**ANTIMICROBIAL EFFICACY
OF CARVACROL LOADED
ANTIMICROBIAL PACKAGING
IN FOOD SYSTEMS:
INFLUENCE OF THE FOOD MATRIX**

LI WANG

Propositions:

1. Including food properties in the design of antimicrobial packaging is essential for the design of effective antimicrobial packaging.
(this thesis)
2. The efficacy of antimicrobial packaging needs to be studied in real foods or model foods instead of on bacterial media.
(this thesis)
3. Broad learning produces opportunities, and deep learning ensures sustainable production.
4. Individual identity integration can only be achieved through a variety of social activities
(Kornhauser W, Politics of mass society, 2013, 102-113).
5. Mood management is as important as body weight management.
6. There is no perfect life, only perfect lies.

Propositions belonging to the thesis, entitled

Antimicrobial efficacy of carvacrol loaded antimicrobial packaging in food systems: Influence of the food matrix

Li Wang

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**Antimicrobial efficacy of carvacrol loaded
antimicrobial packaging in food systems:
Influence of the food matrix**

Li Wang

Thesis committee

Promotor

Prof. Dr V. Fogliano
Professor of Food Quality and Design
Wageningen University & Research

Co-promotor

Dr M. Dekker
Associate professor, Food Quality and Design Group
Wageningen University & Research

Dr J. Heising
Researcher, Food Quality and Design Group
Wageningen University & Research

Other members

Prof. Dr MH. Zwietering, Wageningen University & Research
Dr MAI. Schutyser, Wageningen University & Research
Prof. Dr M. Corredig, Aarhus University, Denmark
Prof. Dr F. Devlieghere, Ghent University, Belgium

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Antimicrobial efficacy of carvacrol loaded antimicrobial packaging in food systems: Influence of the food matrix

Li Wang

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Li Wang

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Chapter 1

General introduction

1.1 Food deterioration and spoilage

Food spoilage is considered as any change in a food product that causes it to become unacceptable to the consumer from a sensory point of view (Gram, Ravn, Rasch, Bruhn, Christensen, & Givskov, 2002). These changes can be physical changes (melting, freezing, and creaming), (bio)chemical changes (lipoid oxidation, enzymatic degradation, non-enzymatic browning, etc.) or appearance of off-flavors and off-odors occurring by microbial growth and metabolism in food (Kilcast & Subramaniam, 2011). Microbial spoilage is by far the most common cause of spoilage that can result in undesirable changes in appearance, texture, flavor and odor, which influences food quality depends on the type of food (Doyle, 2009). It is thus of great importance on reducing the quality loss of food by retarding the growth of microorganisms.

1.1.1 Factors affecting the microbial growth in food

The survival and growth of microorganisms in food can be influenced by several factors which can be categorized into intrinsic and extrinsic factors. Intrinsic factors refer to food itself (water activity, pH, composition, microstructure, etc.). Extrinsic factors relate to the environment where food production, transportation and storage are in processes, such as temperature and relative humidity.

Intrinsic factors. It is now widely agreed that water activity (a_w) and pH are mostly relevant for microbial growth and enzyme activity (Scott, 1957). Food products with an a_w of below 0.6 can be considered as having a low risk on deterioration from microbial growth (Kilcast et al., 2011). Regarding to pH, a pH level of 6.6-7.5 is the optimal range for the growth of most microorganisms (Jay, Loessner, & Golden, 2008). On the contrary, a pH below 4.0 is not in favor of growth of microorganisms (Jay, 1998).

Nutritional compositions and biological structures of foods are also directly related to the growth of microorganism. For example, meat and fish have nutritional values that favor multiplication of microorganisms. Therefore muscle foods are highly perishable which require careful protection against multiplication of microorganisms during storage (Yam & Lee, 2012). The microstructure of food is also of considerable influence in the absorption and diffusion of antimicrobial compounds in food, which directly determines to the activity of antimicrobials. *L. monocytogenes* grew faster in liquid medium (low fat milk) than in alginate gels because the fact that cells may become immobilized (Aspidou, Moschakis, Biliaderis, & Koutsoumanis,

2014). The influence of the microstructure on the growth of microorganism was also discussed by Antwi, Geeraerd, Vereecken, Jenné, Bernaerts, and Van Impe (2006) who found that an increase in firmness of gelatin results in a decrease in the growth rate of *Listeria innocua*.

Extrinsic factors. Temperature is one of the most important factors that influences the growth of microorganisms and impacts the shelf life of foods (McMeekin, Bowman, McQuestin, Mellefont, Ross, & Tamplin, 2008). Temperatures above the upper limit of optimal storage temperature results in a decrease in the growth rate of microorganisms. Oppositely, extreme low temperatures (<freezing temperatures) may damage the membrane function of microorganisms, which in turn inhibit their growth (Morita, 1975; Nedwell, 1999).

Microorganisms in different foods require different relative humidities for their optimal multiplication. For example, the growth of microorganisms on most fruits require an optimal relative humidity (RH) ranging from 85% to 95%, and the optimal RH for the growth of microorganisms in most vegetables is 90–98% (Petersen et al., 1999). Microbial growth reduction in onion and basil occurs when the relative humidity (RH) was lower than 60 percent (Keith, Harris, Hudson, & Griffiths, 1997).

1.1.2 Preventing or retarding microbiological spoilage

Food deterioration caused by microbiological spoilage can be retarded by monitoring and controlling the steps through the product chain starting from food manufacturing, processing, packaging, to transportation and storage (Kilcast et al., 2011). Strategies that are often applied in the food chain include improvement of hygiene in food processing, addition of chemicals such as preservatives and salt; control of temperature and water activity; application of heat treatments or new technologies such as high hydrostatic pressure, pulsed electric fields, ohmic heating, etc; or a combination of these with packaging that has potential to inhibit the microbial growth and extend the shelf life of foods (Kong & Singh, 2009; Pérez-Vicente et al., 2002; Ramirez-Suarez & Morrissey, 2006).

1.2 Antimicrobial packaging

Food packaging is essential for food preservation as it could protect food from outside damage and contamination, and retard product deterioration. (Coles, McDowell, & Kirwan, 2003). In general, food packaging could provide protection against chemical, physical and biological deterioration. A package can provide a barrier to prevent or reduce the deterioration caused by oxidation, moisture and light exposure. It can provide physical protection when the product was

exposed to an open environment. And the package can reduce biological deterioration when it provides protection to the food against exposure to microorganisms and other animals. (Marsh & Bugusu, 2007).

The development of packaging aiming at providing food protection has been well studied over recent decades (Ahvenainen, 2003; Souza & Fernando, 2016). Among these, antimicrobial packaging (AP) is able to retard or inhibit microbial growth by incorporation of specific antimicrobial agents in the package (Cha & Chinnan, 2004). It is a form of active packaging that can release those specific antimicrobial compounds to maintain the quality and safety of food products (Jideani & Vogt, 2016). AP can be classified into several types according to the way antimicrobial compounds are included in the package. These types include addition of sachets/pads containing volatile antimicrobial compounds into packages, incorporation of volatile/non-volatile antimicrobials directly into packaging materials, coating antimicrobials onto polymer surfaces, utilizing of polymers with incorporated antimicrobials, etc. (Appendini & Hotchkiss, 2002).

1.2.1 Mass transfer of antimicrobial compounds in a food-package system

In a food package, the growth of microorganisms can be retarded/inhibited either by direct contact between the food surface and an AP (by using polymers that are coated or loaded with non-volatile antimicrobials or polymers that are inherently antimicrobial) or by indirect contact, when the packaging releases volatile compounds into the packaging headspace (L. Wang, Dekker, Heising, Fogliano, & Berton-Carabin, 2020). AP prepared with a non-volatile antimicrobial agent requires a direct contact between a packaging film and the food matrix. In this food package, the active compound can diffuse and solubilize into the food surface (**Figure 1A**). Thereby the antimicrobial performance of AP is directly related by the mass transfer process of active compounds between the packaging film and the food contact surface. Compared with non-volatile antimicrobials loaded packaging, a packaging film containing volatile active agents is appropriate for applications where direct contact between the food and packaging does not occur. This package represents a packaging film/headspace/food system where the active compound is first released from the polymer film into the headspace of the package, through which it is absorbed into the food matrix (**Figure 1B**). It is thus necessary to consider the equilibrated distribution of the active compound between the headspace, packaging material and/or food to understand the activity of the AP (Quintavalla & Vicini, 2002). Unlike the direct contact food package system, the indirect contact may provide a more homogeneous

distribution of antimicrobials through the headspace, especially for those foods with irregular and heterogeneous surfaces (Inouye, Abe, Yamaguchi, & Asakura, 2003). The mass transfer process of antimicrobials between the packaging film, headspace and model/real food will be discussed in detail in **Chapter 2, 3 and 5**.

Figure 1. Food-package systems and the relative mass transport behavior of active agents, adapted from Han (2003).

1.2.2 Factors affecting the efficacy of antimicrobial packaging

To develop an effective AP, it is essential to know the factors that influence or determine the antimicrobial performance of the packaging film. It has been suggested from previous studies that the efficacy of AP depends on some common factors such as packaging polymers, storage environment (temperature and humidity), antimicrobial agents and food matrix (Ahvenainen, 2003).

Packaging polymers. Packaging polymers can be chosen from synthetic materials like polyethylene and polystyrene, or from natural biopolymers that are biodegradable such as starch, cellulose and proteins, or from biobased materials like polylactic acid (PLA) (Corrales, Fernández, & Han, 2014). Their efficacies are closely related to film mechanical property, barrier (gas and water) property and the way of incorporation/immobilization of antimicrobials (Silvestre, Duraccio, & Cimmino, 2011). Good mechanical property of packaging film ensures the integrity of the protected food under the influence of various stresses occurring during processing, transportation and application, which thus provides a protective barrier for food product from outside risks (Bastarrachea, Dhawan, & Sablani, 2011). An improved barrier (gas and water) property may maintain food quality by preventing the oxygen degradation of food

components, retarding microbial growth stimulated by moisture and retaining aroma/flavors (Sanchez-Garcia, Lopez-Rubio, & Lagaron, 2010). The incorporation/immobilization of antimicrobials influences the release and diffusion of active compounds through packaging polymers (da Silva Jr et al., 2016; Younes, Sellimi, Rinaudo, Jellouli, & Nasri, 2014). A rapid release of antimicrobials from the film polymers causes a fast consumption of the antimicrobials in the beginning of storage, after which the minimum concentration required for the inhibition of microbial growth is no longer reached in the food package. On the other hand, a too slow release of antimicrobials from the packaging film may result in the spoilage of foods (Uz & Altinkaya, 2011). It is therefore essential to use a proper way of encapsulation/immobilization of antimicrobials that can provide a controlled/extended release/migration of antimicrobials to ensure an effective inhibition of the microbial growth during storage.

Storage environments. It is commonly assumed that an increased storage temperature cause relaxation of crosslinked both hydrophobic and hydrophilic polymeric material and a high level of humidity loosen the crosslink of hydrophilic polymeric material. (Bierhalz, da Silva, & Kieckbusch, 2012; Kurek, Guinault, Voilley, Galić, & Debeaufort, 2014; Uz et al., 2011). This can promote the release/migration of active compounds from packaging film (L. Wang, Heising, Fogliano, & Dekker, 2020). However, the mechanical properties of a packaging film (especially for hydrophilic polymers) could be negatively influenced in a high humidity environment, while, at the same time, the growth of microorganism could be activated at high humidity and increased storage temperature. Therefore, a balance between the release of active compounds and microbial growth, both influenced by storage environment, should be carefully considered during the development of an AP. Detailed information about the effect of storage environment on the release of antimicrobials will be discussed in **Chapter 5**.

Antimicrobial agents. An important factor for the efficacy of an AP is the choice of the antimicrobial agent. The functionalities and applications of antimicrobial agents have been extensively studied for synthetic and natural compounds, such as metal ions, inorganic nanoparticles (e.g. titanium dioxide and zinc oxide), organic acids and salts, essential oils, plant extracts, enzyme (e.g. lysozyme), chitosan, bacteriocin and peptides, etc. (Sung et al., 2013; Wu, Wang, Hu, & Nerín, 2018). Currently, trends in antimicrobial food packaging focus more on natural antimicrobials instead of synthetic antimicrobials since some of the synthetic antimicrobials are controversial on their potential toxicity to humans, such as butylhydroxytoluene (BHT) or butylated hydroxyanisole (BHA) (Ramos, Jiménez, & Garrigós, 2016). In this study, carvacrol is used as active agent and incorporated into polylactic acid (PLA)

to prepare an AP. Carvacrol, a phenolic compound (**Figure 2**), is the major component (50-86%) of oregano and thyme essential oil (*Thymus* and *Origanum sp.*). (Kulisic, Radonic, Katalinic, & Milos, 2004).

Figure 2. Structure of carvacrol ($C_{10}H_{14}O$; mol. wt. 150.21).

Carvacrol is recognized as a safe food additive in the United States and Europe Union, and it is also commonly used as a flavor agent in sweets, beverages, pizzas and chewing gum (Nostro & Papalia, 2012; Ramos et al., 2016). Because of its hydrophobicity, carvacrol partitions and dissolves in the phospholipid bilayer of microorganisms where it aligns between the fatty acid chains. This results in a dramatic changes in the structure of the membrane that cause expansion and destabilization of the membrane (Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009). As a result of distortion of the membrane, the passive permeation of fluid is increased and the cell contents leak out through the cell membrane (Burt, 2004; Liolios et al., 2009). Carvacrol activity has been tested on various bacteria, mold and yeast, and the results showed that it exhibits promising antimicrobial, antiviral and antitoxigenic properties, which indicates a high potential to extend the shelf-life and protect the quality of foods (Burt, 2004; Guarda, Rubilar, Miltz, & Galotto, 2011; Vergis, Gokulakrishnan, Agarwal, & Kumar, 2015). In addition, carvacrol is highly volatile, which enables carvacrol to protect foods via indirect contact between the packaging film and food matrix (Bagamboula, Uyttendaele, & Debevere, 2004). **Table 1** summarizes several successful cases of application of oregano, thyme essential oil and carvacrol in food packaging for the preservation of foods. The research on the antimicrobial performance of carvacrol loaded PLA film will be discussed in more detail in **Chapter 2, 4 and 5**.

Table 1. An overview of successful applications of oregano, thyme essential oil and carvacrol incorporated in food packaging, adapted from Ataei, Azari, Hassan, Pingguan-Murphy, Yahya, and Muhamad (2020).

Antimicrobial agents	Food products	Applied film material	References
Cinnamon, winter savory, and oregano	Bologna and ham	Alginate	Oussalah, Caillet, Salmieri, Saucier, and Lacroix (2007)
Oregano	Pizza	Cellulosic resin	Botre, Soares, Espitia, Sousa, and Renhe (2010)
Oregano	Ripened sheep; cheese model	Polypropylene (PP) and polyethylene terephthalate (PET)	Otero, Becerril, Santos, Rodríguez-Calleja, Nerín, and García-López (2014)
Oregano and thyme	Fresh ground beef	Soy protein	Emiroglu, Yemis, Coskun, and Candogan (2010)
Oregano	Fresh beef	Whey protein isolate (WPI)	Zinoviadou, Koutsoumanis, and Biliaderis (2009)
Mixture of oregano, pimento berry, and lemongrass (mix A) and mixture of nutmeg, lemongrass, and citral (mix B)	Fresh broccoli	Methylcellulose (MC) and blend of polycaprolactone/alginate	Takala, Vu, Salmieri, Khan, and Lacroix (2013)
Oregano and bergamot	Formosa plum	Hydroxypropyl methylcellulose and limonene constituent EO	Choi, Singh, and Lee (2016)
Carvacrol	Fresh chicken	Chitosan/cyclodextrin	L. Higuera, Lopez-Carballo, Hernandez-Munoz, Catala, and Gavara (2014)
Carvacrol nanoemulsion	Green beans	Modified chitosan	Severino, Ferrari, Vu, Donsi, Salmieri, and Lacroix (2015)
Cinnamaldehyde and Carvacrol	Cooked chicken wrapped	Apple and tomato edible films	Du et al. (2012)
Carvacrol and cinnamaldehyde	Meat and poultry products	Pectin-based apple puree	Ravishankar, Zhu, Olsen, McHugh, and Friedman (2009)
Thymol	Meat	Zein	Aytac, Ipek, Durgun, Tekinay, and Uyar (2017)
Thymol	Fruits	Poly (DL-lactide-co-glycolide) (PLGA) nanofiber	Y. Zhang, Zhang, Zhu, Jiao, Shang, and Wen (2019)

Besides the strategies/factors mentioned above to improve/influence the antimicrobial efficacy of packaging, another important factor that should be considered in the design of AP is the food matrix (composition and microstructure). This factor has not been elucidated well in literature, and thus was studied in this project.

1.3 Impact of food matrix on the efficacy of antimicrobial packaging

Many successes of using new techniques of AP on the enhancement of food quality and safety have been achieved in food antimicrobial research in recent years. However, many of these techniques show a good performance on the inhibition of food pathogens and spoilage organisms in model media but fail to be effective when applied in complex foods. This reflects that the food matrices, including the food composition and microstructure, have a great influence on the antimicrobial activity of AP for food preservation. The issue of the less effective activity in real food matrices needs to be better understood to design and apply AP for food preservation.

1.3.1 Impact of food composition on the antimicrobial efficacy

Food usually consists of numerous components that determine the overall function, structure and behavior of a food system. The co-existent and interactions among components result in different physical properties that in turn influence the absorption and partitioning of active compounds into the food. Among many compounds in foods, two are highlighted below because they are generally believed as the key factors that influence the antimicrobial activities of antimicrobial agents.

Proteins. The influence of proteins on the antimicrobial activity depends on the pH of food matrices for food-package systems that use charged antimicrobials, such as chitosan. In chitosan-based AP, when the pH is lower than the iso-electric point (IEP) of protein, chitosan and proteins are both positively charged where the presence of protein may have less impact on the antimicrobial efficacy because of the restricted interaction between proteins in the food and antimicrobials. When the pH is increased above the IEP of the protein, the neutralization between the negatively charged protein and positively charged chitosan may occur, which consume the charge density of chitosan and further limit the interaction between the chitosan and the negatively charged microbial surfaces. Consequently, the antimicrobial efficacy of chitosan-based film is reduced in a food matrix with a pH above the IEP of the protein (Chung, Wang, Chen, & Li, 2003; Devlieghere, Vermeulen, & Debevere, 2004). Besides the charge

interactions, antimicrobial compounds having phenolic groups (e.g. carvacrol) may bind to the hydrophobic part of proteins. This reduces the access to microorganisms and lowers the antimicrobial activity of antimicrobial compounds. The negative impact of a high protein content on the activity of essential oils was reported by Tserennadmid et al. (2010) who found that the meat extract concentrations in meat extract mediums higher than 1.6% (w/v) protected the bacteria against the growth reducing effect of marjoram oil.

Lipids. The influence of lipids on the antimicrobial activity has been mainly found for hydrophobic antimicrobial compounds. For those hydrophobic antimicrobials, lipids influence the spatial rearrangement of active compounds because of the interaction between antimicrobials (e.g. lauric arginate, cloves, carvacrol, etc.) and lipids. Antimicrobial compounds that dissolve in lipids may decrease the concentration of antimicrobials in the aqueous phase where microorganisms are located and retard the diffusion of the antimicrobial compounds in foods with a high fat content, thereby reducing the antimicrobial efficacy (Hauerlandová, Lorencová, Buňka, Navrátil, Janečková, & Buňková, 2014). In addition, it has been reported that the fat globules in food could form a protective coat around bacteria, which protect them from contact with antimicrobial agents (Farbood, MacNeil, & Ostovar, 1976). The effect of lipids on the partitioning of antimicrobial compounds and activity of carvacrol-loaded PLA film will be discussed in **Chapter 2** and **5**.

1.3.2 Impact of food microstructure on the antimicrobial efficacy

Food microstructure can be defined as the organization and interaction of food components at different length scales resulting in particular structural interactions of different material phases that may explain properties and functionalities of foods (Aguilera, 2019; Karim, Rahman, Pham, & Fawzia, 2018). Multiple food components form a heterogeneous food microstructure that may contain simultaneously liquid, solid and gaseous phases that complicates the partitioning of active compounds. In reality, multicomponent and multidisperse food systems form colloidal structures that may be associated and clustered together again to form larger structures on the microscopic and macroscopic scale (Weiss, Loeffler, & Terjung, 2015). If antimicrobials are partitioning in these complex food systems, unexpected interference by food structure or consumption by food components may occur in the foods (Dickinson, 2015). This hinders/reduces the interaction between active compounds and bacteria and leads to an less effective activity of antimicrobials for food preservation. An example of the influence of the microstructure on the antimicrobials' functionality can be seen in a loss of antimicrobial activity

in rigid solid foods, due to limited diffusion of antimicrobial compounds. It has been commonly agreed that activity was less effective in solid foods than liquid foods (Ahmadi, 2017; Van Tassell, Ibarra-Sánchez, Hoepker, & Miller, 2017; Ye, Neetoo, & Chen, 2008). Several studies indicate the importance of food microstructure on the efficacy of antimicrobial packaging. Misiou, van Nassau, Lenz, and Vogel (2018) applied the combination strategies of endolysin PlyP825, an enzyme which can specifically recognize the host cell-wall and cleave the peptidoglycan to digest the bacterial cell-wall for release of phage progeny during the lytic cycle (Zhou et al., 2020), and high hydrostatic pressure (HHP) processing on inhibition of *Listeria monocytogenes* in several *Listeria*-critical food products (i.e. milk, mozzarella and smoked salmon). The results showed that the combined effect of 3.4 µg/mL PlyP825 and 400 MPa was sufficient to reduce *L. monocytogenes* to below the detection limit in 89% (milk) or 78% (mozzarella) of the samples during storage. On the contrary, no significant effect on bacteria inhibition was detected in smoked salmon. The reason of the lack of antimicrobial efficacy in smoked salmon may be related to the weak diffusion of endolysin PlyP825 in its rigid microstructure. Similarly, *Listeria* phage A511 showed an unsubstantial effect on the inhibition of the growth of *L. monocytogenes* in a cooked ready-to-eat meat product when it was added in the meat. Only when it was in direct contact with the contaminated meat surface, the reduction of *L. monocytogenes* numbers can be found. The loss of activity may attribute to the immobilization of *Listeria* phage within the meat matrix (Ahmadi, 2017). Zinno, Devirgiliis, Ercolini, Ongeng, and Mauriello (2014) studied the antimicrobial effect of Phage P22 on chicken breast and chicken mince. Phage P22 was inoculated in the surface of the chicken breast, and mixed with chicken mince. Interestingly, the phage P22 affected the cell load of chicken breast immediately after the treatment with a significant log reduction of 0.7 log, however, only 0.07 log reduction was found in the treated chicken mince samples. Their results also highlight the importance of considering the physical topology of the food matrix on the activity of antimicrobial compounds. Unfortunately, the mechanisms of the less effective antimicrobial activity due to the influence of microstructure of foods have been largely unexplored because of the highly complex food structures, which requires an in-depth exploration to understand the mechanisms behind. Due to limitations in current understanding, studies (**Chapter 2-4**) in this thesis were carried out to attain further knowledge on the impact of food microstructure on the antimicrobial efficacy of AP.

1.4 Aim and outline of this thesis

This thesis aims to increase the understanding of how the food matrix (food components and microstructures) impacts antimicrobial agent absorption and diffusion in several model/real foods, and how the partitioning/diffusion of the antimicrobial compounds influences the antimicrobial activity of AP. This knowledge can then be used to improve the antimicrobial efficacy by designing an optimal AP tailored to the properties of the specific food product.

A graphical representation of the thesis outline is shown in **Figure 3**. In **Chapter 2**, the release of carvacrol from a PLA film towards liquid model food systems was investigated. The carvacrol partitioning in liquid samples was studied in three types of fine emulsions, one coarse emulsion (with large oil droplets) and a non-homogenized sample consisting of separate layers of aqueous phase and oil. The aim of this study was to investigate the impact of oil droplet size of emulsions on carvacrol partitioning and antimicrobial activity in emulsion-based food products. The study in **Chapter 3** focusses on another important model food system, being hydrogels. Salt-induced gelation was used to prepare hydrogels that are physically different. The colloidal system prepared with protein-polysaccharide complex was completely characterized for its physical properties to understand the effect of the microstructure of semi-solid/solid food models on the absorption of antimicrobial compounds. The relative information of the factor in the hydrogel microstructure that may influence the antimicrobial absorption/diffusion is further investigated in **Chapter 4**. Here, carvacrol diffusion and its efficacy was tested in hydrogels from the surface to 30-mm depth. Carvacrol concentrations in different layers of hydrogels (with a same height of 10mm) were linked to its antimicrobial performance on the inhibition of growth of *Pseudomonas fluorescens* to clearly address the question whether the lack of antimicrobial activity attributes to the activity loss by interaction with food components or insufficient antimicrobial concentrations. A more applied study of the activity of carvacrol loaded PLA film was performed in real foods as described in **Chapter 5**. The effects of the fat contents of meats and the storage environments on the carvacrol release/partitioning in the food-package system were investigated in this chapter. The antimicrobial activity of packaging film was studied in ground lean/fat meats and the shelf life of protected meats were predicted with the help of mathematic modelling. In the end, a general discussion of the results of this thesis is presented in **Chapter 6**.

Figure 3. Graphical outline of the chapters of this thesis. The size of the yellow, red and white dots/ irregularities corresponds to the size of the oil droplets, proteins and pores, respectively. The amount of yellow dots correspond to the amount of oil in the food matrix.

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Chapter 2

Carvacrol release from PLA to a model food emulsion: Impact of oil droplet size

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Abstract

The effect of food structure on the release of a volatile antimicrobial from an active packaging (AP) was investigated by measuring the migration of carvacrol from a polylactic acid (PLA) film to a model food system, namely, an oil-in-water (O/W) emulsion. We aimed to understand the influence of the oil droplet size on the kinetics of carvacrol migration from the PLA film to the emulsions, on its final partitioning and antimicrobial activity. Five model systems were prepared: they had the same composition, i.e., an oil fraction of 20 wt% and an aqueous phase containing 1.5 wt % whey protein isolate, but different structures.

Emulsions with the smallest oil droplets ($d_{3,2} = 0.27 \mu\text{m}$) absorbed a higher amount of carvacrol than emulsions with large oil droplets ($d_{3,2} = 0.34 \mu\text{m}$ and $d_{3,2} = 0.51 \mu\text{m}$). Despite the higher overall carvacrol concentration, inhibition of bacterial growth was less effective in emulsions with the smallest droplet ($d_{3,2} = 0.27 \mu\text{m}$). This can be explained by the highest log K of carvacrol in this emulsion indicating that carvacrol partitioned more into the oil droplet phase than in the continuous phase.

The current study suggests that the spatial distribution of carvacrol in the emulsion determines its actual antimicrobial effect. The combined findings of antimicrobial distribution and activity highlight the need for tailoring active packaging systems based on the physical characteristics of multiphase food matrices.

Keywords: Carvacrol; Antimicrobial packaging; Emulsion; Oil droplet size; Partitioning.

2.1. Introduction

Reduction of food waste is necessary to combat hunger, raise income and improve food security in developing or low-income countries (FAO, 2011). Food loss through microbial spoilage is an important concern to both consumers and food industries, which has resulted in an increased interest in antimicrobial packaging (AP) to increase the shelf-life and safety of foods. AP is a form of active food packaging, which is developed by incorporating specific active agents into packaging films. AP can reduce the growth rate and extend the lag phase of target microorganisms in packed foods, thereby prolonging the shelf-life of foods (Mihindukulasuriya & Lim, 2014). The growth of microorganisms can be inhibited either by direct contact between the food product and a packaging film loaded with non-volatile antimicrobials, or by indirect contact, when the packaging releases volatile compounds in the headspace. In the latter case, evaporation or equilibrated distribution of the antimicrobials between the headspace, packaging material and/or food has to be considered to understand the activity of the antimicrobials (Quintavalla et al., 2002).

Many food systems contain simultaneously liquid, solid and gaseous phases which can form a broad range of colloidal structures. Therefore, food matrices have very different physical properties, which, in turn, may influence the partitioning and mass transport of antimicrobials added in a food packaging system (Weiss et al., 2015). In order to develop an effective AP, several factors should be considered such as the intrinsic properties of the polymer films, the mass transfer coefficients of active substances, the storage conditions and finally the food matrix characteristics (Han, 2003). In the past decades, many AP products were developed combining novel plastic materials and new antimicrobial compounds (Sung et al., 2013). The strategies to control the release and migration of active compounds from packaging films to either model or real foods were investigated in all details (de Azeredo, 2013; Jideani et al., 2016). However, the influence of the food matrix itself (composition and physical structure) on the efficiency of AP is largely unexplored.

Many food products are oil-in water (O/W) emulsions, which consist of lipid droplets dispersed in an aqueous medium, e.g. milk, yogurt or salad dressing. They may have a broad range of droplet sizes, from submicron to around 100 μm (David Julian McClements, 2015). Droplet size plays an important role for the stability, rheology, appearance, color and texture of emulsions (Becher, 2001; Jafari, Assadpoor, He, & Bhandari, 2008; D Julian McClements, 2005). Flavour release studies investigated the effect of droplet size on volatile compounds

release from emulsions (de Roos, 2003; Doi, Wang, & McClements, 2019; Sootittantawat, Yoshii, Furuta, Ohkawara, & Linko, 2003). Benjamin, Silcock, Leus, and Everett (2012) found an increase in volatile organic compound (VOC) release from unstable emulsions (aggregated droplets) at pH 5, compared to stable emulsions (finely distributed droplets) at the same pH. These studies have shown that flavour retention is influenced by the amount of oil-water interfacial area present in emulsions. This is relevant to the present study, as flavour release and retention from emulsions is simply the opposite process of the absorption and partitioning of volatile antimicrobials from packaging materials into emulsions. Antimicrobial distribution in a food matrix is important as it can be hypothesized that the location of antimicrobial molecules determines their efficiency. However, the effect of the oil droplet size on the antimicrobial partitioning in a food package containing emulsions has not yet been reported.

In this work, we used carvacrol as model antimicrobial agents as it is a volatile compound, which has proved useful to prevent the growth of bacteria (Periago, Delgado, Fernández, & Palop, 2004), mold (Daferera, Ziogas, & Polissiou, 2003) and yeast (Arora & Kaur, 1999). Carvacrol has recently been incorporated in methyl cellulose (MC) (Tunc, Duman, & Polat, 2016), wheat gluten (Mascheroni, Chalier, Gontard, & Gastaldi, 2010), and sodium-calcium caseinate films (Altan, Aytac, & Uyar, 2018). As packaging material, polylactic acid (PLA) was chosen: PLA is a renewable and biodegradable source that holds great potential for packaging applications due to its good mechanical and barrier properties (Mlalila, Hilonga, Swai, Devlieghere, & Ragaert, 2018). Carvacrol-loaded PLA films, besides being a good model, also have a good potential to be used on large scale in food preservation (Ahmed, Hiremath, & Jacob, 2016).

In this work, we investigated the migration of carvacrol from a PLA film to a model multiphase food system (emulsions), occurring via a surrounding air phase (headspace). Sunflower O/W emulsions with a fixed fraction of oil but various oil droplet sizes were considered as model foods, and exposed to indirect contact with carvacrol-loaded PLA films. The aims of this work were to unravel the influence of the oil droplet size on carvacrol partitioning between the available phases, and to further examine the impact of the antimicrobial partitioning on its efficiency at the bacterial growth inhibition in the model food matrix.

2.2. Materials and methods

2.2.1. Materials

PLA (4043D Ingeo™) was kindly supplied by the Food & Biobased Research institute of Wageningen University and Research (Netherlands). Carvacrol ($\geq 98\%$), dichloromethane (DCM), hexane and 2-propanol were purchased from Sigma Aldrich (Pty. Ltd., Netherlands). Whey protein isolate (WPI, $\geq 95\%$ protein) was obtained from Davisco (Lancy, Switzerland). Sunflower oil was purchased from a local supermarket and used without further purification. Plate count agar (PCA) was purchased from Merck (Schiphol-Rijk, Netherlands). Peptone physiological salt solution (PFZ) was purchased from Tritium Microbiologie BV (Eindhoven, Netherlands).

2.2.2. Preparation of the carvacrol-loaded PLA film

The solvent-casting method was used to prepare a carvacrol-loaded PLA film (CAR-PLA) (Rhim, Mohanty, Singh, & Ng, 2006). Briefly, PLA pellets (4 g) were completely dissolved in DCM (25 mL DCM/g of PLA) by gentle stirring at 20 °C for 6 hours. One gram of carvacrol was then added to the solution and stirred for 1 hour at room temperature (20 °C). The carvacrol/PLA/DCM solution was sonicated with an ultra-sonicator (HBM Machines, Moordrecht, the Netherlands) at 40 kHz for 30 minutes to further homogenize the solution. Subsequently, 20 mL of the solution was cast on a glass petri dish (size 100×15 mm) and allowed to dry at room temperature overnight. The film was peeled from the glass petri dish, and was stored at 4 °C in a sealed glass container.

2.2.3. Preparation and characterization of liquid samples

Five model liquid samples were prepared, including three types of fine emulsions (with very small, small, and small-medium oil droplets, abbreviated as XS, S, and M, respectively), one coarse emulsion (with large oil droplets, abbreviated as L) and a non-homogenized sample, consisting of separate layers of aqueous phase and oil (**Figure 1B**). All the samples contained the same concentration of emulsifier (WPI) and sunflower oil, they thus differed only in the oil droplet size, and accordingly, in the amount of oil-water interfacial area.

Figure 1. Scheme illustration of the set-up used to evaluate carvacrol partitioning between a packaging film and a liquid food matrix, separated by a gas phase (A), and scheme of the different liquid samples (B).

2.2.3.1. Preparation of emulsions

The fine emulsions (XS, S, and M) were prepared by high pressure homogenization. A whey protein solution was prepared by dispersing 1.5 g of whey protein isolate into 80 mL ultrapure water (pH 6.7). The mixture was dissolved under slow magnetic stirring for 2 h. It was then kept overnight at 4 °C to allow for complete hydration. Sunflower oil was then added to the protein solution to form an oil-in-water emulsion with 20 wt% sunflower oil. The mixture was pre-emulsified by using a rotor-stator homogenizer (Ultra-Turrax, IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 11,000 rpm for 1 min. The obtained pre-emulsion was then passed through a high pressure homogenizer (Microfluidizer Processor MF 110Y with Y-shaped interaction chamber (F12Y; min. internal channel: 75 µm), Microfluidics, Newton, Massachusetts, USA) at 1×10⁴, 4×10⁴ or 8×10⁴ kPa (for M, S, and XS emulsions, respectively) for 5 cycles. For the coarse emulsion (referred to as L), we processed the pre-emulsion with a colloid mill (Homogenizer MagicLab, IKA®-Werke GmbH & Co. KG, Staufen, Germany) equipped with a MK module at 26,000 min⁻¹ for 5 minutes.

2.2.3.2 Physical characterization of emulsions

Particle size distribution. The droplet size and size distribution of emulsions were analyzed by laser static light scattering (Malvern Mastersizer 3000, Malvern Instruments Ltd, Malvern, Worcestershire, UK) after 3 hours (t=3 h) and 7 days (t=7 d) storage. The refractive indices (RI) were 1.46 (oil) and 1.33 (water) with an adsorption index of 0.01. The average droplet size was

expressed as the Sauter diameter ($d_{3,2}$). Emulsions were diluted 10 times in 1 wt % sodium dodecylsulfate (SDS) solution to separate any flocculated oil droplets.

Creaming stability of the coarse emulsion. The kinetics of cream layer formation was determined in the coarse emulsion (for the fine emulsions, no noticeable creaming was detected over the timescale of the experiments). The height of the creamed layer was recorded during 7 days incubation in steady conditions at 20 °C. Equation 1 was used to fit the increase in height of the cream layer as a function of storage time. The maximum cream height in emulsion is noted H_{max} (mm). D_t (day) is the storage time needed to achieve 50% of the maximum cream height.

$$H = \frac{H_{max} * t}{D_t + t} \quad (1)$$

Whey protein surface load. Emulsion samples were centrifuged at 21191×g for 2 hours to separate the creamed and serum phases. Protein content in the serum was quantified by the bicinchoninic acid (BCA) assay (Pierce BCA protein assay, Thermo Fisher Scientific Inc., Rotterdam, Netherlands). The protein surface load (Γ) was determined by considering the initial protein concentration in the starting WPI solution (C_i), the free protein concentration in the serum phase (C_s), the dispersed phase volume fraction (ϕ) and the mean particle diameter ($d_{3,2}$) (Eq. 2) (Berton, Genot, & Ropers, 2011; David Julian McClements, 2004):

$$\Gamma = (C_i - C_s) * d_{3,2} / 6\phi \quad (2)$$

The total interfacial area (S) was obtained following Berton, Ropers, Viau, and Genot (2011) (Eq. 3):

$$S = S_{droplet} * m_{oil} / (V_{droplet} * \rho) \quad (3)$$

In which $S_{droplet}$ represents the surface area of one fat droplet (obtained from the $d_{3,2}$), m_{oil} is the mass of oil in the same emulsion, $V_{droplet}$ is the volume of one oil droplet (obtained from the $d_{3,2}$) and ρ is the density of oil ($\rho = 0.924$ for sunflower oil according to Borges, Díaz, Alvarez-Galván, and Brito (2011)).

2.2.4. Quantification of carvacrol in the different phases of the system

2.2.4.1. Calibration

To quantify the amount of carvacrol in the headspace of the vial and in the liquid sample, a stock solution of carvacrol in n-hexane:isopropanol (3:1) was prepared at a concentration of 2.0 mg/mL. Five concentrations between 0.4 mg/mL and 2.0 mg/mL were prepared by diluting the stock solution. These calibration solutions were then injected and analyzed by GC-FID (see next section). Within this concentration range, a linear calibration curve was obtained with a coefficient of correlation of 0.9996.

2.2.4.2. Headspace gas chromatography (HS-GC-FID)

The carvacrol concentration in the headspace of the vials was measured using gas chromatography coupled with a flame ionization detector (GC-FID) (Thermo Scientific, Waltham, Massachusetts, United States). The method to directly collect carvacrol from the headspace of glass vials was adapted from Laura Higuera, López-Carballo, Hernández-Muñoz, Catalá, and Gavara (2014) with some modifications. Briefly, a 2.5-mL syringe (Hamilton, Bonaduz, Switzerland) was used to pierce the rubber lid of glass vial, then collect 500 μ L headspace which were immediately injected into the GC-FID injection port. The amount of carvacrol present in the headspace was calculated from the aforementioned external calibration curve.

The followings conditions were used to operate GC-FID: the injection temperature was set at 250 °C. The column oven temperature was initially set to 60 °C, then increased to 250 °C with a rate of 15 °C min⁻¹. Helium was used as the carrier gas, running at a constant flow of 2.0 mL min⁻¹. The FID was operated with a hydrogen flow of 35 mL min⁻¹ and an air flow of 300 mL min⁻¹. The total running time was 25.25 min.

2.2.4.3. Extraction and quantification of carvacrol in liquid samples

Carvacrol extraction from liquid samples was optimised based on the method of (Lu, Pickova, Vázquez-Gutiérrez, & Langton, 2018). To extract carvacrol from the whole emulsion, 4 mL solvent (n-hexane-isopropanol, 3:1, v/v) were added to 2 mL sample. For quantification of carvacrol in the creamed and serum phases, the emulsions were first centrifuged for 2 hours at 21191×g to separate the phase of emulsion, as described above. Three or one millilitres extraction solvent (hexane:isopropanol, 3:1 v/v) were then added to the cream or serum phase,

respectively, to extract carvacrol from each phase. The mixtures were vortexed for 1 minute at room temperature (3,000 rpm), followed by heating in a heating block at 60 °C for 30 minutes. Then the organic phase (10 µL) was injected into GC-FID for quantification of the carvacrol concentration. A high extraction yield (>96%) was achieved by using this extraction method, which ensured a complete extraction of carvacrol from emulsion samples.

2.2.4.4. Determination of the partition coefficient of carvacrol between lipid and aqueous phases

The partition coefficient (log K) of carvacrol was calculated as (Eq. 4):

$$\text{Log } K_{\text{cream/aqueous}} = \log \frac{C_{\text{cream}}}{C_{\text{aqueous}}} \quad (4)$$

Where C_{cream} is the concentration of carvacrol in the creamed phase (mg/mL), and C_{aqueous} is the concentration of carvacrol in aqueous phase of samples (mg/mL).

2.2.5. Microbiological analyses of emulsions

2.2.5.1. Microbial strain

The strain *Pseudomonas fluorescens* ATCC 13525 was provided by the Food Microbiology Group of Wageningen University (the Netherlands). It was cultured in nutrient broth (Merck BV, Schiphol-Rijk, the Netherlands) at 30 °C for 48 h from stock culture.

2.2.5.2. Thermal treatment of emulsions

Fine emulsions were sterilized prior to the antimicrobial test by applying a thermal treatment at 72 °C for 10 min. After thermal treatment, samples were immediately cooled in an icebox.

2.2.5.3. Preparation of samples

After thermal treatment, liquid samples were inoculated with *Pseudomonas fluorescens* to obtain a final concentration of 10^4 CFU/mL. Fine emulsions were divided into three groups according to the size of oil droplet (XS, S and M). Each group was then subdivided into antimicrobial group and control group. In each antimicrobial group, an emulsion aliquot (2 mL) was stored with the CAR-PLA film (0.02 g in each vial; **Figure 1A**). All samples were prepared in individual sterilized glass vials for each periodical measurement (0, 0.5, 1, 1.5, 3, 5, and 7 days), and stored at 20 °C.

2.2.5.4. Enumeration of bacteria

The enumeration of *Pseudomonas fluorescens* was performed by using the pour plate method. At each sampling day, bacterial population was determined by serially diluting 1 mL of emulsion in 9 mL of peptone physiological salt solution (PFZ) and plating 100 μ L on plate count agar (PCA). The microplates were cultured at 30 °C for 24 h before counting the number of bacterial colonies.

2.2.5.5 Microbial growth modelling

A modification of the model developed by Gompertz as used to fit the bacterial growth curves. This modified Gompertz equation (Eq. 5) described the total viable count of bacteria in samples as a function of time (Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990).

$$\ln \frac{N}{N_0} = A_s * \exp \left(- \exp \left(\frac{\mu_{\max} e}{A_s} (\lambda - t) + 1 \right) \right) \quad (5)$$

Herein, A_s is the natural logarithm of the asymptotic value of the relative population size (log (CFU/mL)), $\mu_{\max}$ is the maximum specific growth rate (day⁻¹), λ is the duration of the lag phase (day), t is the storage time (day) and N/N_0 represents the ratio of the microbial cell density (CFU mL⁻¹) to the initial microbial cell density (CFU mL⁻¹).

2.2.6. Experimental design

The carvacrol partitioning and antimicrobial activity were studied in a closed set-up designed to mimic a situation where migration of volatile molecules may occur between a packaging film and a food matrix separated by a gas phase (**Figure 1A**). This system thus consisted of a 20-mL VerexTM headspace vial containing 2 mL of liquid sample, and a piece of CAR-PLA film (0.020 ± 0.001 g) mounted on a pipette tip to prevent direct contact with the liquid sample. Vials were sealed by a rubber lid to prevent gas leaking. Several vials with the same weight of packaging film and same volume of sample were prepared for daily measurement of carvacrol absorption in the food matrix. All samples were stored at room temperature (20 °C).

2.2.7. Statistical analysis

All experiments were performed in independent duplicates, and measurements were performed in triplicate. Data were expressed as mean $\pm$ standard deviation of triplicate measurements. For the statistical analysis, the IBM SPSS Statistics software, version 23.0 for Windows (Armonk,

NY: IBM Corp) was used. One-way analysis of variance (ANOVA), with post-hoc pairwise analysis by the Tukey test was used to evaluate the significant differences between samples. A significant difference between results was determined when $p < 0.05$. The parameters of Eq. (1) and (5) were estimated with the lowest mean square error (MSE), where the maximum cream height in emulsion H_{max} (mm), the storage time needed to achieve 50% of the maximum cream height D_t (day), maximum growth rate μ (day^{-1}), lag phase duration λ (day) and A_s (log (CFU/mL)) were obtained.

2.3. Results and discussion

2.3.1. Physical stability of emulsions

The particle size distribution of emulsions shortly after preparation ($t=3$ h) and after 7 days storage is shown in **Figure 2**. The size distributions for the three fine emulsions were unimodal with an average diameter ($d_{3,2}$) ranging from 0.27-0.51 μm . They remained constant over the timescale of the experiment (**Figure 2B**), indicating a good physical stability of those emulsions. For the emulsion produced by colloid mill homogenization, a larger mean particle size was obtained ($d_{3,2} \sim 2.75$ μm). The droplet size distribution of this coarse emulsion, shortly after preparation, shows two distinct peaks, revealing a large polydispersity. After 7 days storage, a shoulder was visible around 50-100 μm and the droplets at this range of scale did not disappeared in the 1 wt% of SDS solution, which means that some coalescence occurred in the coarse emulsion.

Figure 2. Particle size distribution of oil droplets in the emulsions at (A) $t=3$ h and (B) $t=7$ d.

Figure 3 shows the kinetics of the cream layer formation in the coarse emulsion during storage. Fast creaming was observed within the first day following sample preparation, and 50% of the maximum height of the final cream layer were reached after 8.5 h. This is due to the propensity of large emulsion droplets to experience fast gravitational separation in dilute media, according to the Stokes' law (Stokes, 1851). After 2 days storage, the height of the cream layer increased only minimally, mainly owing to smaller oil droplets. This substantial creaming phenomenon in the coarse emulsion had relevant implications for our study. First, creaming brings droplets in close proximity and may promote droplet coalescence, hence altering the droplet size. Second, it leads to macroscopic separation between the serum and creamed phases, and hence to structural heterogeneity of the sample, which may have implications regarding how and how fast molecules partition between the available environments, which will be discussed in more depth later.

Figure 3. Kinetics of creamed layer formation in the coarse emulsion during storage. The black solid line represents the data fit using equation (1). Error bars represent the standard deviation of 3 measurements.

The partitioning of proteins within the emulsion phases was also determined (**Table 1**). In all emulsions, a large fraction of the total protein used remained non-adsorbed in the aqueous phase (from 64% for the XS-sized emulsion, to 89% for the L-sized emulsion). The protein surface

load ranged between 0.92 mg/m^2 and 1.52 mg/m^2 for the fine emulsions. A significantly larger value was measured in the coarse emulsion ($\Gamma = 3.13 \text{ mg/m}^2$) ($p < 0.05$), which could be a result of the different homogenization procedure used between fine and coarse emulsions. These values are in line with data reported in other publications (Berton-Carabin & Schroën, 2015; Dresselhuis, Stuart, van Aken, Schipper, & de Hoog, 2008; Keppler, Steffen-Heins, Berton-Carabin, Ropers, & Schwarz, 2018; David Julian McClements, 2015).

Table 1. Initial protein concentrations, protein concentrations in the continuous phase of emulsions, volume-surface mean diameter ($d_{3,2}$), surface load (J) and total surface area of emulsions (S). Data represent the mean $\pm$ standard deviation of 3 measurements on 2 independent replicates.

Emulsions	Protein initial concentration in a aqueous solution (mg/mL)	Protein concentration in continuous phase of emulsion (mg/mL)	$D_{3,2}$ (μm) at $t=3\text{h}$	$D_{3,2}$ (μm) at $t=7\text{d}$	Γ (mg/m^2) at $t=3\text{h}$	S (m^2/g) at t_0
XS	12.52 ± 0.12^a	8.02 ± 0.16^a	0.27 ± 0.00^a	0.28 ± 0.00^a	1.01 ± 0.06^a	0.30 ± 0.00^a
S	12.46 ± 0.02^a	9.10 ± 0.11^{bc}	0.34 ± 0.00^b	0.35 ± 0.01^b	0.94 ± 0.02^{ab}	0.24 ± 0.00^b
M	12.30 ± 0.33^a	9.12 ± 0.05^c	0.51 ± 0.00^c	0.50 ± 0.00^c	1.35 ± 0.17^c	0.16 ± 0.00^c
L	12.47 ± 0.06^a	11.10 ± 0.25^d	2.75 ± 0.00^d	2.65 ± 0.05^d	3.13 ± 0.54^d	0.03 ± 0.00^d

Different letters (a, b, c and d) in a column indicate significant differences ($p < 0.05$).

2.3.2. Carvacrol partitioning between PLA film, headspace and emulsion

In our experimental set-up (**Figure 1**), carvacrol was initially included in the PLA film, and first migrates to the headspace of the hermetically sealed tube, through which it can then reach the emulsion sample. **Figure 4A** shows the carvacrol concentration in the headspace over 7 days storage. For all samples, a fast release of carvacrol from the PLA-CAR films to the headspace occurred rapidly after the beginning of the storage. The highest concentration of carvacrol in the headspace was found at day 1 for most samples, except for the emulsion prepared by high pressure homogenization at 1×10^4 kPa (M-sized) where it was already reached after 12 h. After this initial stage, the carvacrol concentration in the headspace slowly decreased for all systems as consequence of its absorption in the emulsion phase. At the end of the storage period (7 days), it was around 0.16-0.18 $\mu\text{g/mL}$ in all tubes, suggesting that the systems were getting close to an equilibrium, although for most systems it was still slowly decreasing. The differences between samples regarding the carvacrol concentration in the headspace were most obvious during the first 3 days, and ranked, from high to low, as follows: M, S, XS, L and separate oil-aqueous layer system. Significantly higher concentrations of carvacrol were found in the headspace of the tubes containing the three fine emulsions, compared with the tubes containing the coarse emulsion or separate oil and aqueous layers. The carvacrol in the headspace of emulsion M was significantly higher than that in the headspace of emulsions S and XS during the first 2 days ($p < 0.05$); after day 3 the differences were not significant anymore ($p > 0.05$).

Figure 4. Carvacrol concentration measured in the headspace of the tubes (A) and in the emulsions (B) over 7 days storage, with different liquid samples present at the bottom of the tube (XS-, S-, M- or L-sized emulsions; or “layer” = separate layers of aqueous phase and oil).

Next to the headspace concentration, the concentration of carvacrol absorbed in the liquid samples was quantified (**Figure 4B**). The highest amount of carvacrol was measured during incubation in the sample containing the separate layers. A comparison of carvacrol absorption in XS-, S- and M-sized emulsions showed that the highest concentration of carvacrol at the end of the storage was measured in the XS-sized emulsion ($p<0.05$). The carvacrol concentration in the S-sized emulsion was significantly higher than that in the M-sized emulsion, and it was significantly lower than that in the XS-sized emulsion from day 2 to day 7 ($p<0.05$). The M-sized emulsion absorbed 22% less carvacrol compared to the XS-sized emulsion at day 7. Regarding carvacrol absorption in the L-sized emulsion, a two-step change in the carvacrol absorption pattern was found. A fast-increasing concentration was observed at day 1, followed by a reduced increase rate (period 1). Then a second fast increase started from day 2 until day 5, followed by a slower increase, approaching the equilibrium, after day 5 (period 2). During the first period, the carvacrol absorption in the L-sized emulsion was fairly comparable to that in the S- and M-sized emulsions, and was significantly lower than that in the separate layer sample ($p<0.05$). Yet a large increase of carvacrol concentration in the L-sized emulsion was found over period 2, and the carvacrol absorption became higher than in all fine emulsions after day 3. After 7 days, the carvacrol absorption in the L-sized emulsion was similar to the carvacrol absorbed in the layer sample ($p>0.05$).

Lipid droplets in emulsions are the major reservoirs for volatile antimicrobials, which often have high log P values. Since carvacrol is a hydrophobic compound with a log P of 3.52 (Ben Arfa, Combes, Preziosi - Belloy, Gontard, & Chalier, 2006), it will tend to partition in the oil phase of emulsions. For the data set obtained with fine emulsions (XS-, S- and M-sized emulsions), the carvacrol concentration in emulsions increased with decreasing oil droplet size, that is, with increasing interfacial area. This suggests that in the process of carvacrol transfer from the PLA film, to the headspace, to the emulsion's continuous (aqueous) phase, to the lipid droplets, the contact area between oil and water is the limiting factor. Accordingly, when the liquid sample was a layer of oil on top of a layer of aqueous phase (i.e., same overall composition, but favored contact between oil and the headspace), a rapid uptake of carvacrol in the liquid sample was found. At first sight, the observations made for the coarse emulsion seem to contradict the aforementioned droplet size effect, as the emulsion showed a rapid uptake of carvacrol despite the large droplet size, and hence limited oil-water interfacial area. However, this emulsion underwent rapid creaming, meaning that large oil droplets accumulated at the surface of the emulsion layer. In this case, the promoted contact of the creamed oil droplets

with the headspace, and with neighboring droplets could have counteracted the low oil-water interfacial area, probably by-passing the transfer through the emulsion's continuous phase. The two-stage absorption behavior could therefore be explained by a combination of two mechanisms: droplet size was responsible for the moderate absorption in comparison with fine emulsions during period 1; and the cream layer formation could explain the increase in carvacrol absorption rate observed after 2 days storage.

Literature on the partitioning kinetics of other hydrophobic compounds, such as flavors, provides some relevant insights for comparison with our findings. Yucel, Elias, and Coupland (2012) used electron paramagnetic resonance (EPR) to determine the distribution of a model hydrophobic compound (4-phenyl-2,2,5,5-tetramethyl-3-imidazoline-1-oxyl nitroxide, PTMIO) between phases in emulsions, and they found an increased proportion of PTMIO in the aqueous environment of emulsions with decreasing the droplet size. The results were explained by two mechanisms. First, a greater interfacial area would promote interaction between PTMIO and the protein-covered droplet surface. Alternatively, the Laplace pressure increases with decreasing droplet size, leading to an increased solubility of the lipophilic spin probe in the aqueous layer immediately surrounding the droplets. These results are well in line with ours. Moreover, Carey, Asquith, Linforth, and Taylor (2002) found higher volatile concentrations in the headspace of a coarse emulsion, compared to a homogenized fine emulsion, indicating a the weaker retention of volatiles in the coarse emulsion. This is also comparable to the effect of droplet size on the partitioning of hydrophobic compounds in emulsions found in our present work.

2.3.3. Carvacrol partitioning in lipid and aqueous phase of emulsions

The distribution of carvacrol between oil and aqueous region was studied to further understand its behavior when exposed to a multiphase food matrix. **Figure 5** shows the partition coefficients ($\log K$) of carvacrol in samples at the equilibrium, calculated using Eq.4. The partition coefficient of carvacrol reached a constant value after day 5 (no significant difference between day 5 and day 7 was found ($p>0.05$)), indicating that an equilibrium (or at least a pseudo-equilibrium) of the carvacrol concentrations in the creamed and aqueous phases was reached. The $\log K$'s for fine emulsions were significantly lower than those for the coarse emulsion and separate layer system at day 5 and 7 ($p<0.05$), owing to the promoted contact of the oil with the headspace in the latter cases (creamed droplet layer, or non-emulsified oil layer, respectively). Within the fine emulsions, the $\log K$ of carvacrol was significant higher in the

XS-sized emulsion than in the S- and M-sized emulsions, both at day 5 and day 7 ($p<0.05$). This suggests that a small oil droplet size promotes the absorption of carvacrol in the lipid droplet phase. When a simple WPI solution (1.5 wt%) was used (i.e., no oil in the system), a linear increase of the carvacrol concentration in the WPI solution was observed during incubation ($R^2=0.9953$), and at day 7 a 4-fold higher carvacrol concentration was measured in this WPI solution compared to the aqueous phase of emulsions and of the separate layer system (**Supplementary Figure 1**). This indicates that when an oil phase was present, the concentration of carvacrol in the available aqueous environment did not reach saturation, most likely because carvacrol is largely hydrophobic.

Figure 5. Partition coefficient ($\log K$) of carvacrol between the oil/creamed and aqueous phases of the samples. The higher the $\log K$, the stronger the partitioning of carvacrol in the oil/creamed phase at the expense of the aqueous phase.

These results can be explained by the fact that the partitioning behaviour was led by the interactions of carvacrol with the different components of each available phase (Asnaashari, Farhoosh, & Sharif, 2014; Di Mattia, Sacchetti, Mastrocola, & Pittia, 2009). Carvacrol is expected to be absorbed in both lipid and aqueous phases, as the lipid (droplet) phase is composed of triglycerides and adsorbed whey proteins, and the aqueous phase of water and free whey proteins. It could even be relevant to consider the protein-stabilized interface as a separate pseudophase of the system, in a comparable manner to the model proposed by the group of Romsted and Bravo-Díaz (2013), although this was developed and validated for surfactant-

based emulsions. Compared with the log P of carvacrol determined between octanol and water (log $P=3.52$ (Ben Arfa et al., 2006)), we obtained a lower log K of carvacrol between the creamed and aqueous phases in all samples. This may be explained via different mechanisms. First, in our systems, the free WPI present in the aqueous phase can also bind hydrophobic compounds. In addition, the partitioning of carvacrol is determined by the nature of the oil phase, i.e., octanol or triglyceride oil (Niimi, 1991). The migration rate and eventual partitioning of carvacrol initially loaded in a CAR-PLA film may be important to understand its antimicrobial efficiency in emulsions, and more generally in multiphase foods. In fact, as bacteria grow in aqueous phase environments, the proportion of antimicrobial in lipid/aqueous region can influence bacteria inhibition during storage.

2.3.4. Antimicrobial effect of the CAR-PLA film and modelling of bacterial growth curve

The growth of a model spoilage bacteria in fine emulsion samples exposed to carvacrol (with CAR-PLA film) or not (without CAR-PLA film) is presented in **Figure 6**. In the M-sized emulsion, the presence of the CAR-PLA film in the vial resulted in a significant reduction of 1.02 ± 0.09 log CFU/mL of the *Pseudomonas fluorescens* population at day 7, by comparison with the control system incubated without the CAR-PLA film ($p<0.05$). In the S- and XS-sized emulsions, inhibition of the bacterial growth induced by the presence of the CAR-PLA film was also found, although to a lesser extent (significant reduction of 0.62 ± 0.05 and 0.52 ± 0.06 log at day 7, respectively).

Figure 6. Growth curves of *Pseudomonas fluorescens* in (A) XS-sized emulsion (B) S-sized emulsion (C) M-sized emulsion in antimicrobial/control group at 20 °C.

The bacterial growth data were fitted with a modified Gompertz model by non-linear regression. The model showed a good fit as can be seen from the small size of the residuals (average deviation 6.7%) and the low sum of squares of residuals (< 6.67). The maximum growth rate (u_{max}) and the logarithm of the asymptotic values of the relative population size (A_s) were estimated; no lag phase was observed ($\lambda=0$) for all conditions (**Table 2**). The growth of bacteria in XS- and S-sized emulsions did not reach to the asymptotic population size during incubation, yet a slight increase in the population size was found after day 5 in both samples, thus we only estimated the asymptotic population size in M-sized emulsions. The results showed that the asymptotic population size for antimicrobial-exposed samples (9.68 ± 0.27 Ln CFU/mL) was significantly lower than the values found in control groups (12.01 ± 0.31 Ln CFU/mL) ($p<0.05$) in M-sized emulsions. Besides, for this emulsion, the maximum growth rate was significantly lower in the antimicrobial-exposed sample compared to the control group ($p<0.05$). However, in the S- and XS-sized emulsion, the presence of the CAR-PLA film in the closed vial did not significantly lower u_{max} during incubation, revealing a weak antimicrobial effect of carvacrol.

Table 2. Estimated growth rate as calculated from a modified Gompertz equation fitted to the *Pseudomonas fluorescens* experimental plate counts in fine emulsions stored at 20 °C.

Parameters	XS-sized emulsion		S-sized emulsion		M-sized emulsion	
	Antimicrobial group	Control group	Antimicrobial group	Control group	Antimicrobial group	Control group
Maximum specific growth rate (days ⁻¹)	9.71 ± 1.25 ^a	11.26 ± 1.71 ^a	9.10 ± 0.97 ^a	10.52 ± 1.21 ^a	9.40 ± 0.86 ^a	12.42 ± 0.86 ^b
Sum of squares of residuals (SS _{res})	4.42	6.67	3.05	4.38	1.91	2.65

Different letters (a and b) indicate significant differences between the antimicrobial and control group ($p < 0.05$).

It can be concluded from the bacterial reduction values and the estimated A_s and u_{max} that amongst the three fine emulsions tested, the antimicrobial effect of the CAR-PLA film was the greatest in the emulsion containing relatively large oil droplets (M-sized emulsion). This finding can be explained by the partition behavior of carvacrol between the creamed and aqueous phases. The Log K of carvacrol in the XS-sized emulsion was relatively high, compared to that in the S- and M-sized emulsions, indicating that more carvacrol was proportionally absorbed in the droplet phase in the XS-sized emulsion. It is possible that the incorporation of carvacrol in the lipid droplet phase segregates it from the bacteria which are present in the aqueous phase, thus resulting in a lower antimicrobial efficiency. Many studies have reported that antimicrobial activity is often low in food matrices with high fat content, as the high solubility of antimicrobials in the fat phase impedes the interplay between these agents and bacteria. For example, Gaysinsky, Taylor, Davidson, Bruce, and Weiss (2007) found that the minimum inhibitory concentrations against food pathogens significantly increased with the increase of fat content in milk. Smith-Palmer, Stewart, and Fyfe (2001) concluded that the direct addition of thyme essential oil in foods had less antimicrobial effect on full-fat soft cheese than on low-fat soft cheese. However, the antimicrobial activities on the emulsions with a fixed fraction of oil but various oil droplet sizes had not been reported yet, and we herein show that the oil droplet size modulates the efficiency of antimicrobial agents.

2.4. Conclusion

This study demonstrates that the oil droplet size in emulsions has an impact on the partitioning and antimicrobial activity of carvacrol initially loaded in a food packaging film. Emulsions with a large interfacial area (very small droplets) absorbed a significantly higher carvacrol amount than emulsions with smaller interfacial areas (larger droplets). This trend is, however, only applicable until a certain droplet size, above which droplet creaming becomes substantial over the timescale of the experiment. Decreasing oil droplet size increased the partitioning of the absorbed carvacrol towards the lipid droplet phase, at the expense of the aqueous phase, which decreased the antimicrobial efficacy of CAR-PLA film. This work provides insightful information for the application of antimicrobials in food emulsions: the effect of antimicrobial-based AP depends more on the spatial distribution of the absorbed antimicrobial molecules within the emulsion, rather than on the total amount absorbed.

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Supplementary materials

Methods

Extraction and determination of carvacrol in packaging film

Ten milliliter of dichloromethane was used to dissolve the carvacrol loaded PLA packaging film at 20°C. After 2 hours stirring at 600 rpm, the whole films were fully dissolved, and the samples (1 µL) were injected into the GC system. A calibration curve with standard solutions of carvacrol in dichloromethane was prepared and a linear relationship ($R^2 = 0.9993$) between the concentration of carvacrol and the area of the corresponding peak was obtained.

Results

The mass balance of carvacrol in each phase of the food-package, including headspace of the package, food matrix and film was calculated and the results are shown in **Figure S2**. It shows that the amount of carvacrol in headspace was very low compared to that in the film and solution. In addition, still a large amount of carvacrol was determined in film for all samples at the end of the storage days. The desorbed carvacrol at the end of storage ranges from 18-29% of the initial carvacrol in packaging films. The results of high residue of carvacrol in PLA film indicates the developed antimicrobial packaging is able to provide a prolonged release of antimicrobial compounds at room temperature (20 °C). We found a mass loss during storage for all samples. A relevant study conducted by Higuera, Lopez-Carballo, Hernandez-Munoz, Catala, and Gavara (2014) found that the adhesive coating and lid was absorbing considerable amounts of carvacrol. Therefore, the carvacrol loss in our study might attribute to the carvacrol absorption in the plastic pipette tip and rubber lid in the setup.

Figure captions

Figure S1. Concentrations of carvacrol in creamed/oil and aqueous phases, over 7 days storage, for A) M-sized, B) S-sized, C) XS-sized, D) L-sized, E) separate oil-aqueous layers and F) simple WPI solution.

Figure S2. Mass balance of carvacrol partitioning in each phase of the package system including the film, liquid samples and headspace of setups containing (A) M-sized emulsion, B) S-sized emulsion, C) XS-sized emulsion, D) L-sized emulsion and E) separate layers in 20°C.

Figure S1. Concentrations of carvacrol in creamed/oil and aqueous phases, over 7 days storage, for A) M-sized, B) S-sized, C) XS-sized, D) L-sized, E) separate oil-aqueous layers and F) simple WPI solution.

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Chapter 3

Volatile antimicrobial absorption in food gel depends on the food matrix characteristics

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Abstract

The aim of this study was to investigate the influence of the food microstructure on the release, absorption and partitioning of the volatile antimicrobials from antimicrobial packaging film. Carvacrol loaded polylactic acid (PLA) film was prepared and tested on a food gel model simulating products like jellies, jams and dressings. Whey protein isolate/carrageenan gels with different microstructures were prepared by varying the NaCl concentration (50-250 mM): the higher the salt concentration the lower the gel consistency. Results showed carvacrol partitioning in the gel increase at higher concentration of NaCl. A 41% increase of carvacrol absorption after 7 days was found in gels prepared with 250 mM of NaCl, compared with the gels at 50 mM NaCl. The results can be explained by the differences in gel microstructures: gels with higher NaCl concentrations showed the small WPI-containing aggregates and large pores from confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) images, in turn facilitating carvacrol absorption by proteins in the gels. The physical properties of gels revealed that gels with higher NaCl concentrations showed lower strength and water holding capacity, pointing to a more loose structure of the gel. The results demonstrate that the microstructure of gels have a clear effect on carvacrol absorption and this factor should be taken into account for designing antimicrobial packaging for the preservation of the gel-like foods.

Keywords: Carvacrol, Whey protein isolate, Carrageenan, Microstructure of gel, Antimicrobials absorption in gel, Antimicrobial packaging design.

3.1. Introduction

Antimicrobial packaging (AP) is developing as a response to consumer demands or industrial production trends towards foods with prolonged shelf-life and controlled quality (Dainelli, Gontard, Spyropoulos, Zondervan-van den Beuken, & Tobback, 2008). The development of an effective AP can be influenced by several factors such as materials and intrinsic properties of the polymer films, mass transfer coefficients of active substances, storage conditions and the preserved food matrix (Han, 2003). In the past decades, large numbers of AP products were developed from novel plastic materials and active compounds (Sung et al., 2013). The strategies to control the release and migration of active compounds from packaging films to either model or real foods were also well known (de Azeredo, 2013; Jideani & Vogt, 2016). However, the influence of the food structures/compositions on the design/development of AP is largely unexplored, which is a research gap needed to be better understood.

Carvacrol, an aromatic and volatile compound, is the major component (50-86%) of oregano and thyme essential oils (Guarda, Rubilar, Miltz, & Galotto, 2011). It has been broadly studied as a food preservative to extend the shelf life of foods as carvacrol is able to effectively inhibit the growth of a wide range of microbes (Avila-Sosa et al., 2012; Fernandez-Saiz, Lagaron, Hernandez-Muñoz, & Ocio, 2008; Garrido Assis & de Britto, 2011). Several studies demonstrated that a good bacterial growth inhibition could be achieved by utilization of the volatile antimicrobials in a vapour phase (Goni, López, Sánchez, Gómez-Lus, Becerril, & Nerín, 2009; Tyagi & Malik, 2011; Velázquez-Nuñez, Avila-Sosa, Palou, & López-Malo, 2013). Volatile antimicrobials in a vapour phase provide a homogeneous distribution onto the surface of food matrix, which improves the interaction between the compound and the (surface) bacteria. Development of AP via incorporation of a volatile antimicrobial compounds is a promising strategy to protect foods by effectively inhibiting or retarding the growth of microorganisms. Polylactic acid (PLA) has attracted most attention due to its renewable resources, high mechanical strength and low prices in market (Auras, Harte, & Selke, 2004; Mukherjee & Kao, 2011; Urayama, Kanamori, & Kimura, 2002). The incorporation of carvacrol in PLA film for the preparation of antimicrobial food packaging was expected to have a good performance on foods preservation.

Food matrices having different physical properties may influence the partitioning and mass transport of antimicrobials added in a food packaging system (Weiss, Loeffler, & Terjung, 2015). Therefore, the structure of the food to be preserved should be considered a concept to

design an effective AP, which can be well explored in gel matrix. Many food products have gel-like structures and many of them contain more than one gelling biopolymers, typically proteins and/or polysaccharides, to obtain essential texture and sensory characteristics (de Jong & van de Velde, 2007). Protein/polysaccharide mixed gels are formed in confectionery products, desserts, and products based on fruits and vegetables (Banerjee & Bhattacharya, 2012). In particular, whey protein isolate (WPI) and carrageenan are commonly used in food products to increase viscosity, to stabilize emulsions or foams and to hold water during food processing and application (Alavi et al., 2018; De la Fuente, Hemar, & Singh, 2004; Hosseini et al., 2013). The interaction between whey protein isolate and carrageenan is applicable to simulate the real foods as a wide range of microstructures and rheological properties can be produced representing diverse food products (Alizadeh-Pasdar, Nakai, & Li-Chan, 2002). Among different food products with gel structure, products like jellies, jams and salad dressings have a weak gel structure having structural features in between solutions and strong gels. Generally, they are formed due to non-covalent interaction between proteins and polysaccharides, like hydrogen bonds, electrostatic and hydrophobic interactions, which are weak and reversible (Dickinson, 2008; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). The presence of salt can control the electrostatic interaction and affect the network strength. Turgeon and Beaulieu (2001) investigated the effect of Na^+ and K^+ concentration on the gelation of k-carrageenan in the presence of WPI and showed how the concentration of these cations modify the gel formation. Adding Na^+ and K^+ up to 50 mM, the firmness of gels increased. However, higher concentration of salt (>100 mM) resulted in a decreasing of the firmness of the gels. Therefore, adapting the concentration of NaCl is a straightforward way to influences the microstructure of WPI-carrageenan gels.

Different antimicrobials applications for the preservation of food gels are investigated from previous studies (Costello et al., 2018; Xu, Tao, Yang, & Chu, 2002). However, the bacteria inhibition effect of antimicrobials (i.e., efficiency) influenced by the food gels is not clear. To understand the efficacy of an antimicrobial packaging, the concentration and location of active compounds in an multiphase food matrix are important to know, which are determined by the antimicrobials absorption and diffusion (Weiss et al., 2015). Carvacrol may interact with WPI via different binding sites: a central hydrophobic cavity and sites located on the protein surface of β -lactoglobulin, and two binding sites in native bovine serum albumin (BSA) (Burova, Grinberg, Grinberg, & Tolstoguzov, 2003; Considine, Patel, Singh, & Creamer, 2005). After attaching at the surface of the gels, carvacrol can diffuse from the surface into the gel if the

complex is reversible. The microstructure of gels may cause differences in carvacrol absorption and diffusion, which would influence the efficacy of AP. The aim of this work was to study how antimicrobials absorption by gel samples is affected by the microstructure of gels. The insights provided by this study are helpful for designing the effective AP for the preservation of gel foods.

3.2. Materials and Methods

3.2.1. Materials

Poly(lactic acid) (4043D Ingeo™) was kindly supplied by Wageningen Food & Biobased Research Institute. Carvacrol ($\geq 98\%$), dichloromethane (DCM), hexane, 2-propanol, sodium chloride and carrageenan (predominantly κ and lesser amounts of λ carrageenan, specification: PRD.1.ZQ5.10000042025) were purchased from Sigma Aldrich Pty. Ltd., Netherlands. The supplier provided compositional information of carrageenan that consists of $\leq 11\% \text{ K}^+$, $\leq 3.5\% \text{ Ca}^{2+}$ and $\leq 2.0\% \text{ Na}^+$. Whey protein isolate (97.5% protein, dry basis) was obtained from Davisco (Bipro, Davisco, lot # JE 198-1-420, USA). The ion contents per 100 g WPI powder are 750 mg sodium, 60 mg potassium, 130 mg calcium, 75 mg phosphorus and 25 mg magnesium (Peng et al., 2019).

3.2.2. Carvacrol loaded PLA antimicrobial film preparation

Carvacrol incorporated PLA film was prepared with the solvent-casting method (Rhim, Mohanty, Singh, & Ng, 2006). Briefly, four grams of PLA pellets were dissolved in one hundred millilitre of DCM solvent. The solution was stirred at 30 °C under magnetic stirring until PLA pellets were completely dissolved. Carvacrol (CAR) was added at the level of 50% (w/w) with respect to PLA content. The mixture was homogenized using an ultra-sonicator (HBM Machines, Moordrecht, the Netherlands) for 30 min. Twenty millilitre of film forming solution was cast on a glass petri dish (size 100×15 mm) and the petri dishes were dried overnight in a fume hood at room temperature.

3.2.3. WPI-carrageenan gel formation

WPI (20% w/v), carrageenan (4% w/v) and sodium chloride (1 M) stock solutions were prepared in ultrapure water under stirring by a magnetic stirrer for 4 hour at 20 °C. Stock

solutions of WPI and carrageenan were kept overnight at 4 °C to promote complete hydration. For preparation of gel solutions, each individual sample was at a constant protein concentration (10% w/v), a constant carrageenan (0.4% w/v) and with varying concentrations of sodium chloride of 50 mM, 100 mM or 250 mM. The carrageenan stock solution was heated at 80 °C in a water bath for 30 min before mixing with WPI solution. The WPI solution was heated to 40 °C and subsequently mixed with the carrageenan solution. During the stirring of the WPI and carrageenan solution at 40 °C on a magnetic stirrer (30 min), sodium chloride solution was gradually added to the mixture. A control solution of WPI/Carrageenan without salt addition was used to compare the effect of sodium chloride on gel formation.

Five millilitre of solution was pipetted into a glass tube (20 mL) and closed with a rubber lid. Tubes were stored at 4 °C in a fridge overnight for gel formation.

3.2.4. Food-Packaging system preparation

The process of carvacrol partition in a food-packaging system was studied in a closed set-up (20 mL verex headspace vial) (**Figure 1**). CAR-PLA film (0.02 g) and WPI-carrageenan gel (5 mL) were the main components in this system. To avoid a direct contact, a pipette tip was used to isolate the film and food matrix. Each set-up was fully sealed with a rubber lid in case of the leaking. Packaging setups were stored at 20 °C to characterize the partitioning of carvacrol in the headspace of the glass vial, carvacrol absorbed in gel and carvacrol left in film during storage. Several single setups consisting of the same weight of CAR-PLA film and the same volume of gel solution were prepared for a 7-day continuous sampling for quantification of carvacrol absorption in gels.

Figure 1. Setup used to study carvacrol partitioning in Food-Packaging system.

3.2.5. Characterization of pre-gel solution and gels

3.2.5.1. Microstructure

Confocal laser scanning microscopy (CLSM)

CLSM was used to visualize the gel microstructure at room temperature according to Zhang and Vardhanabhuti (2014) with some modifications. To stain WPI in gel samples, rhodamine B solution (20 μ L of a 0.2% w/v solution/mL of sample) was added into gel solution immediately prior to gel formation overnight. Gel samples were cut into small pieces for CLSM analysis. CLSM images were recorded at room temperature with a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss, Jena, Germany) with 63 $\times$ and 40 $\times$ oil immersion objectives. A He laser was used with an excitation wavelength of 543 nm. Digital image files were acquired in 512 $\times$ 512 pixels. The average volume of aggregates were calculated using ImageJ software (1.52, National Institutes of Health, USA) (Zhang et al., 2017) (see supplementary information). Four of independent images were used for estimation of the size of aggregates for each gel.

Scanning electron microscope (SEM)

SEM was used to analysis the porous structure changes in gels according to Munialo et al. (2016) with modifications. Gels were cross-linked in 2.5% (v/v) glutaraldehyde in phosphate/citrate buffer for 7 h followed by washing three times in phosphate/citrate buffer. The washed samples were dehydrated with ethanol in steps of 10-30-50% for 10 min, 70% for overnight and 80-90-100% for 10 min, followed by dehydration once with 100% ethanol for 20 min. The critical point drying (CPD 030 BalTec, Liechtenstein) was then used to dry and break the samples into small pieces. The samples were then stored overnight under vacuum. After drying of the carbon cement, the samples were sputter coated with 10 nm iridium in SCD 500 (Leica EM VCT 100, Leica, Vienna, Austria) prior to imaging. All samples were analysed with a field emission scanning electron microscope (Magellan XHR 400L FE-SEM, FEI, Eindhoven, The Netherlands) at room temperature (20 ± 2 °C) at a working distance of 4 mm with SE detection at 2 kV. The average pore size was calculated using ImageJ software (1.52, National Institutes of Health, USA) (Pugnaloni, Matia-Merino, & Dickinson, 2005) (see supplementary information). Four of independent images were used for estimation of the average pore size for each gel.

3.2.5.2. Water holding capacity (WHC) of gels

WHC of gels were determined based on the centrifugation procedure developed by Kocher and Foegeding (1993). Four grams of gel were placed on the bottom of the centrifuge tube (50 mL). Centrifugation was performed at 1000g for 10 min at 4 °C. After centrifugation, the exuded serum from the gel was collected and discarded using a 2 mL of syringe. The WHC was the percentage of the gel's weight after centrifugation relative its initial weight.

3.2.5.3. Surface hydrophobicity

The anionic fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANSA) was used to measure the hydrophobicity of proteins in pre-gel solutions (Saricay, Wierenga, & de Vries, 2014). An ANSA stock solution (2.4 mM) was prepared in 10 mM sodium phosphate buffer at pH 7.0 and the solution was stirred overnight at 4 °C for a complete dissolution. The pre-gel solutions were diluted to a final concentration of 0.1% by using a sodium phosphate buffer (10 mM pH=7.0). During measurements, aliquots of 10 µL ANSA solution were sequentially added into 1 mL pre-gel solution until a maximum fluorescence was obtained indicating that the ANSA concentration was saturated. After each addition of ANSA, fluorescence emission spectrum was recorded at wavelengths between 400 and 650 nm using Shimadzu RF-6000 fluorimeter (Shimadzu, Kyoto, Japan). The excitation wavelength was 385 nm and the scan rate was 120 nm/min. The relative hydrophobicity of each sample was obtained by comparing the maximum area of an integrated curve between 400 and 600 nm after blank subtraction.

3.2.6. Quantification of carvacrol in packaging system

3.2.6.1 Gas Chromatography–Flame Ionization Detector (GC-FID) calibration

Two calibration curves were used for quantification of carvacrol in packaging system. For carvacrol in headspace and in gels, a stock solution (2 mg/mL) was prepared by adding carvacrol in a mixture of n-hexane and isopropanol (n-hexane : isopropanol = 3:1). To determine the amount of carvacrol left in packaging film, another stock solution (2 mg/mL) was prepared by adding carvacrol in chloromethane. Five concentrations for both stock solutions between 0.4 mg/mL and 2.0 mg/mL were prepared by diluting the stock solution. Carvacrol concentration was integrated using the calibration curve of the carvacrol standards ($R^2=0.9996$ for carvacrol in both calibration curves).

3.2.6.2 Carvacrol analysis in the headspace of the setup

The concentration of carvacrol at each time measurement in the headspace of the setup was determined by Headspace Gas Chromatography–Flame Ionization Detector (HS-GC-FID) (Thermo Scientific, Waltham, Massachusetts, United States). The headspace sampling technique used was that previously described by Higuera, López-Carballo, Hernández-Muñoz, Catalá, and Gavara (2014). For carvacrol compound sampling, a gas-tight Hamilton microliter syringe (2.5 mL) was used to absorb 500 μL of gas, then the gas sample was immediately analysed by using GC-FID equipped with a semi-capillary RTX-1301 (30 m, 0.53 mm, 0.5 μm) column (Restek, Teknokroma, Barcelona, Spain). The FID was operated with a hydrogen flow of 35 mL min^{-1} and air flow of 350 mL min^{-1} . To operate GC-FID, an initial oven temperature of 60 $^{\circ}\text{C}$ was used and it increased to 250 $^{\circ}\text{C}$ with a speed of 15 $^{\circ}\text{C min}^{-1}$. The injection temperature was set to 250 $^{\circ}\text{C}$. Carrier gas was helium with a flow rate of 2 mL min^{-1} . Injections were set in split mode with a split flow of 10 mL/min . The total running time was 25.25 min for each sample.

3.2.6.3 Extraction and determination of carvacrol in gel samples

To quantify the concentrations of carvacrol absorbed in gel samples, carvacrol was extracted from the gel samples before analysing by GC-FID. Ten millilitre of organic solvent mixture (n-hexane:isopropanol=3:1) was added into each gel sample (5 mL). The mixture was warmed in a shaking water bath at 40 $^{\circ}\text{C}$ for 20 min at the speed of 60 rpm for dissolving the gel and for carvacrol extraction. The mixtures were then centrifuged for 10 min at 1000g to obtain the top organic layer. Ten microliter of organic solution was subjected into GC-FID for liquid analysis. Extraction efficiency of this method was tested by extraction carvacrol from gel samples by adding known amounts of carvacrol in the gels, and a high extraction yield (>95%) was achieved by using this method.

3.2.6.4 Extraction and determination of carvacrol in packaging film

The following extraction procedure was used to quantify the residual amount of CAR-PLA film. Each CAR-PLA film was immersed in ten millilitre of dichloromethane. The mixture was then stirred on a magnetic stirrer at 600 rpm for 2 hours (20 $^{\circ}\text{C}$). Ten microliter of mixture was injected into the GC-FID for liquid analysis.

3.2.7. Statistical analysis

All experiments were performed in triplicated. The IBM SPSS Statistics version 23.0 for Windows (Armonk, NY: IBM Corp) was used for the statistical analysis. One-way analysis of variance (ANOVA), with post-hoc pairwise analysis by the Tukey test was used to assess the significant differences between samples, and the level of significance was set at $p < 0.05$. The data were presented as mean $\pm$ standard deviation (SD) values.

3.3. Results and discussion

3.3.1. Microstructure of food gel model system

Figure 2 shows the CLSM 3D images of WPI-Carrageenan gels prepared with different salt concentrations (50-250 mM). The red areas represent protein phase. As shown in **Figure 2**, the size of aggregates decreased with the increasing in NaCl concentration. The average volume of aggregate is larger in 50 mM of NaCl ($384 \pm 83 \mu\text{m}^3$), intermediate in 100 mM NaCl ($182 \pm 37 \mu\text{m}^3$) and smaller in 250 mM NaCl ($141 \pm 9 \mu\text{m}^3$) (average of volume on 4 independent images for each sample) (**Figure 2D**). This may indicate that a network with large micron sized of clusters present at low salt of gel while particle like polymer complexes with more uniform appearance in high salt content. Aggregates could be formed between protein and polysaccharide complexes under certain conditions (depending on pH, ionic strength, protein/polysaccharide type, etc) (Duran, Spelzini, Wayllace, Boeris, & da Silva, 2018; Girard, Turgeon, & Gauthier, 2002; Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004). Considering the fact that the location and interaction of the carrageenan with the protein phase could not be visualised by CLSM due to the low resolution of CLSM and lack of the specific dye that is capable of binding to carrageenan, we proposed that the observed clusters from CLSM images were WPI-containing aggregates. Upon the addition of NaCl in gel, a smaller cluster containing WPI was observed for the gel at highest NaCl concentration in **Figure 2C**. The reason might be due to the strong shield effect exerted by the Na^+ ions that at higher concentration effectively screened the charged reactive groups on proteins and polysaccharides (Ako, Nicolai, Durand, & Brotons, 2009; Turgeon et al., 2001). The microstructure of the WPI-carrageenan gel is a result of the competition between gelation of the protein aggregates or carrageenan and the phase separation between protein aggregates and polysaccharide molecules (De Jong, Klok, & Van de Velde, 2009; Zhang & Vardhanabhuti, 2014). At pH 6.8, both WPI

($pI \sim 5.2$ (Swaigood, 1982)) and carrageenan are negatively charged. Salt addition favours protein-protein and polysaccharide-polysaccharide interactions (charge neutralization) (Kuhn, Cavallieri, & Da Cunha, 2011). Incorporation of non-gelled WPI with carrageenan in the presence of Na^+ may promote a competition of binding of Na^+ between self-association of carrageenan by coil-helix conformational transition and self-aggregation of WPI by shielding repulsive charge.

Figure 2. CLSM 3D images of WPI-carrageenan gels at three ionic strengths (A) 50 mM, (B) 100 mM and (C) 250 mM. (D) Average volume of aggregates in gels. Different letters (a, b and c) above the columns indicate significant difference between the three groups ($p < 0.05$).

For a higher resolution investigation of WPI-carrageenan network as a function of NaCl, gels were visualized by SEM images as shown in **Figure 3**. In SEM images, black areas are the pores of the gel while the rest of area represents WPI-carrageenan network. Compact microstructure with even and small pores was observed in gel samples prepared with 50 mM of NaCl (**Figure 3A 1-3**). When the salt concentration increased, the network shows ‘short string’ with fine strands containing relatively large pores (**Figure 3B 1-3**) (see circles). Heterogeneous domains in grey area caused by the sphere-like WPI-biopolymer aggregates were found in this sample. A coarse surface with uneven and large cavities was found in gel prepared with 250 mM NaCl (**Figure 3C 1-3**), where the domain sizes of network structures

represented ‘long strings’ (see circles). ‘Ditches’ were observed at several places (see rectangles) where were composed of small spherical beads and filaments. As suggested by Van den Berg, Rosenberg, Van Boekel, Rosenberg, and Van de Velde (2009), the spherical beads were proteins while filaments (see red arrows) corresponded to polysaccharide, therefore we proposed that the continuous phase was rich in WPI, and carrageenan was located in the protein-rich continuous phase, while some clusters of WPI-containing aggregates were dispersed in protein-rich continuous phase. The average pore area, measured by image analysis, increased from $457 \pm 27 \text{ nm}^2$ to $881 \pm 91 \text{ nm}^2$ with the increase in salt concentration from 50 mM to 250 mM. The different porous network in gels might be due to the combined mechanisms of ion-induced micro-phase separation of proteins and protein–polysaccharide phase separation (Çakır et al., 2012; Yan et al., 2019). Proteins have been shown to be incompatible with anionic polysaccharides at high salt concentrations at neutral pH (Goh, Sarkar, & Singh, 2014; Harrington, Foegeding, Mulvihill, & Morris, 2009). Therefore, a heterogeneous particulate gel network consisting of micro-phase separated spherical beads and WPI-carrageenan phase separation is likely to occur at these conditions due to the strong shielding effect (Ako et al., 2009; Chantrapornchai & McClements, 2002; Renard, van de Velde, & Visschers, 2006). These heterogeneous networks will lead to larger pores compared to homogeneous network (Chantrapornchai et al., 2002; Dumay, Laligant, Zasytkin, & Cheftel, 1999; Khalesi, Emadzadeh, Kadkhodaei, & Fang, 2019). Previous studies indicated the addition of salt to protein-polysaccharide complexes can either increase or decrease the pore size, depending on the concentration of salt (Khemakhem, Attia, & Ayadi, 2019; Wang et al., 2018). Similar results were obtained from Pan, Guo, Li, Song, and Ren (2017) who found that homogeneous network

with small pore size was observed in porcine myosin- κ -carrageenan gel at 0.04 M CaCl_2 while large pores occurred in gel with the addition of 0.12 M CaCl_2 .

Figure 3. SEM images of WPI-carrageenan gel with (A 1-3) 50, (B 1-3) 100 and (C 1-3) 250 mM NaCl. The magnification of the SEM images is $\times 5000$ (first row) and $\times 25000$ (second row) and $\times 80000$ (third row).

The rheological properties of WPI-carrageenan gels as a function of ionic strength were discussed in more detail in the supplementary materials. The results showed that gels with higher NaCl concentrations showed lower strength.

3.3.2. Water holding capacity of gels

The water holding capacity of a food gel is direct relevant to gel stability, microbial safety, functional properties and texture during a storage period (de Kruif, Anema, Zhu, Havea, & Coker, 2015). As seen in **Figure 4**, the gel in 50 mM of NaCl shows a significant higher WHC during storage ($p < 0.05$), compared with WPI-carrageen-250 mM. The WHC of gel WPI-carrageen-100 mM was in general in-between of WPI-carrageen-50 mM and WPI-carrageen

250 mM during measurement days. In addition, it was found that the WHC of all samples decreased during storage. The decrease in WHC might attribute to the formation of insoluble protein or large protein aggregates during storage at 20 °C, which indicated by the decreasing in soluble protein concentration in supernatant after centrifugation (**Figure S5**) (Bouhrara, Clerjon, Damez, Kondjoyan, & Bonny, 2012; Urbonaite, De Jongh, Van der Linden, & Pouvreau, 2014). WHC of gel samples relates to the microstructures since it connects to the gel shrinkage and texture change of gels. Low WHC can be explained by the weak stability of gel caused by the weak network of samples. The gels prepared in high concentration of NaCl leads to a more heterogeneous microstructure in submicron level (**Figure 3**). This coarse network (in submicron level) is expected to have a low capacity to hold aqueous phase during centrifuge (Chen, Chassenieux, & Nicolai, 2018; Kharlamova, Nicolai, & Chassenieux, 2018). A similar effect that WHC of gels was low in gel prepared with high salt concentration was also found from previous studies (Çakır et al., 2011). A negative correlation between WHC and the size of pores in gels was found in several studies. Gel with smaller pore size shows higher WHC (Kuhn et al., 2011; Munialo et al., 2016; S. Zhang, Zhang, & Vardhanabhuti, 2014). Zhang, Zhang, et al. (2014) concluded that the small pores in WPI-xanthan gel could hold more water, which was expected as the reason of the higher swell ratio. According to this correlation, it was expected that the largest pore size was found in gel in 250 mM NaCl, followed by gel in 100 mM NaCl. While the gel in 50 mM NaCl showing the highest WHC had the smallest pore size. This conclusion was in line with the pore size estimation from SEM images.

Figure 4. Changes in water holding capacity (WHC) of gels.

3.3.3. Surface hydrophobicity

Surface hydrophobicity is considered as an underlying parameter that influence carvacrol absorption: gels with higher surface hydrophobicity may have higher ability to bind carvacrol. Results of surface hydrophobicity of pre-gel solutions (WPI-carrageenan-50,100 and 250 mM NaCl) that was measured by using fluorescent probe ANSA showed that adding NaCl at concentrations between 50 mM and 250 mM did not cause a significant increase in the surface hydrophobicity of pre-gel solutions ($p>0.05$) (Data not shown). The results suggest that salt addition did not have a clear impact on unfolding or partially denaturing of the proteins in pre-gel solutions and the different salt concentration is therefore expected not to have an effect on the carvacrol absorption ability.

3.3.4. Quantification of carvacrol partition in each part of a food-package system

The process of carvacrol diffusion and absorption is schematized in **Figure 5**. Carvacrol starts to be released from the PLA film to the headspace of a food-package at the beginning of the storage (t_0). The headspace of the package is the intermediate zone between packaging film and food matrix, and therefore illustrates the dynamic behaviour of carvacrol in the whole package. Once carvacrol is released in the headspace at $t>0$, a dynamic process occurs then in the food-package, where carvacrol continues to be released from the packaging film, while in the meantime carvacrol from the headspace will be absorbed into the gel.

Figure 5. Migration of carvacrol from PLA film and its partitioning in the food pack system (film, headspace and food gel).

3.3.4.1. Carvacrol concentrations in the headspace of the package

Figure 6 shows the carvacrol concentrations in the headspace of package measured as a function of storage days. An immediate large increase of the carvacrol concentration in the headspace was observed in the first day of storage for all gels. The carvacrol concentrations in the headspace of package reached a maximum at day 1 (t_1). Then the concentrations were slowly decreasing in all packages containing the physically different gels. After 4 days (t_4) storage, a relative steady state of carvacrol in the headspace was found for all groups. This illustrates a dynamic equilibrium between carvacrol being released from the film and absorbed into the gels. Thus, a three-stage could be classified for description of carvacrol in headspace of package during a 7-day storage. From t_0 to t_1 , the release of carvacrol from PLA film into the headspace was dominant. The second stage could be defined from t_1 to t_4 , where the decreasing concentration reflects the migration of carvacrol into the gels was higher than the amount of carvacrol released from PLA film. After t_4 , the third stage was found where a dynamic equilibrium in carvacrol concentration in headspace was reached. It was observed that the highest concentration of carvacrol in the headspace was in the presence of the WPI-carrageen-50 mM gel, followed by the WPI-carrageen-100 mM. A significantly lower concentration of carvacrol was found in the setup containing the gel of WPI-carrageenan-250 mM, compared with the carvacrol concentrations in package of WPI-carrageenan-50 mM during the first two stages ($p < 0.05$).

Figure 6. The concentration of carvacrol in headspace of package containing gels prepared with different concentrations of NaCl.

3.3.4.2. Carvacrol concentrations in the gels

The carvacrol concentration in gels prepared with NaCl from 50 mM to 250 mM during storage was shown in **Figure 7A**. The absorption rate of carvacrol in the WPI-carrageen-250 mM gel was faster than the rates in WPI-carrageen-50 mM and 100 mM. It took 6 days to reach an equilibrium between carvacrol release from film and absorption in gels. In terms of WPI-carrageen-100 mM, the absorption rate decreased after day 6, and it was expected that the equilibrium point would be observed after day 7. A linear increase of carvacrol concentration was found for the WPI-carrageen-50 mM gel ($R^2=0.97$). The results of the carvacrol absorption in the gels are in line with the concentration profile in the headspace. At higher salt concentration, the headspace carvacrol concentration is lower and the gel concentration is higher compared to the lower salt concentration. Carvacrol absorption in WPI-carrageen-250 mM was significant higher than the concentration in WPI-carrageen-50 mM for each measuring day ($p<0.05$). 40% more carvacrol was found in gel prepared with 250 mM of NaCl than in gel at 50 mM of NaCl at day 7. The carvacrol absorption in WPI-carrageen-100 mM gel was significant higher than WPI-carrageen-50 mM gel but lower than WPI-carrageen-250 mM ($p<0.05$). To find a direct relationship between carvacrol absorption and gel microstructure, the correlation between the average pore size of gel and the final carvacrol concentration during storage was studied. As shown in **Figure 7B**, carvacrol concentration decreased linearly with the decrease in the pore size ($R^2=0.96$). This suggests that the pore size of gel was an important factor that showed a direct influence on carvacrol absorption, and large pores were favourable for carvacrol absorption in gel matrix during storage.

Figure 7. (A) The absorptions of carvacrol in gels in presence of various amounts of NaCl from 50 mM to 250 mM. (B) The relationship between carvacrol absorption in gel at the day 7 and pore size of gel.

The desorbed carvacrol was calculated by comparing the residual amount on film determined by extraction procedure. Still a high amount of residue carvacrol left in films after 7 days storage. The average desorbed mass of carvacrol in films was 3.14 ± 0.47 mg. However, there was no significant difference among samples as the initial amounts of carvacrol in film were about 7.0-fold higher than the final absorptions in gels. The high residue of carvacrol in PLA film showed that the PLA-carvacrol film is able to provide a prolonged release of antimicrobial agents (20 °C) (**Figure S6**). An extended-release of antimicrobials from AP is important to maintain a concentration of antimicrobials over minimum inhibitory concentration (MIC), which is the key factor to inhibit the bacteria growth in foods during food storage period (Sanla - Ead, Jangchud, Chonhenchob, & Suppakul, 2012).

The results of carvacrol partition in a package showed that there is a clear effect of the microstructure of gel on carvacrol absorption in gel samples. Carvacrol topological polar surface area is $20.2 \text{ \AA}^2$ (Kim et al., 2019), which indicates the molecule is able to absorb and diffuse to the gel that contains the pores ranging from $457 \pm 27 \text{ nm}^2$ to $881 \pm 91 \text{ nm}^2$. Once carvacrol released from PLA film to the surface of gels, the obstruction by the gel network interferes the Brownian motion, and the interaction with the binding sites of protein further impedes the diffusion (Lorén et al., 2009; Mao, Roos, Biliaderis, & Miao, 2017; Wang & Arntfield, 2015). Besides, according to Ghanbarian, Hunt, Ewing, and Sahimi (2013): tortuosity in porous media is inversely proportional to pore radius. This means that carvacrol diffusion can be affected by interaction with the pore walls and the tortuosity of the pores. Therefore smaller a pore size will result in a lower effective diffusion. This highlights the importance of large pores on the diffusion. The gel that was prepared with 250 mM NaCl absorbed the highest amount of carvacrol, which can be explained with its high loose microstructure indicated by large pores and weak physical properties. In addition, the relative smaller WPI-containing aggregates were observed in WPI-carrageen-250 mM, resulting a large surface area that provided more binding sites for carvacrol. In contrast, the compact structure of WPI-carrageen-50 mM with relative small pores and strong physical properties was unfavourable of carvacrol entrapment by the gel.

Since the information of the influence of food matrix on antimicrobials absorption is scarce, it is appropriate to compare the current finding with previous studies on related processes. A possible reference is the flavour release and retention from foods, which is the opposite process of the binding/trapping of volatile antimicrobials by food matrix. The effects of texture and (micro) structure of food/food model on the retention of aroma compounds during processing

and storage on the aroma release and perception during consumption were already well studied (de Roos, 2003). Carr et al. (1996) found that increasing gel hardness reduced the release of flavour compounds in the headspace. A similar finding was described by Weel et al. (2002) who studied the flavour release and perception of flavoured whey protein gels. The results showed that the perceived intensity of flavours (diacetyl and ethylbutyrate) decreased with an increased in gel hardness, which showed that a change in texture (gel hardness and water holding capacity) determines the perception of flavour intensity in a psychophysical way. In addition, the importance of porous structure on the food ingredients (e.g. sodium) release from food matrix has been proposed by several studies in recent years. Kuo and Lee (2017) suggested that the porosity of non-fat containing solid lipoproteic colloids with 1.5% and 3.5% NaCl correlated positively with serum release and the maximum rate of sodium release. In the present study, we observed the loose gel of WPI-carrageenan-250mM NaCl absorbed more carvacrol than gels of WPI-carrageenan-100 and 50 mM NaCl, which is in line with the results of the strong release behaviour of flavour from weak gel. And an important factor that shows the direct correlation between antimicrobials absorption and the microstructure of foods was suggested as the pore size, which was also in line with the previous study.

3.4. Conclusion

This study demonstrated that volatile antimicrobial compound absorption in WPI-carrageenan-NaCl gels was significantly affected by the texture/ microstructure of the food gel models. The most dense gel (WPI-carrageenan-50 mM), showed the lowest carvacrol absorption rate. Whereas the highest amount of carvacrol was measured in the softest gel (WPI-carrageenan-250 mM) that was recognized as the weakest gel with the large pores. A combination of meso and micro factors can explain this behaviour. At meso level the rheological properties and the WHC of gels indicates a more loose structure favour carvacrol absorption. At micro level the large pores and the small WPI-containing aggregates favour a better entrapment carvacrol in gels. As a whole, this study contributes to the understanding of the mechanistic aspects of the antimicrobial effect of PLA-carvacrol packaging film when it is applied to preserve food gels with different microstructures. A tailored design of antimicrobial AP can significantly contribute to increase the shelf life of packaged products and to reduce the food waste at domestic sites, which is the main cause of food waste in the Western countries (Roodhuyzen, Luning, Fogliano, & Steenbekkers, 2017).

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Supplementary materials

Methods

CLSM image analyse methods

ImageJ software was used to determine the average size of protein aggregates in CLSM 3D images. MaxEntropy method was first used to determine the threshold values of aggregates in the images. Images were then converted to binary images where the protein aggregates were solid white and the rest of area were black. The volume (μm^3) of protein aggregates was estimated using a 3-D objects counter plugin. The average protein aggregate V_{av} was calculated as follows (Kenyon, et al., 1989)

$$V_{av} = \sum_{i=1}^n p_i V_i \quad (1)$$

Where n is the total number of the aggregates, p_i is the proportion, and V_i is the volume of each aggregate (μm^3).

SEM image analyse methods

To analyse the pore size of samples from SEM images, the threshold level is the most crucial factor that determines whether a particular pixel in the image is part of the objects (pores in this study) or simply part of the background of the image. We determined the threshold based on a simple thresholding technique developed by (Pugnali, Matia-Merino, & Dickinson, 2005).

Figure S5 shows the total number of pores (including all sizes of pores) identified in the image as a function of the selected threshold level. At the $TL=0$, no pixel in the image is considered as part of the white background, which means all the pixels are considered to be part of the gel network, therefore no pores are detected. With the increasing TL , the number of pores increases because of the increasing proportion of background (“white”) pixels incorporated into the image. At $TL=TL_p$, the number of pores reaches a maximum and it decreases for further increase in TL . The reason of decreased pores in high TL can be explained as some pores connect through channels with neighbouring pores (to become a larger single pore) as more pixels are considered to become part of the background. At the extreme high TL , the entire image is considered to be a single big pore. The value of $TL=TL_p$ is considered as threshold level that all individual pores were detected in the image, corresponding to the maximum number of pores measured (see **Figure S5**). We used this method to determine the threshold level and we applied a same criterion to all the raw images. The pores identified in this method were highly in line with the

pores founds in image by “eye approach”. After the threshold was set, the binary image was processed with noise reduction. The imageJ software was then applied to analyse the average size of pores. The average pore size S_{av} was calculated as follows

$$S_{av} = \sum_{i=1}^n p_i s_i \quad (2)$$

Where n is the total number of the pores, p_i is the proportion, and s_i is the area of each pore (μm^2).

Small strain rheological properties

Rheological characterization was performed according to the methods described by De Jong, Klok, and Van de Velde (2009) with some modifications. Analysis was carried out by using a Physica MCR 302 Rheometer (Anton Paar GmbH, Graz, Austria) equipped with a concentric cylinder geometry (stator inner radius 16.66 mm, rotor outer radius 24.95 mm). Approximately 4.7 mL of pre-gel solution was added to the cylinder. Time-sweep measurements were carried out throughout the gelation process (4 °C) at an angular frequency of 1 Hz and with an applied strain of 1%. The sweep measurements were taken every 10 s for 10 hour. When the gelling process was completed, the sample was subjected to frequency sweep (varied angular frequency from 0.01 Hz to 10 Hz) and strain sweep (increased strain from 0.1% to 1000%) to understand the structural dependency of the gels at 20 °C. The range of frequency and strain independence of the elastic and viscous moduli is characterized as linear viscoelastic range (LVR). The fixed strain (1%) and frequency (1 Hz) were used at this study for oscillatory tests of the samples.

Large strain (fracture) rheological properties

To determine the gel strength, gels (size 22.5×37.5 mm) were subjected to the single cycle compression test at TA-XT Plus Texture (Stable Micro System Co., UK). Briefly, the gels was pressed by a 60 mm diameter cylindrical probe. The test parameters were set at 25 mm distance, 0.05 N trigger force, 1.0 mm/s pre-test speed, 1.0 mm/s test speed and 5.0 mm/s post-test speed. The compression force required to break down the gel were used to indicate gel hardness of the samples.

Protein solubility analysis

Changes in protein aggregates during storage were determined according to the method of (Visessanguan, Benjakul, Riebroy, & Thepkasikul, 2004; Zhou, Liu, & Labuza, 2008) with

certain modifications. Gel sample (5 mL) were extracted with (50 mL) of phosphate buffer (10 mM, pH 7) using a rotor-stator homogenizer (Ultra-Turrax, IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 8000 rpm for 1 min, followed by centrifugation at 5000×g for 15 min. The extraction was repeated twice. The suspension was stirred at room temperature at a speed of 400 rpm for 60 min, followed by centrifugation at 20000×g for 15 min. The supernatants were diluted with phosphate buffer (10 mM, pH 7) to a final concentration of 1%. The concentration of soluble proteins in the supernatant was then determined using the BCA Protein Assay Kit (Pierce BCA protein assay, Thermo Fisher Scientific Inc., Rotterdam, Netherlands).

Results and discussion

Small strain rheological properties

Frequency and strain sweep oscillatory tests were used to measure the structural aspects of the gels. The G' and G'' curves of the samples with NaCl addition show a low dependency on the frequency in the range of 0.01 to 10 Hz (**Figure S3A**), indicating the dominant elastic behaviour of the gel. However, the G' and G'' of no salt sample was strong influenced by the applied frequency, which proved that there was no gel formation at this mixture. **Figure S3B** shows that an increased concentration of NaCl decreased the applied strain for the crossover point (σ) of G' and G'' . The sample prepared with 50 mM NaCl required a highest strain to obtain the σ value. Whereas the σ of samples in 250 mM NaCl appeared at a low strain that less than 10 %. The σ is defined as the critical stress point where the LVR ends, which was recognized as the moment when structured viscoelastic samples undergo a gel-solution transition under increasing oscillatory shear (Patel et al., 2015). The results of frequency sweep (**Figure S3A**) and strain sweep (**Figure S3B**) confirmed that all the salt-induced gel samples are elastic gels as higher values of G' over G'' in the range of frequency stress within LVR. The gel prepared with 50 mM of NaCl required the highest strain to deform from gel-like to solution-like, followed by the gel containing 100 mM of NaCl.

The storage modulus is an indicator for the overall stiffness of a gel (Dagdas, Tombuloglu, Tekinay, Dana, & Guler, 2011). The results that storage modulus (G') decreased with the concentration of NaCl increasing from 50 mM to 250 mM revealed that salt induced WPI-carrageenan gel had the highest stiffness in 50 mM NaCl, followed by the gel in 100 mM NaCl. The lowest stiffness of gel was the sample in 250 mM of NaCl which indicated by the highest

ratio between G'' and G' ($\tan \delta$). The possible reasons could be that the decreased size of WPI aggregates and large cavities in high concentration of NaCl solution led to a looser structure of gel, giving a low G' (Wang et al., 2018). A similar effect that gel stiffness increased with NaCl addition, however it decreased when NaCl concentration was further increased was also found from previous studies. Liu et al. (2019) concluded that an increase in G' and G'' of whey protein isolate-lotus root amylopectin composite gels at the concentration of NaCl from 0 M to 0.05 M. Whereas the values of G' and G'' decreased when NaCl concentration was further increased from 0.05 M to 0.2 M. The results from current study were in line with the previous studies.

Large strain (fracture) rheological properties

Fracture properties are important to reflect the network structures of gels (Renkema, 2004). **Figure S4** shows the changes in hardness of the salt induced WPI-carrageenan gel at different concentrations of NaCl during a 7-day storage at room temperature (20 °C). The hardness of gel prepared with 50 mM NaCl was significantly higher than the gel in 100 mM NaCl ($p < 0.05$). The gel prepared with 250 mM NaCl showed a significant lowest hardness, compared with other two samples ($p < 0.05$). The reason for the decreased hardness when increasing NaCl can be explained from the morphology of gel samples (**Figure 3**). SEM images show a compact microstructure with small pores in WPI-carrageenan-50 mM NaCl gel. This dense structure contributes to a high strength. The fluctuations of hardness of gels during storage might be due to the formation of insoluble protein or large protein aggregates (**Figure S5**) (Alting, Hamer, de Kruif, Paques, & Visschers, 2003). A similar trend was also found by Çakır et al. (2011), who concluded that the fracture strain of gel decreased with the increasing of the NaCl in carrageenan concentration of 0.4% (although only a slight increase at 50 mM NaCl).

Figure captions

Figure S1 Visual observations of gels at three ionic strengths (a) 50 mM, (b) 100 mM and (c) 250 mM

Figure S2 Storage (G') and loss modulus (G'') as a function of time for WPI-carrageenan-50 mM NaCl, WPI-carrageenan-100 mM NaCl and WPI-carrageenan-250 mM NaCl.

Figure S3. Frequency dependence (A) and strain dependence (B) of the storage and loss modulus of gel samples.

Figure S4. Changes in hardness (N) of gels.

Figure S5 Changes in soluble protein concentration in supernatant after centrifuge as a function of time during storage at 20 °C.

Figure S6 Carvacrol partitioning in each phase of the package system including the film, food matrix and headspace of setup containing a) WPI-carrageenan-50 mM NaCl, b) WPI-carrageenan-100 mM NaCl and c) WPI-carrageenan-250 mM NaCl.

Figure S7 Number of pores, N , as a function of the grey level threshold, TL , applied to the image. The threshold levels used for the identification of pores, TL_p , are indicated.

Figure S1. Visual observations of gels at three ionic strengths (a) 50 mM, (b) 100 mM and (c) 250 mM

Figure S2. Storage (G') and loss modulus (G'') as a function of time for WPI-carrageenan-50 mM NaCl, WPI-carrageenan-100 mM NaCl and WPI-carrageenan-250 mM NaCl.

Figure S3. Frequency dependence (A) and strain dependence (B) of the storage and loss modulus of gel samples.

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Figure S6. Carvacrol partitioning in each phase of the package system including the film, food matrix and headspace of setup containing a) WPI-carrageenan-50 mM NaCl, b) WPI-carrageenan-100 mM NaCl and c) WPI-carrageenan-250 mM NaCl.

Figure S7. Number of pores, N , as a function of the grey level threshold, TL , applied to the image. The threshold levels used for the for the identification of pores, TLp , are indicated.

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Chapter 4

The effect of pore size on the diffusion of volatile antimicrobials is a key factor to preserve gelled foods

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Abstract

The present study aimed to understand how the microstructure of gelled foods impacts the diffusion of a volatile antimicrobial compound and its efficacy at different depths from the surface. Carvacrol-loaded polylactic acid film was used to inhibit the growth of *Pseudomonas fluorescens* inoculated in the NaCl induced WPI-carrageenan gels during storage at 4 °C. Gel characteristics, like stiffness and pore size, were varied by changing the ionic strength. A direct relationship between carvacrol concentrations in layers of the gel at different depths and average pore size of gels was found, indicating that the diffusion of antimicrobials was increased in gels having larger average pore size. Regarding the results of in-depth diffusion of carvacrol, antimicrobial efficacy was dependent on the distance from the surface of the gels. The inhibition effect of carvacrol decreased with the increase in depth of WPI-carrageenan-100 mM NaCl gel. Weaker inhibition effect of carvacrol was observed in the WPI-carrageenan-50 mM NaCl gel than that in the WPI-carrageenan-100 mM NaCl gel due to the lower carvacrol concentrations in the gel containing small pores. Our study demonstrates the importance of considering the diffusion of antimicrobials in solid/semi-solid foods in the antimicrobial packaging design.

Keywords: Carvacrol, Whey protein isolate, Carrageenan, Gel pores, Diffusion, Antimicrobial effect, Antimicrobial packaging.

4.1. Introduction

Antimicrobial packaging (AP) has received a great deal of attention in recent years because of the potential application to reduce, retard and inhibit the growth of spoilage and pathogenic microorganisms (Soares et al., 2016). A number of studies showed that the AP could provide a prolonged release of antimicrobial compounds and maintain their activity during long-time storage (Cha & Chinnan, 2004; Jideani & Vogt, 2016), thereby extending the shelf life of perishable foods. AP can be in contact with the surface of foods or be in indirect contact with foods via the headspace of the food package. The efficacy of AP depends on several factors such as the nature and type of antimicrobial agents, the mass transfer coefficients of active substances, the target microorganisms (*i.e.* foodborne pathogens, spoilers or populations), and the physicochemical characteristics of the food products to be protected (Fernández-Pan, Maté, Gardrat, & Coma, 2015; J. H. Han, 2003). In the past decades, many studies were conducted dealing with the development of novel AP with various materials and active agents (Sung et al., 2013). The relevance of the mass transfer coefficients of active substances at different food package systems was also highlighted (Jideani et al., 2016; Singh, ho Lee, Shin, & Lee, 2016). However, the influence of the microstructure of the preserved food matrix on the antimicrobials absorption and diffusion in foods is largely unexplored, while it can be reasonably hypothesized that it has a direct influence on the antimicrobial efficacy of the AP.

Gels made of protein-polysaccharide interacting through electrostatic interactions can be designed to simulate food such as jellies, gummies yoghurt, processed meats, etc. In particularly model systems made up by common food ingredients such whey protein isolate (WPI) and carrageenan are preferred as they are robust and versatile (Alavi, Emam-Djomeh, Yarmand, Salami, Momen, & Moosavi-Movahedi, 2018; De la Fuente, Hemar, & Singh, 2004; Hosseini et al., 2013). Gels prepared with WPI and carrageenan can be obtained by the addition of salt and their microstructure can be adjusted by increasing or decreasing of the ionic strength (Campo, Kawano, da Silva Jr, & Carvalho, 2009; Turgeon & Beaulieu, 2001). The growth of microorganisms can occur on the surface as well as in the internal part of the model system depending on the origin of contamination, contaminant species, food composition, stiffness (density) and microstructure (Cao-Hoang, Grégoire, Chaîne, & Waché, 2010). It is thus of great importance to inhibit the growth of microorganisms, not only on the surface but also in the interior, to enhance the quality and extent the shelf life of gelled food during storage. Unfortunately, the diffusivity of antimicrobial agents in the complex colloidal systems has not

been extensively studied. Only few studies reported the diffusion of lysozyme, nisin and sorbic acid in agarose gels (Chollet, Swesi, Degraeve, & Sebti, 2009; Giannakopoulos & Guilbert, 1986; Mattisson, Roger, Jönsson, Axelsson, & Zacchi, 2000; Ripoche, Chollet, Peyrol, & Sebti, 2006; Sebti, Blanc, Carnet-Ripoche, Saurel, & Coma, 2004). Thereby, the mechanism that causes the differences of bacterial inhibition in protein-polysaccharide composite gels that are physically different is still lacking.

With regard to the antimicrobial agents, several compounds can be used for antimicrobial food packaging (Uzunlu & Var, 2018). Carvacrol ($C_{10}H_{14}O$) is a monoterpenoid phenolic volatile compound, which is the major constituent of oregano and thyme essential oil (Fachini-Queiroz et al., 2012). The antimicrobial effect of carvacrol in model food systems or in real food is well documented (Vergis, Gokulakrishnan, Agarwal, & Kumar, 2015). Carvacrol implements its antimicrobial activity by disrupting the microbial cytoplasmic membrane and thereby compromising its function (Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009). A great number of studies have reported that the incorporation of carvacrol into the packaging polymers is an effective way to protect foods (Vergis et al., 2015). Among potential polymer carriers, polylactic acid (PLA) based films are often used for the incorporation of active agents into polymer film because of its good properties (biobased, mechanical, barrier, optical and thermal) (Tawakkal, Cran, Miltz, & Bigger, 2014). Antimicrobial packaging prepared with carvacrol incorporated PLA film is expected to have a good performance on gels preservation (L. Wang, Dekker, Heising, Fogliano, & Berton-Carabin, 2020). Since the spatial distribution of antimicrobial compounds in the foods determines the antimicrobial efficacy of AP, the diffusion behaviour of carvacrol in food is important to know (Blot, Pea, & Lipman, 2014). It is particularly important to evaluate the depth of efficiency of the antimicrobial activity within the food product. To our knowledge, no work has been reported regarding the quantification of diffusion of a volatile antimicrobial in food gels and its effect on the antimicrobial efficacy.

In the present study, we used a carvacrol loaded PLA antimicrobial packaging to inhibit the growth of the spoilage bacteria (*Pseudomonas fluorescens*) in WPI-carrageenan gels that were physically different during a 10-day storage at 4 °C. The carvacrol concentrations at different depths of the gels were quantified and the antimicrobial effect within layers at different depths was investigated to assess the effectiveness of the antimicrobial films in the preservation of semi-solid foods.

4.2. Materials and methods

4.2.1. Materials

Poly (lactic acid) (4043 D Ingeo TM) was kindly supplied by Wageningen Food & Biobased Research Institute. Carvacrol ($\geq 98\%$), dichloromethane (DCM), hexane, 2-Propanol, sodium chloride, ringer solution $1/4$ strength tablets, carrageenan (predominantly κ and lesser amounts of λ carrageenan, specification: PRD.1.ZQ5.10000042025) were purchased from Sigma Aldrich Pty. Ltd., Netherlands. As the statement from the manufactory, carrageenan consists of $\leq 11\%$ K^+ , $\leq 3.5\%$ Ca^{2+} and $\leq 2.0\%$ Na^+ . Whey protein isolate (97.5% protein, dry basis) was obtained from Davisco (Bipro, Davisco, lot # JE 198-1-420, USA). The ion contents per 100 g WPI powder are 750 mg sodium, 60 mg potassium, 130 mg calcium, 75 mg phosphorus and 25 mg magnesium (Peng et al., 2019). The Plate Count Agar (PCA) was purchased from Merck (Schiphol-Rijk, Netherlands). Peptone Physiological Salt Solution (PFZ) was purchased from Tritium Microbiologie BV (Eindhoven, Netherlands).

4.2.2. Carvacrol loaded PLA film preparation

The inclusion complex of carvacrol and PLA was prepared using a solvent-casting method (Rhim, Mohanty, Singh, & Ng, 2006). Briefly, 4 g (± 0.01 g, dry basis) of PLA pellets dissolved in 100 mL of DCM and stirred under magnetic stirring for 6 hour at 30 °C. Two gram of carvacrol was then added to the solution and stirred for 1 hour at room temperature (20 °C). The resulting mixture was sonicated with an ultra-sonicator (HBM Machines, Moordrecht, the Netherlands) at 40 KHz for 30 minutes to further homogenize the solution. Subsequently, 20 mL of dissolved solution was pipetted onto a glass petri dish (size 100×15 mm) and dried in a fume hood at room temperature for overnight. The dried film was peeled from the glass petri dish, and was stored in a sealed glass container at 4 °C for further analyse.

4.2.3. WPI-carrageenan gel preparation

4.2.3.1. Sterilization of the raw materials of gels

The raw materials of gels and tools used for gel preparation, including WPI, carrageenan powder, 1 M NaCl solution, ultrapure water, magnetic stirrers, graduated cylinders (250 mL), beakers (200-400 mL), pipette tips (1-5 mL), glass tubes with double openings and cutter were sterilized before the preparation of gels. For the sterilization of WPI and carrageenan, dry

powders were separately placed at 100 °C in an incubator for 30 min. The NaCl solution, ultrapure water and experiment tools were sterilized by autoclave at 121 °C for 15 min.

4.2.3.2. Gel preparation and bacteria inoculation

Na⁺ induced gelation of WPI and carrageenan gel was prepared according to our previous work (L. Wang, Fogliano, Heising, Meulenbroeks, & Dekker, 2020). WPI (20% w/v) and carrageenan (4% w/v) stock solutions were prepared in ultrapure water (pH 6.7) under stirring by a magnetic stirrer for 4 hour at room temperature (≈ 20 °C). Stock solutions were kept overnight at 4 °C to promote complete hydration. In case of WPI/carrageenan gels, the stock solution of WPI and carrageenan was diluted with ultrapure water and NaCl solution (1 M) to obtain the final mixtures with 10% (w/v) WPI, 0.4% (w/v) carrageenan and 50 mM or 100 mM of NaCl. Carrageenan stock solution was heated at 80 °C in a water bath for 30 min before mixing with WPI solution. The WPI solution was heated to 40 °C and subsequently mixed with the carrageenan solution. During the stirring of the WPI and carrageenan solution at 40 °C on a magnetic stirrer (30 min), sodium chloride was gradually added to the solution. The prepared pre-gel solutions were cooled down to 37 °C and inoculated with *Pseudomonas fluorescens* to obtain a final concentration of 10⁴ CFU/mL. The mixtures were stirred at a magnetic stirrer for 1 min to ensure the bacteria was evenly distributed in gels.

Four millilitres of solution was pipetted into a glass tube (20-mL) with double openings and closed with two polypropylene lids. Samples were stored at 4 °C in a fridge overnight for gel formation.

4.2.4. Experimental design

Carvacrol diffusion and antimicrobial activity was studied in a glass tubes (20-mL) with openings at the top and bottom (**Figure 1**). This system contains a piece of carvacrol incorporated PLA film (0.100 ± 0.001 g), WPI-carrageenan gel (4 mL) and a pipette tip that was used to prevent the direct contact between packaging film and gel. Each glass tube was closed by the polypropylene lids at the two openings and fully sealed with tape (crepe paper masking tape, tesa® 4323, Germany). Three cylindrical slices of gels (15 mm diameter and 10 mm thickness) were obtained after the incubation by cutting a whole gel with a sterile cutter, abbreviated as top, middle and bottom layer, respectively. Several tubes consisting of the same weight of CAR-PLA film and the same volume of gel were prepared for a 7-day continuous

sampling for diffusion study and a 10-day sampling for antimicrobial study. For each timepoint, a separate tube was used. Packaging setups were stored at 4 °C during storage period.

Figure 1. Setup used to study carvacrol diffusion and antimicrobial efficacy in Food-Package system.

4.2.5. Microstructure of gels

Scanning electron microscope (SEM)

Gels for SEM were prepared according to Munialo, van der Linden, Ako, Nieuwland, Van As, and de Jongh (2016) with modifications in which the samples were cross-linked in 2.5% (v/v) glutaraldehyde in phosphate/citrate buffer for 7 h followed by washing three times in phosphate/citrate buffer. The washed samples were dehydrated with ethanol in steps of 10-30-50% for 10 min, 70% for overnight and 80-90-100% for 10 min, followed by dehydration once with 100% ethanol for 20 min. The samples were then dried by critical point drying (CPD 030 BalTec, Liechtenstein) followed by breaking the gels to smaller pieces and coating with 10 nm iridium in SCD 500 (Leica EM VCT 100, Leica, Vienna, Austria). All samples were analysed with a field emission scanning electron microscope (Magellan XHR 400L FE-SEM, FEI, Eindhoven, The Netherlands) at room temperature (20 ± 2 °C) at a working distance of 4 mm with SE detection at 2 kV. The average pore size was calculated using ImageJ software (1.52, National Institutes of Health, USA) (Pugnaroni, Matia-Merino, & Dickinson, 2005) (see supplementary information). Four of independent images were used for estimation of the average pore size for each gel.

4.2.6. Quantification of carvacrol diffusion in gels

4.2.6.1. Gas Chromatography–Flame Ionization Detector (GC-FID) calibration

The calibration was prepared with known amounts of carvacrol in a mixture of organic solvent (carvacrol:2-propanol=3:1). Briefly, 8 concentrations between 1 µg/mL and 30 µg/mL were prepared by dilution a stock solution (100 µg/mL) and injected (10 µL) into the Gas Chromatography–Flame Ionization Detector (GC-FID) (Thermo Scientific, Waltham, Massachusetts, United States). A linear calibration curve was obtained ($R^2 = 0.9994$) for carvacrol.

4.2.6.2. Quantification of carvacrol diffusion in slices of gels

Carvacrol was extracted from the slice of gels using a method from L. Wang, Fogliano, et al. (2020) with some modifications. The 1.5 mL of organic mixtures (hexane:2-propanol=3:1) was added into each slice of gel. The mixture was warmed in a shaking water bath (JULABO GmbH, Seelbach, Germany) at the speed of 60 rpm at 40 °C for 20 min to dissolve the gel and extract carvacrol. The mixture was then centrifuged at 1000 g for 10 min to obtain the top organic layer. The organic solution in top layer was collected into the amber glass vial (2.0 mL) for liquid analyses by GC-FID. In order to test the extraction efficiency of carvacrol from gel samples, we added known amounts of carvacrol in gels and extracted carvacrol from gels using the aforementioned method. A high extraction ratio (>97%) was achieved by using this extraction method.

For carvacrol compound quantification, 10 µL of organic sample was analysed by GC-FID equipped with a semi-capillary RTX-1301 (30 m, 0.53 mm, 0.5 µm) column (Restek, Teknokroma, Barcelona, Spain). To operate GC-FID, the temperature of column oven was initially set to 60 °C, and it increased to 250 °C with a speed of 15 °C min⁻¹. The temperatures for the detector was 250 °C. Carrier gas was helium with a flow rate of 2 mL/min. Injections were set in split mode with a split flow of 10 mL/min. The total running time was 25.25 min for each measurement.

4.2.6.3. Modelling for absorption assay

The absorption of carvacrol in food matrix was fitted by a zero-order model (X. Liu, Yang, Yu, Zhu, Zhao, & Williams, 2019):

$$C_t = kt \tag{1}$$

where C_t (mg/mL) is the absorbed carvacrol at any given time t ; k is the absorption rate constant (mg/mL/day), and t indicates the storage time (day).

4.2.7. Microbiological analysis of gels

4.2.7.1. Microbial strain

Pseudomonas Fluorescens ATCC 13525 was kindly provided by the Food Microbiology Group (Wageningen University & Research, the Netherlands). It was cultured in nutrient broth (Merck BV, Schiphol-Rijk, the Netherlands) at 35 °C for 24 hours after inoculation with the stock culture.

4.2.7.2. Antimicrobial experimental design and enumeration of bacteria

Samples were divided into two groups as the antimicrobial and the control group. Concerning the antimicrobial group, gels (4 mL) were stored with carvacrol loaded PLA film (0.100 ± 0.001 g) during storage. In the control group, the growth of *Pseudomonas fluorescens* was evaluated without packaging films. The enumeration of *Pseudomonas fluorescens* was conducted by using the drop-plate method (Bremer, Osborne, Kemp, & Smith, 1998). Layers of gels in both the antimicrobial and control groups were mixed with 5 mL of sterilised ringer solution into a sterilized stomacher bag. The mixtures was homogenized with the stomacher 400 circulator for 2 minutes at 230 rpm at room temperature. Appropriate serial decimal dilutions were obtained in Peptone Physiological Salt Solution (PFZ). Counts of *Pseudomonas fluorescens* were determined in triplicate by plating 100 μ L of solution on plate count agar (PCA). Colonies were counted after 24 h incubation at 30 °C. The enumeration of bacteria was conducted at day 0, 1, 3, 5, 7 and 10.

4.2.7.3. Microbial growth modelling

The modified Gompertz model (eq 2) (Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990) was fitted to the experimental counts of *Pseudomonas fluorescens* growing in gels stored at 4 °C.

$$\ln \frac{N}{N_0} = A_s * \exp \left(- \exp \left(\frac{\mu_{max} * e}{A_s} (\lambda - t) + 1 \right) \right) \quad (2)$$

where N/N_0 represents the ratio of the microbial counts at time t (CFU/mL) to the initial (t_0) microbial counts (CFU/mL), A_s is the asymptotic log count as time decreases indefinitely

(CFU/mL), μ_{max} is the maximum specific growth rate (day^{-1}), λ is the lag phase (day), t is the storage time (day).

4.2.8. Statistical analyses

All experiments were performed in independent duplicate batches, and measurements were repeated in triplicate. Data are presented as mean values $\pm$ standard deviations (SD) for all data points. Significant differences were evaluated by using SPSS (version 25.0, International Business Machines, Armonk, NY, USA), one-way analysis of variance (ANOVA) with post-hoc pairwise analysis by the Tukey test to assess the significant differences between samples, the significance level was set at $p < 0.05$. An independent samples t -test was used to compare the differences between antimicrobial and control groups in microbiological analysis. A multi-factorial ANOVA was performed to analyse the effect of two factors (storage days and layers of gels in two types) on bacteria reduction values through a general linear model with the univariate analyse, two-way ANOVA with post-hoc pairwise analysis by the Tukey test to examine the significant differences between samples. The parameters of Eq. (1) and (2) were estimated with the lowest mean square error (MSE), where k , μ_{max} , A_S and λ were obtained.

4.3. Results and discussion

4.3.1. Microstructure of the food gel model

The microstructure of the gel at the submicron level is visualized by SEM images shown in **Figure 2**. The physical analyses of WPI-carrageenan gels as a function of ionic strength, including the rheological properties and water holding capacity, is reported in detail in the supplementary materials. Pore morphology in different layers (top, middle and bottom) was assumed to be a uniform distribution with a similar average pore size along the sample, according to Savina et al. (2011). As shown in **Figure 2**, a dense and homogeneous network with even and small pores was formed in gel samples with 50 mM NaCl, where a well crosslinked protein-carrageenan network was seen, suggesting that the liquid phase was greatly entrapped in a continuous network (Uruakpa & Arntfield, 2006). A coarse-stranded network with large pores was observed at higher salt concentration (WPI-carrageenan-100 mM). The resulting network is composed of small spherical beads. The surface area of pores was estimated using ImageJ software (see supplementary information), and the results suggest that the average pore size increased significantly from $267 \pm 30 \text{ nm}^2$ to $403 \pm 73 \text{ nm}^2$ with increasing NaCl

concentration from 50 mM to 100 mM (average of pore surface area on 4 independent images for each sample) ($p < 0.05$). The loosely crosslinked WPI-carrageenan network with large pores in the gel sample can be explained by the lack of a consistent structural uniformity that is reflected by the micro-phase separated and heterogeneous network gel (Ako, Nicolai, Durand, & Brotons, 2009; Chantrapornchai & McClements, 2002; Chin, Go, & Xiong, 2009). At neutral pH, high levels of incompatibility between proteins and polysaccharides tends to occur at high salt concentration (Goh, Sarkar, & Singh, 2014; Harrington, Foegeding, Mulvihill, & Morris, 2009). The strong shielding effect in high salt concentration leads to the formation of a particulate gel network consisting of connected spherical beads of WPI (Çakır & Foegeding, 2011). This heterogeneous network has been shown large pores than the homogeneous network (Chantrapornchai et al., 2002; Dumay, Laligant, Zasytkin, & Cheftel, 1999; Khalesi, Emadzadeh, Kadhodae, & Fang, 2019). From the visualization of the network by SEM (Figure. 2) and estimation of the average pore size, we can conclude that the gel prepared with 50 mM of NaCl forms an ordered gel network with small pores while addition of 100 mM of NaCl in gels results in a heterogenous network containing large pores. The same effect was also reported by K. Liu, Li, et al. (2019) who found that high concentrations (0.1 M, 0.15 M and 0.2 M) of NaCl induced an irregular WPI-lotus root amylopectin gel network with large pores.

Figure 2. SEM micrographs of WPI-carrageenan-50 mM NaCl (A) and WPI-carrageenan-100 mM NaCl (B) gels. The magnification of the SEM images is $\times 25000$ (first row) and $\times 80000$ (second row). The black areas correspond to the pores of the gel while the rest of area is the WPI-carrageenan network.

4.3.2. Carvacrol absorption/diffusion in layers of gel

The increase in carvacrol concentration in the gels during storage at two ionic strengths is shown in **Figure 3**. The diffusion behaviour was studied in the gels by cutting them into three cylindrical slices with each a height of 10 mm. For both ionic strengths, carvacrol absorption was significantly higher in the top layer than in the middle and bottom layers from day 1 to day 7 ($p < 0.05$); the concentrations in the middle layer were significantly higher than those in bottom layer from day 2 to day 7 ($p < 0.05$). The top layer of the gel prepared with 50 mM NaCl had a final absorption of $(4.28 \pm 0.69) \times 10^{-2}$ mg/mL, this is more than 4-fold higher than the middle layer $(0.96 \pm 0.02) \times 10^{-2}$ mg/mL which is around 4-fold higher than the bottom layer $(0.25 \pm 0.02) \times 10^{-2}$ mg/mL. With regards to the gel with 100 mM NaCl, the final absorption in the top layer $((5.07 \pm 0.74) \times 10^{-2}$ mg/mL) was about 4.2-fold higher compared with carvacrol in middle layer and 14-fold higher compared to the bottom layer at the end of storage. The total absorptions in 7 days in all layers of samples in 100 mM NaCl was about 19% higher than that in gels prepared with 50 mM NaCl. For the gel with 50 mM NaCl, 79% of the total amount of absorbed carvacrol was entrapped in the top layer, while this was about 75% for the gel with 100 mM NaCl after the 7 days storage.

Figure 3. Carvacrol diffusion in top, middle and bottom layers of gels in presence of 50 mM (A) and 100 mM NaCl (B).

Carvacrol is a small molecule with a topological polar surface area (TPSA) of $20.2 \text{ \AA}^2$ (<https://pubchem.ncbi.nlm.nih.gov/compound/10364#section=Clinical-Trials>, Kim et al., 2019). The molecule size (M_s) of carvacrol was estimated of 0.49 nm^2 for a 2D area. The calculation was shown in detail in the supplementary information. Once carvacrol released from the PLA film to the headspace, it was absorbed in the gel that has pore size from $267 \pm 30 \text{ nm}^2$

to $403 \pm 73 \text{ nm}^2$. The diffusion of carvacrol in the gel occurs by the concentration differences between surface and lower layers. Interestingly, the obstruction by the gel network interferes the Brownian motion thus effectively decreases the rate of diffusion (Lorén, Shtykova, Kidman, Jarvoll, Nydén, & Hermansson, 2009). The interaction with the binding sites that are located on the protein surface of β -lactoglobulin, and located on native bovine serum albumin (BSA) further impedes the diffusion behaviour of carvacrol in protein-polysaccharide complex (Burova, Grinberg, Grinberg, & Tolstoguzov, 2003; Considine, Patel, Singh, & Creamer, 2005; Mao, Roos, Biliaderis, & Miao, 2017; K. Wang & Arntfield, 2015). This causes a diffusion limitation of carvacrol in the gels, and results in the bigger differences between the layers of top/middle/bottom. It is known that the antimicrobial efficacy of AP is less effective against food-borne pathogens and spoilage bacteria when applied to real foods compared with model media (e.g. broth and agar). This is generally believed to be due to the activity losses of antimicrobial agents in complex food matrix by complexation with various components in the matrix (Ali, Noh, & Mustafa, 2015; Guo, Yadav, & Jin, 2017; Gutierrez, Barry-Ryan, & Bourke, 2008). However, another mechanism to explain this is the fact that differences in concentrations within the food can cause the concentration of an antimicrobial to be below the inhibiting concentration in certain parts of the food product. As suggested from this study, the slow diffusion of carvacrol in gelled foods may have implications regarding the difference of the antimicrobial efficacy of AP in the different location (from the surface to the centre parts) of solid/semi-solid foods. This mechanism should be taken into account when designing AP for specific product applications.

The absorption rate constant (k) and the final concentration of carvacrol ($C_{\text{carvacrol}}$) in each layer were listed in **Table 1**. The zero-order model has good performance on fitting the experiment data by high R^2 values ranging from 0.9565 to 0.9977, suggesting a constant absorption behaviour in all layers of both gels. For each layer of the gel at 100 mM NaCl, a significant higher absorption rate constant was found compared to the gel at 50 mM NaCl, indicating a stronger diffusion ability of carvacrol in the gel prepared with 100 mM NaCl. The highest absorption rate constants (k) were calculated for the top layers of WPI-carrageenan-50 mM and WPI-carrageenan-100 mM with $(62.76 \pm 1.43) \times 10^{-4}$ and $(72.55 \pm 0.77) \times 10^{-4} \text{ mg/mL/day}$, respectively. For the absorption rate constants in middle layers, about 1.3-fold higher k was found for the gel prepared with 100 mM NaCl compared to k $((12.68 \pm 0.44) \times 10^{-4} \text{ mg/mL/day})$ for the gel in 50 mM NaCl. The k value in the bottom layer of the 100 mM NaCl gel was 1.6 times higher in the bottom layer of the 50 mM NaCl gel ($k = (2.89 \pm 0.37) \times 10^{-4} \text{ mg/mL/day}$).

In addition, the final absorptions in middle and bottom layer of the gel with 100 mM NaCl were significantly higher than those in middle and bottom layer of the gel with 50 mM NaCl ($p<0.05$). The significant higher absorption rate constants in the gels with 100 mM NaCl than those in the gels with 50 mM NaCl show the importance of the larger pores on the antimicrobials diffusion in the gels (pore size: from $267 \pm 30 \text{ nm}^2$ to $403 \pm 73 \text{ nm}^2$).

Table 1. Carvacrol absorption rate constant¹ (*k*), correlation coefficients (*R*²) and final concentration (*C*_{carvacrol}) in layers.

	Top layer		Middle layer		Bottom layer	
	Gel-50mM	Gel-100mM	Gel-50mM	Gel-100mM	Gel-50mM	Gel-100mM
<i>k</i> (mg/mL/day)	(62.76 ± 1.43) × 10 ^{-4 a}	(72.55 ± 0.77) × 10 ^{-4 b}	(12.68 ± 0.44) × 10 ^{-4 a}	(15.98 ± 0.64) × 10 ^{-4 b}	(2.89 ± 0.37) × 10 ^{-4 a}	(4.69 ± 0.19) × 10 ^{-4 b}
Final <i>C</i> _{carvacrol} (mg/mL)	(4.28 ± 0.69) × 10 ^{-2 a}	(5.07 ± 0.74) × 10 ^{-2 a}	(0.96 ± 0.02) × 10 ^{-2 a}	(1.10 ± 0.08) × 10 ^{-2 b}	(0.25 ± 0.02) × 10 ^{-2 a}	(0.37 ± 0.03) × 10 ^{-2 b}
<i>R</i> ²	0.9881	0.9977	0.9824	0.9783	0.9565	0.9613

¹ Obtained by fitting the data from Figure 3 with a zero-order model.

Different letters (a and b) indicate significant differences between the gels at two ionic strength (*p* < 0.05).

The diffusion mechanism in a polymer network has been described by several models based on obstruction, hydrodynamic interaction and entropic barriers (Amsden, 1998; de Gennes, 1971; Nykypanchuk, Strey, & Hoagland, 2005; Walther, Lorén, Nydén, & Hermansson, 2006). In heterogeneous food matrix, the entropic barrier transport model suggests that the diffusate could pass through the food matrix from one cavity/opening to the next (Blomqvist et al., 2015; Silva, Lortal, & Floury, 2015). It has been reported that the pore sizes within gels ranging from 5 nm to 200 nm in diameter is sufficiently large to allow the diffusion of small molecules such as glucose (M_s : 0.41 nm² (see Table S1), molecular weight (M_w): 180.16 g/mol, TPSA: 110 Å² (D-Glucose), 118 Å² (L-Glucose)) and ethanol (M_s : 0.26 nm² (see Table S1), M_w : 46.07 g/mol, TPSA: 20.2 Å²) in the gel matrix (Gombotz & Wee, 1998; S. Kim et al., 2019). The gel prepared with 100 mM NaCl shows larger pores (403 ± 73 nm²) than those in the gel with 50 mM NaCl (267 ± 30 nm²), which will therefore result in a faster diffusion of carvacrol (M_s : 0.49 nm², M_w : 150.22 g/mol, TSPA: 20.2 Å²) in the gel with 100 mM NaCl. This is in line with several previous studies that show the microstructure of a gel or an emulsion-gel, in particular, pore size plays an important role in the diffusion/absorption/retention of small molecules in the food matrix. Sarkar, Juan, Kolodziejczyk, Acquistapace, Donato-Capel, and Wooster (2015) found that the rate of fatty acid release increased with the increasing of pore size of gelatine-gelled emulsions, suggesting a direct relationship between the lipase diffusion to the interface of fat droplets and average pore size. Zhang, Zhang, Zou, and McClements (2016) suggested that the larger pores of a gel network at higher pH values (pH 5 and 7) might be the reason of faster release of whey protein from alginate beads. Benjamin, Davidovich-Pinhas, Shpigelman, and Rytwo (2018) demonstrated that salt release from model fresh cheese can be modified by changing the cheese porosity.

4.3.3. Inhibition of microbial growth in the gels

Antimicrobial experiments were performed to study the influence of the diffusion of carvacrol on the antimicrobial activity of AP on the gels that are physically different. The growth of *Pseudomonas fluorescens* in the top layers of gels exposed to the CAR-PLA film (antimicrobial group) or without film (control group) is presented in **Figure 4**. The growth of spoilage bacteria in the middle and bottom layers of gels at antimicrobial and control groups can be found in **Figure S4**. During the 10 days storage, for all samples in the antimicrobial groups, the multi-factorial two-way ANOVA analyse shows significantly highest values of bacteria reduction in the top layers, followed by the reductions in middle layers and the lowest bacteria reductions in the bottom layers, compared to the other two layers ($p < 0.05$). Significant higher bacterial

reduction values were found in the top layers of WPI-carrageenan-100 mM NaCl gels than those in gels prepared with 50 mM NaCl in antimicrobial groups ($p<0.05$); a stronger growth inhibition effect was significantly found in the middle layers of gels in 100 mM NaCl, compared with the middle layers of gels in 50 mM NaCl ($p<0.05$); a (non-significant) trend of stronger bacteria reductions in the bottom layers of gels was observed in samples prepared with 100 mM NaCl. For the top layers of gels in the antimicrobial group with the presence of the CAR-PLA film, a significant 0.53 ± 0.07 and 0.64 ± 0.06 log reduction, compared to control groups, was found in the gel prepared with 50 mM and 100 mM of NaCl at the end of storage, respectively ($p<0.05$). For the middle layers in the antimicrobial group, a significant 0.42 ± 0.07 and 0.53 ± 0.10 log reduction was observed in the gel with low (50 mM) and high salt (100 mM) concentration, respectively ($p<0.05$). The CAR-PLA film has little effect on the growth inhibition of *Pseudomonas fluorescens* in the bottom layer of both gels, where a 0.31 ± 0.16 and 0.41 ± 0.11 log reduction was achieved for the gel in 50 mM and 100 mM NaCl, respectively.

Figure 4. Growth curves of *Pseudomonas fluorescens* in the top layer of WPI-carrageenan-50 mM NaCl gel (A) and top layer of WPI-carrageenan-100 mM NaCl gel (B) in antimicrobial/control group at 4°C fitted with the modified Gompertz equation.

The results of bacterial growth were fitted with a modified Gompertz model by non-linear regression, through which the maximum growth rate (u_{max}), logarithm of the asymptotic values of the relative population size (A_s) and lag phase (λ) were determined (Table 2). For both gels exposed to CAR-PLA film, the film has a significant effect on prolonging the lag phase and lowering the relative population size of the top layers ($p<0.05$). With regards to the middle layers, only the gel in 100 mM NaCl has a significant lower asymptotic relative population size

in the antimicrobial group than that in control group ($p<0.05$). However, the presence of the CAR-PLA film in the tubes did not significantly affect any of the growth parameters of the bottom layers of both gels, indicating no substantial antimicrobial effect of carvacrol in the layer furthest away from the surface of the gels.

Table 2. Estimated the logarithm of the asymptotic value of the lag phase duration, relative population size and growth rate for modified Gompertz equation as calculated from the models fitted to the experimental *Pseudomonas fluorescens* counts of gels with 50 mM NaCl (A) and gels with 100 mM NaCl (B) stored at 4 °C.

(A)

Parameters	Top layer		Middle layer		Bottom layer	
	Antimicrobial group	Control group	Antimicrobial group	Control group	Antimicrobial group	Control group
Lag phase (days)	1.04 ± 0.17 ^a	0.65 ± 0.16 ^b	0.97 ± 0.17 ^a	0.67 ± 0.16 ^a	0.85 ± 0.14 ^a	0.70 ± 0.15 ^a
Asymptotic value (Ln CFU/mL)	12.77 ± 0.38 ^a	14.18 ± 0.50 ^b	12.68 ± 0.48 ^a	13.68 ± 0.47 ^a	11.79 ± 0.32 ^a	12.38 ± 0.34 ^a
Maximum specific growth rate (days ⁻¹)	2.21 ± 0.13 ^a	2.26 ± 0.15 ^a	2.26 ± 0.18 ^a	2.28 ± 0.15 ^a	2.26 ± 0.15 ^a	2.34 ± 0.16 ^a
Sum of squares of residuals (<i>SS_{res}</i>)	0.33	0.52	0.59	0.51	0.34	0.38

(B)

Parameters	Top layer		Middle layer		Bottom layer	
	Antimicrobial group	Control group	Antimicrobial group	Control group	Antimicrobial group	Control group
Lag phase (days)	0.98 ± 0.17 ^a	0.52 ± 0.18 ^b	1.19 ± 0.21 ^a	0.96 ± 0.16 ^a	0.89 ± 0.20 ^a	0.86 ± 0.13 ^a
Asymptotic value (Ln CFU/mL)	12.57 ± 0.46 ^a	14.09 ± 0.51 ^b	11.74 ± 0.48 ^a	12.92 ± 0.23 ^b	12.27 ± 0.51 ^a	13.27 ± 0.35 ^a
Maximum specific growth rate (days ⁻¹)	2.11 ± 0.15 ^a	2.30 ± 0.17 ^a	2.54 ± 0.28 ^a	2.75 ± 0.13 ^a	2.17 ± 0.19 ^a	2.37 ± 0.13 ^a
Sum of squares of residuals (<i>SS_{res}</i>)	0.49	0.61	0.88	0.21	0.65	0.32

Different letters (a and b) indicate significant differences between the antimicrobial and control group ($p < 0.05$).

The results of bacterial reduction values and the estimated A_s and λ show that the efficacy of CAR-PLA film was greater in the top layers of both gels. This can be expected as more than 75% of the absorbed carvacrol was present in the top layers during storage. In the contrary, low absorptions of carvacrol in bottom layers of both gels (~5-7% of total absorption) is not effective in inhibiting the bacteria growth. This demonstrates that the antimicrobial effect is dependent on the location (depth) of a solid/semi-solid food matrix, which can be explained by the diffusion behaviour of active compounds. Previous studies mainly focused on the application of AP on the surface of solid/semi-solid foods for protecting foods during storage (Cazón, Velazquez, Ramírez, & Vázquez, 2017; J. W. Han, Ruiz - Garcia, Qian, & Yang, 2018). However, considering the fact that for many food products the growth of microorganisms is possible to occur in the interior of foods, the inhibition of microorganisms' growth in the interior of foods should be considered. The effects of antimicrobials/AP at different depth/location of solid food matrix were discussed by some studies (Chollet et al., 2009; Sebti, Delves-Broughton, & Coma, 2003). Cao-Hoang et al. (2010) found that a nisin loaded sorbitol-plasticized sodium caseinate films lost the antimicrobial effect on the growth of listeria in the interior of cheese (> 2 mm from the surface of the active film). However, the reason of the absence of inhibitory effect in the interior part caused either by the inactivation of activity by food compounds and environmental conditions (e.g. pH, temperature, ionic strength) or insufficient concentration was not clarified from these studies. The results from current study highlight the importance of studying the diffusion of antimicrobial and its consequent effect on the bacterial growth inhibition inside foods. Possible factors that can influence the migration/diffusion of antimicrobial compounds from packaging film to food matrix have been studied: storage environments, purity of active compound and the composition of polymer film and the food matrix, although no microstructural properties of the foods were included in these studies (Chollet et al., 2009; Cushen, Kerry, Morris, Cruz-Romero, & Cummins, 2014; Y.-M. Kim, Lee, Paik, & Lee, 2000). In our study, we demonstrated the effect of microstructural properties, particularly pore size, of gels on carvacrol absorption and diffusion that can explain the observed antimicrobial effects at different depth from the surface of the gel. Consequently, different carvacrol concentrations were the direct reason for differences in the antimicrobial effect of carvacrol in different location of a solid/semi-solid food. As a result, we propose to consider the fact that structure-related diffusional limitations of active compounds occur in all the food systems to a greater or lesser extent and should be considered during the antimicrobial packaging design.

4.4. Conclusions

Carvacrol loaded PLA film most effectively inhibits the growth of *P. fluorescens* in the top layer of gels. It was demonstrated that pore size of gels affect the diffusion of carvacrol to deeper locations in the gel and thereby reduced the antimicrobial activity at these locations. Overall, this work has demonstrated that the absorption/diffusion of antimicrobials was increased in gels having larger average pore sizes. This further influences the inhibition effect of antimicrobials on the growth of microorganism in the interior of foods. The insights provided from this study can be useful in the design of AP for the preservation of solid/semi-foods.

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Supplementary materials

Methods

Characterization of the gels

Gel rheological properties

Small strain rheological properties. Small strain rheological properties were performed according to the methods described by De Jong, Klok, and Van de Velde (2009) with some modifications. The oscillatory time sweep, frequency sweep and strain sweep were carried out by using a Physica MCR 302 Rheometer (Anton Paar GmbH, Graz, Austria) equipped with a concentric cylinder geometry (stator inner radius 16.66 mm, rotor outer radius 24.95 mm). Time sweep measurements were carried out to approximately 4.7 mL of the gel pre-solution throughout the gelation process (4 °C) at an angular frequency of 1 Hz and with an applied strain of 1%. The measurements were taken every 10 s for 10 hours. When the gelling process was completed, the sample was subjected to frequency sweep (varied angular frequency from 0.01 Hz to 10 Hz) and strain sweep (increased strain from 1% to 1000%) to understand the structural dependency of the gels at 4 °C. The range of frequency and strain independence of the elastic and viscous moduli is characterized as linear viscoelastic range (LVR). The fixed strain (1%) and frequency (1 Hz) were used at this study for oscillatory tests of the samples as the preliminary work has shown that the strain (1%) and frequency (1 Hz) are within the LVR.

Large strain rheological properties. Changes in the texture of the gels during storage period were measured by large deformation compression testing on the TA-XT Plus Texture (Stable Micro System Co., UK) using a 60 mm diameter cylindrical probe. The test parameters were set at 25 mm distance, 0.05 N trigger force, 1.0 mm/s pre-test speed, 1.0 mm/s test speed and 5.0 mm/s post-test speed. The compression force that is able to break down the gel was defined as the hardness of the samples.

Water holding capacity (WHC) of gels

WHC of the gels were measured according to the centrifugation procedure developed by Kocher and Foegeding (1993). The known weight gels (~ 4 g) (W_1) were placed in a centrifuge tube (50 mL), and then centrifuged at 1000g for 10 min at 4 °C. The released weight of water (W_2) was considered as WHC for calculation by the following equation:

$$\text{WHC (\%)} = \left[(W_1 - W_2) / W_1 \right] \times 100 \quad (1)$$

SEM image analyse methods

The threshold level was determined based on a simple thresholding technique developed by (Pugnaroni, Matia-Merino, & Dickinson, 2005). **Figure S5** shows the total number of pores (including all sizes of pores) identified in the image as a function of the selected threshold level. At the Threshold Level is equal to zero ($TL = 0$), all pixels are in the dark background, therefore all the pixels are considered to be part of the gel network, and no pores are detected. With the increasing TL , the proportion of background (“black”) pixels incorporated into the image increases, leading to the increased number of pores. The number of pores reaches a maximum at $TL=TLp$. Further increase in TL decreases the pore numbers. At the extreme high TL , the entire image is considered to be a single big pore. The value of $TL=TLp$ is used as threshold level that all individual pores were detected in the image, corresponding to the maximum number of pores measured (see **Figure S5**). The same criterion was applied to the raw images and the pores identified in this method were highly in line with the pores found in image by “eye approach”. After finalizing the threshold setting, the binary image was processed with noise reduction. The imageJ software was then applied to analyse the average size of pores.

Estimation of the molecular size of a molecule

The size of a molecule can be estimated by using algebra and the experimental density formula (Triggle & Taylor, 2006). First calculate the number of moles (M) in one cubic meter by using the molecular weight (M_w) and density (ρ):

$$M = \frac{\rho}{M_w} \quad (1)$$

Then calculate the number of molecules (N) in one cubic meter by using the Avogadro number (N_A):

$$N = N_A \times M \quad (2)$$

The volume (V) of one molecule can be obtained by:

$$V = \frac{1}{N} \quad (3)$$

Then assuming this molecule is confined in sphere of diameter L , estimate the size of molecule by calculating the L value:

$$L = \left(\frac{3 \times V}{4 \times \pi} \right)^{\frac{1}{3}} \times 2 \quad (4)$$

The 2D area (M_s) of one molecule:

$$M_s = \pi \frac{L^2}{2} \quad (5)$$

Results and discussion

Physical analyse of food gel model

Gel rheological properties

Small strain properties. Gelation of WPI-carrageenan-NaCl, as characterized by the change in storage modulus (G') and loss modulus (G'') in 10 hours, is presented in **Figure S1 A**. The final G' at the end of measurement shows that storage modulus G' decreased with the concentration of NaCl increasing from 50 mM to 100 mM. This reveals that salt induced WPI-carrageenan gel had the higher stiffness in 50 mM NaCl than the gel in 100 mM NaCl as the storage modulus is an indicator for the overall stiffness of a gel (Dagdas, Tombuloglu, Tekinay, Dana, & Guler, 2011). In addition, it was found that G' and G'' of gel in 100 mM NaCl first reached to the peak and then there was little change, tends to approach a plateau, compared with gel in 50 mM. This can be explained as a faster gelation happened in gel solution with high salt concentration (Chen, Zhao, Chassenieux, & Nicolai, 2017; Maltais, Remondetto, & Subirade, 2008; Marangoni, Barbut, McGauley, Marcone, & Narine, 2000). **Figure S1 B** shows the frequency dependence of G' and G'' on WPI-carrageenan gels at 50 and 100 mM of NaCl. The measurements showed that both gels behaved as elastic materials with the G' values higher than G'' values, and both G' and G'' showing low dependence on frequency over entire frequency scope. Results obtained from strain sweep (**Figure S1 C**) shows that the crossover point of G' and G'' occurs at higher applied strain in sample prepared with higher amount of NaCl. This crossover point is recognized as the moment when structured viscoelastic samples undergo a gel-solution transition under increasing oscillatory shear (Patel, Dumlu, Vermeir, Lewille, Lesaffer, & Dewettinck, 2015). This indicates that gel prepared with 50 mM of NaCl required the higher strain to deform from gel-like to solution-like, compared with the gel containing 100 mM of NaCl.

The possible reason to explain the greater small strain properties of the gel in 50 mM NaCl than that of the gel in 100 mM NaCl could be the dense structure indicated by the small pores in SEM images (**Figure 2**) of gel in 50 mM NaCl (Liu et al., 2019; Wang, Zeng, Qin, Adhikari,

He, & Chen, 2018). Similar findings were also found in the effects of salt addition on G' and G'' variation of gels prepared with WPI-polysaccharide complex (Bertrand & Turgeon, 2007; Harrington, Foegeding, Mulvihill, & Morris, 2009).

Large strain properties. The fracture properties were sensitive to changes in NaCl addition (**Figure S2**). Increasing NaCl concentration from 50 to 100 mM reduces the hardness of the gel during the storage period. The differences in the hardness of WPI-carrageenan gels corresponded to the differences in the microstructure (**Figure 2**). As can be seen in SEM images, homogeneous microstructure with even and small pores was observed in gel in 50 mM NaCl, indicating a dense gel network. The homogeneous dense network would contribute to a higher strength (Salvador, Toldrà, Saguier, Carretero, & Parés, 2009). The small pores in the gel at low salt concentration enhance both small and large rheological properties of gel complex in this study. This trend is similar to the changes in hardness of gels (Çakır, Vinyard, Essick, Daubert, Drake, & Foegeding, 2012; Tan & Joyner, 2018).

Water holding capacity (WHC)

The WHC of samples at different salt concentrations is shown in **Figure S3**. As the ionic strength increased, the stiffness and hardness of gels decreased (**Figure S1** and **S2**), accompanied by a decreased in held water. It has been reported from previous studies that the gels prepared with low ionic strength have a homogeneous structure, whereas high concentration of NaCl leads to a micro-phase separated structure with a coarse network (Chen, Chassenieux, & Nicolai, 2018; Kharlamova, Nicolai, & Chassenieux, 2018). The homogeneous structure is expected to have a better capacity to hold aqueous phase during centrifuge. A decrease in WHC was observed in gels prepared with 100 mM NaCl during storage, which might be related to the formation of insoluble protein or large protein aggregates (Bouhrara, Clerjon, Damez, Kondjoyan, & Bonny, 2012; Urbonaite, De Jongh, Van der Linden, & Pouvreau, 2014). The results from current study were in line with the previous study that showed the water holding capacity of gel decreased at high salt addition (Montero & Pérez-Mateos, 2002).

Water holding capacity represents the fraction of total moisture that is held by the gel network under a given force (Urbonaite et al., 2014). It is correlated with gel permeability (Pascua, Koç, & Foegeding, 2013), which in turn relates to the pore size in gel (Kuhn, Cavallieri, & Da Cunha, 2011; Munialo, van der Linden, Ako, Nieuwland, Van As, & de Jongh, 2016; Zhang, Zhang, & Vardhanabhuti, 2014). The decrease in the WHC of the gels can be attributed to the increase in

pore size in the gels at higher salt concentrations (Barbut, 1995; Kuhn et al., 2011; Munialo et al., 2016; M Verheul & Roefs, 1998a; Marleen Verheul & Roefs, 1998b; Zhang et al., 2014). This indicates that the pores of gels increase with the increase in salt concentration, which is in line with the pore size estimation from SEM images. Similar results were suggested from Chantrapornchai and McClements (2002), who concluded that whey protein isolate gels associate into fine-stranded structures that have higher water holding capacity at intermediate salt concentrations (60 to 150 mM NaCl), whereas at high salt concentrations (>150 mM NaCl) the gels associate into particulate structures that have low water holding capacity.

Figure captions

Figure S1. Storage (G') and loss modulus (G'') as a function of time for WPI-carrageenan gels (A) frequency dependence (B) and strain dependence (C) of the storage and loss modulus of gel samples.

Figure S2. Changes in hardness (N) of gels.

Figure S3. Changes in water holding capacity (WHC) of gels.

Figure S4. Growth curves of *Pseudomonas fluorescens* in the middle layer of WPI-carrageenan-50 mM NaCl gel (A) middle layer of WPI-carrageenan-100 mM NaCl gel (B) bottom layer of WPI-carrageenan-50 mM NaCl gel (C) bottom layer of WPI-carrageenan-100 mM NaCl gel (D) in antimicrobial/control group at 4°C fitted with the modified Gompertz equation.

Figure S5. Number of pores, N , as a function of the grey level threshold, TL , applied to the image. The threshold levels used for the identification of pores, TL_p , are indicated.

Figure S1. Storage (G') and loss modulus (G'') as a function of time for WPI-carrageenan gels (A) frequency dependence (B) and strain dependence (C) of the storage and loss modulus of gel samples.

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Figure S5. Number of pores, N , as a function of the grey level threshold, TL , applied to the image. The threshold levels used for the for the identification of pores, TL_p , are indicated.

Table caption:

Table S1. Estimation of the molecular size of a molecule.

	Carvacrol	Ethanol	Glucose
ρ (kg/m ³)	977	789	1560
Mw (g/mol)	150.22	46.07	180.16
L (nm)	0.79	0.57	0.72
Ms (nm ²)	0.49	0.26	0.41

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Chapter 5

Fat content and storage conditions are key factors on the partitioning and activity of carvacrol in antimicrobial packaging

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Abstract

The ability of carvacrol loaded polylactic acid (PLA) films to improve ground beef preservation was assessed. The mass transfer processes of carvacrol partitioning in a food packaging system between the PLA film, headspace and food product was studied. Carvacrol release was studied on packed ground beef having a fat content of 5 or 12% at a temperature between 5 and 30 °C and an humidity between 43 and 94% for up to 12 days.

Results showed the release rate of carvacrol from the PLA film into the headspace increased with the storage temperature while the humidity in the packaging headspace had no effect on the release rate of carvacrol from the PLA film. The fat content of ground beef has a profound effect on the partitioning of carvacrol: when the system is stored at 5 °C the carvacrol absorption in the 12% fat ground beef was about 1.3-fold compared with the carvacrol concentration observed in 5% fat ground beef. Despite this higher carvacrol absorption in the regular beef, the PLA/carcacrol films had a stronger antimicrobial effect on the lean beef suggesting that partitioning of carvacrol into the fat phase of the beef reduced its antimicrobial activity. Results highlight the importance of considering the food matrix composition in the design of antimicrobial packaging based on natural volatile components.

Keywords: Antimicrobial packaging; carvacrol partition; controlled release; food composition; ground beef.

5.1. Introduction

Food safety and food spoilage is a major problem: a better preservation of food can reduce food waste especially in industrialized country (Roodhuyzen, Luning, Fogliano, & Steenbekkers, 2017) and contribute to achieve the sustainability goals placed in the political agendas of many countries. Fresh product of animal origin are highly susceptible to microbial spoilage. Microbial growth is the main factor in meat, fish and dairy spoilage which results in off-odour and off-flavour as well as defects in texture and appearance (Sun & Holley, 2012). Antimicrobial packaging provides options to inhibit microbial growth and extend the shelf life of foods by incorporating specific antimicrobial compounds into the packaging (Cha & Chinnan, 2004).

Several essential plant oils have antimicrobial activity; they have been incorporated into packaging film to inhibit microbial growth. Essential oils from clove, oregano, rosemary, thyme, sage and vanillin are very effective as antimicrobial agents (de Azeredo, 2013; Kouchak, Ameri, Naseri, & Boldaji, 2014). The antimicrobial activity of essential oils mainly depends on the presence of various terpenic and phenolic compounds. Usually compounds having phenolic groups have strong antimicrobial activity and carvacrol is one of the most powerful (Marchese, et al., 2016). Carvacrol is reported to have antimicrobial activity against a wide spectrum of bacterial strains (Fernandez-Saiz, Lagaron, Hernandez-Muñoz, & Ocio, 2008; Kristo, Koutsoumanis, & Biliaderis, 2008), moulds (Garrido Assis & de Britto, 2011) and yeasts (Avila-Sosa, et al., 2012). Among polymer films, polylactic acid (PLA) is recognised as one of the most promising bio-based and biodegradable materials for food packaging applications (Peelman, et al., 2013). PLA based packaging has been commercially applied for many food products for both short-shelf life products and long-shelf life products, such as PLA Bags for potato chips in PepsiCo's Frito-lay, PLA Bowls for fresh salads in McDonald's and Dannon™ yogurts (Armentano, et al., 2015). Therefore the development of a bio-based PLA film containing carvacrol is a logical choice to extend the shelf-life and improve the safety of perishable foods (Martínez-Camacho, et al., 2010; Sebtí, Martial - Gros, Carnet - Pantiez, Grelíer, & Coma, 2005).

The controlled carvacrol release in a packaging system is the key for developing an efficient antimicrobial package, in fact keeping a concentration of antimicrobials that is continuously above the MIC is important for preserving food products (Vergnaud & Rosca, 2006). The controlled release strategies will be affected by the storage environment, the structure of the releasing films and the composition of food products (Mastromatteo, Mastromatteo, Conte, &

Del Nobile, 2010). The effect of environmental conditions has been demonstrated in studies showing an increased storage temperature and humidity can accelerate the release of the active agents from packaging films (Bierhalz, da Silva, & Kieckbusch, 2012; Kurek, Guinault, Voilley, Galić, & Debeaufort, 2014; Mascheroni, Guillard, Gastaldi, Gontard, & Chaliér, 2011). The effect of food composition on the active compounds release and partitioning within the food-packaging system, however, has been rarely tested, while is expected to be an important factor to determine the antimicrobial efficiency. There is a knowledge gap on the influence of food composition and food matrix structure on the volatile antimicrobials partitioning in food packaging system.

Several mass transfer processes occur in an antimicrobial packaging system, including antimicrobial compounds release into the headspace, absorption of active compounds in the food and microbial growth in the food products (Begley, et al., 2005). To understand the performance and efficacy of an antimicrobial packaging, the process of partitioning of antimicrobial compounds into the headspace and into the food product and the resulting effect on microbial growth should be investigated. The knowledge of partitioning of antimicrobials in active packaging systems helps to design and optimise the packaging system to have a desired antimicrobial effect for specific food categories. Higuera, López-Carballo, Hernández-Muñoz, Catalá, and Gavara (2014) studied the partitioning of the active agent within a food/package system. The authors developed an antimicrobial packaging by incorporating carvacrol into chitosan/cyclodextrin films for chicken fillets preservation. They studied the partitioning of the carvacrol within each phase of the food package system, including tray, lid, film and chicken. Results showed the volume/weight ratio between the package and the film was the main factor influencing the carvacrol partitioning thus the bacterial inhibition.

Several researchers tested the antimicrobial activity of carvacrol incorporated packaging films on beef products preservation, and different materials and structures of polymers were used to entrap carvacrol (Emiroğlu, Yemiş, Coşkun, & Candoğan, 2010; Zinoviadou, Koutsoumanis, & Biliaderis, 2009). However, the combined effect of the packaging environment and fat content of the food product has not been investigated for carvacrol antimicrobial packaging systems, thus far.

In this paper, the influences of the storage environment (temperature and humidity) and the fat content of ground beef on the carvacrol partitioning and antimicrobial activity in a ground meat - PLA food packaging system were studied.

5.2. Materials and methods

5.2.1. Materials

PLA (4043D Ingeo™), produced by company NatureWorks LLC, was provided by Wageningen Food & Biobased Research. Carvacrol ($\geq 98\%$), dichloromethane, hexane, ringer tablets, potassium carbonate, sodium chloride and potassium nitrate were purchased from Sigma Aldrich, Netherlands. The saturated solutions were prepared with potassium carbonate, sodium chloride and potassium nitrate to fix the relative humidity (RH) at 0.43, 0.75 and 0.93 respectively (Blanchard, Gouanvé, & Espuche, 2017; Kim, Kim, Yi, Oh, & Lee, 2015). Peptone Physiological Salt Solution (PFZ) was purchased from VWR, Netherlands.

Ground regular and lean beef products were purchased from a local supermarket. According to the products' label, the fat contents of ground regular and lean beef are 12/100g and 5/100g, respectively.

5.2.2. Carvacrol loaded poly (lactic) acid (PLA) film preparation

PLA films were prepared according to the solvent-casting method of Rhim, Mohanty, Singh, and Ng (2006) with some modifications. Four gram of PLA pellets were dissolved in 100 mL of dichloromethane. The mixture was stirred by a magnetic stirrer at 40 °C until the polymer was totally dissolved. Two gram of carvacrol was then added to PLA/dichloromethane mixture, and the mixture was stirred for 1 hour at room temperature. Subsequently, the mixture was homogenized using an ultra-sonicator (HBM Machines, Moordrecht, the Netherlands) for 30 min at room temperature (20 °C). In the end, the solution was distributed into glass round bottomed flasks and dried in fume cupboard. The films were peeled from the glass flask after 24 h, and were stored at 4 °C in a sealed container.

5.2.3. Packaging system preparation

The carvacrol partitioning between film and headspace was studied in a closed system (**Figure 1**). The 10 mL verex headspace glass vials were used to simulate the closed food system with a package and a food product. Each cell consisted of a glass vial sealed by a rubber lid, packaging film (0.020 ± 0.001 g) and ground beef product (0.100 ± 0.007 g) for sampling. The packaging film was inserted onto a pipette tip to ensure that the film could not have direct contact with the ground beef, which was at the bottom inside of the glass vial. Three exposure

temperatures, including 5 °C, 20 °C and 30 °C, were used to study the temperature effect of carvacrol release and partitioning to food products in the closed packaging film. Several cells with ground beef and film were prepared for daily measurement to characterize the process of carvacrol release from PLA packaging film and partitioning to headspace and food.

Figure 1. Setup used for evaluation of carvacrol partitioning within food/package system.

5.2.4. Carvacrol analyse in the packaging/headspace/ground beef system

5.2.4.1. Calibration

The stock solutions (2.0 mg/mL) of carvacrol in the mixture of hexane and 2-propanol (n-hexane : isopropanol = 3:1) were prepared for quantification of carvacrol in beef products. To determine carvacrol left in film, another stock solution (2 mg/mL) was prepared by adding carvacrol in chloromethane. Spiked standard solutions at different concentrations (0.4, 0.8, 1.2, 1.6 and 2.0 mg/mL) were obtained by diluting the stock solutions to create standard curves for quantification of carvacrol in the system.

5.2.4.2. Carvacrol analyse in headspace in 10 mL verex headspace vials

The carvacrol in headspace was measured with headspace solid phase microextraction (HS-SPME) technology followed by Gas Chromatography – Flame Ionization Detector (GC-FID) (Thermo Scientific, Waltham, Massachusetts, United States). The extraction method of carvacrol from headspace of glass vials was according to Zhang, Qi, Shao, Zhou, and Fu (2007) with some modifications. Briefly, a SPME fiber assembly Polydimethylsiloxane (PDMS) with

needle size of 23 ga was pierced in the rubbery lid of glass vial, then the fibre of SPME was outstretched through the needle and absorbed the carvacrol from the headspace. The fibre was withdrawn into the needle after 15 minutes extraction, and injected to the port of GC-FID immediately. The desorption of carvacrol in GC-FID was 5 minutes. A preliminary experiment was performed to ensure the extraction and desorption time were long enough to absorb all the analytes from headspace and absorbed onto the chromatographic column.

The following conditions were set to operate GC-FID: The injection temperature was adjusted at 250 °C. The column oven temperature was initially set at 60 °C, and then increased to 250 °C with the speed of 15 °C min⁻¹. Helium was used as the carrier gas, running at a constant flow of 2.0 mL min⁻¹. The FID was operated with a hydrogen flow of 35 mL min⁻¹ and air flow of 350 mL min⁻¹. Injections were set in split mode with a split flow of 10 mL/min and a split ratio of 5. The total running time was 25.25 min.

5.2.4.3. Extraction and quantification of carvacrol concentration in ground beef products

Carvacrol was extracted from the ground beef products (0.1 ± 0.007 g) before analysing by GC-FID. Carvacrol was extracted by adding 0.5 mL of organic mixture (n-hexane : 2-propanol = 3:1) in meat sample. The mixture was warmed in a shaking water bath at 40 °C for 20 min at the speed of 60 rpm for carvacrol extraction. The mixtures were then centrifuged for 10 min at 1000 g to obtain the top organic layer. Ten microliter of organic solution was subjected into GC-FID for liquid analyse. Extraction efficiency of this method was tested by extraction carvacrol from beef samples by adding known amounts of carvacrol, and a high solvent-to collect ratio (>97%) was achieved by using this method. The concentration of carvacrol in meat was determined using a calibration curve of carvacrol in the mixture of hexane and 2-propanol (n-hexane : 2-propanol = 3:1) ($R^2 = 9995$).

5.2.4.4. Extraction and determination of carvacrol left in packaging film

To quantify the carvacrol left in the packaging film at each measuring time point, the carvacrol loaded PLA packaging film was dissolved in 5 mL dichloromethane solvent at 20 °C by 2 hours stirring at 600 rpm. Ten microliter of organic solution was subjected into GC-FID for liquid analyse. The concentration of carvacrol in films was determined using a calibration curve of carvacrol in dichloromethane ($R^2 = 9996$).

5.2.5. Ground beef samples preparation and storage

PLA film with higher carvacrol concentration were prepared with two gram of carvacrol and four gram of PLA to test its antimicrobial efficacy. Fresh beef for microbial analyses were divided in two parts, one part was used to test the antimicrobial effect of the developed film, and the other part served as a control. All beef samples ($5.00 \pm 0.05\text{g}$) were stored in airtight sterilised 500 mL plastic containers in a refrigerator (5°C). In the antimicrobial group, $1.00 \pm 0.01\text{g}$ of active film was added to each sample. The samples were prepared in individual containers for each periodical measurement (0, 1, 3, 6, 8 and 12 days). All experiments were done in duplicate.

5.2.6. Microbiological analyses

At each sampling day, beef samples ($5.00 \pm 0.05\text{g}$) were taken from the sealed plastic containers aseptically in a flow cabinet and put into a sterilized stomacher bag together with 45 mL of Ringers solution. The stomacher 400 circulator (Seward Limited, Worthing, United Kingdom) was used to homogenize the beef samples for 2 minutes at 230 rpm at room temperature. Resulting turbid solutions were 10-fold serially diluted in Peptone Physiological Salt Solution (PFZ) and 100 μL was brought onto Plate Count Agar by the spread technique (PCA; Merck, Darmstadt, Germany) in duplicate, incubated at 20°C for 48 h, after which the number of colonies at the appropriate dilutions was counted manually.

5.2.7. Microbial growth modelling

Two empirical microbial growth models were tested in this study, the modified Gompertz equation (Eq. (1)) and the modified logistic model (Eq. (2)) (Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990)

$$\ln \frac{N}{N_0} = A_s * \exp \left(- \exp \left(\frac{u_{max} * e}{A_s} (\lambda - t) + 1 \right) \right) \quad (1)$$

$$\ln \frac{N}{N_0} = \frac{A_s}{1 + \exp \left(\frac{A_s * u_{max}}{A_s} (\lambda - t) + 2 \right)} \quad (2)$$

in which: A_s is the natural logarithm of the asymptotic value of the relative population size, u_{max} is the maximum specific growth rate (day^{-1}), λ is the lag phase (day), t is the storage time (day) and N/N_0 represents the ratio of the microbial cell density (CFU g^{-1}) to the initial microbial cell density (CFU g^{-1}).

5.2.8. Statistical analysis

All the GC analyses were performed in triplicates and the microbiological analyses were performed in duplicates. The IBM SPSS Statistics version 23.0 for Windows (Armonk, NY: IBM Corp) was used for the statistical analysis of the results from GC. One-way analysis of variance (ANOVA), with post-hoc pairwise analysis by the Tukey test was used to assess the significant differences between samples. Data fitting by nonlinear regression was completed with the aid of Solver add-in in Excel by minimizing the sum of the squared residuals (SSR) to obtain the parameters of the empirical microbial growth models. The standard deviation of antimicrobial results and correlation of all parameters was calculated by the macro SolverAid (de Levie, 1999). A level of $p < 0.05$ was defined to test the statistical significance of experimental results. The resulting data were presented as means $\pm$ standard deviation. To discriminate the model performance, the corrected Akaike's Information Criterion (AIC) was used for model discrimination:

$$AIC_c = n \ln(s^2) + 2(p + 1)[n/(n - p)] \quad (3)$$

Where, n is the number of data points, p represents the number of parameters, and $s^2 = SS_{res}/n$. SS_{res} is the residual of sums of squares between measured and estimated values. The model giving the lowest AIC value is the best fitting model (Motulsky & Christopoulos, 2004).

5.3. Results and discussion

5.3.1. Carvacrol analyse in the packaging/headspace/ground beef system

5.3.1.1. Carvacrol in the headspace of the package

The carvacrol intensity in the headspace of the package setup, measured as a function of time, storage temperature and fat content of the ground beef, is reported in **Figure 2**. The headspace of the package is the intermediate zone between packaging film and food matrix, and therefore illustrates the dynamic behaviour of carvacrol in the whole package. At 30 °C, an immediate large increase of the carvacrol intensity in the headspace was observed already at the first day. This intensity remained high during the first 4 days of storage. After 4 days, the intensity of carvacrol started to decrease and after 8 days of storage, it reached a dynamic equilibrium between carvacrol release from film and absorption into the beef. At 20 °C, carvacrol intensity in the headspace was stable during the whole storage period after the initial peak in the release

during the first day. The initial peak area at 20 °C was similar to the equilibrium achieved at 30 °C after 4 days. Data collected by storing at 5 °C, showed that the intensity of carvacrol in the headspace is lower, after the initial increase during the first day; it reached a steady state after 4 days at intensity of about 1/3 of the carvacrol observed at 20 °C and 30 °C.

Figure 2. The carvacrol intensities in the headspace of package containing (A) ground lean beef (B) ground regular beef.

The peak area of carvacrol in the headspace in the system containing lean meat (**Figure 2A**) was significantly different from the regular meat (**Figure 2B**) at 5 °C for 4 days: In fact the initial peak area of carvacrol released from packaging to the headspace was about 1.3-fold higher in the regular beef system. However, the peak area of carvacrol in fat beef was significant lower than the lean beef from day 1 to day 4 ($p < 0.05$). This suggests that at low temperature the reduced vapour pressure of carvacrol, the absorption rate into the meat becomes dominant. This absorption is expected to be faster for meat with a higher fat content due to the high solubility of carvacrol into fat. For longer storage time the intensity of carvacrol in the headspace became the same for the two products suggesting that on the long-term storage (> 9 days) the release of carvacrol from the packaging is controlling the headspace intensity.

The finding that carvacrol released more easily from PLA film at higher temperatures are consistent with Kuorwel, Cran, Sonneveld, Miltz, and Bigger (2013) who observed that the increased temperature plays a significant role on the migration of antimicrobial compounds from films. Various other studies showed that the temperature is an effective factor to control the antimicrobials release from polymer films (Kashiri, et al., 2017; Rubilar, Cruz, Zuñiga, Khmelinskii, & Vieira, 2017; Tunç & Duman, 2011). The amount of volatile compound release depends on the surrounding environment because the high temperature results in high diffusion

coefficients of volatiles. This effect might be strong or weak depending on the polymer matrix used for encapsulation of compounds. In addition, the partitioning of carvacrol into headspace is favoured at higher temperature because of its high vapour pressure (Kurek, et al., 2014).

The effect of relative humidity (RH) on carvacrol released from the PLA film at room temperature (20 °C) was also analysed in this work. However, during the 6-day measuring period, the release of carvacrol from PLA films were not significant influenced by the storage RH for most of the measuring days (results were not shown). Since PLA film has a good water resistance applying for food packaging, the low absorption of water has less effect to trigger the release of the loaded compounds.

5.3.1.2. Carvacrol absorption in meats and desorption from films during storage

The concentration of carvacrol in ground lean and regular beef during storage in different conditions was measured to study the effect of fat content and storage temperature on the absorption of carvacrol released from the package. The samples were stored at different temperatures (5, 20, 30 °C). **Figure 3A** and **3B** shows a plot of the carvacrol concentrations in time and the partitioning of carvacrol from PLA film to lean/ regular beef products at the three storage temperatures. The amount of carvacrol absorbed in food products increased with the storage days at all temperature conditions. In accordance to the results that more carvacrol was released from the PLA films at higher temperature, also more carvacrol was found in both lean and regular beef products with increasing temperature. At 30 °C, the fastest diffusion of carvacrol into the meat samples were observed. After about 8 days a steady state of carvacrol absorption for ground regular beef was observed. Accordingly, when the samples (lean and regular beef) were stored at 20 °C, the time required to achieve the steady state was longer than 12 days. In the products stored at 5 °C, the amount of carvacrol measured in the samples was significant low ($p < 0.05$), and the concentration increased lineally for both lean and regular beef during measurement ($R^2 = 0.95$ for regular beef and $R^2 = 0.98$ for lean beef).

Figure 3. The carvacrol concentrations in (A) ground lean and (B) regular beef products, and the amount of carvacrol in the film of package containing (C) ground lean and (D) regular beef.

For the effect of the food composition on carvacrol migration, it was found that the fat content has significant effects on carvacrol absorption in ground beef products in all temperatures. For the samples stored at 5 °C, the carvacrol absorption in regular beef was significant higher than the absorption in lean beef at every measurement day ($p < 0.05$). At day 12, the ratios of carvacrol in regular/lean beef for 5, 20 and 30 °C was 1.34, 1.12 and 1.30, respectively. The effect of fat on carvacrol uptake by the meat is more pronounced under refrigeration (5 °C).

The carvacrol left in film at each measuring day were measured, and the results are shown in **Figure 3C and 3D**. The initial carvacrol into PLA film was 9.93 ± 0.41 mg. It was lower than the estimated amount (10 mg) as carvacrol was evaporated during film drying process. The overall desorbed mass of carvacrol from the films was less than half of the total amount at the begin of storage. The maximum residual carvacrol in the films could be found for storage at 5 °C (86.28% for lean beef and 82.79% for regular beef), followed by 20 °C (78.92% for lean beef and 69.71% for regular beef). The films stored at 30 °C for both lean and regular beef lost a significant higher amount of carvacrol than the films at 20 °C and 5 °C ($p < 0.05$). The results

of carvacrol left in films also show an effect of the fat content of food on the release of carvacrol from PLA film. For regular beef less than 36% of the carvacrol was desorbed from the PLA films after 12 days of storage at 30 °C, for the lean beef this was less than 42%. This can be explained by the bigger driving force of carvacrol absorption in ground regular beef that promotes carvacrol release from the PLA film.

The intrinsic chemical properties of the antimicrobials (hydrophobicity and volatility), the composition and structure of food medium and environmental conditions (temperature and RH) are the main factors that determine carvacrol partitioning in a food packaging system. In this food-package system, carvacrol released from the PLA film first migrated to the headspace of the package before it was absorbed by the food matrix. The results obtained from **figure 3** clearly indicate that carvacrol was more easily absorbed in meats with high fat contents. This can be explained by the affinity between carvacrol and the fat part of the food products. Both fat and carvacrol are non-polar molecules, and molecules with same chemical polarity have better affinity with each other (Chambin, et al., 2009; Ma, Yang, Yao, Xu, & Tang, 2017). In this case, carvacrol shows a high preferences for hydrophobic phases with the partition coefficient in octanol-water ($\log P$) of 3.52 (Ben Arfa, Combes, Preziosi - Belloy, Gontard, & Chalier, 2006). It is generally recognized that compounds with $\log P$ higher than 1 are hydrophobic and are thus more retained in oil than water, which leads to more carvacrol absorption in ground regular beef products. Although few studies were conducted to study the antimicrobial absorption in real foods, some researches that have tested different food simulants found results in line with this study. Ramos, Beltrán, Peltzer, Valente, and del Carmen Garrigós (2014) studied the migration of carvacrol and thymol from polypropylene (PP) films into different food simulants, including distilled water, acetic acid ethanol and isooctane. Based on their results, the calculated migration coefficients were reduced when the polarity of the simulant increased. The higher migration into isooctane could be attributed to the affinity of apolar antimicrobials with isooctane. This behaviour has also been suggested by Sánchez-González, Cháfer, González-Martínez, Chiralt, and Desobry (2011) who studied the release kinetic of limonene from chitosan films to different food simulants. Aqueous solutions with 0%, 10%, 50% and 95% of ethanol and isooctane were tested at room temperature (20 °C). The polarity of these food simulating solutions decreased with the increase in ethanol concentration. According to their results, the release kinetic constants of limonene for chitosan films increased exponentially when the ethanol concentration increased in the aqueous system.

However, despite the fact that hydrophobic olive oil has high affinity to carvacrol, less carvacrol was detected in pure olive oil compared to ethanol and acetic acid according to the results from Fernández-Pan, Maté, Gardrat, and Coma (2015). The reason attributes to the direct/indirect contact between food stimulates and film. In their study, the aqueous solutions of ethanol and acetic acid cause a loss of the structural integrity of the hydrophilic edible (chitosan-carvacrol) films when the films were in contact with the solutions; however, olive oil has a limited access into the film matrix leading to a closed structure of chitosan film. In our study, the migration process of carvacrol started from PLA film to the headspace of setup, followed by absorption into foods. The food products were not able to swell/loosen the structure of packaging film due to the indirect contact between film and the ground meats. Additionally, the moisture in closed vials has less impact on the structure of PLA film due to its hydrophobicity, and the higher diffusion of carvacrol mainly because of the high temperature. Therefore, the higher amount of carvacrol in ground regular beef products were mainly because of the higher molecule affinity between carvacrol and fat food matrix. The results of carvacrol partitioning in the packaging system show that antimicrobials diffusion is dependent on the packed food product characteristics, therefore different food products require tailored food packaging solutions.

5.3.2. Antimicrobial effect of the carvacrol/PLA film and modelling of bacterial growth curve

The total viable count (TVC) changes of ground beef samples that were treated with and without packaging films are presented in **Figure 4**. The ground regular beef samples with 12% of fat contents showed a 0.52 log reduction of TVC population in samples treated with antimicrobial films at day 12, compared with the control samples. For ground lean beef, the TVC population of antimicrobial groups at day 12 the reduction was larger with a 1.1 log reduction of TVC compared to the control group.

Figure 4. Growth curves of total bacterial count in (A) ground lean beef and (B) ground regular beef in antimicrobial/control group at 5°C fitted with the modified Gompertz equation.

Mathematical modelling was used to predict the potential for growth of bacterial in food products during longer storage. The modified Gompertz and modified Logistic model were used to describe the microbial growth in the ground beef samples. Both models gave reasonable fits. According to the AIC values from two models, the modified Gompertz model gives the better performance as the AIC value of the Gompertz model ($AIC_c = -2.3$) is lower than the modified Logistic model ($AIC_c = 7.6$). Therefore, the Modified Gompertz model was selected for further analysis (Figure 4). The lag period λ (day) and maximum growth rate μ_{max} (day^{-1}) for bacterial growth in beef samples packaged within/without developed setup were estimated by Gompertz model (Figure 5). The value of upper asymptote for all samples was estimated of 14.8 ± 0.6 log CFU/g, according to the modified Gompertz model. In particular, the lag phase of bacteria growth was longer in regular beef. The estimated growth rate of bacteria in lean beef packaged with PLA film was much lower than the growth rate in control group ($p = 0.07$), indicating the antimicrobial package concept has a clear effect on bacterial growth inhibition in lean beef in terms of the growth rate estimation.

Figure 5. Estimated (A) lag phase duration and (B) growth rate for modified Gompertz equation as calculated from the models fitted to the total viable count experimental points of ground beef stored at 5 °C.

From estimated parameters and bacterial reduction values could be concluded that the efficacy of carvacrol loaded PLA film was greater in food products with low fat content than in high fat foods. According to the results of carvacrol absorption in lean/regular beef products and carvacrol desorption from films (**Figure 3**), more carvacrol was found in ground regular beef at the same storage temperature. However, the microbiological analyses in antimicrobial and control groups indicated that a 50% larger log reduction of TVC population was observed in ground lean beef groups compared to regular beef samples. This phenomenon might be related to the effect of fat content on the antimicrobials migration into the food matrix. Carvacrol has a high log *P* value of 3.52 (Ben Arfa, et al., 2006); therefore, it will mainly be present in the fat of the food. The solubility of carvacrol in fat limits the interaction of carvacrol with bacteria that actually grow in the aqueous portion of the meat. Studies on adding herb extracts to beef products to reduce bacterial growth demonstrated that the antimicrobial activity of herb extracts might be weaker in beef products with high fat content, pointing to the influence of the way the fat is distributed in the food matrix on the antimicrobial effect of hydrophobic compounds (Cutter, 2000). Similarly, Singh, Singh, Bhunia, and Singh (2003) concluded that the direct addition of thyme essential oil in foods reduced *L. monocytogenes* populations significantly in zero- and low-fat hotdogs, but not in full-fat hotdogs. The results of this research confirms the influence of fat content of foods on the bacteria inhibition of antimicrobials.

With the estimated parameters A_s , λ and $\mu_{\max}$ the shelf life extension of ground lean and fat beef in the antimicrobial group could be predicted using the Gompertz equation (Table 1). The

spoilage level of $9 \log_{10}$ CFU/g at 5°C was used as the value for the end of shelf life for ground meat (Koutsoumanis, Stamatiou, Skandamis, & Nychas, 2006). The PLA film with carvacrol had a significant positive effect on prolonging the shelf life of lean beef products at chilled chain conditions (5°C), compared to the control group. According to the model prediction, the shelf life of lean beef preserved with antimicrobial film was extended by 2.5 ± 0.9 days compared with the beef in the control group. A 30% longer shelf life was achieved in ground lean beef stored with carvacrol loaded PLA film. In terms of the regular beef in antimicrobial group, the carvacrol loaded PLA film was able to extend 1.1 ± 1.5 days of shelf life for the test sample.

Table 1. Predicted values for the shelf life extension of ground lean and fat beef packaged within antimicrobial film at 5°C .

	Shelf life extension of lean beef (day)	Shelf life extension of regular beef (day)
Shelf life (day) $\pm$ SD	2.5 ± 0.9	1.1 ± 1.5
<i>p</i> -value	0.007	0.486

5.4. Conclusions

In the present study, a PLA film with carvacrol was developed to study the correlation between the carvacrol partitioning and its antimicrobial efficacy. It was demonstrated that the storage temperature and fat content of foods have significant effect on carvacrol partitioning within the package, while relative humidity did not have an effect. For both ground lean and regular beef, the carvacrol concentrations in the headspace of package at 30°C and 20°C were higher than the carvacrol quantity of sample at 5°C . In the headspace of packages containing ground lean beef, 2.9-fold higher carvacrol was found in the samples at 30°C than the sample stored at 5°C . The higher release of carvacrol from PLA film is “intelligent” when the temperature increase. If for any reason the chill chain is interrupted the system reacts releasing more antimicrobials. In addition, 1.3-fold carvacrol was found in ground regular beef with a high fat content compared to ground lean beef at 5°C . Despite this higher carvacrol concentration in regular beef the antimicrobial activity was more effective in lean beef. This can be explained by the absorption of carvacrol occurring mainly in the fat phase of the products. The shelf life of

ground lean beef stored with carvacrol loaded PLA film was 2.5 days longer compared to the control. According to the results from this study, it can be concluded that the developed food packaging film is a feasible application to provide a prolonged carvacrol release from PLA film within a longer period of storage time. As a whole, this study contributes to the understanding of the mechanistic aspects that are useful in the design of antimicrobial packaging for a specific food composition.

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Chapter 6

General discussion

6.1. Introduction

The quality and safety of food products need to be ensured to meet consumer expectations. This can be achieved by retarding/inhibiting the growth of microorganisms in foods by application of antimicrobial packaging. An important but easily overlooked fact in the design of antimicrobial packaging is the effect of the food matrix, relating to the food components and microstructures, on the performance of AP. Important in this respect is the role of the food matrix on the partitioning/diffusion of the antimicrobial agent and the resulting effect on the bacterial growth inhibition.

The aim of this thesis was to unravel the relations between the food matrix and the antimicrobial activity of antimicrobial packaging (AP). More specifically, the aim was to understand the influence of foods with different microstructures in relation to the antimicrobial effect of antimicrobial packaging prepared with carvacrol loaded PLA film (**Figure 1**). By varying food microstructures in dispersed particle sizes (oil droplets and protein aggregates), a more fundamental understanding of the influence of the disperse phase on the absorption, partitioning and final antimicrobial activity of carvacrol was gained (**Chapter 2** and **3**). By tuning the interactions between the food components (protein and polysaccharide) and other food ingredients (salt), food model systems with different physical properties (including rheological properties and water holding capacities) and porous structures were obtained, in which the absorption/partitioning and activity of carvacrol in semi-solid food models were studied (**Chapter 3** and **4**). By measuring carvacrol absorptions in real foods (ground lean and regular beef), interesting relationships between the carvacrol concentrations and carvacrol antimicrobial activities were found (**Chapter 5**). In this general discussion, main findings and conclusions of this thesis are presented as shown in **Table 1**, along with the discussion and interpretations of the main results. Following this, guidelines in the design of suitable AP properties for specific food products are first provided based on the knowledge obtained in this thesis. Finally, recommendations for future research are given.

Figure 1. Approach of the thesis: Linking properties of food composition and structural features to antimicrobial activity of antimicrobial packaging. The size of the yellow, red and white dots/irregularities corresponds to the size of the oil droplets, proteins and pores, respectively. The amount of yellow dots correspond to the amount of oil in the food matrix.

6.2. Discussion and interpretation of the results

The main findings obtained in this thesis are summarized in **Table 1**.

Table 1. Summary of the main findings of the studies described in this thesis.

Chapter	Aim	Key factors investigated	Main findings
2	To unravel the influence of the oil droplet size on carvacrol partitioning between the available phases, and to further examine the impact of the antimicrobial partitioning on its efficiency of the bacterial growth inhibition in the model food matrix.	Oil droplet size	<ul style="list-style-type: none"> • Carvacrol absorption and partition coefficient were higher in emulsions containing smaller oil droplets. • The antimicrobial activity of carvacrol was not the highest in the emulsion containing the smallest oil droplets. • The antimicrobial activity of small bioactive molecules depends on the colloidal structure
3	To study how antimicrobials absorption by gel samples is affected by the microstructure of gels.	Microstructure of gel; Pore size	<ul style="list-style-type: none"> • The size of aggregates depends on ionic strength of the gel. • High ionic strength caused a looser gel structure with large pores. • Antimicrobial absorption was increased in loose gels with large pores.
4	To understand how the microstructure of hydrogel food impacts diffusion of a volatile antimicrobial compound and its efficacy from the surface to the 30-mm depth of hydrogel.	Carvacrol diffusion	<ul style="list-style-type: none"> • Carvacrol diffusion depends on the pore size (microstructural) differences of hydrogels. • Increased depth of hydrogels decreased carvacrol concentrations. • Ineffective activity in the deep layer was in line with the low carvacrol concentrations.
5	To investigate the influences of the storage environment and the fat content of ground beef on the carvacrol partitioning and antimicrobial activity in a ground meat - PLA food packaging system.	Fat contents; Storage temperature	<ul style="list-style-type: none"> • Carvacrol release from PLA film is enhanced at higher storage temperature. • A higher fat content of ground beef increases carvacrol absorption. • Carvacrol absorption in samples with higher fat content reduces its antimicrobial activity.

6.2.1. Mechanisms underlying absorption/diffusion behaviour of antimicrobial compounds in multi-component (model) food systems

This thesis considers liquid, semi-solid model systems and a food system, intending to resemble a wide range of food products. The food model systems consisted of particles dispersed in liquids and semi-solid hydrogels. Particles used in this thesis include fat droplets, proteins and carbohydrates. They vary in amount, size and the way of interaction with the continuous phase. For the serum/continuous phase, proteins in liquid and semi-solid food models were studied. These model systems allowed us to investigate the influence of the food microstructure/composition on the interaction with antimicrobial components and on the antimicrobial activity of AP. The influence of both the dispersed particle fillers and serum/continuous phase on the antimicrobials absorptions are discussed in the following paragraphs.

Fats and proteins can accommodate hydrophobic components and have high retention ability of hydrophobic compounds (Ammari & Schroen, 2018; Ayed, Martins, Williamson, & Guichard, 2018; Doyen, Carey, Linforth, Marin, & Taylor, 2001; Giroux, Perreault, & Britten, 2007; van Ruth & Roozen, 2002). Besides hydrophobic interaction, proteins can bind with a wide range of substances, such as aldehydes, ketones, phenyl groups, ionones and esters, via reversible binding of hydrogen bond (Guichard, 2002; Han, Shen, Zhao, & Sun, 2018; Liu, Powers, Swanson, Hill, & Clark, 2005; Zhou, Zhao, Su, & Sun, 2014). The presence of fats and proteins provides the possibility of absorbing the hydrophobic antimicrobial compound to foods when the antimicrobials release from the AP. Consequently, it can be reasonably hypothesized that a high fat or protein content shows the strong binding activity with the hydrophobic compounds (Lesme, Rannou, Famelart, Bouhallab, & Prost, 2020). Indeed this was the case when carvacrol was absorbed in meats that contain different amounts of fat contents (**Chapter 5**). The concentrations of carvacrol in regular beef were higher than those in lean beef at the end of storage at the different temperatures. This showed a direct link between the fat content in foods and the absorption concentrations of the hydrophobic antimicrobial agent.

When the antimicrobials are absorbed in the surface of the foods, the diffusion occurs by the concentration differences between the surface and the inner part of foods. The location of antimicrobials during the diffusion and the total absorptions during storage depends highly on the microstructure of the foods. Food microstructure involves all the rheological and structural (geometric and surface) attributes of the food products (Chen & Opara, 2013). It can be defined

as the organization and interaction of food constituents resulting in a particular microscopically visible spatial partition of different material phases (Verboven, Defraeye, & Nicolai, 2018). Variations of the particle size of the dispersed phase, such as the fat droplets in emulsions and the WPI-containing aggregates in gels, influence the total carvacrol absorptions during storage (**Chapter 2 and 3**). This can be attributed to the “droplet size effect”. The “droplet size effect” can be explained by the fact that, for a given mass of compound, a smaller droplet size offers a larger surface area of the droplets, which influences the morphology and functionality of the food system (Armand et al., 1999).

Fat droplets range in size from 0.1 μm , for example for fat globules in milk, to 140-240 μm , for example for fat droplets in meat batter (Lin & ZAYAS, 1987; Mulder & Walstra, 1974; Salminen, Herrmann, & Weiss, 2013). In liquid emulsions, the size of fat droplets is an important physicochemical property affecting not only the appearance, texture, stability and sensory properties of the emulsion itself, but also the gastrointestinal function and satiety of consumers (Hussein et al., 2015). Carvacrol absorption was the highest in the emulsion containing the smallest oil droplets. This can be interpreted by two mechanisms: “droplet size effect” and Laplace pressure. The Laplace pressure can impact the solubility of carvacrol in the aqueous layer that promotes carvacrol absorption during storage. Specifically, a decreased droplet surface results in an increase in Laplace pressure, which increases the solubility of lipophilic compounds in the aqueous layer immediately surrounding the oil droplets (Yucel, Elias, & Coupland, 2012).

Similar to the oil “droplet size effect”, smaller WPI-containing clusters in the gel prepared with the highest NaCl concentration contributed to a higher carvacrol absorption, compared with the gels containing larger WPI-containing clusters. The smaller size of WPI aggregates may increase the exposure of binding sites that are located on the protein surface of β -lactoglobulin, and located on native bovine serum albumin (BSA) (Considine, Patel, Singh, & Creamer, 2005; Wang & Arntfield, 2015), leading to a high chance of interaction between WPI proteins and carvacrol.

When the hydrophobic compound partitioned itself between the two phases (e.g. oil phase and aqueous phase containing proteins) in emulsions, the effect of protein on the binding of carvacrol was not significant. This can be explained by the fact that carvacrol is largely hydrophobic and shows a higher preference to be absorbed into the oil when oil is present in the system. This indicates that in an oil suspended in aqueous phase (o/w) emulsion, the

dominant hydrophobic compound absorber is oil (fat). It thus can be reasonably hypothesized that the oil “droplet size effect” is of importance to the partitioning of carvacrol in the lipid and aqueous phase of emulsions. Not only the highest total absorption, but also the highest partition coefficients (ratio of carvacrol concentration in oil phase versus serum phase) were measured in the emulsions containing the smallest oil droplets. This indicated that a small oil droplet size promotes the absorption of carvacrol in the lipid phase in the emulsion.

Variations of the physical states of foods also influence the absorption and partitioning of antimicrobials in foods. The absorption/diffusion of antimicrobial compounds in a semi-solid food model is more complex than that in a liquid model food. This can be explained as a fact that the Brownian motion is more interfered because of the limited particle driving forces available in a semi-solid food, compared with that in liquid foods (Moschakis, 2013). In a semi-solid/solid food, the transportation/diffusion of molecules can be achieved via the pores that are formed during food processing as a result of the existence of air volume and water droplets (Ma, Cui, Song, & Hao, 2011). The large pores acting as a passageway can promote movement of molecules, which contribute to a high concentration of antimicrobial agents absorbed in food products. In a highly interconnected porous hydrogel with the pores that ranged from $457 \pm 27 \text{ nm}^2$ to $881 \pm 91 \text{ nm}^2$, carvacrol total absorption was 40% higher in the hydrogel with large pores than that in hydrogel with small pores (**chapter 3**). Similar results were obtained in **chapter 4**, where the carvacrol concentrations in different layers of a hydrogel with large pores were higher than that in respective layers of a hydrogel with small pores. The results above help to elucidate the mechanisms responsible for carvacrol absorption/diffusion in relation to the microstructure of a semi-solid model food (gel): in brief, carvacrol concentrations in the semi-solid model foods are directly linked to the pore size of the model foods (in the range of 237-972 nm^2).

It has been found that pores in materials play a critical role in various aspects, including in clinical application of biomaterials (Dadsetan et al., 2008), development of drug delivery devices or other encapsulation devices (Varghese, Chellappa, & Fathima, 2014), understanding/prediction of food processing operations such as drying, smoking, blanching, frying, evaporative cooking, extrusion and cooking (Rahman, 2001), development of a food product with specific mechanical, textural and sensorial properties (Loisel, Lecq, Ponchel, Keller, & Ollivon, 1997; Vincent, 1989), etc. Our study is the first study that has proved pores to have a great influence on the antimicrobial effect of the antimicrobial compounds in AP. To obtain information about the pore network, numerous techniques can be used such as the

microscopic techniques including qualitative three-dimensional (3-D) imaging techniques such as X-ray micro-tomography and magnetic resonance imaging (MRI), and two-dimensional (2-D) imaging techniques such as scanning electron microscopy (SEM) and transmission electron microscopy (Gueven & Hicsasmaz, 2013), and the capillary penetration techniques including mercury intrusion porosimetry and liquid extrusion porosimetry (Datta, Sahin, Sumnu, & Keskin, 2007; Hicsasmaz & Clayton, 1992).

Considering the combined effect of the proteins effect and porosity effect, it is reasonable to understand that the carvacrol concentration in the top layer of hydrogels was about 4-fold and 11-fold higher than those in the middle and bottom layer, respectively (**chapter 4**). Different mechanisms that influence the absorption/diffusion of antimicrobial compounds may co-exist, enhance, suppress, or compensate each other in a multi-component food system. To understand or estimate the effect of the food matrix on the partitioning of an antimicrobial compound in a food, the first step is to identify and classify all the factors that might strengthen or weaken the interactions between the antimicrobial compound and the food, thereafter analyze which factors play a key role. After pinpointing the key factors, it should be possible to understand the partitioning behavior of the antimicrobial compound and further estimate its activity in a food matrix.

6.2.2. Effect of the food matrix on antimicrobial activity

The effects of carvacrol-loaded PLA film on the growth of bacteria were investigated in various model/real foods, along with measurements of the carvacrol concentrations in the food matrix (**Figure 2**). In an oil-in-water emulsion with fine oil droplets (**chapter 2**), the antimicrobial activity of a packaging film was low in emulsions containing the smallest oil droplets, although the highest amount of carvacrol was found in the same samples, compared with other fine emulsions with larger droplets. Similarly, carvacrol concentration in ground regular meat was 1.34-fold than that in ground lean meat. Yet no substantial effect could be found in the inhibition of bacteria in the ground regular meat (**chapter 5**). In another case of a colloidal structural food model system without an oil phase, a positive correlation between carvacrol concentration and antimicrobial activity of packaging film was found: the higher carvacrol concentrations were measured in food hydrogel, the higher antimicrobial activity of the antimicrobial packaging was achieved (**chapter 4**). These results show that in some cases the antimicrobial activity was positively correlated with the concentrations of antimicrobials while in other cases a negative correlation was found. This indicated that the antimicrobial activity can be suppressed by the

presence of the food components, and highlighted the necessity of considering the available dosage of antimicrobial compounds in the food matrix in the design and application of antimicrobial packaging film.

Either a positive or negative antimicrobial effect caused by the interaction between food compounds and antimicrobials depends on the spatial distribution of antimicrobial compounds and microorganisms. More specifically, antimicrobials that have a preference for the aqueous phase are less affected by the hydrophobic food components, and their antimicrobial activity is increased with the increase of dosage. For the hydrophobic antimicrobial compounds, their location of presence in the food matrix is more important for the antimicrobial activity than the total concentration in a food matrix. The partitioning of these hydrophobic antimicrobial compounds can be strongly influenced by the existence of the major food compounds such as fats and proteins. This was seen in a study when a large amount of antimicrobial compounds were stuck in a hydrophobic part of a multi-phase food mixture, which phase was, however, unfavorable for microorganism growth. The availability of antimicrobials that were present in parts of the food matrix that can reach bacteria was relatively low, resulting in an activity loss and low utilization of the antimicrobial compounds of the antimicrobial package.

Consideration of the available dosage and location of antimicrobial compounds in the food matrix can avoid occurrences of some issues. For example, although high doses of active compounds had been used, the antimicrobial performances were less effective in some applications, concurrent with an unacceptable sensory impact (Muriel-Galet, Cerisuelo, López-Carballo, Lara, Gavara, & Hernández-Muñoz, 2012; Wiecznyńska, Luca, Kidmose, Cavoski, & Edelenbos, 2016).

Figure 2. Summary of the relationship between the performance of AP and the antimicrobials absorption and diffusion in the food matrix. The green particles represent carvacrol; yellow particles represent fat droplets; white irregularities within the semi-solid system represent pores in the food matrix; red curves represent ground meats. The model/real foods in **Chapter 2, 4** and **5** are emulsions that defer in the size of oil droplets, WPI-carrageenan gels that differ in pore sizes and meats with different amounts of fats, respectively. AP did not perform well on the emulsion with small oil droplets, the gel with small pores, and the meats with high-fat contents.

This thesis covered model/real food systems, ranging from simple particle dispersions in liquid samples to polymer hydrogels, and ended with experiments on real foods (meat products). When the system becomes more complex, as can be seen in the existence of multiple food components and in the multi-phase of the food microstructure, the complexity in the efficacy of antimicrobials increases. Studying the antimicrobial activity firstly in a liquid sample helped in understanding the influence of food compounds on the diffusion of antimicrobial compounds, since enough particle driving forces available in a liquid sample may reduce the interference from the spatial arrangement of the food components in the food matrix. After introducing protein-polysaccharide complexes in the food system, colloidal gels may be formed depending on factors in systems such as pH, temperature and ionic strength (Duran, Spelzini, Wayllace, Boeris, & da Silva, 2018; Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004). This food system underwent a sol-gel transition, resulting in a semi-solid/solid structure. In contrast to the high mobility of antimicrobial compounds in a liquid food system, a limited Brownian motion occurs in a semi-solid/solid food system, leading to an immobilization and un-even distribution of antimicrobial compounds in foods. This highlights the impact of the microstructure of foods on the diffusion of antimicrobial compounds, and may enhance the impact of food components on the interaction with antimicrobial compounds. Due to food complexity and the fact that microstructure complexity is related to the interactions between food macromolecules (Druaux & Voilley, 1997), it is often difficult to have conclusive results on the determination of antimicrobial activity is mainly caused by either food microstructure or food components. In all cases, the influence on antimicrobial activity is a result of the combined effect from both food components and food microstructures.

6.3. Selection criteria for antimicrobial packaging

The amount of AP options that are being developed with various antimicrobial compounds and with different technologies are steadily increasing. A selection tool to choose the right package for the right food is essential to make an efficient use of antimicrobial packaging and enhance the qualities of foods. In the previous chapters, we have discussed how the partitioning and activity of antimicrobial agents in a food system are influenced by: the composition of the food matrix, the location where food components are present, and the resulting food microstructure caused by the interactions between food components. It is obvious that the selection of a suitable AP is a burdensome task. The roadmap that suggests criteria to select the tailored AP for a specific food product was developed and is shown in **Figure 3**.

Figure 3. The roadmap for AP selection from the viewpoint of food matrix.

The roadmap helps food manufacturers and developers to determine the application of AP by using the following criteria:

- **Physical state and microstructure of food (i.e. liquid vs solid).** To develop and apply an antimicrobial food packaging, differences in the physical state (i.e. liquid vs solid) of food products should be firstly considered, since the physical state influences the driving force of mass transfer processes of antimicrobial compounds in a food-package system. In liquid foods, the molecular diffusion is more random. However, diffusion in semi-solids/solids is far more complex than in liquids, because (1) the compounds diffusing through a semi-solid/solid may actually be diffusing through a liquid or gas contained within the pores of the solid (Callaghan, Coy, MacGowan, Packer, & Zelaya, 1991); and (2) molecules may have a preferential movement path in semi-solid/solid foods, which largely depends on the microstructure of food products, and can be easily affected by the spatial arrangement of food components. Considering the facts above, it can be suggested to use antimicrobial packaging prepared with a relatively low amount of antimicrobial compound, depending on the fat content of foods, and with a packaging polymer that shows a good water barrier property to preserve aqueous liquid foods. When an antimicrobial package for semi-solid foods with a porous structure is considered, a packaging film prepared with volatile antimicrobial compounds is more suitable to provide a homogeneous distribution of antimicrobials to the surface of food, and a relatively high dosage of antimicrobials can be recommended in the application since the diffusion of antimicrobials is easily limited by the microstructure of semi-solid foods. For rigid foods (e.g. fruits) where the multiplication of microorganisms hardly occurs in the internal part of the food, providing an edible coating might be a good choice since the food deterioration is mainly caused by the growth of microorganisms on the surface of foods.
- **Food composition.** When designing antimicrobial packaging, it should be considered that the properties (i.e. hydrophobicity and ionicity) of antimicrobial agents should not affect the performance of antimicrobial packaging, in the presence of major food components such as proteins and polysaccharides. For instance, it is suggested not to apply antimicrobial compounds that have a high affinity to fats, such as essential oils, in an antimicrobial packaging to protect a food product containing a high fat content. For products with a high fat content it is advised to use a hydrophilic antimicrobial agent (e.g. organic acids) instead of hydrophobic antimicrobials. An alternative strategy for

improving the antimicrobial efficacy when using hydrophobic antimicrobial agents in foods with a high fat content would be to encapsulate antimicrobial compounds in micrometric size capsules (Castro-Rosas et al., 2017). When the capsules reach the food surface, the encapsulated antimicrobials can release off from the capsules by specific stimuli which include rupture of the capsule wall by dissolving triggered by temperature, pH or enzymatic activity. The choice of the different capsule materials is the key factor that determines the release rate of antimicrobials from capsules. This technique can increase the concentration of the antimicrobials in food areas where microorganisms are preferably located (Weiss, Gaysinsky, Davidson, & McClements, 2009), and enhance the transport mechanisms of antimicrobial compounds through the cell membrane of the bacteria (Donsì, Annunziata, Sessa, & Ferrari, 2011).

Regarding to the preservation of a food product that is rich in proteins and polysaccharides, the choice of chitosan-based antimicrobial films should be carefully considered in food applications since the diffusion of chitosan in foods and the interactions with microorganisms that were influenced by the presence of proteins and polysaccharides might differ at different pH values. For food products with moderate to high sodium concentration, such as ham, endolysin LysSA11 and LysH5, which are cell wall-hydrolyzing enzymes synthesized during late gene expression in the lytic cycle of multiplication of most phages, would be a good candidate as antimicrobial compound (Chang, Kim, & Ryu, 2017; García, Martínez, Rodríguez, & Rodríguez, 2010). Besides, these antimicrobial compounds were found to have a good performance on the preservation of food products that are slightly acidic to above neutral pH (e.g. milk). On the other hand, endolysin PlyP100, an endolysin derived from the *L. monocytogenes* phage P100 that was effective against *L. monocytogenes* (Van Tassell, Ibarra-Sánchez, Hoepker, & Miller, 2017), is not suitable for foods that are acidified and containing higher NaCl levels (>150 mM) (Van Tassell et al., 2017). For the preservation of acidic foods (e.g. dairy products and some canned soups), nisin is an alternative antimicrobial agent as its antimicrobial activity is stronger at acidic pH (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016; Hurst, 1981). This means that when antimicrobial packaging is prepared with bacteriocins, bacteriophages and endolysins as antimicrobials, parameters like pH and salt concentration in food products should be taken into account in the design.

- **Product storage environment.** Another important aspect to consider when designing an antimicrobial package is the storage (internal and external) environment of the food-package. The release and diffusion rate of antimicrobials from the packaging film

depends highly on the storage environment (e.g. temperature). And the growth of microorganisms is influenced by similar factors like temperature, pH and water activity of the food product. Therefore, applications of controlled release of antimicrobials that are in response to a particular environmental trigger (e.g. pH, ionic strength, or temperature) can be considered as the effective way to preserve foods (Balasubramanian, Rosenberg, Yam, & Chikindas, 2009; Weiss et al., 2009). For example, aroma volatiles encapsulated in pullulan and β -cyclodextrin would be triggered to release from the nanofibrous membranes in an environment with a relative humidity beyond a threshold of 90% (Mascheroni et al., 2013). This development is expected to be extended in the food packaging area for controlling the release of antimicrobials depending on the external conditions.

When an AP for food products that are stored at a low temperature is designed, it is suggested to increase the freedom of motion of antimicrobials immobilized in polymers by lowering the degree of crosslinking of polymers, to enable the antimicrobial compound to be easily released from packaging films at low temperatures (Appendini & Hotchkiss, 2002; Shao, Yan, Chen, & Xiao, 2018). This ensures that the concentrations of antimicrobials in the food are higher than the minimum inhibitory concentration (MIC) during storage. However, a decrease in the degree of crosslinking may lead to a reduction in mechanical and barrier properties of polymer films. If a tightly crosslinked polymer has to be chosen for the preservation of food products like milk, antimicrobials that are specifically active at lower temperatures should be selected, for example, LysH5 endolysin (Obeso, Martínez, Rodríguez, & García, 2008).

When the protection of foods with a high water activity (or the storage environment has a high humidity) is required, the application of hydrophilic polymers should be carefully considered. On one hand, this application is beneficial in inhibiting the growth of microorganisms in a high humidity environment since antimicrobials can be easily released from hydrophilic materials in this situation (Ganewatta & Tang, 2015). On the other hand, a hydrophilic polymer may be damaged in a humid environment, resulting in a burst release of antimicrobials from the packaging film (Assarar, Scida, El Mahi, Poilâne, & Ayad, 2011). As an option, multilayer films can be used to protect foods against microorganisms and to fulfill multiple expectations from customers. By combining different layers, a multilayer film that takes advantage of the barrier properties of one polymer and the antimicrobial properties of another layer to develop the “multifunction” package for the application (McKeen, 2016).

- **Other selection criteria.** In addition to the above-mentioned evaluation criteria, industries and researchers may also consider a number of other factors such as, economic aspects, environmental impact of packaging materials and other considerations (i.e. sensory, toxic effects for some specific antimicrobial compounds). Questions regarding the development scenario analysis may include: (1) does the active compounds used in the packaging film endanger human health, (2) is the packaging polymer environmentally friendly, (3) what is the cost and benefit of the implementation of AP, and (4) can it bring some negative impacts like pungent flavor and unacceptable texture for foods.

6.4. Recommendations for future work

The knowledge obtained in this study contributed to the improvement of the antimicrobial efficacy of AP by knowing the influences of the food matrix. Various model/real food systems have been taken into consideration to investigate the antimicrobial compounds partitioning in a food-package and the efficacy of the developed AP. In this section, aspects are mentioned that need further research for the development of AP.

From the viewpoint of scientific investigation. This study developed a food package system consisted of carvacrol as antimicrobial agent and PLA as an antimicrobial carrier to unravel the relations between the food matrix and antimicrobial performance of AP. Knowledge obtained from this system can be widely utilized in the design of AP incorporated with antimicrobial compounds that are rich in volatile terpenoids and phenolic particles, such as a broad range of essential oils and plant extracts (e.g. allyl isothiocyanate, rosemary, oregano and garlic essential oils, etc.). However, to obtain a comprehensive understanding of the influences of food matrix on the activity of AP, other antimicrobial agents, such as enzymes (e.g. lysozyme and endolysins), chitosan, bacteriocins (e.g. nisin) and inorganic nanoparticles (e.g. titanium dioxide and zinc oxide), are encouraged to study in future research.

To design an effective AP, the mechanism/process of diffusion and release of the antimicrobials, their partitioning to the headspace and the food products, their interactions with food components, their diffusion in foods affected by the microstructure of foods, and the resulting effect on microbial growth are important to understand the performance and effectiveness of an AP. Since these processes will be affected by many factors, such as environmental conditions, food composition, packaging materials, packaging dimensions, etc., it will be very costly and time-consuming to investigate all possible combinations. Thus, to describe the main

physicochemical and microbial processes involved in the antimicrobial packages, mathematical modelling can be used as it is an advantageous way to simulate and optimise the effects of design parameters of antimicrobial packages, which in turn can improve the antimicrobial effect of AP.

From the viewpoint of industrial application. From the results of this thesis it is clear that the efficacy of an antimicrobial compound strongly depends on the properties of the food composition and of the food matrix. The inhibition of the bacterial growth was relatively weak in some experiments in this thesis, compared with previous studies that caused a higher than 2 log reductions of colonies (Sung et al., 2013). This shows the effect of differences in the media that were used to inoculate bacteria. For example, the bacteria were inoculated in model or food media that are structural complex and multi compositional like emulsions and hydrogels in this thesis, instead of a model medium that composed of basic enrichments. For industrial applications it is required that AP is not only effective on pure bacterial media, it should also be effective in reducing microbial growth in real foods. This research shows that improvements could be needed in the antimicrobial efficiency when using an antimicrobial package for foods. Potential technologies that may contribute to improvement in the protection of foods against microorganisms are suggested as followings:

- AP that incorporates multiple antimicrobial compounds could be utilized to make the best use of their antimicrobial activities on the inhibition of the growth of various microorganisms in foods (Iamareerat, Singh, Sadiq, & Anal, 2018). A single antimicrobial agent may display its efficacy in a limited range of microorganisms, while the combined multiple antimicrobial compounds may display a synergic activity against broad range of microorganisms in foods. In addition, the multiple compounds show different behaviors based on their properties, which can inhibit the growth of microorganisms in the different locations of food in long term storage, e.g. one antimicrobial agent is designed for the surface of food, one that is fast released, and one that is slowly released, etc.
- A combination of AP and other technologies (e.g. high-pressure processing, refrigeration and modified atmosphere packaging) may provide a synergistic effect (Belletti, Kamdem, Patrignani, Lanciotti, Covelli, & Gardini, 2007; Mastromatteo, Incoronato, Conte, & Del Nobile, 2011). The lowered activities of antimicrobial compounds caused by the interactions with the food compounds can be compensated by inhibiting the growth of

microorganisms by technologies that are not easily affected by the presence of food components.

- Multilayer AP can be considered as a strategy to fulfill a multiple requirements of a packaging (Gherardi, Becerril, Nerin, & Bosetti, 2016). Multilayer AP consists of several thin films that can optionally be selected based on its individual properties and grouped to a multilayer AP with the multi-functional properties. It provides a good protection of foods from food spoilage by combining the benefits of each monolayer in terms of antimicrobial properties, improved water/gas barrier properties and mechanical properties (Anukiruthika, Sethupathy, Wilson, Kashampur, Moses, & Anandharamakrishnan, 2020). However, the negative environmental impact of a multilayer polymer film due to the difficulties of reuse or recycle should be considered and evaluated before application.
- Application of nanotechnologies, such as nanocarrier, nanoparticle and electrospun nanofibrous film, is expected to improve the functional properties of AP (Ataei, Azari, Hassan, Pingguan-Murphy, Yahya, & Muhamad, 2020; Bahrami, Delshadi, & Jafari, 2020). The incorporation of antimicrobials in nanocarrier or nanoparticle has been proved to protect antimicrobial compounds from physical and chemical damages from the environment. Upon deposition and exposure to the aqueous phase of the food matrix, the nanocarrier or nanoparticle dissolves and releases the antimicrobials from the carrier, which provides a high chance for good interaction between antimicrobials and microorganisms that are present in the aqueous phase of foods (Gao, Chen, Zhang, Zhang, & Zhang, 2018). The electrospun fibers have a good property of a large surface area to volume ratio due to the high porosity, which improves the encapsulation efficiencies of antimicrobial compounds and provides a sustained release of antimicrobials from the polymer films to the foods (Ataei et al., 2020).

To conclude, a proof of concept that the consideration of the effect of the food matrix in the design of AP is effective in improving the antimicrobial activity of AP was demonstrated in this thesis. This concept is versatile in design, and it is especially important for designing AP that can be used to preserve real foods. Ultimately, the outcomes of this thesis yield roadmap that can be used as the selection criteria of AP for specific foods.

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Summary

Nowadays, antimicrobial packaging (AP) can be considered as a promising technology that is helpful to improve food quality and safety in the food supply chain. The indirect-contact AP is effective in retarding the growth of microorganisms by releasing the incorporated volatile antimicrobials to the headspace of the package through which the antimicrobials diffused to the surface of foods. During food storage, both the composition and microstructure of the food matrix are relevant for antimicrobial compounds absorption and diffusion in foods. Understanding the influences of the food matrix on the efficacy of a volatile antimicrobial compound is important in the antimicrobial packaging design, which is largely ignored in previous studies. Hence, this thesis aimed to link the physical-chemical properties of model/real foods to the partitioning of volatile antimicrobials in a food-package, and in this way, contribute to an efficient design of antimicrobial packaging for specific foods.

After providing a brief introduction to the fundamental aspects of the relationship between food spoilage, antimicrobial packaging and food matrix (**Chapter 1**), the partitioning of antimicrobial compounds (using carvacrol as an example) in liquid model foods that consist of lipid droplets dispersed in an aqueous medium is first described in this thesis (**Chapter 2**). At macro level, the alteration of the size of oil droplets influenced the partitioning of the antimicrobial compound carvacrol in a food package which was reflected in the highest absorptions in emulsions containing the smallest oil droplets while relatively low antimicrobial concentrations in the headspace of food package. At meso level, the alteration of the size of oil droplets determined the spatial distributions of antimicrobials in the emulsions that can be reflected in the highest log K (equilibrium concentration of carvacrol between the oil/creamed and aqueous phases of samples) for carvacrol measured in emulsions that contains the smallest oil droplets. The spatial distribution of antimicrobials in the colloidal structure explained why carvacrol was less effective in the inhibition of growth of *Pseudomonas fluorescens* in emulsions containing the smallest oil droplets, although these samples absorbed the most carvacrol, compared with other emulsions containing larger oil droplets.

The obtained insights in the influence of the dispersed phase in model foods with a colloidal structure on the antimicrobial performance of packaging helped to further explore semi-solid model foods. Weak hydrogels were prepared with WPI and carrageenan, and the partitioning of carvacrol in the food package containing these weak hydrogels was studied (**Chapter 3**). The morphologies shown from the CLSM and SEM and the rheological properties indicated that the microstructure of hydrogels were tuned by adding different amounts of NaCl. High ionic strength (250 mM NaCl) resulted in a loose hydrogel structure with large pores and small

WPI-containing aggregates. This microstructure favored absorption of antimicrobials in the hydrogel by enabling an efficient interaction to the binding sites of the gel proteins. This study highlighted the importance of considering the porous structure in semi-solid foods on the active compounds' absorption, however how the active compounds diffuse within the foods and how the microstructure of hydrogel influences antimicrobial activity of active compound were still unknown. Thereby studies on hydrogels in **Chapter 4** were undertaken in which carvacrol diffusion and antimicrobial activity in hydrogels from the surface to 30-mm depth were analysed. It became clear that pore size is a key factor that impacts on both carvacrol diffusion and its antimicrobial activity. In loose hydrogel containing large pores, the carvacrol concentrations in top, middle and bottom layers (each layer with a same height of 10mm) were significantly higher than the concentrations in the respective layers in hydrogels containing small pores. This caused the higher antimicrobial activity in each layer of the hydrogels with large pores, compared with hydrogels containing small pores. Regarding the carvacrol concentrations in each layer of the hydrogels, >75% of carvacrol was retained in the top layer of the hydrogels (<10 mm from the surface of hydrogel). As a result, carvacrol activity was effective in retarding the bacteria growth in the top layers of all samples. On the contrary, only 5-7% of the total amount of absorbed carvacrol diffused from the top to the bottom layers, resulting to an insubstantial inhibition of the growth of *Pseudomonas fluorescens* in the bottom layers.

Carvacrol partitioning and antimicrobial activity in a food package containing real foods were measured in **Chapter 5**. The results showed that carvacrol was faster released from PLA film at 30 °C, compared to 20 °C and 5 °C. However the release profile was not influenced by water activity of the storage conditions. When the food package was stored at 5 °C, carvacrol absorption in 12% fat ground beef was about 1.3-fold higher compared with the carvacrol concentration in 5% fat ground beef after a 12-days storage. Interestingly, despite this higher carvacrol absorption in the regular beef (12% of fat), the PLA/carcacrol films had a stronger antimicrobial effect on the lean beef (5% of fat). This phenomena could be explained by the partitioning insights in the model studies.

In the general discussion of this thesis (**Chapter 6**), the main findings and a general discussion about the underlying mechanisms of the absorption/diffusion behaviour of the antimicrobial compounds in multi-component model/real food systems were summarized and discussed. The effect of the concentration of antimicrobials in the food matrix on the inhibitory effect of antimicrobial packaging on the growth of bacteria were linked using the insights from this thesis.

An explanation of the observation that antimicrobial packaging performs greatly on the inhibition of microorganisms in model media, but easily fails to be effective in real foods, was obtained by considering the effective antimicrobial concentration in the aqueous phase that can interact with the microorganisms. Selection criteria for antimicrobial packaging are proposed based on the main findings obtained from this thesis. These selection criteria emphasize the effect of the food matrix on the antimicrobial activity, and it provides a guide for the selection of a tailored antimicrobial packaging for specific characteristics of food products. Finally, follow-up researches that may be helpful for designing an effective AP were recommended.

The most important conclusion from this thesis is that the characteristics of food matrices are crucial for the effectiveness of antimicrobial packaging. A proof of concept provided by this thesis shows that the antimicrobial efficacy of AP can be improved by applying a customized packaging film for specific food products.

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发生的都有意义，答案就在未来。

Li Wang 王荔

10 August, 2020

Guangzhou, China

About the author

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Curriculum Vitae

Li Wang was born on the 22nd of October 1991 in Wuhai, Inner Mongolia Autonomous Region, China. She completed her Bachelor study in Henan University of Technology in 2014. In the same year, Li started her Master study Food Technology at Wageningen University & Research. Her master thesis was completed at the Food Quality & Design group on the topic of “The extraction and optimization of chitin from insects”. During her internship at International Flavors & Fragrances, Hilversum, the Netherlands, she developed and validated a proof of principle for fragrance performance model for liquid detergent. She obtained her Master degree in 2016.

Li continued studying as PhD candidate at the Food Quality & Design at Wageningen University & Research in 2017. During this research, she worked on the antimicrobial packaging designed by consideration of the influence of the food matrix, and the results of this research are described in this thesis.

Contact: li1.wang@outlook.com

List of publications

Wang, L., Dekker, M., Heising, J., Fogliano, V., & Berton-Carabin, C. C. (2020). Carvacrol release from PLA to a model food emulsion: Impact of oil droplet size. *Food Control*, 107247.

Wang, L., Fogliano, V., Heising, J., Meulenbroeks, E., & Dekker, M. (2020). Volatile antimicrobial absorption in food gel depends on the food matrix characteristics. *Food Hydrocolloids*, 105933.

Wang, L., Heising, J., Fogliano, V., & Dekker, M. (2020). Fat content and storage conditions are key factors on the partitioning and activity of carvacrol in antimicrobial packaging. *Food Packaging and Shelf Life*, 24, 100500.

Wang, L., Fogliano, V., Heising, J., & Dekker, M., The effect of pore size on the diffusion of volatile antimicrobials is a key factor to preserve gelled foods. (paper submitted to journal).

Overview of completed training activities

Discipline specific activities

Courses

Management of Microbiological Hazards in Foods, VLAG, Wageningen, NL, 2017

Reaction kinetics in Food Science, VLAG, Wageningen, NL, 2018

Healthy food design, VLAG, Wageningen, NL, 2018

Summer Course Glycosciences, VLAG/GBB, Wageningen, NL, 2018

VLAG online lectures, VLAG, Online, 2020

Sustainable Packaging in a Circular Economy, edX Remote Access Program, Online, 2020

Conferences

32nd EFFoST International Conference¹, Nantes, France, 2018

2nd FOOD CHEMISTRY¹, Seville, Spain, 2019

34th EFFoST International Conference¹, Online, 2020

General courses

VLAG Statistics courses 2017 – 1. Introduction to R, VLAG, Wageningen, NL, 2017

PhD week, VLAG, Baarlo, NL, 2017

The Essentials of Scientific Writing and Presenting, WGS, Wageningen, NL, 2018

Scientific Writing, WGS, Wageningen, NL, 2018

Searching and Organizing literature, WGS, Online, 2020

Career Assessment, WGS, Online, 2020

Brain Friendly Working and Writing, WGS, Online, 2020

Working on Your PhD Research in Times of Crisis, WGS, Online, 2020

Optional courses and activities

Scientific meetings, seminars, colloquia, FQD, Wageningen, NL, 2017-2020

Preparation of research proposal, FQD, Wageningen, NL, 2017

FQD PhD trip to Australia, FQD, Australia, 2018

Teaching obligation

Mentoring MSc- and BSc-students, Wageningen University and Research, Wageningen, NL, 2017-2020

Food Packaging and Design, FQD-36306, FQD, Wageningen, NL, 2017-2018

Advanced Fermentation Science, FHM-30806, FHM, Wageningen, NL, 2019-2020

¹ oral presentation;

VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences;

WGS: Wageningen Graduate School;

FQD: Food Quality and Design group;

FHM: Food Microbiology group.

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