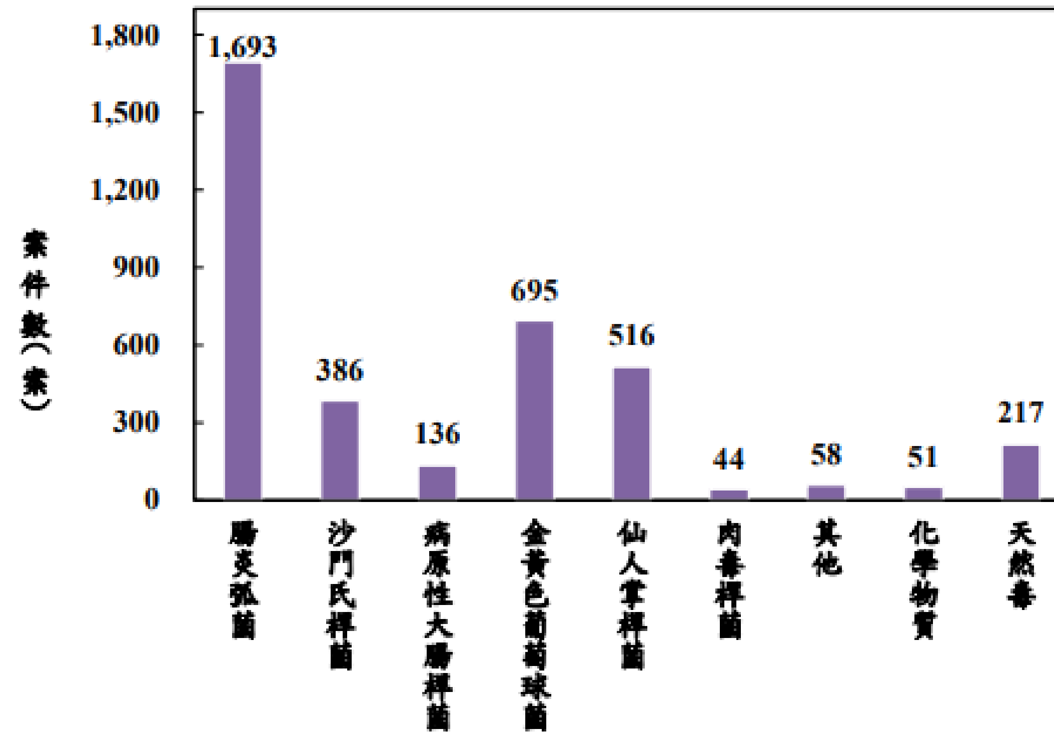

Figure 5.The etiology of collective foodborne disease outbreaks from years 1981 to 2021 in Taiwan.

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Figure 6. Antimicrobial activity of formulated chitosan-gelatin-caprylic acid coating.

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Figure 7. Minimum inhibitory concentration of caprylic acid against foodborne pathogens by visual turbidity.

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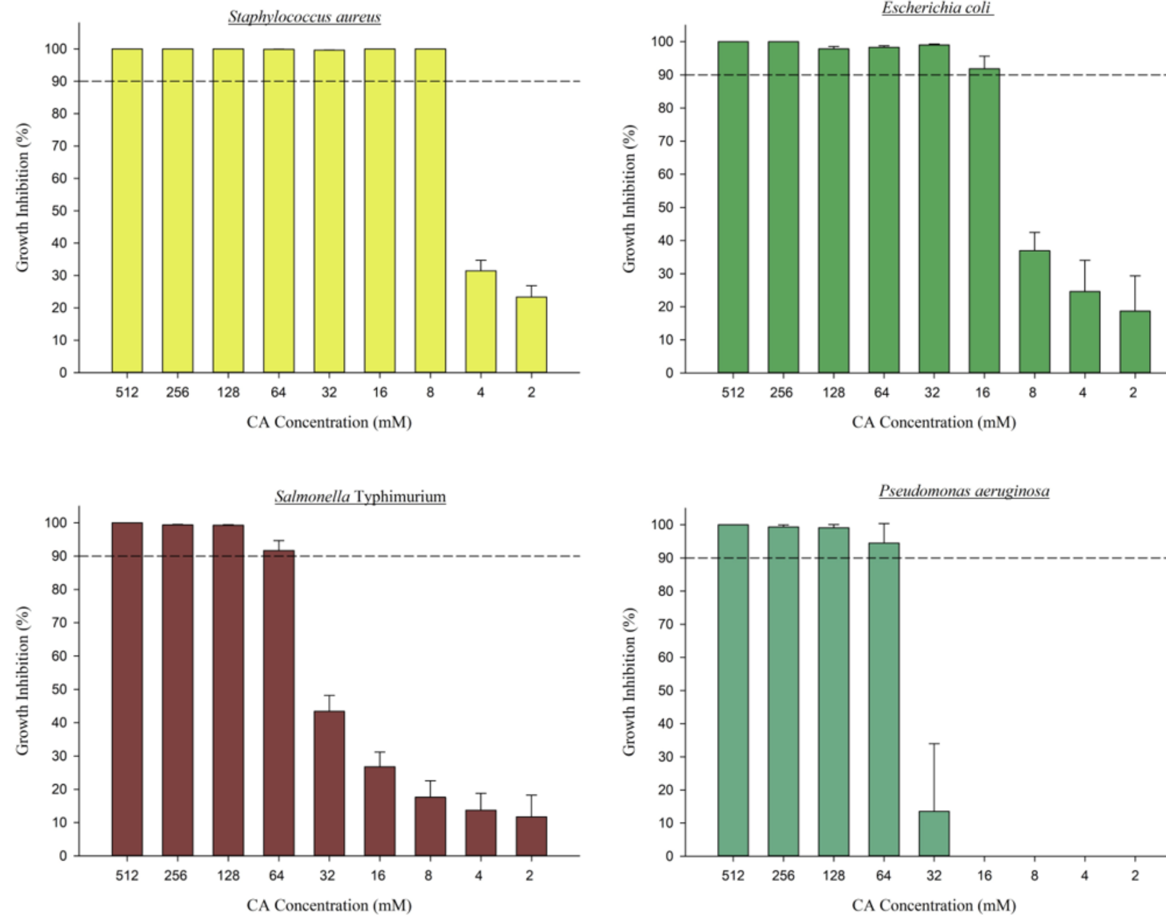

Figure 8. Minimum inhibitory concentration of caprylic acid against foodborne pathogens by spectrophotometric assay.

Error bars represent standard errors of the mean (8 independent replicates) for the % inhibition of the bacterial population after 24 hr at 37 °C.

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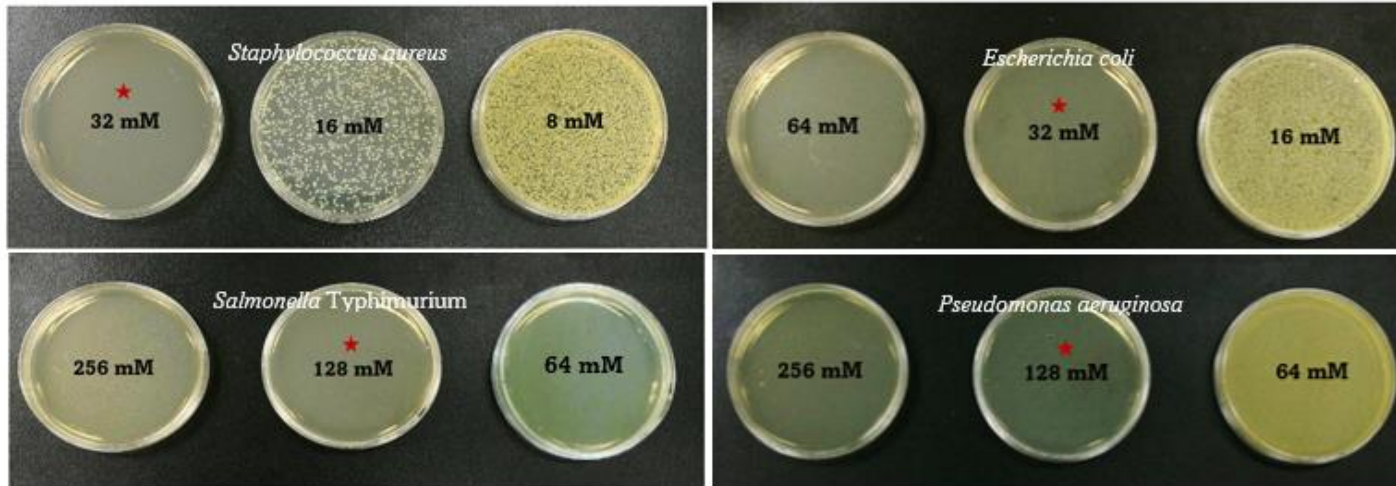

Figure 9.. Minimum bactericidal concentration of caprylic acid against foodborne pathogens.

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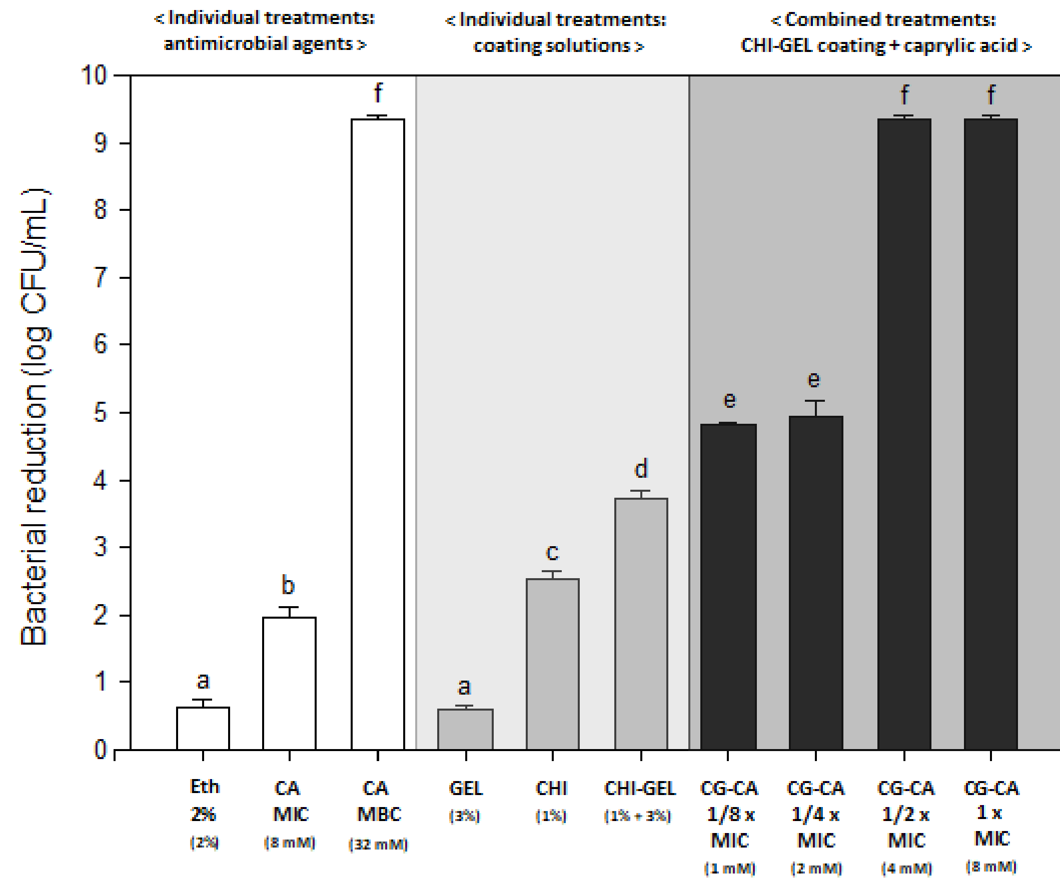

Figure 10. Bactericidal effects of antimicrobial agents alone, coating solutions alone, or combined treatments against *Staphylococcus aureus*.

Error bars represent standard errors of the mean (3 independent replicates) for bacterial growth reduction in log CFU/mL after 24 hr.
Analysis was conducted using one-way ANOVA with Tukey's test.
Significant difference ($P < 0.05$) were indicated with bars having different letters.

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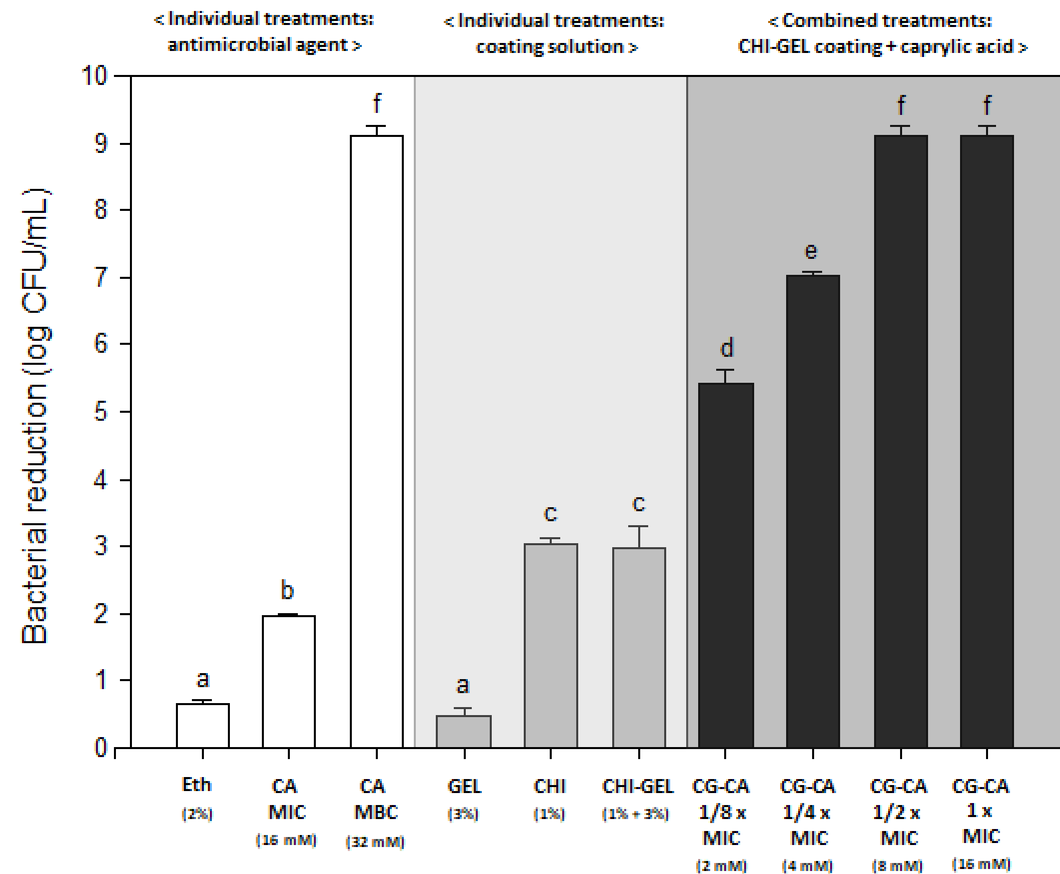

Figure 11. Bactericidal effects of antimicrobial agents alone, coating solutions alone, or combined treatments against *Escherichia coli* O157:H7.

Error bars represent standard errors of the mean (3 independent replicates) for bacterial growth reduction in log CFU/mL after 24 hr.
Analysis was conducted using one-way ANOVA with Tukey's test.
Significant difference ($P < 0.05$) were indicated with bars having different letters.

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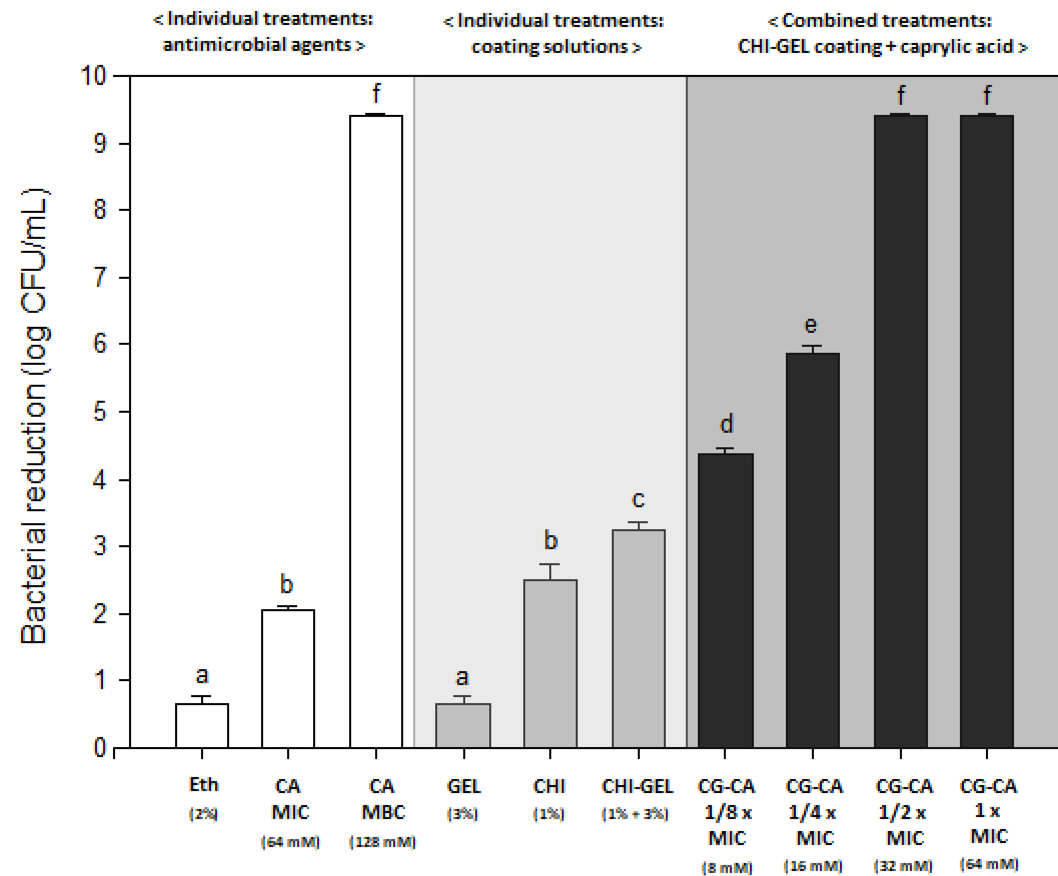

Figure 12. Bactericidal effects of antimicrobial agents alone, coating solutions alone, or combined treatments against *Salmonella* Typhimurium.

Error bars represent standard errors of the mean (3 independent replicates) for bacterial growth reduction in log CFU/mL after 24 hr.
Analysis was conducted using one-way ANOVA with Tukey's test.
Significant difference ($P < 0.05$) were indicated with bars having different letters.

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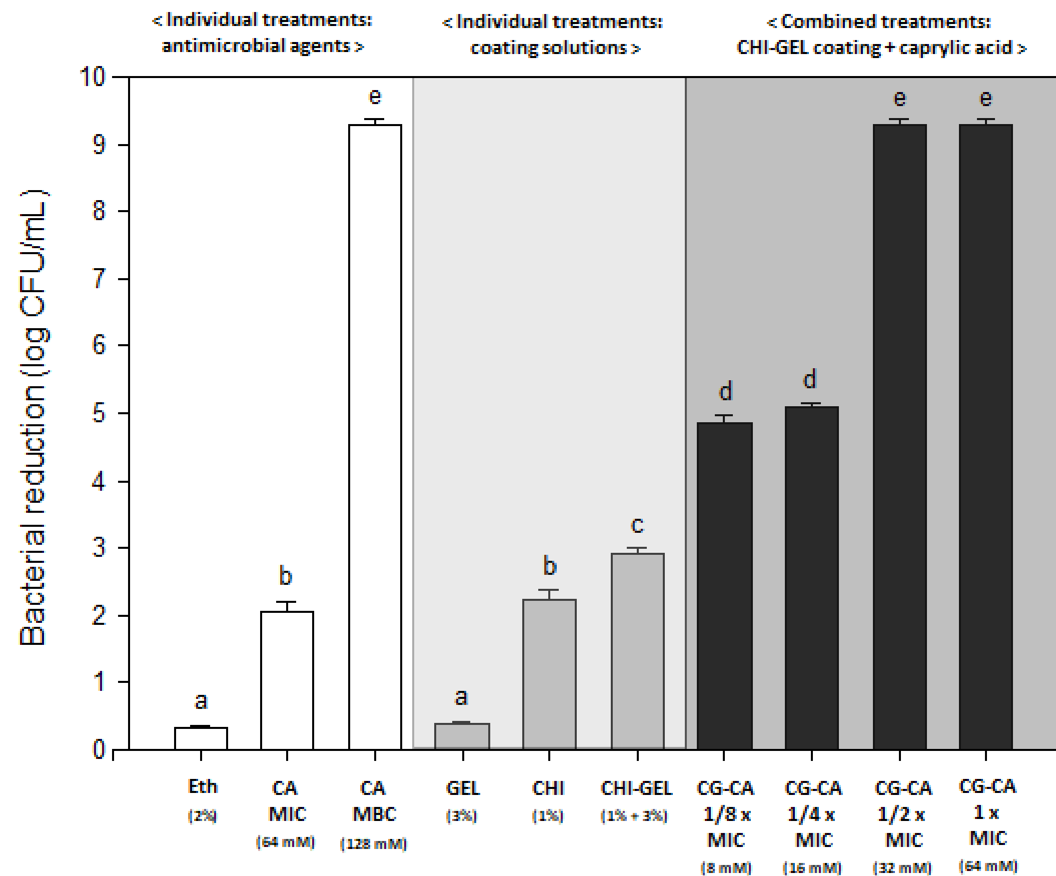

Figure 13. Bactericidal effects of antimicrobial agents alone, coating solutions alone, or combined treatments against *Pseudomonas aeruginosa.*

Error bars represent standard errors of the mean (3 independent replicates) for bacterial growth reduction in log CFU/mL after 24 hr.
Analysis was conducted using one-way ANOVA with Tukey's test.
Significant difference ($P < 0.05$) were indicated with bars having different letters.

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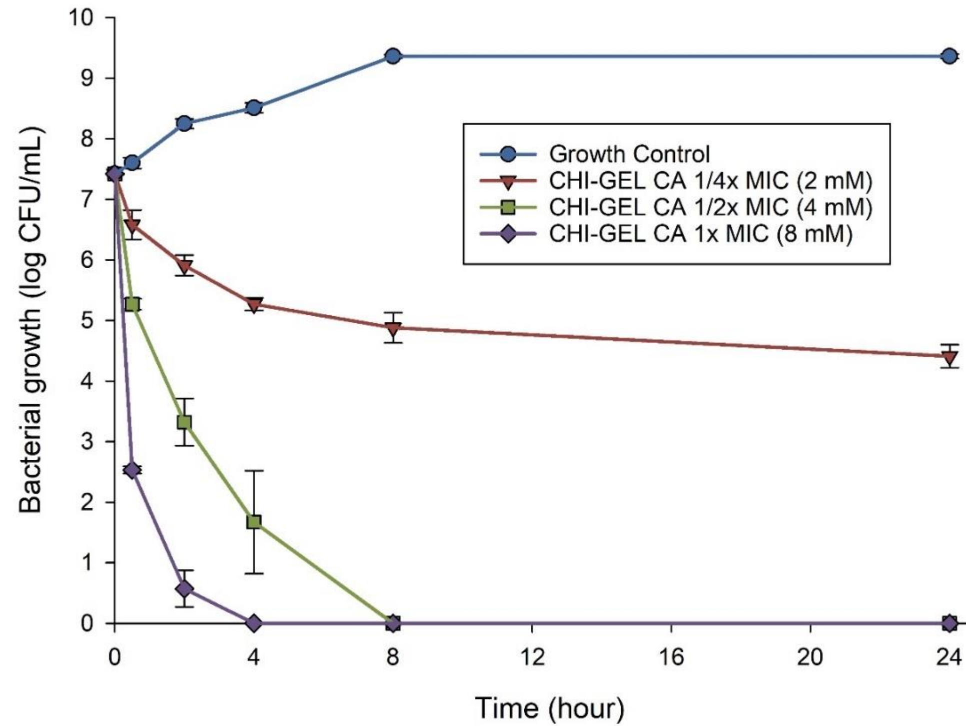

Figure 14. Time-kill curve of *Staphylococcus aureus* under chitosan-gelatin coating with different incorporated CA MIC concentrations.

Error bars represent standard errors of the mean (3 independent replicates) for the number of culturable cells, as measured in CFU/mL at each time point at 37 °C. Analysis was conducted using one-way ANOVA with Tukey's test ($P < 0.05$).

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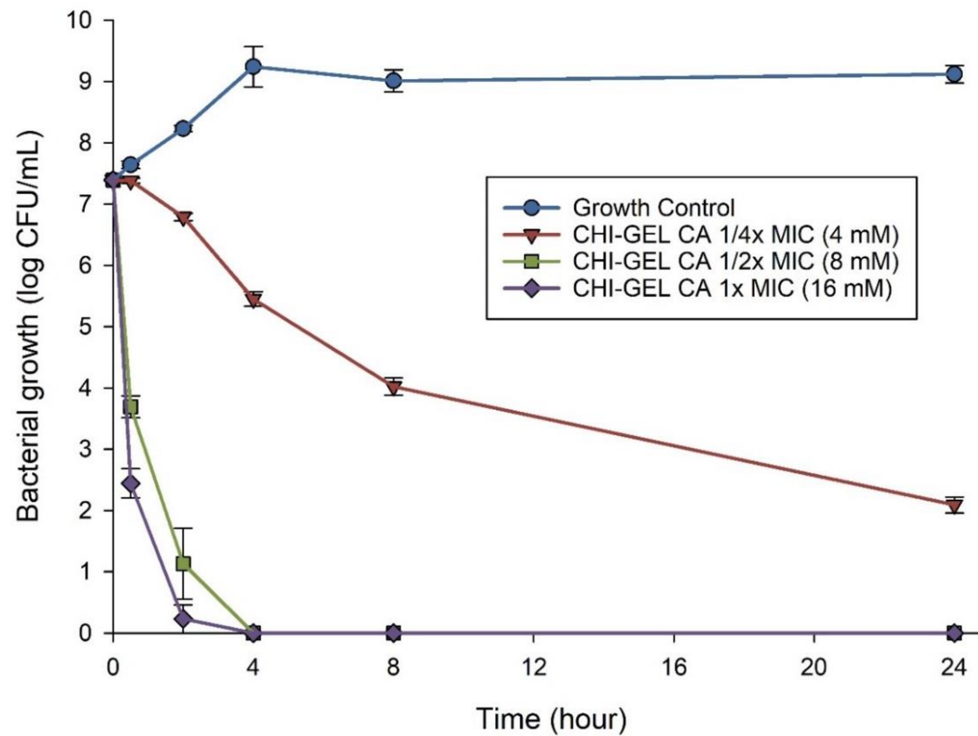

Figure 15. Time-kill curve of *Escherichia coli* O157:H7 under chitosan-gelatin coating with different incorporated CA MIC concentrations.

Error bars represent standard errors of the mean (3 independent replicates) for the number of culturable cells, as measured in CFU/mL at each time point at 37 °C. Analysis was conducted using one-way ANOVA with Tukey's test ($P < 0.05$).

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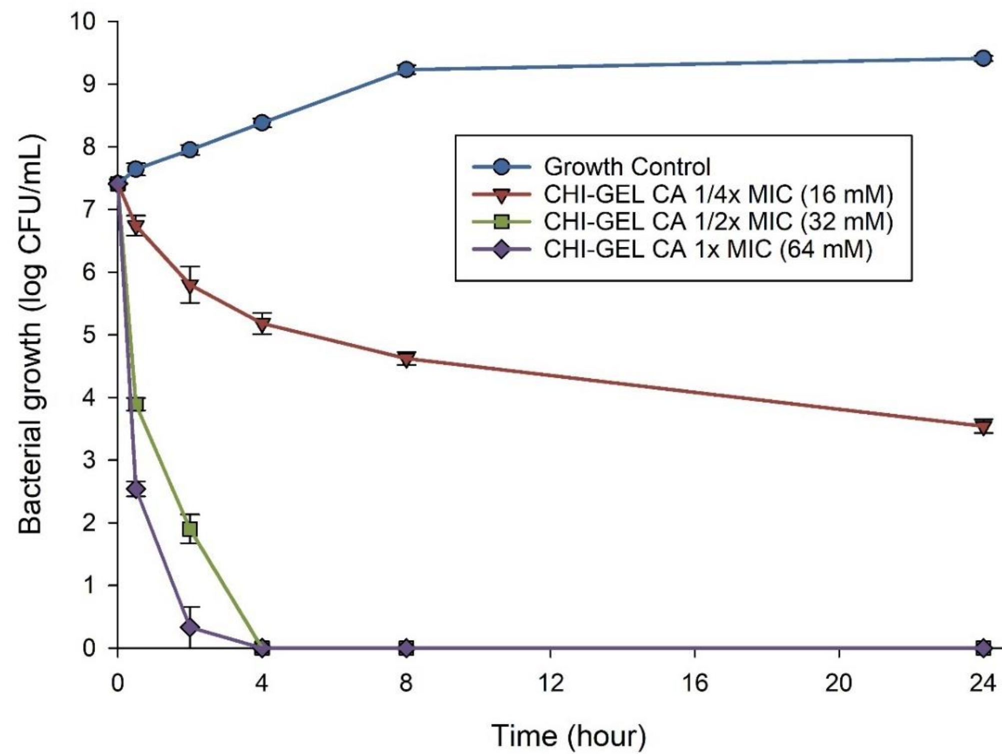

Figure 16. Time-kill curve of *Salmonella* Typhimurium under chitosan-gelatin coating with different incorporated CA MIC concentrations.

Error bars represent standard errors of the mean (3 independent replicates) for the number of culturable cells, as measured in CFU/mL at each time point at 37 °C. Analysis was conducted using one-way ANOVA with Tukey's test ($P < 0.05$).

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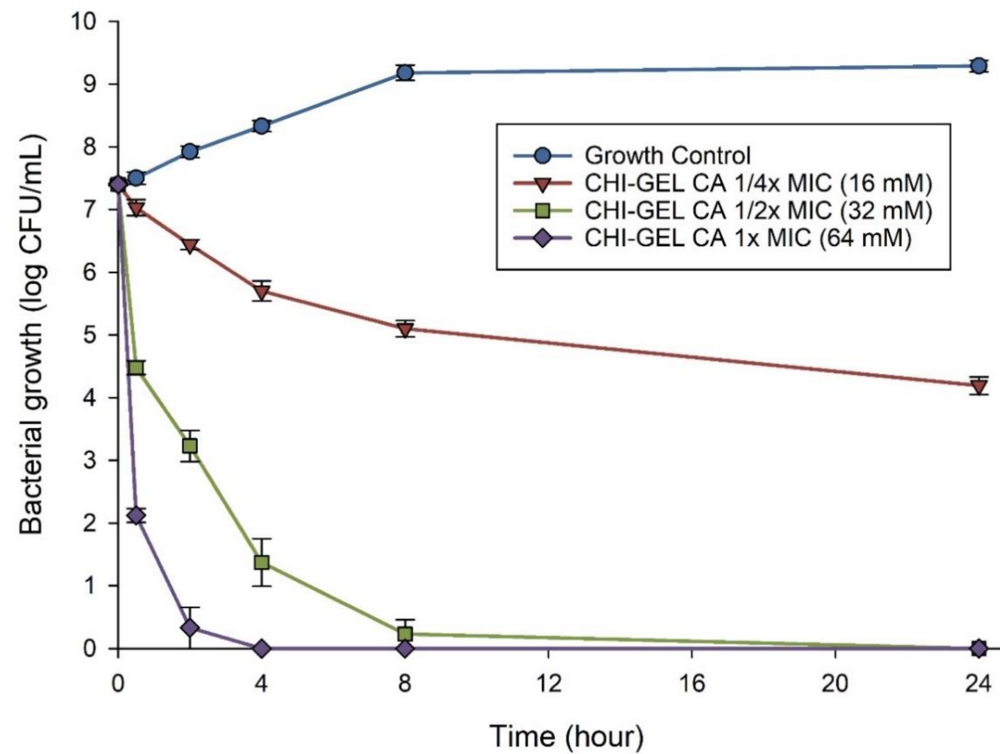

Figure 17. Time-kill curve of *Pseudomonas aeruginosa* under chitosan-gelatin coating with different incorporated CA MIC concentrations.

Error bars represent standard errors of the mean (3 independent replicates) for the number of culturable cells, as measured in CFU/mL at each time point at 37 °C. Analysis was conducted using one-way ANOVA with Tukey's test ($P < 0.05$).

doi:10.6342/NTU202300594

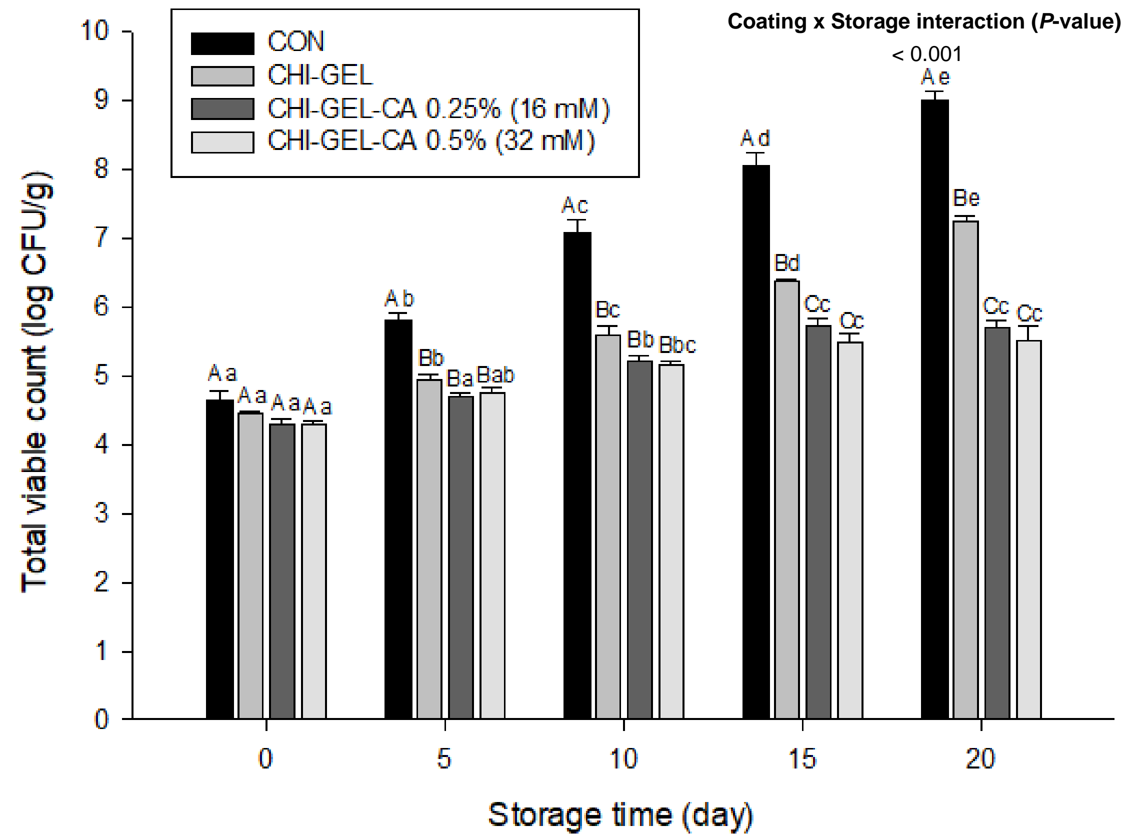

Figure 18. Total viable count in aerobically packaged beef samples during cold storage (4 °C).

Error bars represent standard errors of the mean (3 independent replicates).
Analysis was conducted using two-way ANOVA with Tukey's test.
Significant difference ($P < 0.05$) were indicated with bars having different letters.
Capital letter represent comparison between treatments, while small letter represent comparison between days.

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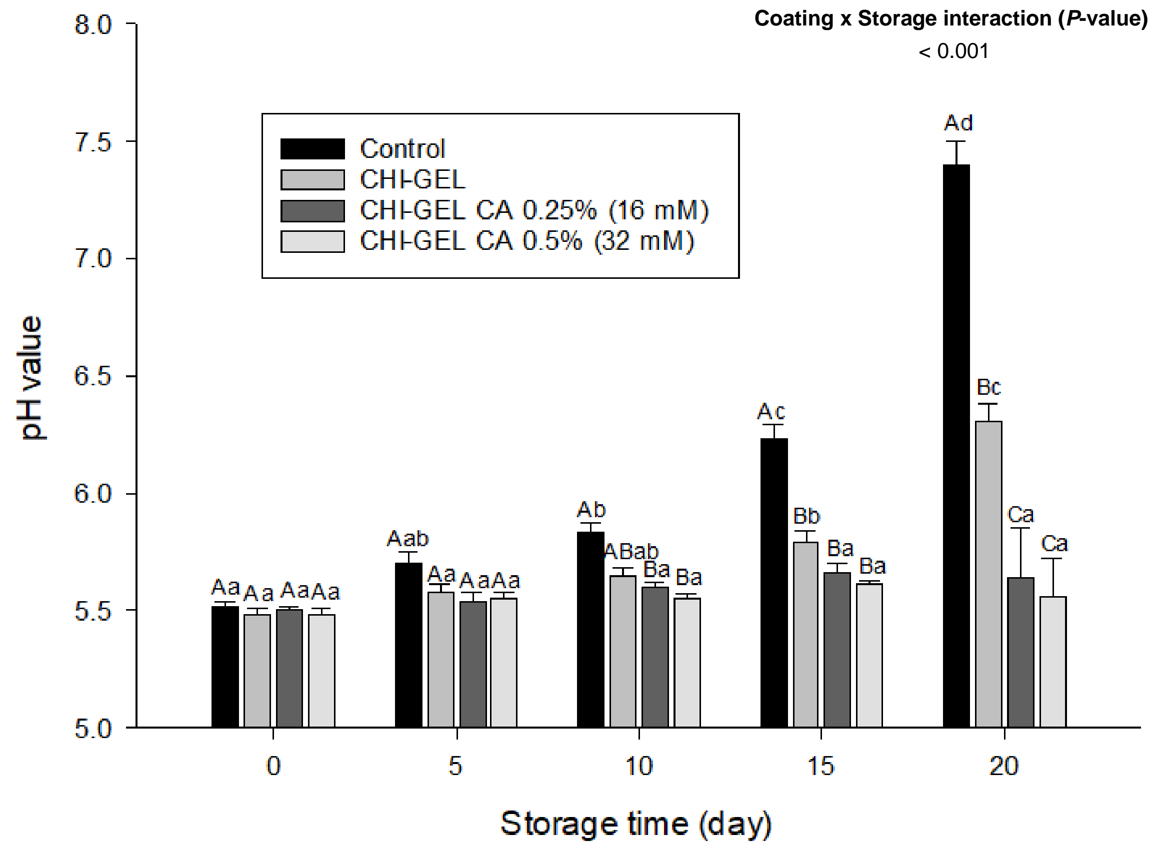

Figure 19. Changes of pH value in aerobically packaged beef samples during cold storage (4 °C).

Error bars represent standard errors of the mean (3 independent replicates).
Analysis was conducted using two-way ANOVA with Tukey's test.
Significant difference ($P < 0.05$) were indicated with bars having different letters.
Capital letter represent comparison between treatments, while small letter represent comparison between days.

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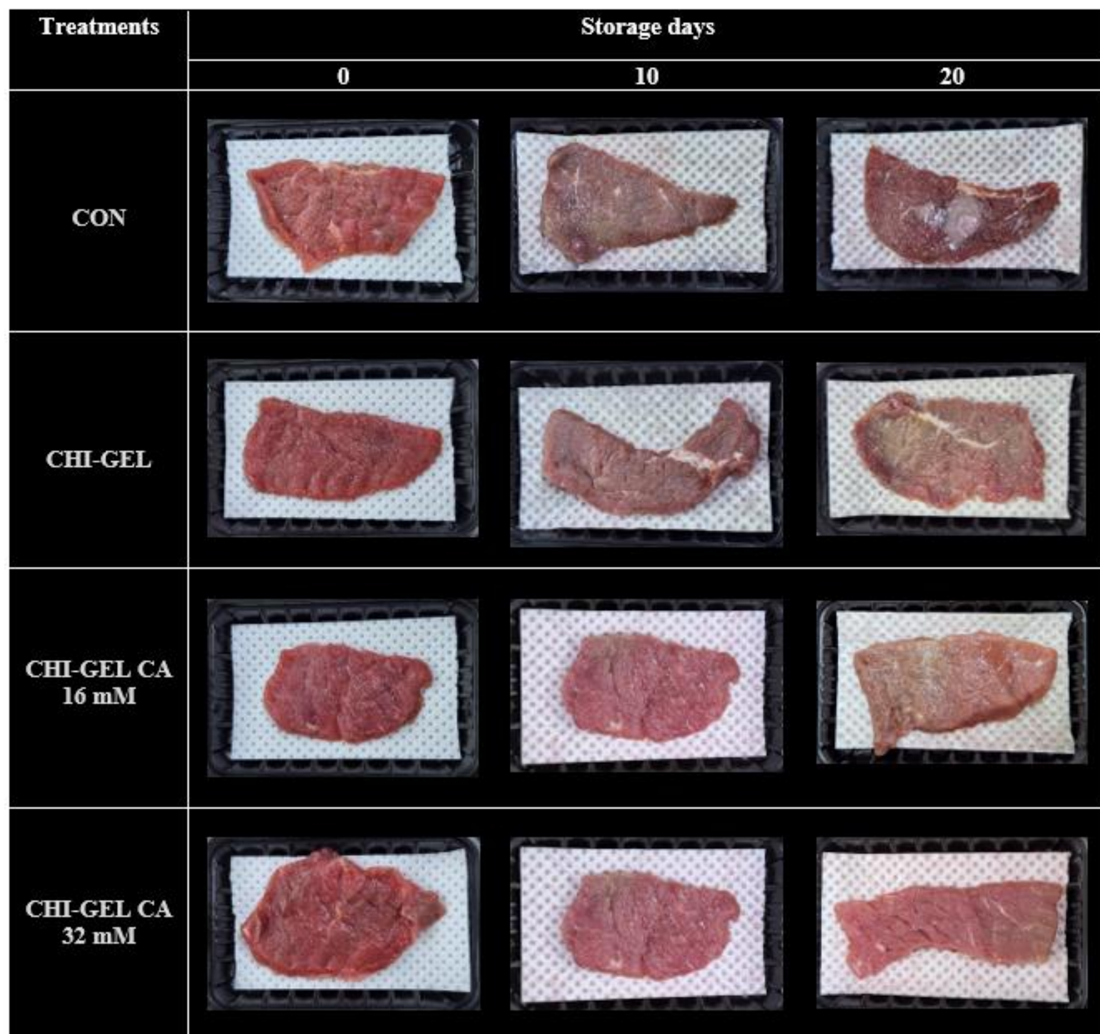

Figure 20. The visual representative images show the effects of different coating formulations on microbial growth and color of beef samples under aerobic packaging conditions during cold storage (4 °C).

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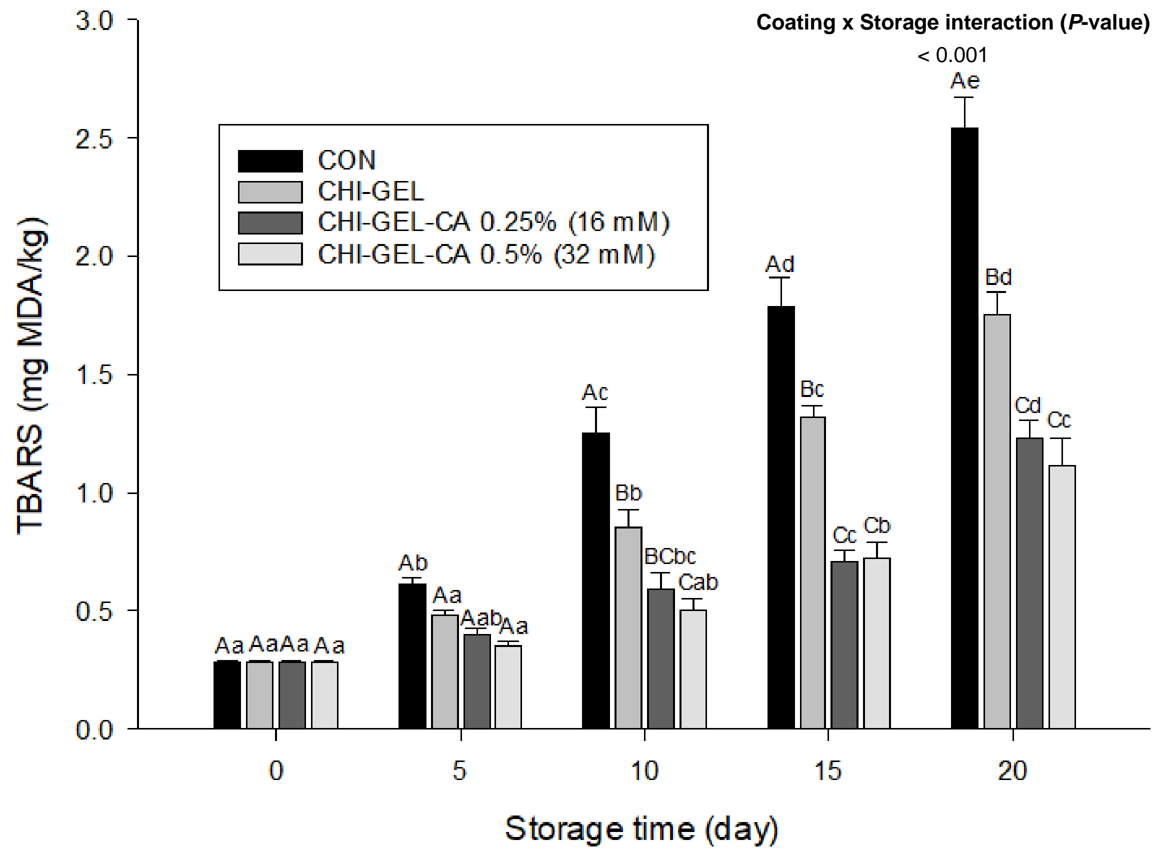

Figure 21. Thiobarbituric acid reactive substances (TBARS) in aerobically packaged beef samples during cold storage (4 °C).

Error bars represent standard errors of the mean (3 independent replicates).
Analysis was conducted using two-way ANOVA with Tukey's test.
Significant difference ($P < 0.05$) were indicated with bars having different letters.
Capital letter represent comparison between treatments, while small letter represent comparison between days.

doi:10.6342/NTU202300594

Coating x Storage x Temperature interaction (*P*-value)

0.007

Staphylococcus aureus count (log CFU/g)

Storage time (day)

CON
CHI-GEL
CHI-GEL-CA 0.25% (16 mM)
CHI-GEL-CA 0.5% (32 mM)

Staphylococcus aureus count (log CFU/g)

Storage time (days)

CON
CHI-GEL
CHI-GEL-CA 0.25% (16 mM)
CHI-GEL-CA 0.5% (32 mM)

Figure 22. Growth of *Staphylococcus aureus* in beef samples during storage at refrigerated (4 ℃) and abusive (10 ℃) temperatures.

Error bars represent standard errors of the mean (3 independent replicates) for the number of culturable cells, as measured in CFU/g at each time point. Analysis was conducted using three-way ANOVA with Tukey's test ($P < 0.05$).

doi:10.6342/NTU202300594

Coating x Storage x Temperature interaction (*P*-value)

< 0.001

Escherichia coli O157:H7 count (log CFU/g)

CON
CHI-GEL
CHI-GEL-CA 0.25% (16 mM)
CHI-GEL-CA 0.5% (32 mM)

Storage time (day)

Escherichia coli O157:H7 count (log CFU/g)

CON
CHI-GEL
CH-GEL-CA 0.25% (16 mM)
CH-GEL-CA 0.5% (32 mM)

Storage time (day)

Figure 23. Growth of *Escherichia coli* O157:H7 in beef samples during storage at refrigerated (4 °C) and abusive (10 °C) temperatures.

Error bars represent standard errors of the mean (3 independent replicates) for the number of culturable cells, as measured in CFU/g at each time point. Analysis was conducted using three-way ANOVA with Tukey's test ($P < 0.05$).

doi:10.6342/NTU202300594

Coating x Storage x Temperature interaction (*P*-value)

< 0.001

Salmonella Typhimurium count (log CFU/g)

Storage time (day)

CON
CHI-GEL
CHI-GEL-CA 0.25% (16 mM)
CHI-GEL-CA 0.5% (32 mM)

Salmonella Typhimurium count (log CFU/g)

Storage time (day)

CON
CHI-GEL
CHI-GEL-CA 0.25% (16 mM)
CHI-GEL-CA 0.5% (32 mM)

Figure 24. Growth of *Salmonella* Typhimurium in beef samples during storage at refrigerated (4 ℃) and abusive (10 ℃) temperature.

Error bars represent standard errors of the mean (3 independent replicates) for the number of culturable cells, as measured in CFU/g at each time point. Analysis was conducted using three-way ANOVA with Tukey's test ($P < 0.05$).

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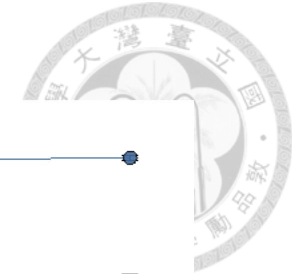

Coating x Storage x Temperature interaction (*P*-value)

0.007

Pseudomonas aeruginosa count (log CFU/g)

Storage time (day)

CON
CHI-GEL
CHI-GEL-CA 0.25% (16 mM)
CHI-GEL-CA 0.5% (32 mM)

Pseudomonas aeruginosa count (log CFU/g)

Storage time (day)

CON
CHI-GEL
CHI-GEL-CA 0.25% (16 mM)
CHI-GEL-CA 0.5% (32 mM)

Figure 25. Growth of *Pseudomonas aeruginosa* in beef samples during storage at refrigerated (4 ℃) and abusive (10 ℃) temperature.

Error bars represent standard errors of the mean (3 independent replicates) for the number of culturable cells, as measured in CFU/g at each time point. Analysis was conducted using three-way ANOVA with Tukey's test ($P < 0.05$).

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Tables

Table 1. The daily consumption of beef in Taiwan arranged by sex and age group.

Age Group	Sex	Daily Consumption (g/day)
4–12	Male and female	LN [1] (52.69, 2.60)
13–18	Male	LN (78.34, 2.10)
13–18	Female	LN (61.32, 2.24)
19–65	Male	LN (74.53, 2.41)
19–65	Female	LN (53.08, 2.75)
>65	Male	LN (40.14, 2.21)
>65	Female	LN (70.44, 1.33)
19–49	Female	LN (54.71, 2.81)

[1] LN = log normal distribution.

Reprinted from Lien et al. (2020).

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Table 2. Foodborne pathogens related to meat and their characteristics.

Pathogen	Natural habitat	Associated meat sources	Infective/ Intoxication dose	Growth temperature (°C)	Growth pH	Growth aW
Staphylococcus aureus	Human and animal skin	Raw meat and poultry, fermented sausage	Intoxication (10^6)	7 to 48	4.2 to 9	0.95
Escherichia coli O157:H7	Ruminant intestine	Raw beef	Infection (10-100)	8 to 43	4.4 to 9	0.95
Salmonella spp.	Animal intestine, processing environment	Raw meat and poultry	Infection (10^4 to 10^7)	6 to 46	4 to 9.5	0.94
Listeria monocytogenes	Animal intestine, moist processing areas, soil	Raw meat	Infection (10^2 to 10^3)	0 to 44	4.6 to 9.2	0.93
Pseudomonas aeruginosa	Environment	Raw meat	Infection (10^8 to 10^9)	4 to 42	4.5 to 9.5	0.97
Campylobacter jejuni	Animal intestine, soil	Raw poultry	Infection (<500)	27 to 45	4.9 to 9	0.98

Adapted from Wallace et al. (2011); Kniel et al. (2017).

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Table 3. The shelf life of various meat products under storage temperatures and conditions.

Meat Product	Storage condition	Storage temperature (°C)	Shelf life (days)	Reference
Fresh ground beef	High oxygen atmosphere	4.3 (Refrigerated)	9	Limbo et al. (2010)
		8.1 (Household)	3-4	
		15.5 (Abusive)	2	
Fresh beef loin	Aerobically packaged	4 (Refrigerated)	9	Qian et al. (2018)
		-1 (Super chilling)	23	
		-6 (Subfreezing)	84	
		-9 (Subfreezing)	126	
		-12 (Subfreezing)	168	
		- 18 C (Freezing)	168	
Fresh chicken breast	Vacuum packaged	2 to 4 (Refrigerated)	8	Kaewthong et al. (2019)
		-1.3 (Subfreezing)	18	
Fresh beef	Aerobically packaged	3 to 5 (Refrigerated)	3	Rudy et al. (2020)
	Vacuum packaged		8	
Fresh pork	Aerobically packaged		2	
	Vacuum packaged		6	
Fresh chicken	Aerobically packaged		1-2	
	Vacuum packaged		7	

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Table 4. Microbial standards for fresh beef products.

Meat type	Bacteria	Microbial limits	Regulatory bodies	Regulation
Meat, before chilling	Aerobic plate count	10^6 CFU/g	ICMSF (International)	Microorganisms in Foods
Meat, chilled or frozen	Aerobic plate count	10^7 CFU/g		
Meat, before chilling	*Salmonella* spp.	absence		
Cattle meat, before chilling	Aerobic plate count	5 log CFU/cm^2	EU Regulations (Europe)	EC No. 2073/2005: Microbiological criteria for food
	Enterobacteriaceae	2.5 log CFU/cm^2		
	Salmonella spp.	absence		
Boneless beef	Aerobic plate count	10^5 CFU/g	USDA (USA.)	Microbiological testing of AMS purchased meat
	Total coliform	10^3 CFU/g		
	Escherichia coli	10^2 CFU/g		
	Escherichia coli O157:H7	absence		
	Salmonella spp.	absence		
Meat, chilled	Aerobic plate count	$< 3 \times 10^6$ CFU/g	TQF (Taiwan)	TQF-PST-001
	Escherichia coli	50 MPN/g		
	Salmonella spp.	Absence		

ICMSF stands for International Commission on Microbiological Specifications for Foods.
USDA stands for United States Department of Agriculture.
TQF stands for Taiwan Quality Food Association.

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Table 5. Common biopreservatives added in chitosan-based coating for fresh meat preservation.

Biopreservative		Coating type	Food Matrix	Storage study	Target microorganism	Reference
Classification	**Compound**					
Plant extract	Tomato extract (0.1%, 0.3%)	Chitosan coating (1%)	Fresh pork loin	21 days at 4°C, Aerobic packaging	Total coliform, mesophiles and psychrophiles	Ramirez-Guerra et al. (2018)
Organic acid	Gallic acid (0.2%, 0.4%)	Chitosan coating (2%)	Fresh pork loin	20 days at 4°C, High oxygen, Modified atmospheric packaging	Total aerobic bacteria	Fang et al. (2018)
Essential oil	Oregano oil (0.25%)	Chitosan coating (1.5%)	Fresh chicken breast	21 days at 4 °C, Modified atmospheric packaging	Total aerobic bacteria, LAB, molds, yeasts, *Pseudomonas* spp., *Brochothrix thermosphacta*, *Enterobacteriaceae*	Petrou et al. (2012)
	Oregano and Thyme oil (0.5%, 1%)	Chitosan film (1.5%)	Fresh beef round	30 days at 4°C, Aerobic packaging	Psychrophiles, Pseudomonads, LAB *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus*	Gaba et al. (2022)
Combination	Nisin (0.1%) with Grape seed extract (0.5%)	Chitosan (1%) + Gelatin (3%) composite coating	Fresh pork loin	20 days at 4°C, Aerobic packaging	Total aerobic bacteria	(Xiong et al., 2020)
	Nisin (0.2%) with Gallic acid (0.2%)	Chitosan coating (2%)	Fresh pork loin	20 days at 2°C, High oxygen, Modified atmospheric packaging	Total aerobic bacteria	Cao et al. (2019)

Note: * stands for inoculated pathogen at 6 log CFU/cm^2

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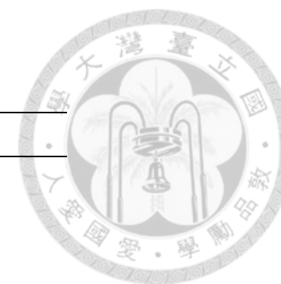

Table 6. Available studies of fatty acid incorporation in chitosan-based coating for fresh meat and seafood preservation.

Biopreservative	Coating type	Food Matrix	Storage study	Target microorganism	Reference
Lauric acid (1, 3 mM)	Chitosan coating (2%)	Fresh beef striploin	21 days at 4°C, Aerobic packaging	Total aerobic bacteria, lactic acid bacteria, *Pseudomonas* spp.	Hoa et al. (2022a)
Oleic acid (0.5%, 1%)	Chitosan coating (2%)	Fresh pork shoulder	21 days at 4°C, Aerobic packaging	Total aerobic bacteria , *Pseudomonas* spp. Total *E. coli*	Hoa et al. (2022b)
Caprylic acid (1%) with Carvacrol (0.5%)	Chitosan coating (1%)	Fresh shrimp	10 days in ice, Aerobic packaging	Total plate count	Wang et al. (2018)

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Table 7. Minimum inhibitory concentration of caprylic against selected pathogens.

Pathogen	Strain	Solvent	MIC	Endpoint determination	Reference
Staphylococcus aureus	ATCC 25923	Ethanol	32 mM	Visual turbidity	Pangprasit et al. (2020)
	ATCC 25923	Ethanol	19.79 mM	Visual turbidity	Hulankova and Borilova (2011)
	ATCC 25923	Ethanol	10 mM	OD value (600 nm)	Nobmann et al. (2010)
	ATCC 33592	Ethanol	20 mM	OD value (600 nm)	Nobmann et al. (2010)
	ATCC 6538P	Not specified	18.5 mM	Visual turbidity	Ruiz-Rico et al. (2015)
Escherichia coli	ATCC 25922	Ethanol	64 mM	Visual turbidity	Pangprasit et al. (2020)
	ATCC 25922	Not specified	20 mM	Visual turbidity	Ruiz-Rico et al. (2015)
	ATCC 25922	Ethanol	10 mM	OD value (600 nm)	Nobmann et al. (2009)
	ATCC 25922	DMSO	13.87 mM	Visual turbidity	Skrivanova et al. (2006)
	ATCC 35218	DMSO	13.87 mM	Visual turbidity	(Skrivanova et al., 2006)
Escherichia coli O157:H7	ATCC 700728	Ethanol	22.26 mM	Visual turbidity	Hulankova and Borilova (2011)
	ATCC 700728	Ethanol	10 mM	OD value (600 nm)	Nobmann et al. (2009)
Salmonella Typhimurium	K3	DMSO	20.80 mM	Visual turbidity	Skrivanova et al. (2006)
	ATCC 14028	Ethanol	> 20 mM	OD value (600 nm)	Nobmann et al. (2009)
Salmonella Enteritidis	ATCC 13076	DMSO	20.80 mM	Visual turbidity	Skrivanova et al. (2006)
	ATCC 13076	Ethanol	29.43 mM	Visual turbidity	Hulankova and Borilova (2011)
Salmonella Choleraesuis	ATCC 13312	Not specified	20 mM	Visual turbidity	Ruiz-Rico et al. (2015)

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Table 8. Summary of the used bacterial pathogen strains with their characteristics and cultivation methods.

Strain	**Number**	**Origin**	**Culture temperature (°C)**	**Growth medium (Brand)**
Staphylococcus aureus	ATCC 25923	Clinical isolate	37	MSA (Becton Dickinson)
Escherichia coli O157:H7	BCRC 15374	Human feces	37	SMAC (Becton Dickinson)
Salmonella Typhimurium	BCRC 12947	Food poisoning in man	37	XLD (Becton Dickinson)
Pseudomonas aeruginosa	ATCC 27853	Blood culture	37	Cetrimide (Himedia)

ATCC stands for American Type Culture Collection
BCRC stands for Bioresource Collection and Research Center

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Table 9. Formulated coating solutions to be applied in fresh beef samples.

Treatments	**Formulae**
CON	Control group, samples without coating application
CHI-GEL	1% chitosan + 3% gelatin
CHI-GEL-CA 16 mM	1% chitosan + 3% gelatin + 0.25% caprylic acid
CHI-GEL-CA 32 mM	1% chitosan + 3% gelatin + 0.50% caprylic acid

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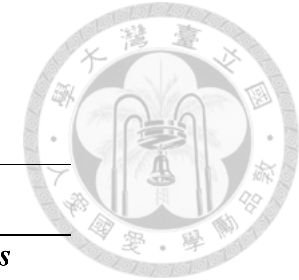

Table 10. The impact of ethanol concentration on the growth of bacterial pathogens.

Concentration	**Pathogen growth**			
	Staphylococcus aureus	***Escherichia coli* O157:H7**	***Salmonella* Typhimurium**	***Pseudomonas aeruginosa***
0%	100^{a}	100^{a}	100^{a}	100^{a}
1%	98.93 ± 0.34^{a}	99.71 ± 0.23^{a}	99.49 ± 0.37^{a}	99.22 ± 0.15^{ab}
2%	97.41 ± 0.20^{ab}	98.18 ± 0.73^{ab}	98.68 ± 0.42^{a}	97.65 ± 1.23^{ab}
4%	95.32 ± 0.95^{b}	96.76 ± 0.49^{b}	97.61 ± 0.77^{ab}	96.31 ± 0.81^{b}
8%	89.10 ± 0.77^{c}	91.73 ± 1.05^{c}	95.17 ± 0.84^{b}	92.67 ± 0.44^{c}

Data presented were based on calculations from optical density (600 nm).
Error bars represent standard errors of the mean (3 independent replicates).
Analysis was conducted using one-way ANOVA with Tukey’s test.
Significant difference ($P < 0.05$) were indicated with bars having different letters.

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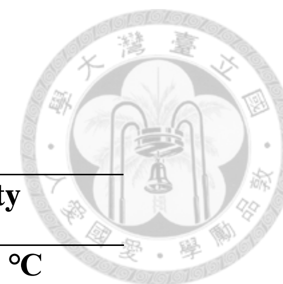

Table 11. Changes in properties of formulated coatings at 30 days of storage.

Treatment	pH change		Microbial growth		Storage stability	
	Initial	Final	Initial	Final	25 °C	35 °C
CHI-GEL	5.55	5.55	ND	ND	5	5
CHI-GEL-CA 0.25%	5.54	5.57	ND	ND	5	5
CHI-GEL-CA 0.5%	5.56	5.60	ND	ND	5	4

ND stands for no detected growth.
Data presented were measured at day 0 for initial and day 30 for final.
The pH and microbial growth were measured from treatment groups stored at 25 °C.
Storage stability grading is based on 20 mL of solution.
A score of 5 stands for excellent stability (without separation), 4 for good stability (0.25 mL separation), 3 for slightly stable (1 mL separation), 2 for quite stable (1.5 mL separation), 1 for moderately unstable (2 mL separation), 0 for extremely unstable.

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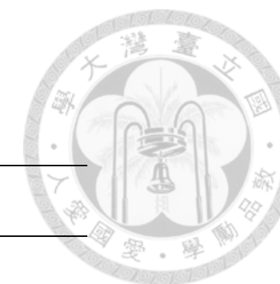

Table 12. Pathogen count and total viable count averages of UV and non-UV treated fresh beef samples.

Types of Bacteria	**Bacterial Count (log CFU/g)**	
	Non-UV treated	**UV treated**
Staphylococcus aureus	2.36 ± 0.06	ND
Salmonella Typhimurium	ND	ND
Pseudomonas aeruginosa	ND	ND
Escherichia coli O157:H7 and other non-lactose fermenters	2.97 ± 0.09	ND
Staphylococcus epidermis	3.42 ± 0.14	1.67 ± 0.84
Escherichia coli	3.21 ± 0.31	2.46 ± 0.24
Total viable count (TVC)	5.50 ± 0.09	4.74 ± 0.16

Other non-lactose fermenters include *Shigella flexneri*, *Escherichia coli* serotype O11 and O55.

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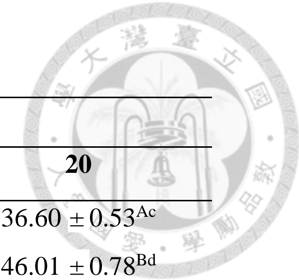

Table 13. Color loss in aerobically packaged beef samples during cold storage (4 ℃).

Attributes	Coating treatments	Storage days 0	5	10	15	20
L* (lightness)	CON	40.73 ± 0.27^{Aa}	42.46 ± 0.38^{Aab}	43.37 ± 1.13^{Ab}	40.50 ± 0.68^{Aa}	36.60 ± 0.53^{Ac}
	CHI-GEL	40.79 ± 0.27^{Aa}	41.60 ± 0.74^{Aab}	43.38 ± 0.75^{Abc}	44.62 ± 0.74^{Bcd}	46.01 ± 0.78^{Bd}
Coating x storage interaction (P value)	CHI-GEL CA 16 mM (0.25%)	40.64 ± 0.29^{Aa}	41.45 ± 0.87^{Aab}	43.29 ± 0.62^{Abc}	44.28 ± 0.23^{Bc}	45.02 ± 0.49^{Bc}
< 0.001	CHI-GEL CA 32 mM (0.5%)	40.84 ± 0.30^{Aa}	41.56 ± 0.73^{Aab}	42.91 ± 0.64^{Aabc}	43.69 ± 0.30^{Bbc}	44.13 ± 0.21^{Bc}
a* (redness)	CON	17.71 ± 0.16^{Aa}	15.09 ± 0.41^{Ab}	11.84 ± 0.35^{Ac}	7.89 ± 0.16^{Ad}	7.11 ± 0.45^{Ad}
	CHI-GEL	17.81 ± 0.20^{Aa}	16.23 ± 0.15^{Bb}	13.61 ± 0.24^{Bc}	12.54 ± 0.16^{Bd}	10.62 ± 0.38^{Be}
Coating x storage interaction (P value)	CHI-GEL CA 16 mM (0.25%)	17.73 ± 0.11^{Aa}	17.03 ± 0.07^{Ba}	15.68 ± 0.20^{Cb}	13.76 ± 0.37^{Cc}	12.44 ± 0.30^{Cd}
< 0.001	CHI-GEL CA 32 mM (0.5%)	17.82 ± 0.09^{Aa}	17.12 ± 0.19^{Ba}	15.82 ± 0.25^{Cb}	15.30 ± 0.10^{Db}	14.20 ± 0.19^{Dc}
b* (yellowness)	CON	8.80 ± 0.55^{Aa}	7.93 ± 0.16^{Aab}	7.41 ± 0.08^{Aab}	6.57 ± 0.19^{Abc}	5.73 ± 0.23^{Ac}
	CHI-GEL	8.83 ± 0.62^{Aa}	7.95 ± 0.41^{Aab}	7.44 ± 0.19^{Aab}	7.37 ± 0.09^{Bab}	6.64 ± 0.26^{Bb}
Coating x storage interaction (P value)	CHI-GEL CA 16 mM (0.25%)	8.87 ± 0.55^{Aa}	8.52 ± 0.27^{Aa}	8.21 ± 0.10^{Ba}	8.09 ± 0.11^{Ca}	7.56 ± 0.17^{Ca}
0.107	CHI-GEL CA 32 mM (0.5%)	8.91 ± 0.42^{Aa}	8.74 ± 0.13^{Aa}	8.29 ± 0.12^{Ba}	8.02 ± 0.06^{Ca}	8.03 ± 0.10^{Ca}
Loss of redness (%)	CON	0^{Aa}	14.84 ± 1.61^{Ab}	33.16 ± 1.56^{Ac}	55.45 ± 0.82^{Ad}	59.85 ± 2.52^{Ad}
	CHI-GEL	0^{Aa}	8.86 ± 0.79^{Bb}	23.60 ± 0.85^{Bc}	29.62 ± 0.25^{Bd}	40.41 ± 1.80^{Be}
Coating x storage interaction (P value)	CHI-GEL CA 16 mM (0.25%)	0^{Aa}	3.90 ± 0.71^{Ca}	11.54 ± 1.02^{Cb}	22.40 ± 1.62^{Cc}	29.84 ± 1.58^{Cc}
< 0.001	CHI-GEL CA 32 mM (0.5%)	0^{Aa}	3.95 ± 0.55^{Ca}	11.23 ± 0.95^{Cb}	14.16 ± 0.09^{Db}	20.34 ± 0.66^{Dc}

L* = lightness ranging from 0 = black to 100 = white, a* = green/red hue component, b* = yellow/blue hue component.
Error bars represent standard errors of the mean (3 independent replicates).
Analysis was conducted using two-way ANOVA with Tukey's test.
Significant difference ($P < 0.05$) were indicated with bars having different letters.
Capital letter represent comparison between treatments, while small letter represent comparison between days.

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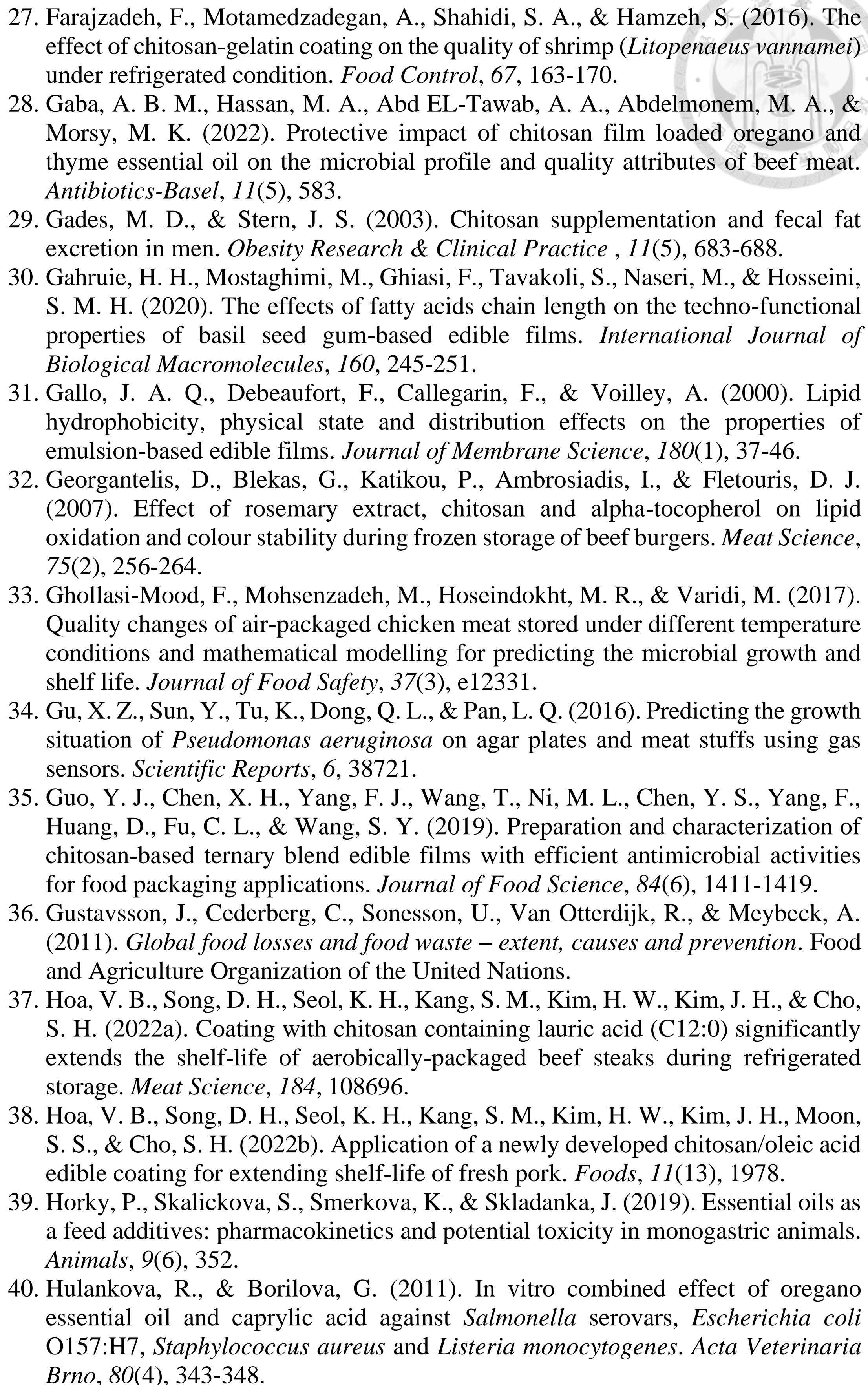

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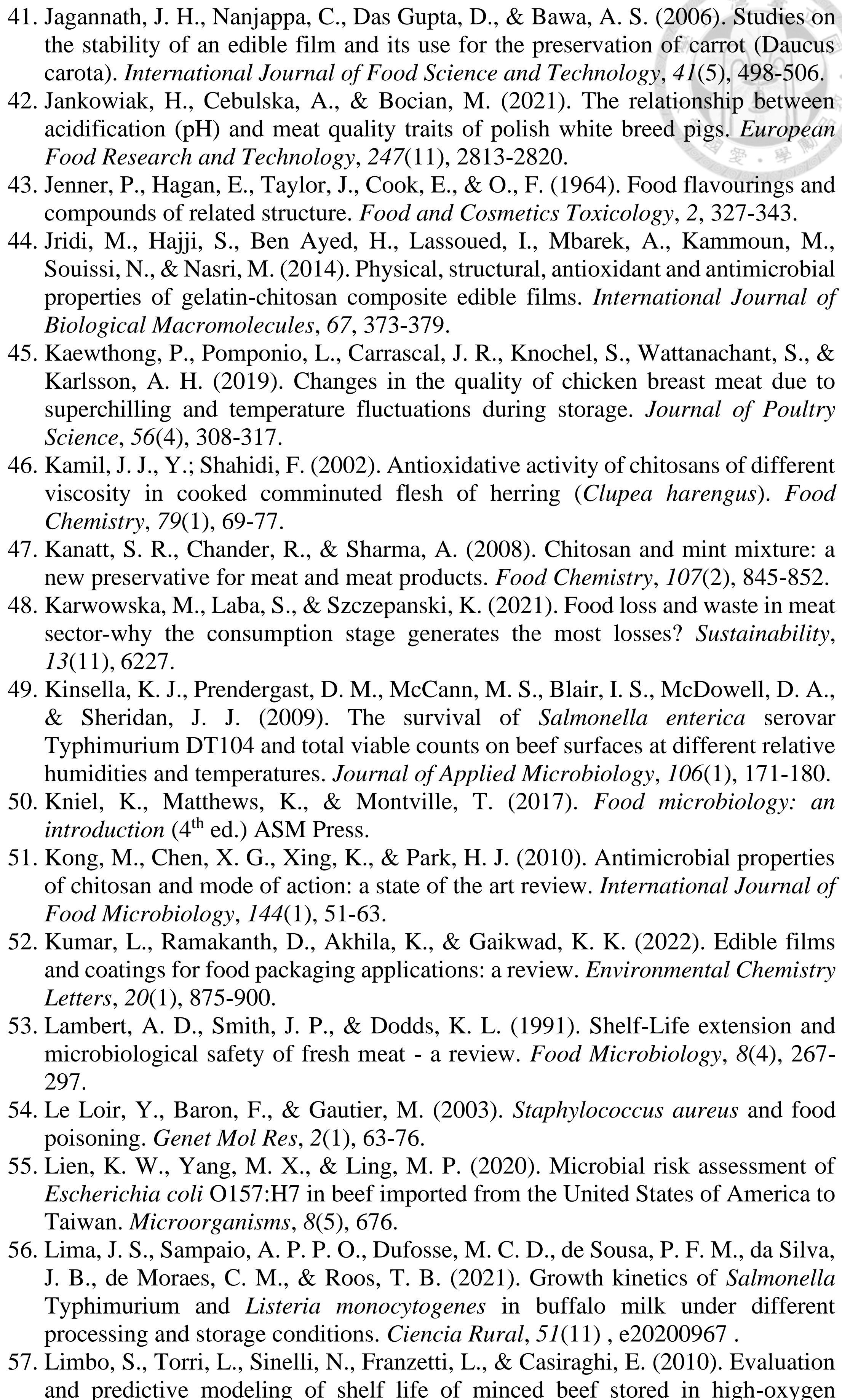

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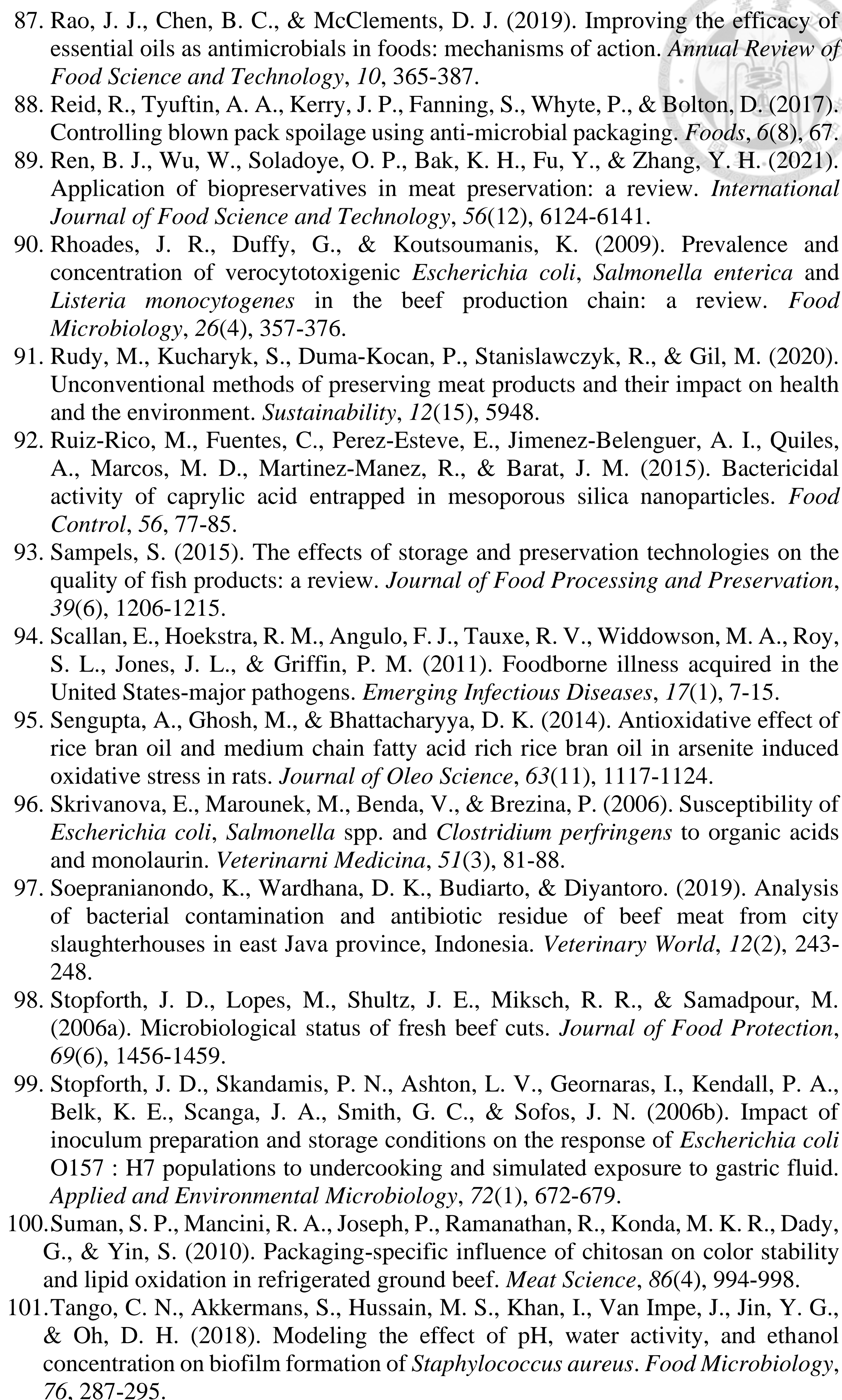

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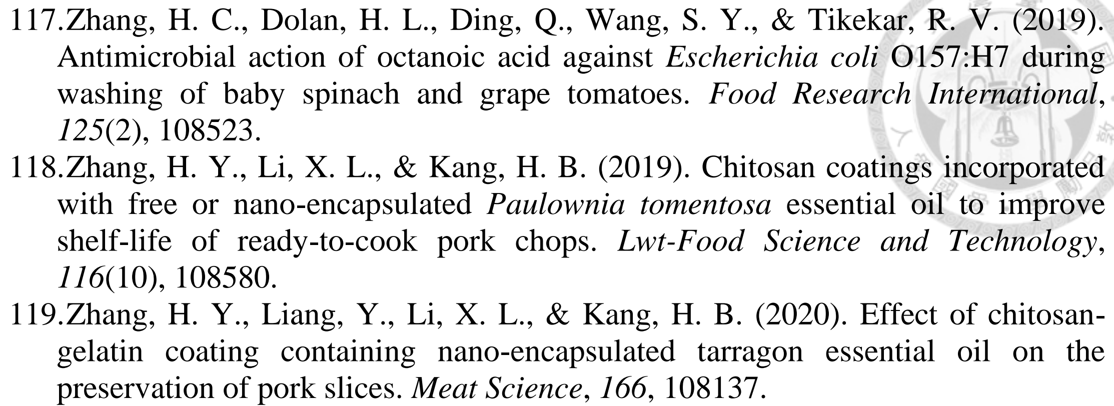

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Appendix

Table 14. Water activity of fresh beef after coating application.

Treatments	**a_w**
CON	0.989 ± 0.02^a
CHI-GEL	0.987 ± 0.02^a
CHI-GEL-CA 0.25%	0.988 ± 0.01^a
CHI-GEL-CA 0.5%	0.986 ± 0.01^a

Error bars represent standard errors of the mean (3 independent replicates).
The mean values denoted by different letters indicate significant difference ($P < 0.05$).
One-way ANOVA with Tukey's test at 95% confidence level.

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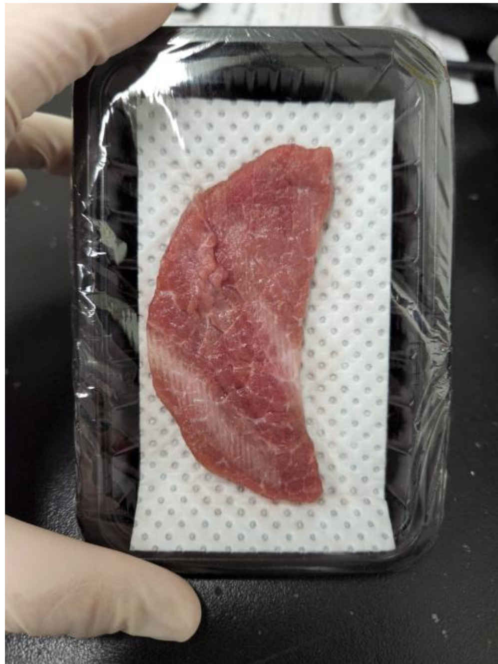

Figure 26. Image of aerobically packaged fresh beef (8 x 5 x 2 cm) with coating application.

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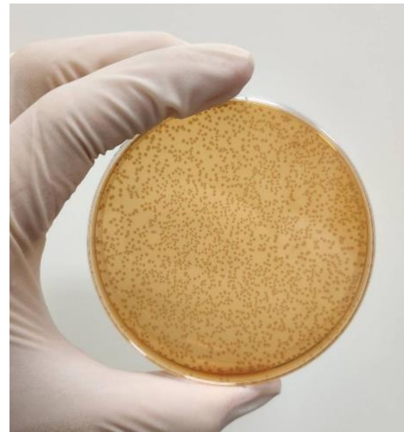
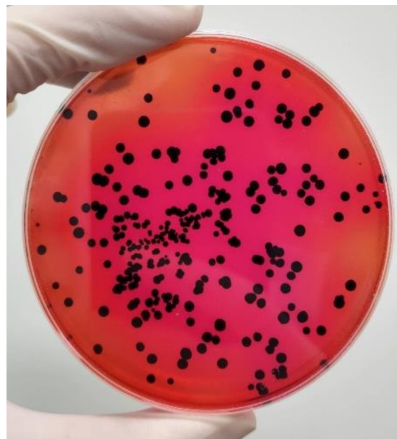
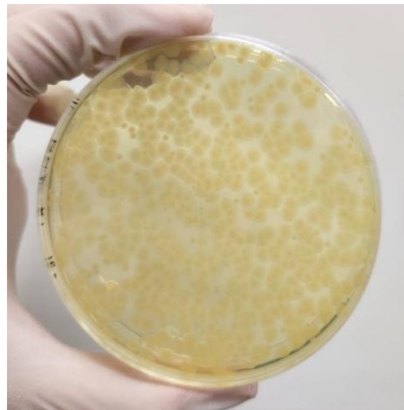

Figure 27. Pathogenic bacterial growth.

Staphylococcus aureus (top left), *Escherichia coli* O157:H7 (top right), *Salmonella* Typhimurium (bottom left), *Pseudomonas aeruginosa* (bottom right).

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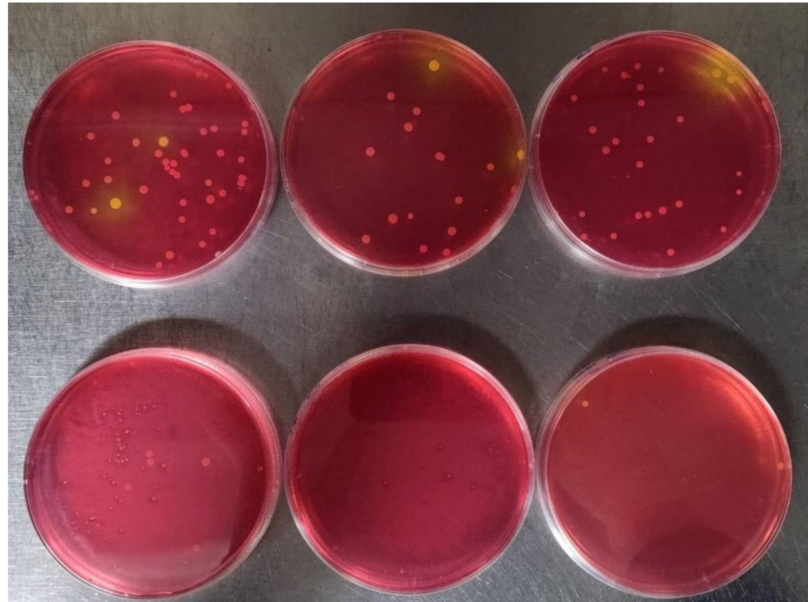

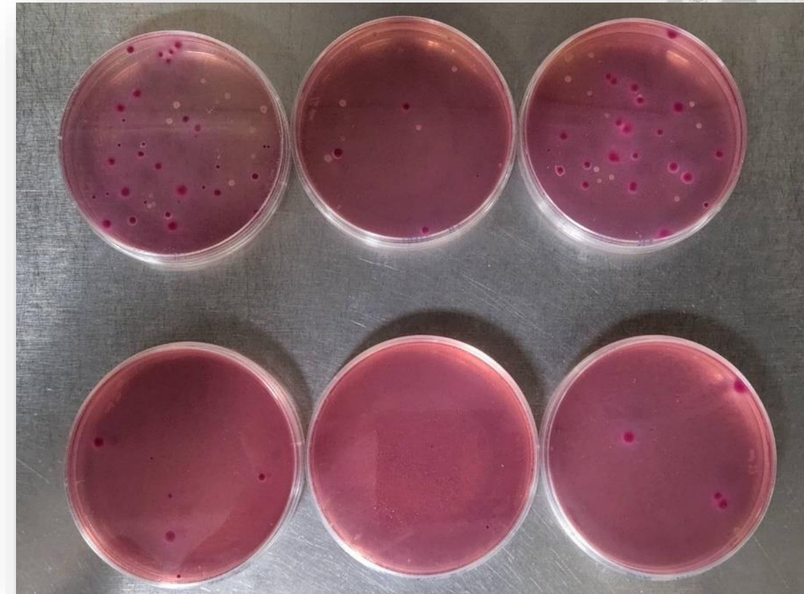

Figure 28. Initial pathogenic bacterial count in collected fresh beef (I).

Staphylococcus aureus count (left) and *Escherichia coli* O157:H7 count (right) in fresh beef; non-UV (top), UV (bottom).

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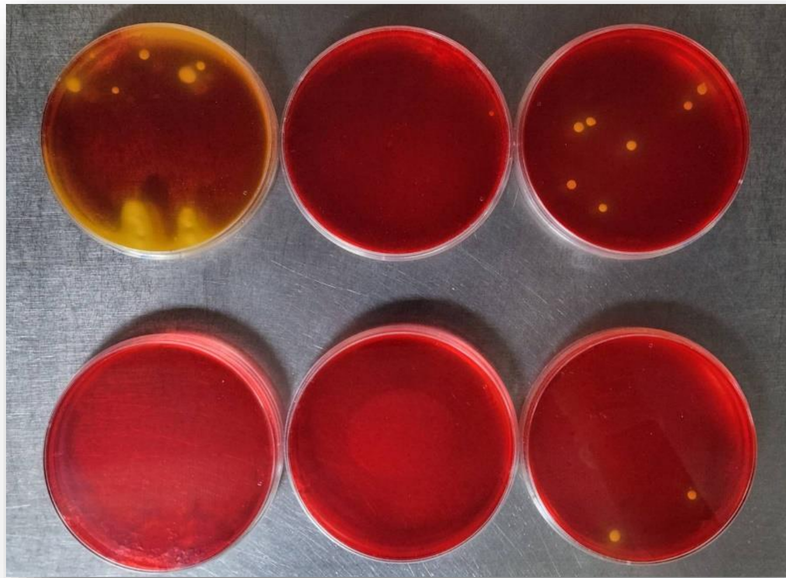

Figure 29. Initial pathogenic bacterial count in collected fresh beef (II).

Salmonella Typhimurium count (left) and *Pseudomonas aeruginosa* count (right) in fresh beef; non-UV (top), UV (bottom).

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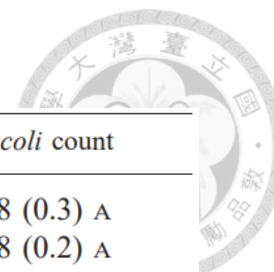

Table 15. Microbial profile of fresh beef cuts.

Type of cut	IMPS notation	No. of samples	Aerobic plate count	Total coliform count	*E. coli* count
Club ends		60	6.2 (1.1) A	1.3 (0.8) A	0.8 (0.3) A
Strip loins	175	52	5.9 (1.6) A	1.6 (1.2) A	0.8 (0.2) A
Top sirloin butt	184	113	5.9 (1.0) A	1.8 (1.3) A	0.9 (0.7) A
Bottom sirloin butt	185A	35	5.6 (1.3) A	1.1 (0.7) A	0.8 (0.2) A
Shoulder clod	114	117	5.0 (1.4) AB	1.4 (0.8) A	0.8 (0.3) A
Short loins	174	238	4.8 (1.5) B	1.2 (0.8) A	0.9 (0.5) A
Clod, top blade	114D	57	4.3 (1.4) B	1.2 (0.8) A	0.8 (0.4) A
Rib eye roll	112A	133	4.0 (1.6) B	1.3 (0.9) A	1.0 (0.7) A
Butt, butt, ball tip	185B	94	4.0 (1.4) B	1.5 (1.0) A	1.0 (0.6) A
Miscellaneous cuts		123	5.4 (1.7) AB	1.6 (1.1) A	1.0 (0.7) A
Total		1,022	5.0 (1.6)	1.4 (1.0)	0.9 (0.5)

[a] Values are mean log CFU per gram (standard deviation). Means within a column with different letters are statistically ($P < 0.05$) different. IMPS, International Meat Purchase Specifications.

Reprinted from Stopforth et al. (2006a).

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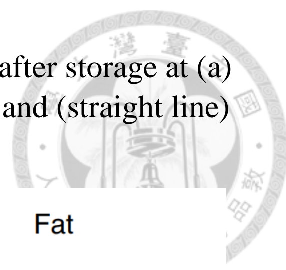

Table 16. The survival of total viable counts stored on beef surfaces after storage at (a) 5 °C and (b) 10 °C and under relative humidity of (broken line) 96% and (straight line) 75% determined using overlay counts.

(a)

Lean and fascia
Lean
Fat

Total viable counts ($\log_{10}$ CFU cm^{-2})

0 1 2 3 4 5 6

0 24 48 72 0 24 48 72 0 24 48 72

(b)

Lean and fascia
Lean
Fat

Total viable counts ($\log_{10}$ CFU cm^{-2})

0 1 2 3 4 5 6

0 24 48 72 0 24 48 72 0 24 48 72

Time (h)

Reprinted from Kinsella et al. (2009).

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Table 17. End products produced by major meat spoilage microorganisms.

Micro-organism	Substrates used for growth[b]		Major end products of metabolism	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Pseudomonas	Glucose[1] Amino acids[2] Lactic acid[3]	—	Slime Sulfides, esters, acids, amines	—
Acinetobacter / Moraxella	Amino acids[1] Lactic acid[2]	—	Esters, nitriles, oximes, sulfides	—
Alteromonas putrefaciens	Glucose[1] Amino acids[1,2] Lactic acid[3]	Glucose[1] Amino acids[1]	Volatile sulfides	H_2S
Brochothrix thermosphacta	Glucose[1] Amino acids[2] (glutamate only)	Glucose[1]	Acetic acid Acetoin Isovaleric acid Isobutyric acid	Lactic acid, volatile fatty acids
Enterobacter	Glucose[1] Glucose 6-phosphate[2] Amino acids[3] Lactic acid[4]	Glucose[1] Glucose 6-phosphate[2] Amino acids[3]	Sulfides Amines	Lactic acid, CO_2, H_2 H_2S Amines
Lactobacillus	—	Glucose[1] Amino acids[2]	—	Lactic acid, volatile fatty acids

[a] Adapted from Dainty et al. (1983), Gill (1986) and McMeekin (1982).
[b] The number in superscript indicates the order of utilization of this substrate.

Reprinted from Lambert et al. (1991).

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