Antimicrobial Effects of Natural Ingredients against Spoilage Microbiota of Chicken and Sausage

by

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A Thesis Submitted to the Graduate and Post-Doctoral Studies Office

in partial fulfillment of the requirements of the degree of

Master of Science

July 2018

ABSTRACT

Fresh chicken and Italian pork sausage are highly perishable due to their high water activities, high protein and nutrient content, combined with a pH range of 5.8-6.2, which allow good growth of spoilage microorganisms. Refrigerated chicken products generate exudates, in which aerobic spoilage bacteria can grow and generate off-odors. In contrast, due to the absence of exudates, the spoilage microbiota of sausage could grow on the external surface and inside the product. The aim of this study is to delay the growth of initial spoilage microbiota from chicken exudates and sausage itself by natural antimicrobial ingredients, in response to the preference of consumers for clean-label food preservatives.

The natural ingredients tested include food by-products (cranberry pomace, sauerkraut juice powder) and plant extracts (green tea, grape seed and blueberry). An automated spectrophotometry (AS) method was used to ascertain the effectiveness of each tested ingredient under different concentrations. The growth kinetics of an undefined mixture of spoilage bacteria of chicken or sausage were followed in "meat-based" media prepared from sterilized chicken exudates or diluted sausage homogenates. The bacterial growth kinetics in AS assays were characterized by the lag time (LagT) and the maximum growth rate (μ_{max}) calculated from optical density (OD) readings. Data from AS assays showed that, the powder of sauerkraut juice at 20 g/L, grape seed extract at 10 and 20 g/L significantly delayed the growth of chicken spoilage microbiota. Meanwhile, all ingredients at 20 g/L had antimicrobial effects against spoilage population from sausage, and the inhibitory effects of extracts of grape seed and blueberry were higher, since inhibition was still detected when the concentration was reduced to 5 g/L. Traditional plate count assays were carried out for chicken and sausage media with the maximum tested concentrations (20 g/L) to confirm the conclusions from AS assays. Correlations between the average growth rates (μ_{avg}) during the early stage of storage obtained from AS assays and those of plate count assays were obtained. .

Further tests adding natural ingredients at their effective concentrations to absorbent pads were carried out for chicken exudates. The dry antimicrobial ingredients were distributed inside the absorbent pads to which fresh untreated chicken exudates were added. Effectiveness of these antimicrobial pads was evaluated by recovering bacteria from homogenized pads samples and

enumerating them through traditional plate counts (CFU). In comparison to AS assays, the antimicrobial effect of these selected ingredients was notably reduced when being applied the same concentrations to the absorbent pads. Possible explanations could be the slow and uneven distribution of antimicrobial ingredients in the pads.

RÉSUMÉ

Le poulet frais et la saucisse de porc italienne sont hautement périssables en raison de leur activité d'eau élevée, de leur teneur élevée en protéines et en nutriments, combinées à une zone de pH de 5.8 à 6.2, qui permettent une bonne croissance des microorganismes de d'altération. Les produits de poulet réfrigérés génèrent des exsudats, dans lesquels les bactéries aérobies peuvent se développer et produire des odeurs désagréables. En revanche, en raison de l'absence d'exsudats, le microbiote de d'altération de la saucisse pourrait se développer sur la surface externe et à l'intérieur du produit. Le but de cette étude est de retarder la croissance du microbiote d'altération initial des exsudats de poulet et de la saucisse elle-même par des ingrédients antimicrobiens naturels, en réponse à la préférence des consommateurs pour les additifs alimentaires avec « un clean label ».

Les ingrédients naturels testés comprennent des sous-produits alimentaires (marc de canneberges, poudre de jus de choucroute) et des extraits de plantes (thé vert, pépins de raisin et bleuet). Une méthode automatisée de spectrophotométrie (SA) a été utilisée pour déterminer l'efficacité de chaque ingrédient testé à différentes concentrations. La cinétique de croissance d'un mélange inconnu de bactéries de (d'altération) du poulet ou de la saucisse a été suivie dans des milieux «à base de viande» préparés à partir d'exsudats stériles de poulet ou d'homogénats de saucisses dilués. La cinétique de croissance bactérienne dans les essais de SA a été caractérisée par le temps de latence (LagT) et le taux de croissance maximal (μ_{max}) calculé à partir des lectures de densité optique (DO). Les données provenant des essais de SA ont montré que la poudre de jus de choucroute à 20 g/L et l'extrait de pépins de raisin à 10 et 20 g/L retardaient significativement la croissance du microbiote d'altération du poulet. Pendant ce temps, tous les ingrédients à 20 g/L avaient des effets antimicrobiens sur les saucisses et les effets inhibiteurs des extraits de pépins de raisin et de bleuets étaient plus élevés, puisque l'inhibition était toujours détectée lorsque la concentration était réduite à 5 g/L. Des comptages traditionnels sur plaque ont également été effectués pour des milieux de poulet et de saucisses avec les concentrations maximales testées (20 g/L) afin de confirmer les conclusions des tests de SA. Des corrélations entre les taux de croissance moyens (μ_{avg}) au début du stockage obtenus à partir des tests de SA et celles des comptages sur plaque ont pu être obtenues.

D'autres tests ont été effectués en ajoutant les ingrédients naturels à des tampons absorbants pour les exsudats de poulet. Les ingrédients antimicrobiens secs ont été distribués à l'intérieur des tampons absorbants auxquels des exsudats de poulet frais non traités ont été ajoutés. L'efficacité de ces tampons antimicrobiens a été évaluée en récupérant les bactéries à partir d'échantillons de tampons homogénéisés en les dénombrant à l'aide de comptages traditionnels sur plaque (UFC). En comparaison avec les tests de SA, l'effet antimicrobien de ces ingrédients sélectionnés a été considérablement réduit lorsqu'on a appliqué les mêmes concentrations aux tampons absorbants. Parmi les explications possibles il y aurait la distribution lente et inégale des ingrédients antimicrobiens dans les tampons absorbants.

ACKNOWLEDGEMENT

First, I would like to show my sincere appreciation for my supervisor, Prof. Ashraf A. Ismail. Thank you so much for giving me the precious opportunity to work on this interesting and creative project. His supports and encouragement for my thesis preparation, as well as his patience and considerate mind were valuable for me throughout my Master's program.

Words cannot express my gratitude to my co-supervisor, Dr. Claude P. Champagne at Saint-Hyacinthe Research and Development Centre of Agriculture and Agri-Food Canada (SHRDC-AAFC), for being an extremely knowledgeable supervisor, a father-like mentor, and a sincere friend. I would never discover my potential in research without his guidance, his unconditional supports and encouragements. I am exceedingly lucky to have him as my abecedarian in research. I have learned so much from his enthusiasm and rigorous attitudes to science, as well as his generous care for his students and colleagues. I also gratefully acknowledge Mr. Yves Raymond and Ms. Marie-Josée Lemay, two research assistants of Dr. Champagne, who not only offered me very useful guidance on protocol making, experimental techniques as well as data analysis for AS assays, but also took a great care of me like parents and friends, helping me overcoming my loneliness and settle down in this completely new environment. Thank you again to the "team of three" at SHRDC, for making the one year and a half working at Saint-Hyacinthe the happiest time in my life.

I sincerely thank Dr. Louise Deschênes, Dr. Katherine Bujold and Dr. Christina Calver at SHRDC for their help on the chemical analysis of cranberry pomace as well as the improvement of our bacterial enumeration technique for absorbent pads. In the meantime, I gratefully thank Dr. Tony Savard and Ms. Julie Barrette for their technical assistance in the analyses of organic acids of sauerkraut juice. I would also thank Dominic Lapalme and Mahsa Ravanbakhsh, as CO-OP students from *Université de Sherbrooke* and McGill University, who helped carrying out partial plate count assays in this project.

Great thanks also go to our industrial contributors as Ms. Maryse Dumont of Exceldor and Marie-Claire Pelletier of Olymel for the supplements of chicken and sausage samples for the experiments. I also thank Atoka, Caldwell Fermentations, New Directions Aromatics and DianaFood for providing antimicrobial ingredients for the study. Gratitude is also expressed to

Agriculture and Agri-Food Canada (AAFC), TransfromAction and *Le Conceil de la Transformation Alimentaire du Québec* (CTAQ) for financial support.

I gratefully ackowledge Dr. Salwa Karboune (McGill University) and Dr. Andrea Hill (McGill University) for organizing the McGill-CTAQ network, which secured us to carry out the project successfully. Sincere thanks also go to Dr. Monique Lacroix (INRS Institut Armand-Frappier), Prof. Ismail Fliss (Université Laval) for all the supplementary data on the minimum inhibitory concentrations (MICs) of various plant extracts against a wide range of pathogens, which were valuable information for me to select antimicrobial agents to be tested in this study.

I am also grateful for Prof. Marie-Josée Dumont from Department of Bioresource Engineering, another collaborator in the McGill-CTAQ network who provided the major part of my funds for the M.Sc. program. I also acknowledge the financial supports from the Graduate Excellence Award offered by the Department of Food Science and Agricultural Chemistry.

Special thanks to all the friends I have made at SHRDC: Ms. Marie-Josée Gagné, Mr. Jonathan Lussier, Dr. Barbara Bisakowski and Ms. Chantal Turcotte, for all the generous care, all the happy memories filled with laughters, and all the encouragements when I faced difficulties from the project or from my personal life.

Last but not the least, I would like to thank my parents for the endless love and encouragements, as well as the financial and spiritual supports throughout my studies. I also wish to thank Zibu Zhou and Ruize Qin, my two childhood friends from China. We luckily gathered together in the fantastic journey in Canada and support each other no matter life went up or down.

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THESIS FORMAT

This thesis is submitted in the format of papers suitable for journal publication. This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines: Concerning Thesis Preparation, which are as follows:

“As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis).

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following

(a) A table of contents;

(b) An abstract in English and French;

(c) An introduction which clearly states the rationale and objectives of the research;

(d) A comprehensive review of the literature (in addition to that covered in the introduction to each paper);

(e) A final conclusion and summary;

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers".

CONTRIBUTIONS OF AUTHORS

The following chapters are the manuscripts prepared for publication:

1. **Yao, L.**, Raymond, Y., Lemay, M.-J., Champagne, C.P., Deschênes, L., Bujold, K. & Ismail, A.A. 2018. "Antimicrobial activities of food by-products and plants extracts against spoilage microbiota of refrigerated chicken and their application in absorbent pads" **(draft prepared)**
2. **Yao, L.**, Raymond, Y., Lemay, M.-J., Champagne, C.P., Deschênes, L., & Ismail, A.A. 2018. "Antimicrobial activities of food by-products and plants extracts against spoilage microbiota of Italian sausage" **(draft prepared)**

Parts of this thesis were also presented at the following posters:

1. **Yao, L.**, Raymond, Y., Lemay, M.-J., Champagne, C.P., Lapalme, D., Ravanbakhsh, M., Deschênes, L., & Ismail, A.A, 2018. "Development of antimicrobial absorbent pads with natural ingredients for improved storage of refrigerated chicken". CTAQ-McGill technological meeting, Saint-Hyacinthe Research and Development Centre of Agriculture and Agri-Food Canada, Saint-Hyacinthe, Canada
2. **Yao, L.**, Raymond, Y., Lemay, M.-J., Champagne, C.P., Lapalme, D., Ravanbakhsh, M., Deschênes, L., & Ismail, A.A, 2018. "Antimicrobial activities of food by-products & plant extracts against spoilage microbiota of Italian sausages". CTAQ-McGill technological meeting. Saint-Hyacinthe Research and Development Centre of Agriculture and Agri-Food Canada, Saint-Hyacinthe, Canada

The study reported here was carried out by Lang Yao co-supervised by Dr. Ashraf A. Ismail from the Department of Food Science and Agricultural Chemistry, McGill University, and Dr. Claude P. Champagne at Saint-Hyacinthe Research and Development Centre of Agriculture and Agri-Food Canada (SHRDC-AAFC). The entire research work was carried out at bio-ingredients laboratory, Saint-Hyacinthe Research and Development Centre of Agriculture and Agri-Food Canada, Saint-Hyacinthe, Canada. Lang Yao, as the M.Sc. candidate, planned experimental protocols, conducted most of the experiments, analysed all the experimental results and drafted the thesis and manuscripts for the scientific publications, in consultation with Dr. Claude P. Champagne, a research scientist at SHRDC-AAFC, as well as Mr. Yves Raymond and Ms.

Marie-Josée Lemay, research assistants at SHRDC-AAFC. Dr. Claude P. Champagne guided the planning, protocol developing and the data analysing of the project, reviewed and corrected the thesis and manuscript drafts for publications along with Dr. Ashraf A. Ismail. Mr. Yves Raymond and Ms. Marie-Josée Lemay provided technical supports with the conduction of experimental protocols, the calculation of growth parameters in AS assays and the development of bacterial enumeration method of absorbent pads. Dr. Louise Deschênes, a research scientist at SHRDC-AAFC, as well as Dr. Katherine Bujold, a post-doctoral candidate of McGill University, carried out the chemical analysis of proanthocyanidins of cranberry pomace applied in this study. Dr. Louise Deschênes also provided suggestions on developing bacterial enumeration method of absorbent pads. Dominic Lapalme and Mahsa Ravanbakhsh, as CO-OP students from Université de Sherbrooke and McGill University, respectively, helped carrying out plate count assays testing antimicrobial effects of natural ingredients *in vitro*, followed experimental protocols prepared by Lang Yao. Dominic Lapalme also helped carrying out testing the antimicrobial effectiveness of absorbent pads.

ABBREVIATIONS

ANOVA = Analysis of variance

APHA = American Public Health Association

AS = Automated Spectrophotometry

ATP = Adenosine Triphosphate

Bacterial species

Many bacterial genera cited in this study start by the letter "L": *Lactobacillus, Listeria, Lactococcus, Leuconostoc.* Thus, the abbreviation *"L."* would be confusing. In order to avoid this problem, the instances where *Lactococcus* and *Leuconostoc* and mentioned, the complete names will be used. Furthermore, the following abbreviation system will be used so that the correct genera is identified:

L. = Listeria

Lb. = Lactobacillus

The same strategy was used with genera starting with the letter "S" and "B". Thus:

B. = Brochothrix

Ba. = Bacillus

S. = Salmonella

Staph. = Staphylococcus

Other bacterial abbreviations are:

C. = Carnobacterium

E. = Escherichia

M. = Melaleuca

P. = Pseudomonas

BCP = Bromcresol Purple

BHI = Brain Heart Infusion

CFU = Colony Forming Units

d = days

DGGE = Denaturing Gradient Gel Electrophoresis

EO = Essential Oil

h = hours

HPFB = Health Products and Food Branch - Government of Canada

LAB = Lactic Acid Bacteria

LagT = Lag Time

LPS = Lipopolysaccharides

MAP = Modified Atmosphere Packages

MBC = Minimum Bactericidal Concentration

MIC = Minimum Inhibitory Concentration

MTC = Maximum Tolerate Concentration

MRS = de Man-Rogosa-Sharpe

NA = Not Available

OD = Optical Density

PAC = Proanthocyanindins

PCA = Plate Count Agar

PCR = Polymerase Chain Reaction

PMF = Proton Motive Force

SEM = Standard Error of the Means

SSO = Specific Spoilage Organisms

TSB = Tryptic Soy Broth

TPC = Total Psychrotroph Counts

TVB-N = Total Volatile Basic Nitrogen

VBNC = viable-but-not-culturable

VP = Vacuum Packages

μ_{avg} = Average Growth Rate

μ_{max} = Maximum Growth Rate

CHAPTER 1. GENERAL INTRODUCTION

Several preservation approaches, including refrigeration and modified atmosphere packaging (MAP), have been applied by the meat industry in order to reduce the growth of the initial meat microbiota during refrigerated storage. However, it is still a challenge to further delay the microbial spoilage of fresh chicken and Italian pork sausage, which are highly perishable with high water activities, high nitrogen and nutrient sources combined with non-inhibitory pH values against spoilage microorganisms. To extend the shelf-life of these two products, natural antimicrobial ingredients, as the preference of consumers for clean-label food preservatives, could be applied in food packaging or by direct addition to food products.

Due to the difference between the volume of drips (exudates) formed, the nature of the atmosphere during storage, as well as bacterial species involved in the spoilage of the two products, different preservation strategies with the advantage of these natural ingredients were considered for chicken and Italian sausage samples in this project. Chicken stored in aerobiosis generates a considerable volume of exudates during refrigeration. Aerobic bacterial species, mainly *Pseudomonas* spp., can grow in the chicken exudates, and generate off-odors as a typical spoilage indicator (Charles et al., 2006; Fernández et al., 2010b; Vihavainen and Björkroth, 2010). Traditional absorbent pads maintain the visual appeal of packaged meat products by reducing the presence of exudates, but fail to delay the growth of spoilage bacteria present in these pads. To avoid this, the addition of natural ingredients as antimicrobial agents in absorbent pads was carried out. Antimicrobial contents of these ingredients could be dissolved and distributed in the exudates soaked up by the pads, thus inhibiting the bacteria in the exudates and delaying the generation of off-odors. In contrast, Italian sausage samples tested in this project barely produce exudates during storage. Being stored under modified atmosphere with a high CO_2 content, facultatively anaerobic species such as psychrotrophic lactic acid bacteria and *Brochothrix thermosphacta* overgrow the aerobic species and become dominant. These dominant bacteria could present not only on the external surface, but also inside the product due to the contamination from meat mincing during manufacture. In this case, the ideal approach is to add natural antimicrobial ingredients directly to the product at appropriate concentrations.

Food by-products with high acidity (cranberry pomace, freeze-dried powder of sauerkraut juice), as well as plant extracts rich in phenolic compounds (green tea, grape seed, blueberry), were chosen as antimicrobial agents due to their wide-range antibacterial effects claimed by literatures (Aziz and Karboune, 2018; Das et al., 2017). However, to the best of my knowledge, no previous study focused on either the inhibitory effects of ingredients mentioned above against the spoilage microbiota of spoiled chicken and sausage, or their application in antimicrobial food packages or direct addition as food preservatives.

1.1. Hypothesis

1) The addition of powder-form natural ingredients will inhibit the growth of initial spoilage microbiota of chicken refrigerated aerobically and Italian sausage refrigerated under high CO_2 modified atmosphere, under *in vitro* systems of chicken exudates or sausage homogenates;
2) These antibacterial effects of these ingredients would maintain when being incorporated in absorbent pads (for chicken) and slow down the deterioration under low-temperature storage.

1.2. Overall objective

To delay the growth of spoilage microbiota of chicken and sausage by applying natural antimicrobial ingredients, two main steps will be followed:

1) The selection of natural ingredients as well as their minimum effective concentrations against spoilage microbiota of spoiled chicken or sausage, in the system of chicken exudates or sausage homogenates (*in vitro*);
2) Proof of the effectiveness of selected antimicrobial ingredients in absorbent pads (*in situ*, for chicken exudates only).

In order to keep costs of supplementing the pads or the sausage with natural antimicrobials as low as possible, it is advisable to ascertain the concentration required for the purpose. The effectiveness of antimicrobials is often linked to their concentration. Therefore, it was decided that the role of these antimicrobial ingredients would be to slow down the growth of spoilage microbiota in meat samples instead of inactivating the spoilage bacteria completely.

Growth parameters such as the lag time (LagT) and the growth rate (μ) could describe the growth kinetics of the bacterial population, and therefore, reflect the effects of natural ingredients against the spoilage population.

In order to obtain these growth parameters, screening assays would be carried out using automated spectrophotometry (AS) with a Bioscreen^{TM} unit. This technique has proven useful in evaluating antimicrobial effects of various compounds (Houstma et al., 1996). Plate count assays are also required to verify the accuracy of growth parameters obtained from AS assays. The antimicrobial formulations, which were selected based on their effects on bacterial growth parameters, were tested in absorbents pads (chicken) or added as food preservatives (sausage extract). The effectiveness of these formulations will be confirmed by traditional plate count method.

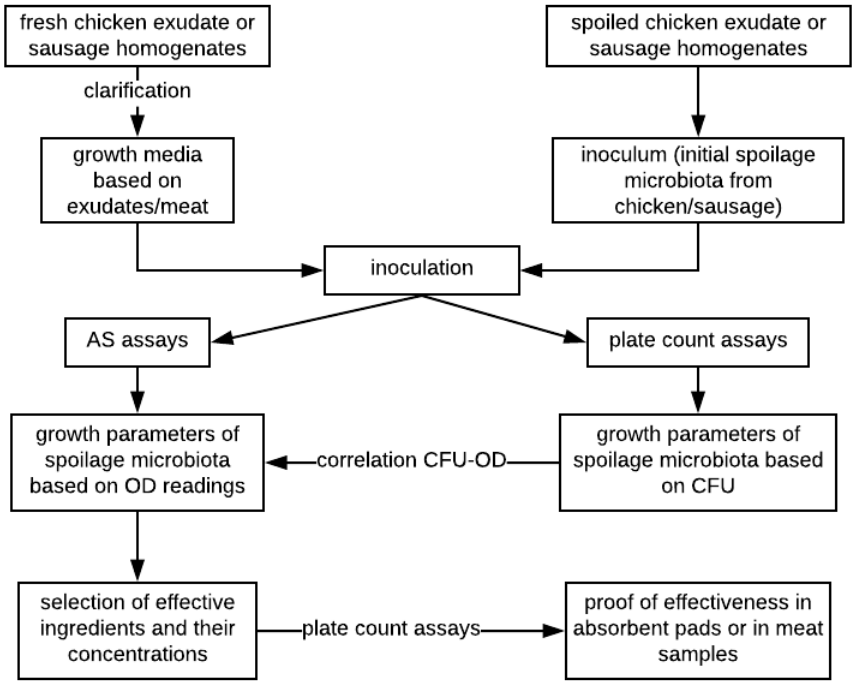


Fig 1.1 Overall activities in this project

CHAPTER 2. LITERATURE REVIEW

2.1. Microbial spoilage in chicken and sausage

Due to the difference of manufacture techniques and incubation conditions, the bacterial species from spoiled chicken and sausage samples tested in this project could vary considerably. The difference of spoilage bacterial species would highly affect the strategies on delaying the deterioration of these two products.

2.1.1. Spoilage microbiota in refrigerated chicken

Fresh chicken is a high a_w, high pH (5.5-6.5) matrix containing water (75%), amino acids (0.35-0.40%), nucleotides (0.10%), glucose (0.05%) and the traces of other glycolytic intermediates (0.05%), supporting the growth of bacteria being able to break down lipids as well as amino acids and other nitrogen sources such as peptide when the carbon sources (normally glucose) are exhausted (Dainty et al., 1989; Lawrie, 2006).

The microbiota appearing in stored meat depends on the characteristics of the products as well as the storage condition. For raw meat and chicken, the initial microflora caused by contamination in slaughtering and cutting line appears mostly on the meat surface instead of within the deeper muscle tissues. Mesophilics are considered as the primarily dominating microbiota until the meat is stored in low temperature. They are subsequently outgrown by psychrotrophs which have shorter lag times and higher growth rates at low temperature. These initial mesophilic microbes are complex populations depending on several factors such as the procedures and hygienic conditions of the production line. Major genera being reported include *Acinetobacter, Bacillus, Corynebacterium, Enterobacteriaceae, Flavobacteria, Lactobacillus, Micrococcus, Pseudomonas, Staphylococcus, Listeria, Campylobacter* and *Salmonella* (APHA, 2001). However, the initial microbiota will be overgrown by the initial psychrotrophs during refrigeration. These psychrotrophs are defined as organisms which can grow at 0-7°C with visible colonies or turbidity in 7-10 days regardless of their optimum growth temperatures (Jay 1992). Bacterial species of dominant microbiota under refrigeration are not easy to identify due to internal factors as the initial bacterial contamination on poultry carcasses after slaughtering and external factors as varieties of handling and storage conditions. Table 2.1 shows the dominant species involved in chicken spoilage reported by several references.

All five references identified *Pseudomonas* spp. as major bacterial species in the latter stage of spoilage under aerobic refrigeration regardless of the identification methods. *Pseudomonas* is considered as the most important spoilage bacteria for muscle foods, usually with a proportion over 50% among the microbiota at latter stage of spoilage (Dainty and Mackey, 1992). Although it may not be the predominated species at the beginning of storage, *Pseudomonas* grows faster than other initial bacteria on meat surface at low temperature due to its ability of attacking and breaking down amino acids, forming ammonia as well as volatile malodorous sulfides, especially when limited source of glucose in chicken muscle is exhausted, (Dave and Ghaly, 2011; Gill and Newton, 1977; James and James, 2002; Viehweg et al., 1989). Therefore, the shelf-life of refrigerated fresh meat and chicken stored aerobically depends on not only the total count of initial bacterial contamination at the beginning, but also the proportion of *Pseudomonas* in the population.

The main *Pseudomonas* species presence on spoiled chicken are *P. fragi, P. fluorescens* and *P. lundensis* (Shaw and Latty, 1982). Since fresh meat is considered as its ecological niche, *P. fragi* actually consist 61% of meat pseudomonads on cutting lines and during storage. (Ercolini, Casaburi, et al., 2010; Molin and Ternström, 1982, 1986). The reason of *P. fragi* being dominant in several types of meat matrixes could be its strong adaptation towards the diverse sources of iron in meat. (Champomier-Vergès et al., 1996) *P. fluorescens*, on the other hand, is mainly dominant from slaughtering to chilling. During this period, it produces biosurfactants in order to utilise and degrade the fat associated with chicken skin, enhancing the availability of the usable substrates in chicken for the growth of other spoilage microflora (Davies and Board, 1998; Mellor et al., 2011).

Other microorganisms, such as *Shewanella putrefaciens*, *Brochothrix* spp., *Carnobacterium* spp., *Staphylococcus* spp., and genus in family *Enterobacteriaceae* were also believed to appear on chicken during storage.

Table 2.1. Dominant bacterial species of chicken stored aerobically

Bacterial species reported	Storage condition	Identification techniques	Reference
Pseudomonas-Moraxella-Acinetobacter group	Refrigeration	NA	(APHA, 2001)
71% *Pseudomonas* spp. (pigmented and non-pigmented), 19% chromogenic Gram-negative bacteria (identical with *Pseudomonas putrefaciens*)	1°C, 10-11 d, aerobically	Traditional plating method on selective media	(Barnes and Thornley, 1966)
33% *Pseudomonas* spp. 26% *Acinetobacter*, and 15% *Enterobacteriaceae*	10°C,3-4 d, aerobically	Same as above	(Barnes and Thornley, 1966)
Main components: *Pseudomonas* spp. (mainly *P. fragi*, *P. fluorescens* and *P. lundensis*); *Shewanella putrefaciens*; Minor components: family *Enterobacteriaceae* (including *Hafnia* spp., *Serratia* spp. *Enterobacter* spp.)	Aerobic storage at low temperature	Traditional plating method on selective media	(Vihavainen and Björkroth, 2010)
Dominant flora: *Carnobacterium* spp., *Pseudomonas* spp. and *Brochothrix* spp. Also detected*: Acinetobacter* spp.*, Rahnella* spp. and *Weissella* spp.	4°C aerobically, for 2,4,6 days for DNA extractions, 0-15 days for physicochemical, microbiological, and	PCR amplification and denaturing gradient gel electrophoresis (PCR-DGGE)	(Liang et al., 2012)

	sensory analysis		
Staphylococcus spp., *Pseudomonas* spp., *Acinetobacter* spp., *Carnobacterium* spp., *Aeromonas* spp., and *Weissella* spp.	At normal (4°C) and fluctuating storage temperatures (0-4°C and 4-10°C),aerobically	PCR-DGGE	(Zhang et al., 2012)

2.1.2. Spoilage microbiota in sausage stored under modified atmosphere

Compared with fresh chicken, sausages are salted and usually stored in oxygen-impermeable conditions such as vacuum packages (VP) and modified atmosphere packages (MAP), leading to the difference of dominant microflora in these two products. Some of the initial microbiota is inhibited during manufacture after the addition of salts as well as spices, leading to the overgrowth of facultative anaerobic or microaerophilic gram-positive microbes such as psychrotrophic lactic acid bacteria (LAB), which are more tolerant to high salt and low-O_2 conditions. The overgrowth of these LAB might also occur because of mincing during sausage manufacture, allowing the microbe to grow not only on the exterior surfaces, but also inside the products with reduced oxygen availability. Data screening spoilage microbiota in sausage is less available but quite comparable to studies of fresh meat (beef and pork, which are the main contents of the fresh sausage sample used in our project) stored under MAP or VP. Thus, dominant species in bacterial communities on fresh meat could largely represent the one in fresh sausages.

9 references claimed the dominance of LAB (mostly *Lactobacillus* spp., *Lactococcus* spp. and *Leuconostoc* spp.) in meat microbial population under VP and MAP with low O_2 concentration (Table 2.2). Meanwhile, dominant microflora under MAP with higher O_2 concentration was more similar with the one under aerobiosis condition. *Pseudomonas* spp. and *Brochothrix thermosphacta* were mentioned to be dominant in studies based on high-O_2 MAP twice and four times, respectively. *Brochothrix thermosphacta* could not only be tolerant with low-temperature and high-salt condition, but also the O_2 limitation caused by the growth of Gram negative *Pseudomonas* at the start of aerobic spoilage, as well as the low-pH condition after *Pseudomonas* being overgrown by LAB. However, the study done by (Susiluoto et al., 2003) indicated that the bacterial count of *B. thermosphacta* was approximately 3 log lower than that of LAB. The *Enterobacteriaceae* were also recognized twice as dominant bacteria in low O_2 conditions by both plate count method and PCR.

Several psychrotrophic LAB species, including *Lactobacillus* groups such as *Lb. sakei, Lb. curvatus,* as well as the genera *Carnobacterium* such as *C. pisciola,* are important competitors among the spoilage microbial populations (Hammes and Hertel, 2006). Generally, *Lb. sakei* is commonly recognized as the major species of lactic acid bacteria meat stored under

MAP, due to its activity to produce ATP, which is one of the energy precursor substances under glucose starvation during meat spoilage (Ercolini, Casaburi, et al., 2010; Montel et al., 1991). However, the dominant species might be various among products, depending on the gas contents in MAP (Castellano et al., 2004; Vihavainen and Björkroth, 2007). A study on meat stored under high-O_2 MAP stimulated *Lactobacillus gasicomitatum* and *Leuconostoc gelidum* as spoilage inhibitors causing the green discolouration and buttery off-odors. (Vihavainen and Björkroth, 2007) Although psychrotrophic LAB are related to sensory downgrades by producing offensive metabolites, their roles in meat spoilage are actually controversial. *Lb. sakei* was noticed to weaken the growth of foodborne pathogens such as *Salmonella enterica* serotype Typhimurium and *Escherichia coli* O157:H7. *Leuconostoc* spp. was reported to inhibit *E. coli* O157:H7 and *Listeria monocytogenes* (Chaillou et al., 2014; Labadie, 1999; Yost and Nattress, 2002). The presence of spoilage LAB might indicate higher sensory deterioration as a warning of spoilage, but also lessen the danger level caused by pathogens.

Some other initial microbes such as genera of *Enterobacteriaceae* family, including *Enterobacter, Citrobacter, Salmonella,* etc. could also grow anaerobically at storage temperature between 7°C and 10°C, being able to grow readily on meat skin tissues along with *B. thermosphacta* (Jiménez et al., 1997; Mossel et al., 1979). *B. thermosphacta* is noticed to cause early spoilage by fermenting carbohydrate sources in meat and subsequently producing odorous metabolites, while enterobacteria normally contribute to spoilage at the latter stage (Gill and Harrison, 1989). However, in some studies *Enterobacteriaceae* are not considered as dominant spoilage bacteria due to their slow metabolism at such storage conditions (Borch et al., 1996).

Table 2.2 Dominant bacterial species of meat stored under modified atmosphere packages (MAP) or vacuum packages (VP)

Dominant species reported	Product and storage condition	Identification techniques	References
Lactobacillus spp. and *B. thermosphacta*	Italian pork sausage stored at 7ºC under 30% CO_2-70% O_2 modified atmospheres packages (MAP)	Plate count and 16S rRNA gene profiling method	(Raimondi et al., 2018)
Lactic acid bacteria	Minced beef samples stored at 0, 5, 10 and 15°C, under 40% CO_2-30% O_2-30% N_2 MAP, with/without 2% (v/w) oregano essential oil	Species-specific PCR, Pulsed-field Gel Electrophoresis (PFGE)	(Doulgeraki et al., 2010)
	Beef samples stored at 1°C after 0-32 d, packed in nisin-activated plastic bags	PCR-DGGE (Temperature Gradient Gel Electrophoresis)	(Ercolini, Ferrocino, et al., 2010)
	Pork under VP stored at 4°C after 3-23 d	PCR-DGGE	(Jiang et al., 2010)
	Beef under VP at 2°C during 6 weeks of storage trial	RAPD-PCR	(Yost and Nattress, 2002)
	Pork under VP stored at 4°C after 0-20 days	Plate counts on selective medium and PCR-DGGE	(Pennacchia et al., 2011)
	Beef stored at 4°C after 0-35 days, under bacteriocin (nisin)-activated antimicrobial package	PCR-DGGE, Species-specific PCR	(Ercolini et al., 2011)
	Beef stored at 5°C after 0-14 days, under MAP with 1) 60% O_2 and 40% CO_2; 2) 20% O_2 and 40% CO_2	Plate counts on PCA (mesophilic aerobic bacteria), violet red bile glucose agar (Enterobacteriaceae), MRS (LAB), STAA medium (*B.thermosphacta*), and PCR-DGGE	(Ercolini et al., 2006)
	Marinated broiler meat strips stored at 6°C, 7–9 days	Plate counts on MRS and Tomato juice agar	(Susiluoto et al., 2003)

	under 20% CO_2 - 80% N_2 MAP	(TJA) for total LAB, Rogosa Agar (SLA) for lactobacilli, PCA for totoal bacterial count and Streptomycin Thallium Acetate Agar (STAA) for *Brochothrix thermosphacta*	
Pseudomonas spp. and *B thermosphacta*	Beef stored at 5°C after 0-14 days, under 60% O_2 - 40% CO_2 MAP	Plate counts on PCA (mesophilic aerobic bacteria), violet red bile glucose agar (*Enterobacteriaceae*), MRS (LAB), STAA medium (*B. thermosphacta*), and PCR-DGGE	(Ercolini et al., 2006)
	Beef stored at 4°C after 0-35 days, under 60% O_2 - 40% CO_2 MAP	PCR-DGGE, Species-specific PCR	(Ercolini et al., 2011)
Enterobacteriaceae	Beef stored at 5°C after 0-14 days, under MAP with 20% O_2 and 40% CO_2	Plate counts on PCA (mesophilic aerobic bacteria), violet red bile glucose agar (*Enterobacteriaceae*), MRS (LAB), STAA medium (*B. thermosphacta*), and PCR-DGGE	(Ercolini et al., 2006)
	Minced beef samples stored at 0, 5, 10 and 15 °C, under modified atmospheres packaging (MAP) consisting of 40% CO_2 -30% O_2 -30% N_2 with MAP, with/without 2% (v/w) oregano essential oil	PFGE, SDS-PAGE	(Doulgeraki et al., 2011)
B. thermosphacta	Raw beef after 7 days of chill storage	Quantitative Real-Time (RTi-) PCR	(Pennacchia et al., 2009)
Lactococcus spp., *Carnobacterium* spp., *Enterococcus* spp.	Poultry meat at 3.5°C for up to 7 weeks, under 20% CO_2 - 80% N_2 MAP	Species-specific PCR	(Barakat et al., 2000)

2.1.3. Sensory deterioration caused by meat spoilage microbiota

Meat spoilage typically results in physicochemical, biochemical and microbiological transformations. Microbial spoilage is by far the one with major sensory concerns including slime production, visual changes such as discoloration, and most importantly, chemical changes such as off-odors.

Off-odors normally would not appear before glucose utilization when the bacterial counts reaches exceeds 10^7 CFU/mL (Gill, 1976; Ingram and Dainty, 1971). The microbes, which grow not only on the exterior surface, but also in the meat exudates formed due to the chemical changes of myofibrillar proteins in muscle tissues (Charles et al., 2006; Fernández et al., 2010a). Those microbes are usually recognized as specific spoilage organisms (SSO), which have lower lag phases than the initial microbiota on the meat and cause sensory rejection. *Pseudomonas* spp. and sometimes *Shewanella putrefaciens* under aerobic storage, as well as LAB and *B. thermosphacta* under anaerobic conditions, are noticed as SSO in meat organoleptic deterioration (Broda et al., 1996; Dainty and Mackey, 1992; Garcia-Lopex et al., 1998; Rukchon et al., 2014; Viehweg et al., 1989).

Chemical inhibitors of flavor deterioration include volatile bases, volatile acidity and biogenic amines (BAs) level. In meat and poultry products with non-inhibitory pH and low glucose content, volatile odors are mainly generated by utilization of amino acids, short chain fatty acids and sulfide compounds after carbohydrate sources depletion (Gill, 1976; in't Veld, 1996; Vihavainen and Björkroth, 2010). One of the main chemical inhibitors related to microbial spoilage is total volatile basic nitrogen (TVB-N), including substances such as ammonia, dimethylamine (DMA) and trimethylamine (TMA) (Byun et al., 2003). TVB-N production has a strong correlation with total psychrotrophic aerobic counts as well as the microbial counts of important SSO in meat preservation (Fraqueza et al., 2008). pH increase in spoiled meat as a reflection of TVB-N generation could be used as a spoilage inhibitor. Other gases, mainly H_2S and CO_2, are also generated due to the degradation of meat amino acids triggered by glucose limitation (Borch et al., 1996). CO_2 is an important spoilage inhibitor especially in products under MAP, due to the high correlation between the SSO (i.e. LAB, *B. thermosphacta*, etc.) under low-O_2 conditions and the CO_2-level detected as the metabolites of these SSO (Rukchon et al., 2014). Therefore, in some case, CO_2 is believed to be more important spoilage inhibitor than

TVB-N. Meanwhile, some spoilage lactic acid bacteria such as *Lactobacillus* spp. is reported as hydrogen sulfide producer in VP but not in aerobic condition, and it could be more active in hydrogen sulphide producing in high-pH meat sample (6.2-6.6) rather in low-pH ones (5.6-5.8) (Egan et al., 1989; Shay and Egan, 1981).

2.2. Application of natural ingredients as food preservatives

Due to the increasing concern about chemical residues from food packages, food-trade preservatives are earning increasing interests. There are two main concerns for the selection of antimicrobial ingredients. First, the natural ingredients will not bring off-flavors to the food products. Second, the active components in these antimicrobial agents should easily diffuse and interact with the products.

2.2.1. Incorporation of natural ingredients with package system of chicken or sausage

During the refrigeration of raw chicken, chemical changes of myofibrillar proteins in meat tissue lead to phenomena called “drip-lost”. The application of traditional absorbent pads (pads alone without adding antibacterial agents) at the bottom of meat packages could maintain the visual appeal by soaking up meat drips (exudates). However, as a nutritionally-rich medium, these exudates allow microbes to grow in the drip within the pads and generate volatile components causing off-odor when the microbial population exceeds approximately 10^7 CFU/mL (Charles et al., 2006; Fernandez et al., 2010; Vihavainen and Björkroth, 2010). The undesirable odors caused by spoilage microbes growth in the pads could be spoilage indicators for the consumers. If the growth of the initial microbes soaked up by pads was successfully inhibited, then the time point when they start to generate off-odors could be delayed, and shelf life is extended. Thus, the addition of antimicrobial ingredients in pads is worth testing in order to inhibit microbes inside these pads. The antimicrobial ingredients would delay the spoilage of packaged meat by 1) dissolving in meat exudates soaked up by the pads and inhibiting bacteria presents in the exudates; and 2) migrating slowly from the pad to the headspace in the packaging and then affect the bacteria at the surface of meat products (Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002).

Both non-volatile and volatile antimicrobial ingredients could be potentially distributed within the absorbent materials (usually cottons) of the pads. The non-volatile antimicrobial agents interact with the exudate microbiota through diffusion and equilibrated sorption, while the volatile antimicrobial agents can also be absorbed by food through evaporation and sorption (Quintavalla and Vicini, 2002). In absorbent pads, previously tested non-volatile antimicrobial agents are nanorized metals and their oxides, bacteriophages, bacteriocins etc., while the most popular volatile agent is essential oil (Lee et al., 2015). There are two problems with the presence of these particular ingredients in pads. First, the non-volatile ingredients which potentially diffuse from the pads might not be "food-grade". Second, the volatile components might bring off-flavors. Therefore, in this study, natural ingredients are preferred to be tested in absorbent pads. The active compounds in these ingredients should be water-soluble, so that they can dissolve in the absorbed exudates and interact with the initial contaminating bacterial cells.

The effectiveness of such antimicrobial absorbent pads depends on several factors, including 1) the choice of antimicrobial agents added to the soaking materials (Sung et al., 2013); 2) the distribution or diffusion of bioactive components within the absorbed exudates (Quintavalla and Vicini, 2002); 3) the drip/juice volumes formed during storage (Lloret et al., 2012). The effectiveness of antimicrobial ingredients highly depends on the dominant spoilage species of the specific meat product (See *2.2.3. Antimicrobial agents selected in this project*). As for the third factor, assuming the amount of antimicrobial ingredients per cm^2 as well as the volume of exudates are constant, exudates soaked up by a pad with relatively smaller absorbency are able to spread in a larger area of the pad and contact with a large quantity of the antimicrobial agent, while exudates absorbed up pad with strong absorbency might stick in a small area with limited interaction with antimicrobial agents added to the pads. On the other hand, if the exudate could distribute all over the pad, as the volume of exudate increases, the actual concentration of antimicrobial agents decrease and the absorbent pads probably become less inhibitory. Therefore, the regular exudate volumes from each package should be well investigated for the selection of pads with appropriate absorbency.

In contrast, the antimicrobial absorbent pads are not applicable for the Italian sausages. The presence of sausage casings significantly reduce the formation of exudates, and the active ingredients from the pads would barely contact with the spoilage bacteria from the exudates and

fail to perform their inhibitory effects. In this case, these food-grade ingredients could be added directly to the minced meat during manufacture (Aziz and Karboune, 2018). The direct addition could not only solve the problem of the absence of sausage exudate, but also inhibit the internal anaerobic spoilage bacteria contaminated from meat mincing.

2.2.2. Antimicrobial agents selected in this project

Various natural ingredients could share a similar mechanism of action to inhibit bacteria if their major active components have a similar structure or contain same functional groups. In this project, antimicrobial agents that inhibit the spoilage bacteria by their acidity or by the action of phenolic compounds will be examined.

2.2.2.1. Inhibition by acidity

Cranberry pomace and the powder of sauerkraut juice are chosen as antimicrobial agents based on their high acidity. Cranberry products contain abundant source of non-phenolic (ascorbic, citric, quinic, etc.) and phenolic (benzoic, phenylacetic, etc.) acid from the cranberry fruit (Hong and Wrolstad, 1986). Sauerkraut juice, as a by-product from sauerkraut manufacture, contains high contents of organic acids thanks to the metabolism of the initial lactic acid bacteria from raw cabbage during fermentation (Hutkins, 2006a). These by-products formed by LAB through homolactic and heterolactic fermentations are mainly lactic acid and acetic acids, but propionic, formic, and butytic acid can be found in traceable amounts (Ouattara et al., 1997).

Organic acids from antimicrobial agents could inhibit microorganisms by weakening the proton motive forces (PMF) of cell membrane through cytoplasm acidification, limiting the energy transformation through membrane due to the trans-membrane osmotic gradients. (Devlieghere and Debevere, 2000; Ouwehand and Vesterlund, 2004). The undissociated molecular of the organic acid would also diffuse and dissociate in the cytosol, inactivating the bacteria by decreasing the intercellular pH which the bacteria might not tolerate to (Tharmaraj and Shah, 2009; Wang et al., 2014). The decrease of pH in the broth could delay the growth of psychrotrophic species involved in chicken spoilage (*P. fragi* and *P. fluorescens*) by extending their lag times (Lebert et al., 1998). It was also observed that different bacterial strains had different resistance against the organic acids. For example, *B. thermosphacta* could be inhibited by various organic acids (lactic, acetic, propionic, citric, etc.), and such inhibition were enhanced with the increase of the concentration of each organic acid. In contrast, for more resistant strains

such as *Lb. sake*, *Serratia liquefaciens* and *P. fragi*, the inhibitory effect only appeared when the concentration reached a critical value (Ouattara et al., 1997).

The by-product from cranberry juice or sauerkraut manufacture with high acidity would lead to environmental and ecological concerns if being disposed to the environment directly without processing (Hamer and Scuse, 2010). Thus, using these by-products as food-trade antimicrobial agents could not only take advantage of the bioactive compounds which still remain in the by-products, but also be as an eco-friendly treatment of the waste from food manufacture. However, problems might occur by applying these by-products. For cranberry pomace, the quantities of bioactive contents, mostly polyphenols, might be significantly lower than cranberry juice or extracts (Barchechath, 2014). For sauerkraut juice, it is difficult to be added to absorbent pads in the liquid form. To freeze dry the by-product and then add them to the pads in a powder form might be a practical solution (Champagne et al., 2017).

2.2.2.2. Inhibition by phenolic compounds

Extracts of green tea, blueberry, grape seed as well as cranberry pomace are chosen as antimicrobial agents due to their abundant phenolic compounds (Das et al., 2017; Perumalla and Hettiarachchy, 2011). The actual major active component of each plant extract depends on the raw material, including the place of origin and the sites of plants collected from (i.e. leaves, roots, fruits, etc.) as well as the extraction and manufacture technique. (Archana and Abraham, 2011) These parameters of extract manufacture would notably influence the antimicrobial effects. For example, the catechin content of green tea extract could be variable due to difference of raw material (fresh tea or dust tea), manufacture reason, fermentation and heat treatment levels during manufacture, as well as the solution used for extraction (water, methanol, acetone, etc.) (An et al., 2004; Archana and Abraham, 2011; Chou et al., 1999; Diker and Hascelik, 1994).

Phenolic compounds present in these ingredients share the same major active group as the hydroxyl group, which could bind with cell membrane proteins and increase the membrane permeability (Archana and Abraham, 2011; Das et al., 2017; Davidson et al., 2005; Lambert et al., 2001; Mitsumoto et al., 2005; Perumalla and Hettiarachchy, 2011; Tiwari et al., 2009; Wang and Ho, 2009). The inhibitory effects are carried out generally through 1) physical membrane action; 2) dissipation of proton motive force (PMF) and 3) the inhibition of membrane associated enzymes (Reygaert, 2014; Shimamura et al., 2007). These phenolic compounds could weaken

the PMF of the cytoplasmic membrane by breaking down the fatty acid chains from the membrane phospholipids, allowing the leakage of ions from the cytoplasm, which leads to the disruption of the transportation of active compounds through the membrane. They could also bind and interact with the hydrophobic functional groups from enzymes and interfere with cell metabolism, inactivate the synthesis of fatty acid or ATP (Chinnam et al., 2010; Sikkema et al., 1995; Zhang and Rock, 2004). Since the bacterial inactivation from phenolic compounds is mostly caused by membrane damage, it is worth noting that the membrane structure would influence the extent of such inactivation. In other words, gram negative bacteria are less susceptible to those antimicrobials based on phenolic compound, due to the higher proportion of lipid compounds (lipoproteins & lipopolysaccharides) presence in their outer membrane, which could be as barriers for the diffusion of hydrophobic phenolic components from plant extracts (Angienda et al., 2010; Ceylan and Fung, 2004).

Some tannin-like phenolic compounds, such as proanthocyanindins (PAC) from berry products, as well as catechins and their gallate esters from green tea or grape seeds, also affect bacterial growth as iron chelators, competing the limited iron source from the meat media and then affecting the heme enzyme formation as well as ATP synthesis of bacterial cells. Such metal chelation could be observed at either acidic or neutral pH (Kylli et al., 2011; Santos‐Buelga and Scalbert, 2000). The growth of aerobic bacteria requires iron for the formation of heme, the reduction of O_2 for ATP synthesis as well as the reduction of ribonucleotide precursor of DNA (Neilands, 1995). When the iron source is exhausted, these bacteria could produce siderophores to bind and solubilize iron from the medium. As stronger iron chelators, tannin-like phenolic compounds compete with bacteria siderophores in iron chelation, making the limited iron source less available to these bacteria. However, lactobacilli may not be affected in such iron competition since they do not generate heme enzyme for their metabolism (Chung et al., 1998; Neilands, 1995; Santos‐Buelga and Scalbert, 2000).

The antimicrobial effects of antimicrobial ingredients based on phenolic compounds against bacterial species potentially present in spoiled chicken or sausage are investigated (Table 2.3). All ingredients rich in phenolic compounds showed inhibitory effects against *Pseudomonas* spp. (Caillet et al., 2012; Chou et al., 1999; Jayaprakasha et al., 2003; Rao et al., 2014; Silva et al., 2013), so they would hypothetically be effective against the *Pseudomonas*-based microbiota

of spoiled chicken. Other hazardous bacterial species which could appear in spoiled chicken such as *Salmonella* spp., *E. coli*, *Staph. aureus* and *L. monocytogenes* were also reported to be inactivated by these extracts (Friedman, 2007; Lacombe et al., 2012; Weerakkody et al., 2010; Yam et al., 1997). However, lactic acid bacteria as the dominant bacteria in spoiled sausage may behave more tolerant against these ingredients based on phenols. Previous studies have reported effectiveness of green tea, grape seed and blueberry against gram positive pathogens such as *Ba. subtilis*, *Staph. aureus* and *L. monocytogenes*, but such effect disappeared or significantly reduced against *Lactobacillus* spp. or *Streptococcus* spp. through different methods including agar diffusion, broth dilution and even *in situ* bacterial enumeration of sausages cased with chitosan film impregnated with antimicrobial ingredients (Ankolekar et al., 2011; Biswas et al., 2012; Horiba et al., 1991; Moradi et al., 2011; Silva et al., 2013; Tajik et al., 2014). *B. thermosphacta,* which is previously stated as the other major bacterial species in meat product under MAP, was poorly studied in previous studies on natural antimicrobial ingredients. However, previous research indeed claimed notable inhibitory effect of green tea, grape seed, as well as the phenolic fraction of cranberry against *Listeria*, which also a genera of family *Listeriaceae,* closely related to *Brochothrix* (Ahn et al., 2004; Garrity and Holt, 2001; Lacombe et al., 2013; Mbata et al., 2008; Moradi et al., 2011; Nshimiyumukiza et al., 2015). Similar stress response genes and antibiotic resistance genes was found between *B. thermosphacta* and *L. monocytogenes*, these genes would play similar role contributed to the survival of these bacteria against food preservatives as well as environmental stress under incubation such as low pH and low temperature (Stanborough et al., 2017b). Therefore, *B. thermosphacta,* as a major species in the spoilage population, might show similar sensitivity as *L. monocytogenes* under the treatments of tested plant extracts.

2.2.3. Interaction between food contents and antimicrobial ingredients

To the best of our knowledge, no previous study used growth media clarified from meat exudates or meat homogenates. Using the meat-based media could not only have a “real situation” microbiota, because the bacteria could grow in a condition with close nutritional contents as the real meat product, but also consider the effects meat matrix on the inhibitory effect of bioactive compounds against spoilage bacteria. The meat contents (proteins, peptides, fats, etc.) may either protect bacterial cells from membrane damage of antimicrobials, or interact with the active components from natural ingredients (Burt, 2004; Holley and Patel, 2005). For example,

polyphenols from plant extracts could combine with proteins and generation colloidal size compounds or precipitates, reducing the actual concentration of free polyphenols in meat matrix and the antimicrobial effects would decrease (Charlton et al., 2002; Von Staszewski et al., 2011). Studies have noticed the significantly lower inhibitory effects of natural ingredients in meat matrix compared with the one obtained from *in vitro* tests using standard laboratory broth (Burt, 2004; Gill et al., 2002; Von Staszewski et al., 2011). For antimicrobial agents rich in organic acids, the buffering effect of meat contents, mostly dipeptides (anserine and carnosine), could delay the pH decrease of the meat-based media treated by acidic ingredients (Lea et al., 1969). The pH in the media would then promote to the dissociation of organic acid and reduce the inhibitory effects, because it was the undissociated molecular of organic acid that was more capable of decreasing the intercellular pH of bacterial cells instead of the H^+ alone (Ouattara et al., 1997; Tharmaraj and Shah, 2009; Wang et al., 2014).

Table 2.3. Studies on antimicrobial effects of selected natural ingredients or the fraction of their major active components

Ingredient(s) tested	Target bacteria	Methodology	Antimicrobial effects	References
Green tea water extract	*Lb. acidophilius* & *Streptococcus mutans*	Agar diffusion	MIC: 0.9 mg/mL and 0.8 mg/mL, respectively	(Tahir and Moeen, 2011)
	L. monocytogenes	Disc diffusion & agar diffusion	MIC: 0.68 mg/mL	(Mbata et al., 2008)
	Ba. subtilis and *Staph. aureus*	Broth dilution	0.1% (w/v) green tea extract inhibited 12% and 30% bacterial cells of *Bacillus* and *Staphylococcus*, respectively	(Chou et al., 1999)
	Two subspecies of *Staphylococcus aureus* with methicillin resistance	Broth dilution	MIC: 400 and 400 µg/mL, respectively	(Radji et al., 2013)
	Lb. acidophilus, Lb. bulgaricus and *Lb. plantarum*	Broth dilution	No inhibition at 10 µg/mL was observed for all LAB strains for 24 h	(Ankolekar et al., 2011)
	Lactobacillus spp. and *Peptostreptococcus* spp.	Broth dilution	No inhibition at 5% (w/v)	(Horiba et al., 1991)
	P. fluorescens	Broth dilution	*P. fluorescens* was 100% inhibited at 0.1% (1 mg/mL)	(Chou et al., 1999)
	P. aeruginosa	Broth dilution	MIC=50 mg/mL	(Rao et al., 2014)
Tannic acid (an antimcriobial component from	*Lb. acidophilus*	Broth dilution	Significant inhibition started to appear at 500 µg/mL	(Chung et al., 1998)

green tea)				
Grape seed extract	Total lactic acid bacteria, *L. monocytogenes*	Plate count: Direct addition in impregnated chitosan film for mortadella sausages, follow the CFU from 0-21days under incubation at 4ºC	0.1-0.7 and 0.8-1.5 log cycle reduction at 10g/kg, respectively	(Moradi et al., 2011)
	Psychrotrophic bacteria, total lactic acid bacteria	Plate count: Direct addition to raw buffalo patty, follow the CFU for 9 days at 8ºC	0.8 and 0 log cycle reduction at 0.1% and 0.2% (w/w), respectively	(Tajik et al., 2014)
	L. monocytogenes, E. coli	*In vitro* test: broth dilution *In situ* test: Direct addition to fresh ground beef inoculated with target bacteria at 1% (w/w), incubated at 4ºC	*In vitro* test: MIC: 8 mg/mL and 6 mg/mL, respectively *In situ* test: *E.coli*: 0.6-1.1 log cycle reduction from day 3 to day 9; *L. monocytogenes*: 0.9-1.0 log cycle reduction from day 3to day 9	(Ahn et al., 2004)
	B. thermosphacta	Disc diffusion: Applied in impregnated films for pork loins	Strong inhibition effects at 1% based on inhibition zones Inhibition was strongly weaken because the migration from the film to meat was low (only surface contact)	(Corrales et al., 2009)
	E. coli and *P. aeruginosa*	Agar diffusion	MIC: 1250 and1500 ppm, respectively	(Jayaprakasha et al., 2003)

Freezed dried extract of blueberry fruit infusions	Lactic acid bacteria (*Lactococcus lactis, Lb. rhamnosus, Lb. bulgaricus*) and *P. aeruginosa*	Agar diffusion and broth dilution	No inhibitory effect observed at 0.5 g/mL against any species of lactic acid bacteria; MIC against *P. aeruginosa*: 50 mg/mL	(Silva et al., 2013)
Pasteuized blueberry juice	*Lb. bulgaricus, L. monocytogenes* and *Campylobacter jejuni*	Plate count: Bacterial strains were inoculated into blueberry juice directly	Inhibitory effect against *Lactobacillus* was notably smaller than that against *L. monocytogenes* and *Campylobacter jejuni*, however, over 2 log cycle reduction of *Lactobacillus* could still be observed at 48 h and 72 h	(Biswas et al., 2012)
Lactic acid	*Lb. sake, P. fluorescens, S. liquefaciens, B. thermosphacta*	Broth dilution	MICs were 0.75% (v/v), 0.5%, 0.75% and 0.5%, respectively.	(Ouattara et al., 1997)
Organic acids (OA) including acetic, benzoic, butyric, citric, lactic acid	*Ba. cereus, Ba. subtilis, E. coli, Lb. fermentum, Lb. plantarum*	Broth dilution	MIC of *Lactobacillus* were 3-300 times higher than that of *Bacillus*; MIC of *E. coli* against each acid were slightly higher than that of *Bacillus*	(Hsiao and Siebert, 1999)
Cranberry pomace	*P. aeruginosa*	Broth dilution	MTC: 11.7-16.0 µg phenol/well; MIC: 32.0-46.8 µg phenol/well	(Caillet et al., 2012)
3 fractions from cranberry: 1) Sugar & OA; 2) Phenolic acid (gallic acid); 3)	*L. monocytogenes, Lb. rhamnosus* and *E.coli*	Broth dilution	MIC of: 1) 5.2 g/L, NA (not observed) and NT (not tested), respectively;	(Lacombe et al., 2013)

Proanthocyanidins (PAC)			2) 2.8 g/L, 11.25 g/L and NT, respectively; 3) 500 g/L, NA & 225 g/L, respectively	
PAC fraction (1 mg/mL) from cranberry	*Staph. aureus, E.coli, S. enterica* and *Lb. rhamnosus*	Broth dilution	Inhibitory effect was only observed against *Staph. aureus*	(Kylli et al., 2011)
Two berry extracts	Total lactic acid bacteria	Direct addition to marinades for marinated broiler chicken cuts stored under MAP	No significant effect was observed	(Alakomi et al., 2017)
American cranberry concentrate	*E. coli* O157:H7, *L. monocytogenes, S.* Typhimurium and *Staph. aureus*	Broth dilution	All bacterial strains tested were sensitive in BHI broth supplied with cranberry concentrate at 100 µL/mL; Cranberry concentrate showed a stronger antibacterial effects compared with the acidic solution with the same concentration of cranberry	(Wu et al., 2008)
Cranberry juice concentrate (CJC)	*E. coli* O157:H7, *L.monocytogenes, S.* Typhimurium	*In vitro*: broth dilution method *In situ* (on pre-cut red peppers and cranberry fruits): plate count method	Resistibility *in vitro*: *L. monocytogenes* > *S.* Typhimurium > *E. coli* CJC showed greater antibacterial effects than the acidic solution because of their phenolic compounds. 1.8-5 log reduction of CFU was observed in presence of CJC *in situ*.	(Harich et al., 2017)

2.3. Microbial analytical methodologies for meat spoilage microbiota

There are different ways to describe the inhibition of antimicrobial ingredients quantitatively. Disc diffusion method and agar diffusion method are a widespread technique for antimicrobial effect assessment based on the diameter of the inhibition zone of the microorganism around the disc soaked up with the tested antimicrobial ingredients. This technique is useful for not only the evaluation of the inhibitory effect of a certain ingredient on a specific bacterial species, but also the comparison of antimicrobial effectiveness among several ingredients. However, antimicrobial effects obtained through disc or agar diffusion methods might lack of accuracy and repeatability. Lacking of a protocol standardizing the incubation condition (medium, time, temperature, agar thickness) and the methods of adding the ingredients (addition in wells or discs, with or without solution/emulsifier, the types and concentrations of the solution/emulsifier) could lead to the variable and unrepeatable results obtained from different studies. Meanwhile, since the microbial inhibitory zone is not always a circle, the fluctuation of the inhibitory zone boundaries brings on the agar though either paper disc or the wells difficulties and the loss of accuracy for measuring the diameters of the inhibitory zones.

Broth dilution method (*in vitro* method) as a more precise alternative, has been widely applied by culturing the objective microbe(s) in the supplied with antimicrobial ingredient(s). Growth curves of the target bacteria treated by different ingredients could be conducted based on the viable counts or the turbidity of the growth medium, and the antimicrobial effect of each ingredient would be determined based on the growth parameters including the lag time (LagT), growth rate (μ) or the change of biomass (ΔOD) (see Table 2.4).

2.3.1. Plate count method

Plate count method is the most direct estimation of the amount of viable bacterial cells present in food samples (Petran et al., 2015). To appropriately evaluate the bacterial population from spoiled meat samples, it is important to determine 1) the target microorganism; 2) the plate incubation parameters (medium applied, temperature, time and atmosphere for petri dish incubation).

To describe the microbial spoilage of refrigerated meat, the ideal approach is to obtain the total psychrotroph counts (TPC). These psychrotrophs are defined as microorganisms which

are able to develop visible colonies when incubated at 7°C in 10 days (Vasavada and Critzer, 2015). In petri dishes incubated at such low temperature, the lag phases of most mesophilic bacteria are highly extended and the bacterial cells usually fail to form visible colonies during incubation. Thus, spreading the sample on PCA at 7°C for 10 days is an advisable and widely-used official method to evaluate the total psychrotrophic populations (APHA, 2001). However, this official method is time-demanding. Since psychrotrophic bacteria could develop optimally at approximately 15°C, and their growth is still active under 20-25°C, studies on spoilage of refrigerated meat could be carried out through incubation conditions using shorter times at higher temperatures (HPFB, 2015). The American Public Health Association (APHA, 2001) suggests to spread the samples on PCA and incubate the dishes at 17°C for 16 h, followed by 7°C for 3 days. This method can not only allow the psychrotrophic colonies to grow faster at the beginning, but also ensure the visible colonies to remain as psychrotrophs by decreasing the temperature back to 7°C during the latter incubation period. Health Canada offers another option by incubating the PCA dishes at the growth temperatures between 15°C and 20°C aerobically for 48 h (HPFB, 2015). Previous studies tried to obtain total psychrotroph counts (TPC) more rapidly by applying higher incubation temperature, extending the incubation time or using nutritionally richer laboratory medium (see Table 2.6). However, these studies did not compare the TPC obtained from standard method and these alternative medium. Thus, further studies might be required to enumerate meat spoilage microbiota by plating not only on alternative media, but also PCA in order to 1) have data with the standard method for CFU of psychrotrophs, and 2) to verify the accuracy of the rapid method on the alternative media.

On the other hand, in numerous studies, total aerobic counts (also named as total plate count, and mesophilic aerobic plate count), which were carried out at higher incubation temperatures commonly as 35-37°C, were chose as the microbial spoilage indicator instead of TPC when the aim was only to have a generic test for spoilage population (Rsyer and Schuman, 2015). Actually, many spoilage species related to stored foods are not strictly psychrophilic organisms, sub-groups of mesophilics with psychrotolerant properties, are able to grow under refrigeration but with optimal growth at higher temperatures. Under the high temperatures applied in total aerobic count, it would only allow the mesophilic bacteria generate visible colonies quicker than incubated at 7°C instead of increasing the proportion of mesophilic cells. Therefore, the total aerobic count could still represent the population of spoilage microbes.

Spreading the sample and incubating the PCA dishes aerobically at 35°C for 48 h is the official method for total aerobic counts, which has been widely used (HPFB, 2015). Other incubation temperatures, such as 22°C, 25°C and 37°C, were also used in previous studies (see Table 2.6). In these assays investigating the spoilage of refrigerated meat, the CFUs obtained from total aerobic counts were normally 1-1.5 log cycle lower than that from psychrotroph counts, meaning the total aerobic counts could partly correspond to the population of spoilage psychrotrophilic bacteria (Delaquis et al., 1992; Nortjé et al., 1990; Thayer et al., 1993).

In some assays, differential medium were also used to describe the microbial spoilage of meat (Table 2.6). In these cases, researchers usually had hypotheses of the probable dominant bacterial species presented in their samples. In this project, lactic acid bacteria (LAB) are believed as the major species among spoilage populations. However, these bacteria have a high demand of complex carbon and nitrogen sources from the media because they lack the ability of synthesizing growth factors such as amino acids (Van Niel and Hahn-Hägerdal, 1999). Compared with PCA, MRS agar could be used to evaluate the total bacterial count of a population whose major species are LAB, due to its abundant sources of lactobacilli growth factors (Difco, 1998). Although some lactobacilli growth factors such as acetate, Magnesium and Manganese in MRS might inhibit the growth of some species, MRS agar in regular pH (6.5) is still a richer media compared with PCA, allowing microorganisms other than lactobacilli to grow (Difco, 1998).

As previously stated, the plate count is the most recognized approach to enumerate the viable bacterial population although it is time-demanding. Thus, the selection of ingredients as well as the ascertainment of their effective concentration of each ingredient could be carried out through a more rapid indirect method (see *2.3.2 Automated spectrophotometry method*), while plate count method could be used to confirm the reliability of data from the rapid approach. Also, after obtaining the effective antimicrobial formula from AS method, plate count method seems to be the only option to confirm the effectiveness of these formula when applied in absorbent pads, since the AS method is only applicable in an aqueous system. Plate count method could evaluate the effectiveness of antimicrobial absorbent pads by enumerating the initial spoilage bacteria recovered from either pads themselves, or the surface of meat samples treated by pads (Table 2.5) Since the goal is to delay the spoilage in the soaked up chicken exudates, the enumeration of

bacterial cells in the pads is more practical. Thus, it is important to find an appropriate technique to recover the bacteria from the pads. Based on this purpose, homogenization in a sterile solution such as peptone water with a stomacher unit was previously tested (Fernandez et al., 2010; Gouvêa et al., 2016; Ren, Qiao, et al., 2018). The enumeration of bacteria from pads themselves is quite applicable for the non-volatile compounds when these compounds make afford by the penetration from the pad to the meat, while the enumeration for meat itself is more applicable for the evaluation of volatile ingredients inhibiting the spoilage microbiota on meat surface by evaporating to the headspace inside of the package.

2.3.2. Automated spectrophotometry (AS) method

The automated spectrophotometry (AS) method evaluates the change of bacterial population as the function of incubation time, by measuring the turbidity of the bacterial growth medium regularly and automatically. The principle of this method is based on the Beer-Lambert's Law, assuming that when the cells in the media are equitably distributed, the absorbance under a certain wavelength has a linear correspondence to the total count of these cells within a linear range of optical density (normally 0.2-1.2) (Begot et al., 1996). The AS method is more sensitive and rapid compared with plate count method, any slight optical density change can be simply accounted by running a blank (an non-inoculated media) and taking all optical density (OD) readings against the blank media (Johnston, 1998).

The detection parameters and incubation conditions were also different among studies. The choices of medium broth, detection temperatures and time were mostly depending on the microorganisms of interest in their initial studies, while the detection wavelength around 600 nm was commonly used with better absorbance for bacterial cells but with less interference of the medium (see Table 2.7). Meanwhile, it is important to decide an appropriate inoculation level of the media through optical density method. Because of the detection threshold of AS method, the density of cells in the system has to reach a level (approximately 10^7 CFU/mL with the OD around 0.2) after which the growth of cells could be detected by the increase of OD. For example, if the bacterial population of the inoculated media is approximately 10^5 CFU/mL at the beginning of measurements, the OD would remain as the same until the population reaches 10^7 CFU/mL instead of the end of the "real" lag phase. Therefore, unless an exact assessment the effect of inoculation level on lag time in media without antimicrobial compounds is established

(McKellar and Hawke, 2006), some studies claimed that the turbidity method would exaggerate the length of lag phase, but the technique is still considered effective to screen the antimicrobial effects of different treatments by comparing the lag phase between samples (Skandamis et al., 2001). If the bacterial population of the inoculum is too low, it would take an unnecessary long time before the OD reaches the detection threshold. On the other hand, if the bacterial population of the inoculum is too high, the OD could exceed the upper limit of the detection threshold at the beginning of measurement and fail to provide accurate growth parameters. Thus, previous studies commonly yield initial bacterial populations of around 10^5- 10^6 CFU/mL after inoculation (see Table 2.7).

It has to be kept in mind that the AS data is only reliable when the OD increase indeed corresponded to the increasing amount of bacterial cells in medium broth (Champagne et al., 2014). Some plate count tests are usually necessary to confirm correlations between OD readings and the real viable counts, by carrying out regression analysis between growth rates obtained from AS data and the one form plate count data (Champagne et al., 2017; Champagne et al., 2014; Champagne et al., 2009).

2.3.3. Correlations between data from AS and total viable count

As previously stated, regression analysis between OD data from AS and CFU data from plate counts are required. Two main reasons would affect the result of the linear regression between OD and CFU: 1) the different working principles of AS and plate count methods; 2) different incubation conditions (temperature, atmosphere) applied in the two assays, if applicable.

The ideal situation having perfect correlation between AS data and the total viable counts is that, the cells are evenly distributed during OD measurements, and the cells presence in the liquid medium are culturable (Begot et al., 1996; Kell and Young, 2000). In AS assays, it could not be guaranteed that all cells contributed to the OD increase were viable. Injured or VBNC (viable-but-not-culturable) cells could had absorbency as the healthy cells but failed to form colonies on agar plate, since they had less active metabolism or had long lag times to repair themselves and might not generate visible colonies within a given time of petri dishes incubation (Champagne et al., 2014; Kell and Young, 2000). An opposite opinion claimed that AS assays might under-estimate the cell population since the cell injury caused by antimicrobial ingredients would be masked during the immediate OD measurement, however, these injured cells could

repair themselves and generate visible colonies during the incubation of petri dishes, although the colony size would be smaller than the non-injured cells (Lambert and Van Der Ouderaa, 1999). Therefore, whether there was an exaggeration of cell population in AS assays compared with traditional plate count method is still not clear.

The second theory is only applicable when AS and plate count were carried out applying different incubation parameters. Samples are usually incubated at 7ºC as the widely recognized incubation temperature for psychrotrophs in all plate count assays, while in AS assays, since the OD measurements are immediate, higher temperatures could be applied as accelerated approaches for the growth of psychrotrophic spoilage microbes (Vasavada and Critzer, 2015). However, the increase of incubation temperature might not only allow some sub-species of mesophilics to grow in the media increasing the OD reading, but also affect the antimicrobial properties of applied ingredients in some extent (APHA, 2001; Ramos-Villarroel et al., 2011). For example, higher temperature of the system might enhance the binding between polyphenols from green tea extract and protein and peptides from the exudates, reducing the real concentration of active phenolic compound which could actually inhibit spoilage bacteria (Von Staszewski et al., 2011)

2.3.4. Description of bacterial growth kinetics

The effect of antimicrobial agents could be bactericidal or bacteriostatic (O'Mahony, 2010). In some circumstances, whether the effect is bactericidal or bacteriostatic could be concentration-dependent (Levison, 1995). As previously mentioned, the goal of this study is to delay the time when spoilage occurs (when the overall bacterial population reaches 10^7-10^8 CFU/mL) (Vihavainen and Björkroth, 2010), by delaying the overall growth of initial spoilage microbiota of both meat products, rather than completely kill these bacteria. In other words, this study will only evaluate the bacteriostatic effect of tested ingredients against meat spoilage microbiota. The growth delay of spoilage microbiota could be described by the change of growth parameters calculated from bacterial growth curves (Quintavalla and Vicini, 2002). Either the extension of the lag phase or the decrease of growth rate at the early log phase leads to the longer period required for the population of initial microbiota to reach the level indicates spoilage.

Other parameters, such as the minimum inhibitory concentration (MIC), usually defined as the lowest concentration of antimicrobial agents to yield complete inhibition (no visible growth) has been commonly used to evaluate antimicrobials against certain target bacterial species potential involved in food spoilage *in vitro* (Ghabraie et al., 2016; Oussalah et al., 2006; Saha et al., 2017; Schirmer and Langsrud, 2010; Turgis et al., 2012). It is also possible to evaluate MIC_{50} or MIC_{90}, as the minimum concentration inhibiting 50% or 90% of the target population, respectively (Mahieux et al., 2018). Other parameters such as minimum bactericidal concentration (MBC) and maximum tolerate concentration (MTC) are seen in previous studies on food preservatives but less common (Mahieux et al., 2018; Oussalah et al., 2006). Due to the purpose of delaying the spoilage instead of completely inhibit the bacterial population present in the meat products, instead of MIC, MBC or MTC, it is more appropriate to evaluate the effectiveness of tested natural ingredients based on the change of bacterial growth parameters.

2.3.4.1. Lag time (LagT)

The presence of lag time (lag phase) in microbial growth kinetics is because that, when the microorganism is inoculated to a new medium, a delay of growth is required to adapt the nutritional condition of the media and generate the enzymes, RNA and other molecules for their further growth (Cohen, 2014). When the medium is inoculated with antimicrobial compounds, the extension of the lag phase is a reflection of the antimicrobial effect, because these compounds act as barriers for the growth of the more sensitive species in the population, but allowing the remaining cells to adapt the medium during a longer period and then generate. However, there was no agreement on the definition of the lag phase. Ideally, in studies using turbidity method, the lag phase of microbe in inoculated sample was defined as the period from time 0 to the latest time point showing the equal absorbance as the blank (Bukvicki et al., 2015; Ghabraie et al., 2016; Turgis et al., 2012). This definition fits well for *in vitro* studies applying pure bacterial cultures and standard laboratory medium supplied with antimicrobial ingredients. In such cases, physico-chemical properties of the standard media were believed to remain relatively stable during incubation without interfering with the OD readings. The growth curves were usually smooth, and the growth parameters determined from these curves were accurate. However, this approach could hardly represent the actual antimicrobial effects of tested ingredients on the complex meat spoilage population in practice.

Due to the preference for simulating the "real situation" in this project, the standard laboratory media would be replaced with a "meat-based" media prepared through homogenization and clarification. Also, the inoculum could be the initial microbiota extracted from spoiled meat sample instead of pure bacterial cultures. However, in such cases, it is hard to maintain a completely repeatable OD value during the lag phase for two reasons: 1) Fluctuation of OD during the lag phase may appear due to the potential degradation or precipitation of the complex meat contents, which might interfere the OD readings and provide false lag time. 2) As a mixture of various bacterial species, each species in the population has their specific lag phase. Those species which adapt in the medium earlier cause a slight increase of OD, but the slight change would not be concerned as symbol of the end of lag phase because the rest of bacteria in the population have not initiated growth at that point. In this case, the lag phase would not appear as a complete horizontal curve, rather, it would probably be a curve with a slight slope or even with several small fluctuations. In order to have the picture of the lag phase of the general population, in some studies the lag phase was considered as from time 0 to the ΔOD ($OD_{real\ time}$-$OD_{time\ 0}$) reaches a certain point. For example, some studies defined lag phase as when the ΔOD exceeded 0.05 (Champagne et al., 2014; Schirmer and Langsrud, 2010).

The lag phase is an important and meaningful parameter for the evaluation of shelf-life extension. The spoilage of refrigerated poultry or meat appears when the microbial count typically exceeds 10^7 CFU/g (with the OD around 0.2), if the density of bacterial cell in the broth is below this value, the growth of bacteria is below the detection threshold. Therefore, the lag time obtained by spectrophotometry method could be considered as the time for the spoilage microbiota to deteriorate the product. 6 out of 9 previous studies ascertain the antimicrobial effects based on lag phase data, and all lag phase data from these 6 studies were calculated based on OD readings rather than CFUs from plate count method, probably because the plate count assays could not be carried out frequent enough to provide precise lag time (Table 2.4).

2.3.4.2. Growth rate (μ)

In addition to the lag phase, the growth rate (μ) is another widely used parameter describing growth kinetics. Due to the presence of the antimicrobial agents, the generation times are affected by the antimicrobial treatments, leading to the reduction of growth rates during the exponential growth period. The growth rate could reflect the bacterial growth kinetics alone or

together with the lag phase. (Gill and Holley, 2003; Hayouni et al., 2008; Saha et al., 2017). In most cases, the $\mu_{max,}$ defined as the slope of the tangent of the growth curve when the fastest growth occurs (Hayouni et al., 2008; Saha et al., 2017). As previously stated, it is hard to carry out plate count assays frequently enough to obtain precise growth parameters, the μ_{max} is preferred to be calculated by OD data.

However, in assays using meat-based media, the accuracy of growth rates might be challenged due to 1) gradual precipitation in the non-inoculated control; and 2) precipitation caused by bacterial metabolism. The first occurrence is unusual since most laboratory media used in AS assays are stable. When a slight OD increase occurs in time within the non-inoculated medium, it would seem necessary to register the OD values of the blank medium at each moment the OD values of the inoculated media are ascertained. Then, corrected OD values are generated by subtracting the OD of the non-inoculated medium from that of the inoculated one. However, this logical methodology remains hypothetical since no study has confirmed it to enable the exact calculation of biomass increases by OD.

The second situation is much more complicated. The growth and metabolism of microorganisms could change the physico-chemical properties (i.e. pH) of the media, and these changes would affect the OD reading and the growth rate calculated based on these OD values. The typical influence on the OD measurement was protein precipitation due to the reduction of pH (Champagne et al., 2014). For example, the dominant spoilage bacteria of sausage tested in this project are believed as lactic acid bacteria. Organic acids (mainly lactic and acetic acid) generated by these acid-producers during their growth would make the pH reach the isoelectric point (pI) of each protein presence in the sausage media and precipitate them successively (Batt, 2014; Champagne et al., 2014; Pothakos et al., 2015; Wang et al., 2014). Such precipitates also have absorbency during OD reading, leading to the over-estimation of bacterial biomass in the broth. Based on this hypothesis, the μ_{max} was sometimes ascertained during the early stage of exponential phase instead of the whole incubation period before the metabolism strongly affects the OD (Champagne et al., 2014).

2.3.4.3. Change in biomass (ΔOD_{max})

In some studies, the changes of biomass (ΔOD_{max}) linked to the presence of the antimicrobial agents were investigated via plate count method or/and turbidity method (Bukvicki et al., 2015; Hayouni et al., 2008; Kanatt et al., 2008; Oussalah et al., 2006). Compared with lag phases and growth rates, the ΔOD_{max} could indicate whether the cells are stressed or completely inactivated. Thus, this parameter is very valuable when screening the bactericidal effect of the antimicrobial ingredient against a certain foodborne pathogen strain in a standard medium since the aim is usually to completely inhibit the pathogen. In this case, the increase of lag phases or the decrease of growth rates are not enough as a proof for the bactericidal effect, since they might only indicate that the cells are stressed instead of completely inactivated, similar total counts level probably be observed compared with the one without adding antimicrobial agents in stationary phase. However, when the goal is only to delay rather than completely inactivate the bacterial growth, the decrease of the change in biomass is not a necessary as a reflection of a bacteriostatic effect (growth delay). In this project, the change of biomass might not be as applicable as lag phase or growth rate as an indicator of shelf-life extension. The spoilage microbiota is a complicated community consisting of different bacteria strains with different susceptibilities against one antimicrobial treatment. In this case, if the antimicrobials would only decrease the generation times of the spoilage microbiota instead of kill them completely, these bacteria would only grow slower with longer lag phases and lower growth rates but being able to reach the same maximum growth level at the end of the logarithmic phase. Thus, extending the lag phase or reducing the growth rate could be corresponded to not only the delay of the sensory deterioration but also the shelf-life extension.

Table 2.4 Methodologies of evaluating the effects of several antimicrobial agents *in vitro*

Product	Antimicrobial agents	Target bacteria	Methodology	Criteria to determine antimicrobial effects	References
NA	6 essential oils (EO) and 4 bacteriocins	5 pathogens (*Ba. cereus, E.coli, L. monocytogenes, S.* Typhimurium and *Staph. aureus*); 2 spoilage bacteria (*Lb. sakei* and *P. putida*)	Broth dilution method based on OD	The lowest EO concentrations showing complete inhibition of the visible growth of bacteria in 24 h	(Turgis et al., 2012)
Meat and meat products	Chitosan and mint extracts mixture	*E. coli, P. fluorescens, S.* Typhimurium, *Staph. aureus* and *Ba. cereus*	Broth dilution method based on total aerobic counts	Surviving population	(Kanatt et al., 2008)
Meat	Lysozyme, chrisin, EDTA, NaCl and nitrite	14 tested bacteria (foodborne pathogens and spoilage bacteria from meat)	Broth dilution method based on OD	The slope (growth rate) of the response predicted by the statistically generated model within 48 h growth	(Gill and Holley, 2003)
Vacuum-Packed Pork Meat	Thymol, cinnamaldehyde, allyl isothiocyanate, organic acids, plant extracts	*Lactobacillus.* spp., *Leuconostoc* spp., *Carnobacterium* spp., *B. thermosphacta*, and *Serratia proteamaculans*	Broth dilution method based on OD	Detection times (lag time, when $\Delta OD > 0.05$) within growth under anaerobic condition for 6 day at 20°C and 3 weeks at 4°C	(Schirmer and Langsrud, 2010)

Vacuum-packaged cold-smoke salmon	Potassium lactate (KL), potassium acetate (KA), and liquid smoke (LS)	5 LAB, three fish spoilage bacteria (FSB), and two strains of *L. innocua*	Broth dilution method based on OD	Lag times (λ) and growth rates (μ) derived via Weibull model parameter equation within 24 h growth	(Saha et al., 2017)
Ground meat	32 EOs	4 pathogens (*E. coli, L. monocytogenes, Staph. aureus,* and *S.* Typhimurium)	Broth dilution method based on OD	The lowest EO concentrations showing complete inhibition of the visible growth in 24 h	(Ghabraie et al., 2016)
NA	*Melaleuca armillaris* EO	6 LAB strains	Broth dilution method based on OD & plate counts	The increase in lag phase (ΔLagT); The maximum growth rate (μ_{max}); The reduction in culture density (ΔOD); The decrease in the percentage of viable cells (via plate counts)	(Hayouni et al., 2008)
Vacuum packed and pasteurized minced (ground) pork	*M. dalmatica* EO	*S.* Typhimurium	Broth dilution method based on OD	*In vitro* assays: The lowest EO concentrations showing complete inhibition of the visible growth in 24 h. *In situ* assays: inhibition%= $[(\Delta OD_{sample}/\Delta OD_{pos\ ctrl})-\Delta OD_{blank}]\times 100\%$	(Bukvicki et al., 2015)

NA	Clove and cinnamon powder	*E.coli, Staph.aureus, B. thermosphacta , Lb. rhamnosus* and *P. fluorescens*	Broth dilution method based on plate counts	The inhibitory effect: I%=[(A-B)/A]×100% Where A=CFU in positive control (no agents in the test tube), B= CFU in the test tube	(Kuang et al., 2011)
Meat	60 EOs	*P. putida* from meat origin	Agar dilution method	The absence of colonies on BHI containing EOs in 48 h	(Oussalah et al., 2006)
Chilled pork stored in antimicrobial pack	Rosemary, angelica root and clove	*Enterobacteriaceae, Staph. aureus, Pseudomonas* spp., Lactic acid bacteria, *B. thermosphacta*	Disc diffusion method	Diameter of inhibition cycle measured after 24 h incubation	(Liu et al., 2012)

Table 2.5 Methodologies of evaluating the effectiveness of antimicrobial absorbent pads (*in situ*)

Product	Antimicrobial compound(s)	Target microbes	Enumeration method		Reference(s)
			For the pad	For the surface of the product	
Beef under MAP	Silver nanoparticles	Total aerobic counts, lactic acid bacteria, *Pseudomonas*, *Enterobacteriaceae*	Absorbent pads (0.5 g) were homogenised in 10 ml of a sterile solution (0.1% peptone water; 0.85% NaCl) with a stomacher	Beef samples (15 g in contact with the polyethylene layer covering the absorbent pad) were homogenized in 10 ml of a sterile solution (1% peptone water and 8.5% NaCl) with a	(Fernandez et al., 2010)

				stomacher	
Packed raw beef	N-halamine, 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC)	Total aerobic counts, lactic acid bacteria, *Pseudomonas*, *Enterobacteriaceae*	Absorbent pads (2 g) from packed beef samples were homogenized in 1% BPW (buffered peptone water) at 1:10 dilution (w/w) for 1 min at 260 rpm with a stomacher	Beef samples were homogenized in 1% BPW at 1:10 dilution (w/w) for 1 min at 260 rpm with a stomacher	(Ren, Qiao, et al., 2018)
Overwrap packed chicken drumsticks stored at 4°C	Oregano essential oil	Total aerobic counts, psychrotrophs, lactic acid bacteria, *Pseudomonas*, *Enterobacteriaceae*	-	Chicken drumsticks were rinsed for 1 min and diluted in physiological saline	(Oral et al., 2009)
Chilled meat	Sodium carboxymethylcellulose (CMCNa)	Total aerobic counts	-	The meat surface tissue sections of 3 cm2 were swabbed with sterile peptone water and the microbial load was resuspended in 4 mL of peptone water.	(Wang et al., 2013)
Sliced roast beef	Cetylpyridinium chloride (CPC), acidified sodium	Total aerobic counts, *E. coli*	-	A beef square in 20 ml of 0.1% sterile peptone water (pH 7.0)	(Lim and Mustapha,

	chlorite (ASC)	O157:H7, *L. monocytogenes*, *Staph. aureus*	was homogenized for 2 min	2007)

Table 2.6 Plate incubation parameters for the evaluation of meat spoilage

Objective	Product	Plating method	Medium	Atmosphere	Temperature	Incubation time	Reference(s)
Psychrotrophic plate count	NA	Pour plate	PCA	aerobic	7°C	10 days	(APHA, 2001)
	Pork	Spread plate	PCA	aerobic	7°C	7-8 days	(APHA, 2001; Delaquis et al., 1992; GREER and MURRAY, 1991)
	NA	Spread plate	PCA	aerobic	17°C for 16 h, followed by 7°C for 72 h		(APHA, 2001)
	NA	Spread plate	PCA	aerobic	15°C	48 h	(Health Canada, 2015)(Yuste et al., 2001)
	Refrigerated pork	Spread plate	TSA	aerobic	10°C	120 h	(Thayer et al., 1993)
Psychrotrophic gram-	Meat	Spread plate	BHI	aerobic	7°C	10 days	(Jay, 1987)

negative bacteria							
		Spread plate	BHI	aerobic	10°C	48 h	(Susiluoto et al., 2003)
Total aerobic counts	NA	Spread plate	PCA	aerobic	35°C	48 h	(Health Canada, 2015)
	Marinated broiler under MAP with 20% CO2- 80% N2	Spread plate	PCA	aerobic	25°C	3 days	(Susiluoto et al., 2003)
	Refrigerated pork	Spread plate	TSA	Aerobic	35°C	96 h	(Thayer et al., 1993)
	Pork preblends	Spread plate	PCA	aerobic	30°C	48 h	(Delaquis et al., 1992)
	Refrigerated minced meat	Spread plate	PCA	Aerobic	35°C	24-48 h	(Nortjé et al., 1990)
		Spread plate	PCA	Aerobic	25°C	2-3 days	
	Chicken breast under VP	Spread plate	PCA	aerobic	37°C	48 h	(Pavelková et al., 2014)
	Sampled food products including fresh raw meat, cooked meat products	Pour plate	PCA	aerobic	37°C	48 h	(Pothakos et al., 2012)

	and read-to-eat vegetable salad						
			PCA	aerobic	22°C	5 days	
Total acid-producer count	Products other than dairy products	Pour plate	PCA+0.02g/L bromcresol purple (BCP)	aerobic	35±1°C	48±3 h	(APHA, 2001)
		Pour plate	Acidified MRS (pH=5.5±0.1)	anaerobic	35±1°C	72±3 h	
Lactic acid bacteria	Pork preblends	Spread plate	MRS	Anaerobic, in a H2+CO2 atmosphere	25°C	48 h	(Delaquis et al., 1992)
	Chicken breast under VP	Spread plate	MRS	aerobic, with 5% CO2	37°C	48-78 h	(Pavelková et al., 2014)
	Sampled food products including fresh raw meat, cooked meat products and read-to-eat vegetable salad	Pour plate	Acidified MRS (pH=5.9)	aerobic	37°C	48 h	(Pothakos et al., 2012)
			Acidified MRS (pH=5.9)	aerobic	22°C	5 days	

Table 2.7 Incubation and detection parameters applied in spectrophotometry assays

Product	Antimicrobial agents	Objective bacteria	Inoculum (CFU/mL)	Inoculated medium	Inoculation level in each well	Detection parameters	Equipment	Reference
NA	6 EOs and 4 bacteriocins	5 pathogens (*Ba. cereus, E. coli, L. monocytogenes,* *S.* Typhimurium and *Staph. aureus*); 2 spoilage bacteria (*Lb. sakei* and *P. putida*)	10^6	Mueller-Hinton medium	15 µL strain + 125 µL medium	37°C, 24 h, at 630 nm	An Ultra Microplate Reader with 96-well microplates	(Turgis et al., 2012)
Meat	Lysozyme, chrisin, EDTA, NaCl and nitrite	14 tested bacteria (foodborne pathogens and spoilage bacteria from meat)	10^5	Either APT or BHI broth (depending on the bacteria), both with 10 g/l MES buffer, titrated to pH 6.0	10 µL strain + 100 µL medium	24°C, 3 days, at 450 nm	A Titretek Multiscanner with 96-well polypropylene micro-titre plates	(Gill and Holley, 2003)
Vacuum-Packed Pork Meat	Thymol, cinnamaldehyde, allyl isothiocyanate, organic acids, plant extracts	*Lactobacillus.* spp., *Leuconostoc* spp., *Carnobacterium* spp., *B. thermosphacta*, and *Serratia proteamaculans*	10^6	Anaerobe basal broth (ABB), (The pH was adjusted to 6.05 ± 0.05) incubated anaerobically	20 µL strain + 160 µL medium	20°C, 6 days, at 600 nm	A microplate photometer with a 96-well plate	(Schirmer and Langsrud, 2010)

Vacuum-packaged cold-smoke salmon	Potassium lactate (KL), potassium acetate (KA), and liquid smoke (LS)	5 LAB, three fish spoilage bacteria (FSB), and two strains of *Listeria innocua*	10^3	TSBYE medium (TSB supplied with 0.6% w/v yeast extract)	Inoculum of 250 µL (total volume), with 10^3 CFU/mL of tested bacteria	20°C, 5-7 days, at 600 nm	A BioScreen C microplate incubator and a reader	(Saha et al., 2017)
Ground meat	32 EOs	4 pathogens (*E.coli, L. monocytogenes, Staph. aureus,* and *S.* Typhimurium) 1 spoilage bacteria (*P. aeruginosa*)	10^6	Emulsified EO consisted of 2.5% EO (v/v), 5.0% Tween 80 (w/v) and 92.5% water (w/w)	15 µL strain + 125 µL medium	37°C, 24 h, at 595 nm	A microplate photometer with a 96-well plate	(Ghabraie et al., 2016)
NA	*Melaleuca armillaris* EO	6 LAB	10^6	MRS + DMSO (dimethyl sulfoxide) +tested EO	150 µL strain in MRS broth (10^6 CFU/mL approximately) + 50 µL EO diluted in DMSO	At the optimal temperature for tested LAB species for 3 days, at 600 nm	A BioScreen C microplate incubator and a reader	(Hayouni et al., 2008)

Vacuum packed and pasteurized minced pork product	*M. dalmatica* EO	*S.* Typhimurium	10^6	*In vitro* assays: TSB with addition of EO	*In vitro* assays: 100 µL strain (10^6 CFU/mL approximately) + 100 µL medium	*In vitro* assays: 37°C, 24 h, at 610 nm	*In vitro* assays: A microplate photometer with a 96-well plate	(Bukvicki et al., 2015)
				In situ assays: Pork meat medium	*In situ* assays: *Salmonella* Typhimurium was adjusted to have 10^6 cells per well (100 µL)	*In situ* assays: 4°C, 7, at 610 nm	*In situ* assays: ELISA plate reader	

CONNECTING TEXT 1

In the comprehensive review, major bacterial species involved in the spoilage of chicken exudates and sausage itself were discussed. Potential natural ingredients were selected based on their antimicrobial activities against dominant bacterial species potential present in spoiled chicken and sausage according to previous studies. The antimicrobial effects of these selected ingredients need to be ascertained by using representative meat or exudate based growth medium inoculated with the contaminating microbiota extracted from chicken exudates or sausage samples spoiled under refrigeration. Due to the preference of evaluating the inhibitory effects under such "real situation", broth dilution method based on OD reading is the best choice for the rapid selection of an effective formula of antimicrobial agents, while traditional plate count method is applicable in 1) confirming the OD reading link to the real bacterial counts; and 2) confirming the effectiveness of formula based on AS data in pad assays.

CHAPTER 3. ANTIMICROBIAL ACTIVITIES OF FOOD BY-PRODUCTS AND PLANT EXTRACTS AGAINST SPOILAGE MICROBIOTA OF REFRIGERATED CHICKEN AND THEIR APPLICATION IN ABSORBENT PADS

Abstract

The aim of this study is to delay the growth of spoilage microbiota from the exudate of refrigerated chicken, by adding natural antimicrobial ingredients to absorbent pad present in the package. The natural ingredients tested were plants extract (green tea and grape seed) as well as food by-products (cranberry pomace and powder of sauerkraut juice). An automated spectrophotometry (AS) method was first applied to ascertain the concentration of antimicrobial ingredients to be tested in pads. In order to ascertain the effectiveness of ingredients in the "real" meat system, in the AS assays, a clarified sterile chicken exudate was inoculated with a microbiota obtained from spoiled exudate. Growth curves were analysed for lag phase (LagT) and maximum growth rate (μ_{max}). Data from AS assays showed that the sauerkraut juice at 20 g/L as well as grape seed extract at 10 and 20 g/L had antimicrobial effects. Observations made from the AS assays were confirmed in classical experimental conditions based on the evaluation of total psychrothroph plate counts in exudates during refrigerated storage. However, the antimicrobial effect of these selected ingredients was notably reduced when being applied the same concentrations to the absorbent pad. Possible reasons could be the more aerobic conditions above absorbent pads than in AS microplates or test tubes, as well as the uneven distribution of antimicrobial contents in exudates applied in the pads.

3.1. Introduction

Raw meat and poultry are highly susceptible for microbial spoilage, due to their high water activity (a_w), non-inhibitory pH and their high contents of growth factors, which would support the growth of a wide range of microorganisms (Dainty et al., 1989; Lawrie, 2006). Refrigeration delays the growth of the initial microbiota from the surface of chicken as well as the exudates inside the package, but psychrotrophic species present in the natural microbiota grow during the refrigerated temperature and become the dominant microbiota (Gill, 2003). The main spoilage genera in refrigerated fresh chicken stored aerobically have been identified as

Pseudomonas spp., mainly *P. fragi*, *P. fluorescens* and *P. lundensis*, which have shorter generation times at low temperature, producing by-products from amino acid and protein utilization such as ammonia, amines and organic sulphides (Byun et al., 2010; Charles et al., 2006; Fernández et al., 2010b; Gill, 1976; in't Veld, 1996; Shaw and Latty, 1982). These volatile metabolites with off-odor presence in the exudates could be as indicators of microbial spoilage from the view of the consumers.

Traditional absorbent pads could improve the visual appeals of packaged chicken by soaking up the exudates. However, spoilage microbes could still grow in the pads and generate compounds with off-odors (Charles et al., 2006; Fernández et al., 2010b). To avoid this, antimicrobial agents can be added to the traditional pads to inhibit the spoilage bacteria from chicken exudates. The antimicrobial agents that have been tested for this purpose include silver nanoparticles, N-halamines, stilbenes, as well as volatile natural antimicrobial agents such as essential oils (Fernández et al., 2010b; Oral et al., 2009; Ren, Hayden, et al., 2018; Ren, Qiao, et al., 2018; Wang et al., 2013) However, the antimicrobial agents applied by these previous studies had two main concerns. First, some of these chemical compounds are not "food-grade", and could leach out of the pads into the foods. This would be undesirable to consumers and food health agencies. Second, volatile compounds from essential oils may bring undesired flavors to the meat product. Thus, there is a need for non-volatile food-grade ingredients that could be added to absorbent pads in the aim of slowing the growth of the spoilage microbiota during refrigerated storage.

In this project, cranberry pomace and the powder of sauerkraut juice, as by-products from food manufacture were tested due to their high acidity. The high acidity of antimicrobial agents was proven to be effective in delaying the growth of bacteria strains such as *P. fragi*, *P. fluorescens, B. thermosphacta*, which could appear in spoiled chicken under refrigeration (Lebert et al., 1998; Ouattara et al., 1997). Moreover, as by-products with high acidity, cranberry pomace or sauerkraut juice would cause environmental and ecological problems if handled inappropriately, such as being disposed into soil directly without additional treatments (Hamer and Scuse, 2010). Thus, using these by-products as food-grade antimicrobial agents could not only take advantage of the remaining bioactive compounds, but also be as an eco-friendly treatment of the by-product.

In addition, the powders of tea and grape plant extracts were evaluated. Such products are also clean-label bioactive ingredients rich in phenolic compounds. Although each plant extract might contain different major active phenolics, these phenolic ingredients have the same functional group to inhibit the growth of various bacteria by physical membrane action, dissipation of proton motive force (PMF) or the inhibition of membrane associated enzymes (Reygaert, 2014; Shimamura et al., 2007; Ultee et al., 2002). Data from the literature suggest that both gram positive and gram negative bacteria with major safety concern, including *Ba. cereus, Staph. aureus, L. monocytogenes, Pseudomonas* spp., *Salmonella* spp. and *E. coli*, could be inhibited by green tea and grape seed in different extent (Chou et al., 1999; Friedman, 2007; Liu et al., 2017; Mbata et al., 2008; Perumalla and Hettiarachchy, 2011; Yam et al., 1997). However, no data are available on the inhibitory activity of green tea and grape extracts on the overall microbiota involved in the spoilage of refrigerated chicken.

In strategies to delay the growth of the initial spoilage microbiota in refrigerated chicken, it was important to evaluate the antimicrobial effect under a "real situation" with the closest nutritional value and microbial communities to that of the spoiled chicken in practice. Thus, it was decided to screen the optimal concentration of each antimicrobial agent in the system of clarified chicken exudates inoculated with a representative chicken spoilage microbiota, instead of using the broth of pure standard medium with addition of these antimicrobial agents inoculated with pure strains of target bacteria. The screening of effective concentrations for each antimicrobial agent in the system of chicken exudates was carried out by analyzing growth parameters (lag phases and growth rates) from bacterial growth curves obtained from automated spectrophotometry (AS) techniques. Afterwards, selected antimicrobial agents under their effective concentration against chicken microbiota were added in absorbent pads to verify their effectiveness in food packaging by evaluation of the total psychrotroph counts (TPC) from pads through plate count method.

3.2. Material and methods

3.2.1. Antimicrobial ingredients

The antimicrobial agents tested in this study were powder of cranberry pomace (ATOKA Cranberries Inc., Manseau, QC, Canada), powder of green tea extract (New Directions Aromatics Inc., Mississauga, ON, Canada), powder of grape seed extract (New Directions

Aromatics Inc.) and the freeze dried powder of sauerkraut juice (Caldwell Bio Fermentation Canada Inc., Ste-Edwidge, QC, Canada).

The lacto fermented sauerkraut juice (Caldwell Bio-fermentation) was poured into a stainless steel pan placed in a freeze-dryer (FTS Systems Dura-Dry, SP Scientific, Warminster, PA, USA), with the tablet temperature pre-set at -40°C. Two freezing steps were applied as: 300 min holding time at -40ºC, followed by 180 min after the temperature of the sample had reached -30°C. The drying process was then followed by the parameters shown in Table 3.1. The lyophilized cake from the drying process was processed into a powder and kept in conical tube under vacuum at 4°C.

Table 3.1. Parameters of freeze-drying as the function of the drying time

Temperature raised (°C/min)	Duration (hours)	Tablet Temperature (°C)	Vacuum applied (mTorr)
2	48	-30	150
2	16	-15	150
2	16	0	75
2	16	15	75
2	24	20	75

3.2.2. Analysis of bioactive compounds from powder of sauerkraut juice

The freeze dried powder of sauerkraut juice was first diluted at a concentration of 31.4 g/L in the mobile phase, passed on a SepPak C18 plus cartridge (Waters) for the cleaning process and then filtered with a 0.45 µm filter before injected into a HPLC unit. Injection (20 µL) was carried out with automatic injectors (Waters 717 plus, Waters, Milford, MA, USA). The organic acids from sauerkraut juice were analysed by HPLC on an ION-300 column (Concise separations, San Jose, California, USA) coupled with a pre-column of the same type, heated at 65°C, with 0.0085N sulphuric acid at 0.4 mL/min as mobile phase. Detection of organic acids was carried out by a UV detector (Waters 2489, Waters) at 208 nm. Empower 2 software (Waters) was used for integration.

3.2.3. Analysis of proanthocyanidins (PAC) from cranberry pomace

The polyphenol-enriched extract of cranberry pomace was first prepared for the HPLC analysis. Dried cranberry pomace (0.50-0.52 g) was weighed and extracted with 10.0 mL acetone/ deionized water/acetic acid (75: 24.5: 0.5, v/v/v) in a glass tube and then sealed with Parafilms. The tubes were vortexed around 10 s and sonicated for 30 min at room temperature by Bransonic Ultrasonic bath (Branson, Danbury, USA), while being covered by a lid to protect the samples from light. Tubes covered with aluminum foil were then shaked at room temperature for 1 h. The polyphenol extract was separated from the pomace using a medium glass frit connected to a vacuum line, and dried under 1) a nitrogen stream (10-20 psi) for 30-60 minutes, and 2) reduced pressure using a rotary evaporator with a water vacuum line and a 40ºC water bath, in order to remove water, acetone and proteins. Such extract was resuspend by 6 mL deionized water filtered by 0.45 µm filters, frozen at -20ºC for 1-2 hours in vials wrapped with aluminum foil and dipped in liquid nitrogen for at least 5 minutes. The sample was then lyophilized at -20 ºC by a FTS Dura-Dry System (SP Scientific, Warminster, PA, USA) for 48 h under vacuum.

The HPLC analysis was carried out with a HPLC unit (1200 series, Agilent Technologies, Ottawa, ON, Canada) equipped with a Diode Array Detector (DAD, G131SB). 15 µL polyphenolic extract (1 mg lyophilized solid in 100 µL deionized water) filtered through 0.45 µm PTFE syringe filter was injected and separated with a Kinetex C18 reverse phase column. The binary gradient consisted of water with 0.5% trifluoroacetic acid (A) and methanol (B). The gradients were operated as follows: 0-3 min, 0% B; 3-7 min, 0-10% B; 7-20 min, 10-17% B; 20-30 min, 17-17.5% B; 30-75 min, 17.5-42% B, 75-80 min, 42-100% B; 80-85 min 100% B; 85-90 min 100-0% B; 90-100 min 0% B. This was followed by a 10 min equilibration. PAC was detected by a DAD detector at 280 nm. An HPLC-System 3 Software was used for integration.

3.2.4. Medium preparation

In each repetition, 20 kg of the whole chicken samples (Exceldor, St-Bruno, QC, Canada) in a container were received and stored at the walk-in fridge at 4°C for 24 h to obtain higher drip (exudate) volume. After 24 h, all chicken samples were transferred in a new bag, and the drips in the original bag were collected with a pipette. The time of drip collection was considered as Time 0. The original drip was separated into two parts during each sample reception: one part (approximately 10 mL) for inoculum preparation, and the rest of the raw exudate for media

preparation. The exudate-based media was prepared from the original chicken drip collected from the whole chicken at time 0. This chicken drip was first centrifuged at 30 000 ×*g* for 20 min at 25°C (Beckman centrifuge model J-20 XPI, Rotor JA-18, Palo Alto, CA, USA) in Beckman tubes #355620. The antimicrobial agents were dissolved in the clarified chicken media in order to obtain serial concentrations of 5, 10 and 20 g/L, respectively, followed by vortexing for 5 s at 2500 rpm by a digital vortex mixer (Fisher, USA) to avoid the powder assemble at the bottom of the tube. All tubes with the addition of the antimicrobial ingredients as well as the control (chicken media without the addition of antimicrobial ingredients) were then kept under 4°C for more than 12 h while being agitating via a Labnet Labroller (H5100, Woodbridge, NJ, USA) at 25 rpm to ensure the complete dissolution of the water-soluble active components in the powder. For the powder of sauerkraut juice only, part of the sample was neutralized with 2N KOH to adjust the pH to the original pH of chicken exudate, to evaluate whether the antimicrobial effect of sauerkraut was based on its acidity.

After this, another centrifugation at 30 000 ×*g* for 20 min at 25°C (Beckman centrifuge model J-20 XPI, Rotor JA-18, Palo Alto, CA, USA) was carried out to remove not only the unsoluble part of these antimicrobial ingredients but also most of microorganisms presence in the media. The supernatants from the second centrifugation were filtered via 0.45 µm filters to be sterilized.

3.2.5. Inoculum preparation

The spoilage microbiota of refrigerated chicken is variable and complex. Thus, in order to best ascertain the effect of antimicrobials on this complex spoilage microbiota, it was decided not to inoculate with pure cultures of psychrotrophic organisms. Rather, it was decided to incubate the food products and allow the natural psychrotrophic microbiota to develop.

In each repetition, the inoculum was prepared from 10 mL of chicken drips collected directly from each reception (the chicken reception was described in *3.2.4. medium preparation*). The initial microbiota of the drip was on the average of 3×10^5 CFU/mL and, as error bars of SEM show, it was surprisingly constant within the 3 repetitions (Fig. 3.1). This initial microbiota was representative of the bacterial contamination of the chicken at the beginning of storage, but not necessarily of the spoilage microbiota of the refrigerated chicken. To obtain this representative psychrotrophic spoilage microbiota, it was therefore decided to incubate the drip

at 7°C to generate the inoculum for the AS assays. Furthermore, a clarification step was needed to remove as much as possible the non-bacterial elements that contributed to optical density. In order to generate an inoculum of spoilage bacteria having an OD reading which would be repeatable, i.e. the initial bacterial concentration is similar, these chicken drips collected from time 0 were incubated at 7°C aerobically for 7-10 days to reach a stable psychrotrophic count of approximately 4×10^8 CFU/g based on total aerobic count on Plate Count Agar (Difco/Becton Dickinson, Sparks, Maryland, USA) at 15°C for 48 h (Fig. 3.1). After the incubation, for the assays evaluating the inhibitory effect of cranberry pomace, powder of sauerkraut juice and green tea extract, a partial clarification of the drip was carried out as centrifugation at 500 ×*g* for 10 min at 5°C (Beckman GS-6R) followed by filtration via 10 µm filter. These steps were required in order to remove the small meat particles and protein aggregates in the drip with the potential to interfere the OD but nevertheless keep the microbiota. However, this inoculum contained soluble proteins, which could precipitate when being added to chicken media supplied with the grape seed extract. Therefore, for assays evaluating the inhibitory effect of grape seed extract, an additional step was carried out to prepare the inoculum. The cell suspension resulting from the previous procedure was centrifuged at 10 000 ×*g* for 10 min at 5°C (Beckman centrifuge model J-20 XPI, Rotor JA-18, Palo Alto, CA, USA). The cell pellet obtained from the centrifugation was then suspended with a sterile 8.5 g/L NaCl solution until the volume reached the original volume prior to centrifugation.

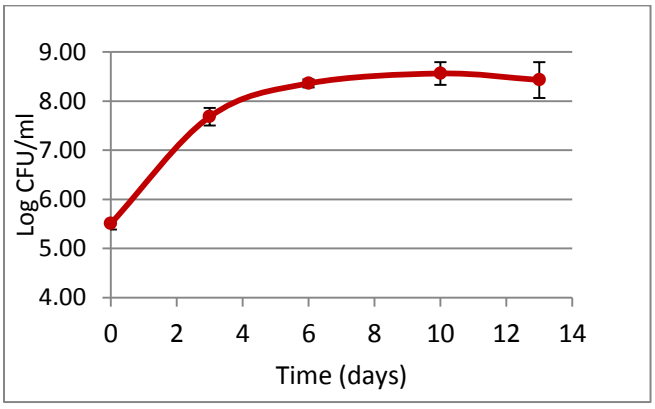


Fig. 3.1. Average growth curve of three repetitions of chicken exudates under 7°C incubation (plated on PCA, incubated at 15°C for 48 h).

3.2.6. Screening of antimicrobial effects *in vitro* through automated spectrophotometry (AS) method

The antimicrobial effects of the by-products and plant extracts were evaluated by the growth parameters obtained by the OD curves from automated spectrophotometry (AS) assays. The serial concentrations of each antimicrobial agent were injected into a Bioscreen microplate (Honeycomb, Labsystems, Helsinki, Finland) with a volume of 180 µL (3 wells for the inoculation). Regardless the antimicrobial agents tested, the inoculum was diluted by 10 times using sterile peptone 1 g/L water (Difco/Becton Dickinson, Sparks, Maryland, USA) right before inoculation to reach a concentration of representative spoilage microbes at 10^7 CFU/mL, approximately. Each well was then inoculated with 20 µL of the diluted inoculum to yield a concentration of approximately 10^6 CFU/g spoilage microbes at the beginning of incubation. This slightly centrifuged and filtered chicken exudate will be referred to as the "chicken media". Non inoculated filter-sterilized chicken media with addition of antimicrobial agents under different concentrations was also placed into the microplate wells with a volume of 200 µL each well as the blank; they served as negative controls. Additionally, BHI broth (Difco/Becton Dickinson, Sparks, Maryland, USA) inoculated with the same inoculum was used as the positive control in order to ensure the chicken inoculum was viable. The microplate was incubated aerobically at 17°C for 42 h as an accelerated approach for psychrotrophic bacteria. The optical density (OD) at 600 nm of each well was measured every 15 min after shaking the microplate at "extra intensive" level by a Bioscreen C unit (Thermo Labsystems, Espoo, Finland). At least 5 independent repetitions were carried out for each antimicrobial ingredient under each concentration.

3.2.7. pH measurement

pH-time curves were made for the control group (chicken media without addition of antimicrobials) during the incubation in a Bioscreen unit at 17°C for the first 30 h. The pH of the inoculated as well as the non-inoculated chicken media was measured by a pH meter (Fisher Scientific, Singapore) at 0, 6, 9, 22, 24, 26 and 30 h, respectively. The pH meter was equiped with the pH probe (Accumet) for small volumes.

3.2.8. Assays based on plate counts

The AS method is an indirect approach for bacterial enumeration, which is only meaningful if the increase of OD correlate with those of plate counts, the standard method of evaluating bacterial growth. Therefore, total psychrotroph count (TPC) of the spoilage microbes obtained from plate count method was required to ensure the high OD indeed corresponded to a high biomass level. In this case, the incubations were carried out under 7°C incubation, to not only ensure the antimicrobial effects indicated in AS assays, but also prove the applicability of the antimicrobials under refrigeration. Each 27 mL of chicken media supplied with each antimicrobial agent at the highest tested concentration as well as the control (media without addition of any antimicrobial agents) was inoculated with 3 mL of clarified inoculum and incubated at 7°C aerobically. The TPC was obtained by plating the inoculated chicken media on PCA at the sampling time of 0, 3, 5, 7 and 10 day(s).

3.2.9. Evaluation of the effectiveness of selected ingredients in absorbent pads *(in situ)*

Based on the data from AS assays, sauerkraut powder as well as grape seed extract was tested in pads by distributing the ingredients in absorbent pads soaked up with chicken exudates, and analysing the microbial loads in these pads. The absorbent pads (Model DT 050.103 Fluff Pulp, Glatfelter, Gatineau, QC, Canada) were cut into round pieces (diameter = 6 cm, 160 mg for each piece of pad) with a circular punch previously soaked in 70% ethanol for 15 min. These pads could absorb liquid at a level of 10 times of their own weight.

The methodology was designed to simulate conditions where chicken is placed in a foam tray carrying an absorbent pad and wrapped in a fashion that air is present in the package. Thus, two pieces of pads (approximately 320 mg total dry weight) were placed at the bottom of a sterile Mason jar (Bernadin Ltd., Richmond Hill, ON, Canada), and up to 64 mg of dried antimicrobial ingredient particles were placed between two the pads (approximately 320 mg). Then, 3.2 mL of the raw chicken exudates collected from time 0 was added on the pads in the jar, which was then capped. Theoretically, when the pad soaked up the exudates at their maximum absorbency (3.2 mL exudate in 320 mg pads), there should be between 0-20 g/L antimicrobial ingredients presence in the soaked-up exudates. A control without absorbent pads was also prepared by adding 3.2 mL of the fresh chicken exudate in the Mason jar.

Three jars were prepared for each treatment (each ingredient at each concentration and controls) and were incubated at 7°C for the three sampling times (days 3, 7 and 10). Additionally, at day 0, the TPC of the raw exudate (without pads and antimicrobials) were analyzed. Control groups which contained 1) two pieces of pads without addition of antimicrobial agents; and 2) exudate only without pads, were also prepared and enumerated in each assay, respectively.

At each sampling time, 96 mL of sterile peptone water (31-fold dilution) was added in the Mason jar, followed by homogenization with a blender (Oster, Model 6802, Mexico) for 30 seconds at "ice crush" level. 1 mL homogenized sample from each jar was carefully collected without taking cotton fibers, respectively. Microbial analysis was then carried out to obtain the CFU of the homogenized pad sample. In preliminary assays (data not shown), it was observed that over 90% of the bacteria in the pads could be recovered with this high shear homogenization method.

3.2.10. Screening of the organic acid distribution from sauerkraut powder in absorbent pads

A qualitative test was carried out to investigate if the organic acid was evenly distributed on the absorbent pad after the addition of chicken exudate by using a pH-indicator dye. After distributing the particles from 64 mg of dried sauerkraut between the pads, 3.2 mL chicken exudate mixed with 0.04% bromocresol purple was sprayed onto two pads. Pictures were then taken of the appearance of the pads after 1, 6, 24, 72 and 120 h of incubation at 7°C. Bromcresol purple (BCP) is yellow at pH 5.2 but purple at pH 6.8. This allowed an examination of the pH of the exudate in the pads. Whether the powder was evenly dissolved by the soaked up exudate was determined by the color appeared on the pad.

3.2.11. Microbial analysis

Samples collected either from *in vitro* assays in tubes or from pad homogenization were diluted subsequently with 1 g/L peptone water, respectively. For all the first 10-fold diluted samples, an intensive homogenization was carried out by a homogenization probe (Omni International, Marietta GA USA) at 27000 rpm for 30s in order to break down the link between cells. Samples at appropriate dilution folds were spread on plate count agar (PCA). The petri dishes were incubated aerobically at 15°C for 48 h as the enumeration method for total

psychrotrophs recognized by Health Products and Food Branch of Canada (HPFB, 2015). All plate count assays were carried out in triplicates.

3.2.12. Kinetic analysis

The creation of the growth curves based on either OD or log CFU, as well as the mathematical analysis of the curves for growth parameters, was carried out by Microsoft Excel 2010. All the OD values of the inoculated media were re-calculated by minus the OD of the blank media at each OD measuring time.

In AS assays, LagT was defined as the period from time 0 to the ΔOD ($OD_{at\ real\ time}$-$OD_{t=0}$) exceeded 0.075 during incubation. Although most previous studies defined the lag phase as the period from time 0 to the latest point showing the equal OD reading as that of the blank, the reason of defining the LagT this way instead was that, the contents in the exudate-based media were complex and uncertain, which might lead to the fluctuation of OD during the lag phase (Bukvicki et al., 2015; Ghabraie et al., 2016; Turgis et al., 2012). Some other research also determined the LagT as the period from the beginning of incubation to the moment when Δ OD exceed an appropriate point such as 0.05 (Champagne et al., 2014; Schirmer and Langsrud, 2010). Meanwhile, μ_{max} was screened during the early exponential growth period, which in our study was from the end of the lag phase to the time when OD reached 0.5, instead of the whole incubation period, because the high increasing rates of OD at the latter exponential growth period might not be accurate due to the chemical precipitates caused by the metabolism of the spoilage bacteria. In addition, the average growth rate (μ_{avg}) from the end of the lag phase to the end of the log phase during Bioscreen assays, was calculated.

In plate count assays, only the μ_{avg} from day 0 to day 3 was calculated, because the plate count were not carried out frequent enough to obtain precise lag phase or μ_{max}. Comparable μ_{avg} data from CFU and OD were used to evaluate the correlation between the OD and the real psychrotroph counts in chicken exudates.

3.2.13. Statistical analysis

For the AS assays, at least 5 independent repetitions were carried out in triplicate for each treatment. For plate count assays, 3 repetitions were carried out in duplicate for each treatment. Statistical analysis was carried out on Sigma Plot 13.0 (Systat Software; San Jose, CA, USA).

One-way ANOVA of each growth parameter was carried out based on the Student-Newman-Keuls multiple comparison test by ingredients. ANOVA was also done for all control groups between each treatment of different ingredients, in order to evaluate the variation of spoilage microbiota from the exudate. Linear regression was used to evaluate the relationships between the μ_{avg} obtained from OD readings and the total psychrotroph counts, in order to ensure the increase of OD reading indeed linked to proportional increase of bacterial counts. Differences were considered significant when $P \leq 0.05$.

3.3. Results and Discussion

3.3.1. Effect of pH change on OD reading of chicken media during incubation

The basis of the AS methodology is that the OD of the medium itself does not change during incubation, and that increases in OD are linked to bacterial growth. However, microbial growth can change the pH of the medium, which can generate precipitation of proteins or minerals (Champagne et al., 2014). Thus, we followed the pH of inoculated chicken media during the 17°C incubation in a Bioscreen unit was to ascertain if pH would change. During the first 9 h of growth, the pH of inoculated chicken media was stable, but subsequently increased (Fig. 3.2). Meanwhile, the pH of the non-inoculated chicken media remained stable during the 17°C incubation (Fig. 3.2), suggesting that the pH increase was linked to bacterial growth rather than physico-chemical change of the chicken media itself. This observation agreed with previous studies showing that *Pseudomonas*, which was believed as the dominant bacterial species in spoiled refrigerated chicken, would hydrolyze amino acid from meat and generate alkaline by-products leading to the increase of pH when their population exceeded 10^9 CFU/mL (Kim et al., 2017; Lea et al., 1969).

Since the chicken exudate contains varies proteins, each of which had its specific isoelectric points (pI), there were concerns that this pH increase could generate protein precipitation. When the pH of non-inoculated chicken media was adjusted between 4.8 and 8.8 by sterile 2N KOH or 40% lactic acid, precipitation occurred (Fig. 3.3). However, within the pH range of 6.0-6.8, OD change was small (Fig. 3.3). In the controls, the pH of 6.8 was reached after approximately 15 h of incubation (Fig. 3.2) and the OD was then of 0.5. As a result, only the data of the growth curves below OD values of 0.5 were used in the LagT and μ_{max} calculations. These pre-tests ensured that the metabolites produced by the inoculated microflora would not interfere

with the OD readings linked to microbial biomass when the data are selected under certain parameters.

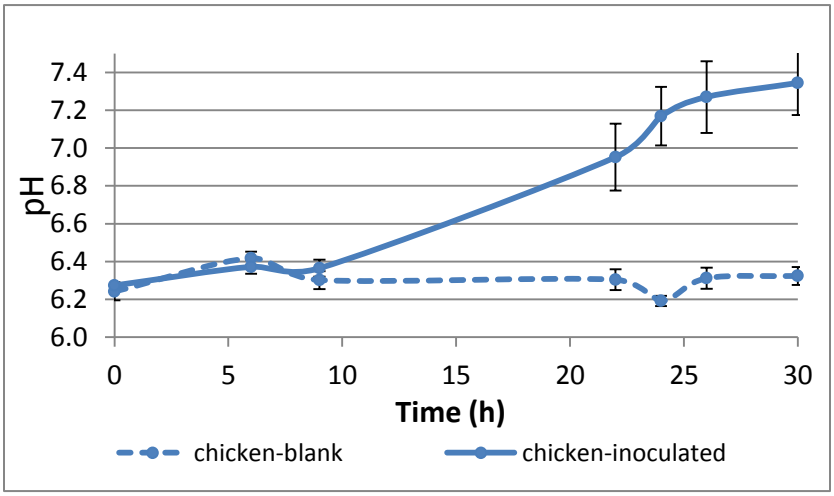


Fig. 3.2. Effect of bacterial growth on the pH of inoculated chicken media during incubation in a Bioscreen unit

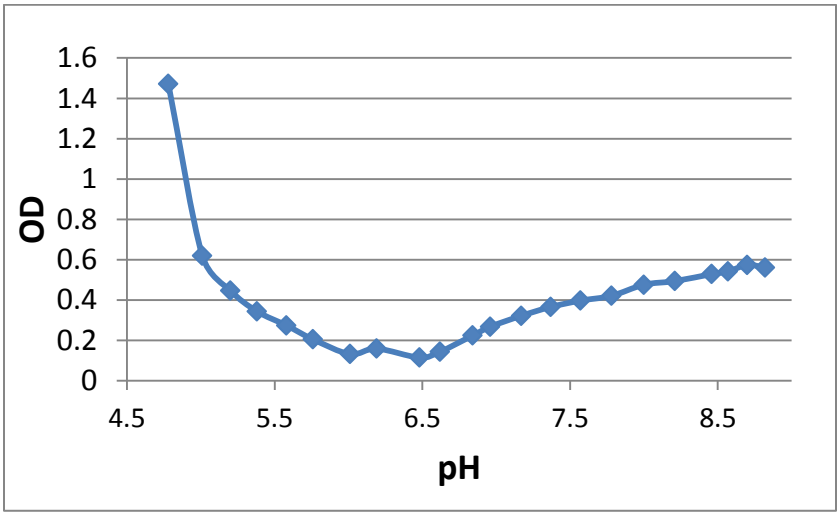


Fig. 3.3. Effect of pH on the OD reading of sterile chicken media

3.3.2. Antimicrobial effect of tested ingredients against chicken spoilage microbiota

It should be reiterated that the strategy used in this study was designed to represent the spoilage microbiota and that each assay potentially had variations in the bacterial composition. In order to reduce the SEM of the results, at least 5 independent repetitions were done with the AS assays for each ingredient.

The growth of the initial microbiota of spoil chicken exudate treated by different antimicrobial agents under different concentrations was analysed for their lag phases (LagT) and maximum growth rates (μ_{max}) (Fig. 3.4). Although AS assays treated by each antimicrobial ingredients were carried out separately, there was no significant difference between the growth parameters of spoilage microbes obtained from the control groups (for LagT: $P = 0.052$; for μ_{max}: $P = 0.207$) (Fig. 3.4), suggesting that 1) our inoculum preparation technique was able to provide active, stable and representative spoilage bacterial population in chicken exudates for AS assays; 2) The nutritional content of the chicken exudate from each trial was relatively stable.

Generally, the extension of LagT linked to the reduction of μ_{max}, either of these two phenomena would suggest an inhibitory effect. Regression analysis carried out between LagT and μ_{max} of all samples showed there was a linear relation between these two parameters ($P \leq 0.001$, $R^2 = 0.56$) Treatments with 20 g/L cranberry pomace, 20 g/L powder of sauerkraut juice, 5, 10 and 20 g/L of green tea extract, as well as 10 and 20 g/L grape seed extract significantly decreased the μ_{max}, while only 20 g/L powder of sauerkraut juice as had significant extension effect on the lag phase. No significant inhibitory effect appeared for the 20 g/L sauerkraut when its pH was adjusted to 6.2 prior to addition to the chicken exudate. This suggests that the efficiency of the 20 g/L sauerkraut treatment was mostly due to its acidity.

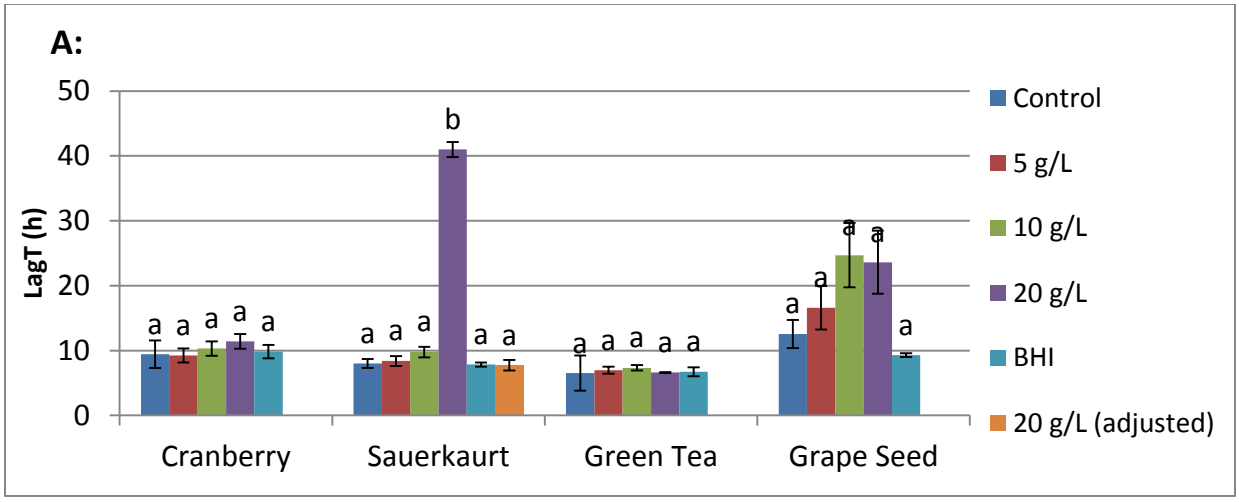
Cranberry pomace and the powder of sauerkraut juice did not show remarkable antimicrobial effect against chicken spoilage microbiota. This was a surprise because, when added to water, these two products have a pH well below 4.0 (Fig. 3.5) which would be strongly inhibitory to a *Pseudomonas*-based microbiota (Gonçalves et al., 2017). However, the chicken exudate had considerable buffering capacity, because the drop in pH was much smaller than in water (Fig. 3.5). The abundant source of amino acids as well as peptides such as anserine and carnosine in the meat media were considered as major contributors to the strong buffering effect (Lea et al., 1969). These data suggest that sauerkraut juice by-product was more effective in slowing the growth of the spoilage microbiota of chicken than the cranberry pomace because of its greater effect on lowering pH of the exudate (Figs. 3.4 and 3.5). In the sauerkraut powder, the concentrations of lactic acid and acetic acid were 30.0 g/100 g and1.1 g/100 g, respectively. Theoretically, chicken media with addition of 20 g/L sauerkraut should contain 0.60% of lactic acid and 0.02% acetic acid. Therefore, it was not surprising that sauerkraut powder only showed

inhibitory effect at 20 g/L, since a pervious study claimed that lactic acid only started to inhibit *P. fluorescens* at a concentration of 0.5% (w/v) (Ouattara et al., 1997). In other studies of the inhibition of *Pseudomonas* based on low-pH, when adjusting the pH of nutrient broth with phosphate buffer from 7.0 to 3.5, which was the pH level of water with addition of 5 g/L cranberry pomace or powder of sauerkraut juice (Fig. 3.5), nearly 70% of the bacterial cells would be inhibited (Oladipo et al., 2010).

It was hoped that antimicrobials other than the organic acids would exert an effect. Indeed, cranberry is an abundant source of bound benzoic and phenolic acids, as well as polyphenols such as proanthocyandins (PAC) (Pappas and Schaich, 2009). According to the literature, cranberry products including the juice, concentrates, as well as bioactive fractions derived from cranberries were proved to have antimicrobial effect against both gram positive and gram negative pathogens by disc diffusion assays and broth dilution method (Caillet et al., 2012; Harich et al., 2017; Vattem et al., 2004; Wu et al., 2008). Data from the pH-adjusted media (Fig. 3.4) suggest that the concentrations in these cranberry antimicrobials were not high enough, even at 20 g/L pomace addition, to affect the psychrotrophic chicken spoilage microbiota. The contents of proanthocyanidins (PAC) in cranberry pomace applied in this study was 2.6% (w/w), meaning that there would be no more than 0.05% (w/w) of PAC in the chicken media with addition of 20 g/L cranberry pomace powder. A study applying cranberry extract containing 210 mg/g PAC failed to detect antimicrobial effect against *E.coli* at 20 mg/mL, although there was theoretically 0.42% (w/v) PAC in the broth (LaPlante et al., 2012). Thus the absence of effect of the cranberry pomace was linked to its insufficient composition in organic acids and phenols. Presumably, extracts and concentrates of the cranberry antimicrobial could be more efficient.

Ideally, inocula prepared for each AS repetition would have been characterized for the bacterial composition, as well as the microbiota of products submitted to the antimicrobials. This would have enabled the identification of the species affected by the antimicrobials. Unfortunately this was not possible with the resources available for the project and in the required timeframe. Further studies are required on this aspect. As a result, assumptions on bacterial species involved must be made based on the literature. Assuming that the major psychrotrophic spoilage microbiota of chicken is *Pseudomonas*-based, the inhibitory effects of plant extracts (extract of green tea and grape seed) were also weaker than previous studies

indicated. Broth dilution assays carried out in nutrient broth supplied with green tea extract showed that complete inhibitory effect against *P. fluorescens* at 1 mg/mL, but 50 mg/mL against *P. aeruginosa* (Chou et al., 1999; Rao et al., 2014). On the other hand, agar dilution assays showing that grape seed extract had minimum inhibitory concentrations (MICs) against gram-positive and gram-negative pathogens at 850-1000 ppm and 1250-1500 ppm, respectively (Jayaprakasha et al., 2003). The lower antimicrobial effects of plant extracts compared with the literatures might be also explained by the interaction between antimicrobial components and food matrix. It was well evidenced that the polyphenols would combine with amino acids chains from proteins and form colloidal size compounds or even precipitates, reducing the actual concentration of active phenolic compounds in the chicken exudate and weakening the antimicrobial activities (Charlton et al., 2002; Von Staszewski et al., 2011). Previous research compared the antimicrobial effect of green tea extract under a certain concentration against *L. innocua*, in the media supplied with whey protein at different concentrations as a model food matrix rich in proteins, showing that higher protein content indeed leaded to the loss of antimicrobial effect of green tea (Von Staszewski et al., 2011). Therefore, it was reasonable that in studies applying standard medium broth with lower contents of proteins would have less interference with antimicrobial effects of plant extracts.



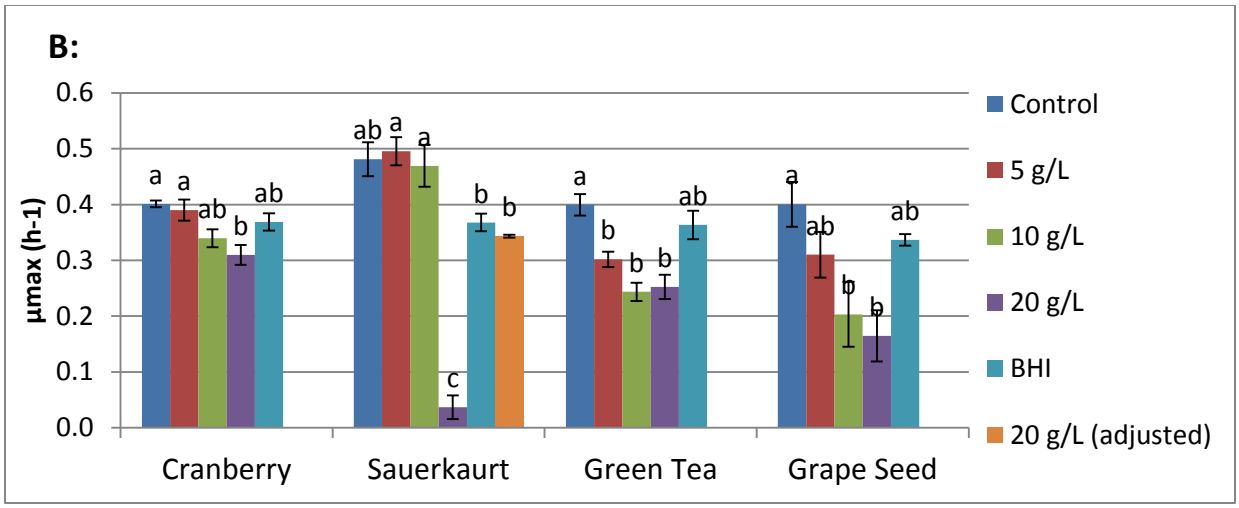


Fig. 3.4. Effect of the addition of the powder of cranberry pomace, sauerkraut juice, green tea and grape seed to chicken media on the lag time (A) and the maximum growth rate (B) of the spoilage microbiota from chicken exudates

Note: The ANOVAs were carried out separately for each ingredient. Within a given antimicrobial ingredient, bar columns headed with the same letter indicate that the data were not significantly different (P > 0.05); Column "20 g/L (adjusted)" represents a treatment where the pH of the sample after addition of the antimicrobial ingredient was adjusted to the level of original media after the addition of 20 g/L ingredients (only applicable with sauerkraut); LagT, lag time; μ_{max}, maximum growth rate.

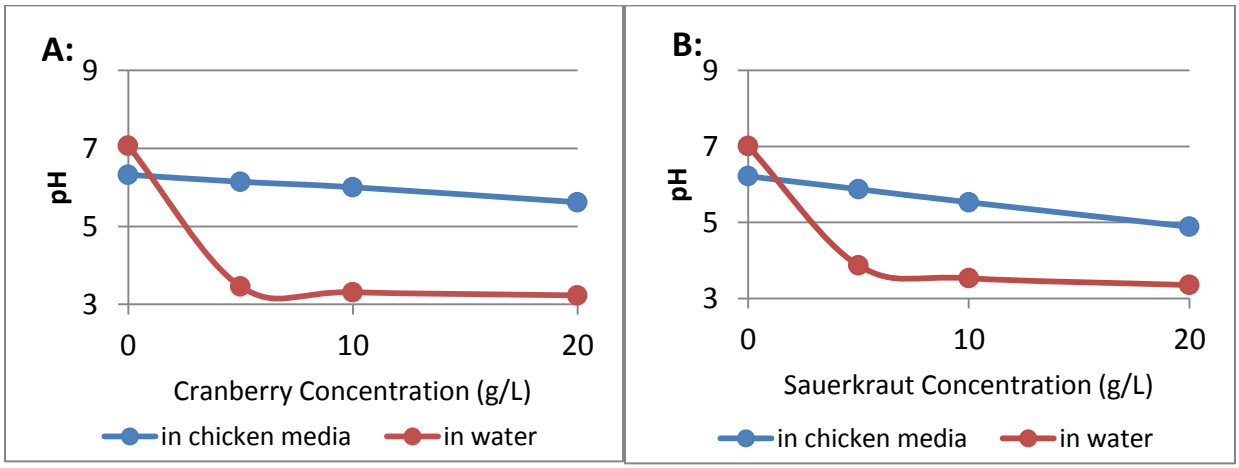


Fig. 3.5. Effect of the addition of cranberry pomace (A) and sauerkraut (B) in chicken media and in water

3.3.3. Correlation between optical density (OD) and total psychrotroph counts (TPC)

The AS methodology rapidly enabled the examination of many antimicrobials and ascertain the effect of concentration. However, optical density is an indirect indicator of cell density in the broth. Thus, to confirm whether the OD data correctly predicted the development of the spoilage microorganisms, classical growth curves from the plate count method were conducted, and the correlation between OD and CFU data were evaluated. It should also be kept in mind that the AS methodology used an incubation temperature of 17°C, while the CFU data were obtained following incubations at 7°C. The 17°C incubation temperature was chosen for the AS assays, because it is a recommended temperature to accelerate analyses of psychrotrophs (APHA, 2001). The 7°C incubation in plate count assays is a standard condition for psychrotrophs (APHA, 2001).

The growth curves of spoilage microbiota treated by two antimicrobial agents based on CFU and OD, as examples are seen in Fig. 3.6. The curves had similar trends, and suggest that CFU data would be in line with OD data. However, good correlations between OD and CFU were essential to obtain good estimation of growth parameters (Francois et al., 2005).

In this study, either lag phase or μ_{max} were inappropriate as parameters for regression analysis between CFU and OD, since the sampling of CFU was not frequent enough to provide precise LagT and μ_{max}. Thus, the average growth rate (μ_{avg}) was used to compare the data between the two techniques. The μ_{avg} could describe the combined action of lag phase and μ_{max} within a certain range of time. For plate count assays, the μ_{avg} was calculated based CFUs between day 0 and day 3, while for AS assays the μ_{avg} was defined as the growth rate from the end of the lag phase to the end of log phase, because in the early stage of the lag phase, the real bacterial growth would not promote to the increase of OD reading until the total bacterial count in the broth reached the detection threshold of an automated spectrophotometer (approximately 10^7 CFU/mL with the OD around 0.2) (Begot et al., 1996). The regression analysis showed that there was a significant linear regression between the μ_{avg} obtained from OD and the one from CFU ($P = 0.007$, $R^2 = 0.87$). The correlation between OD and CFU was good even though 1) different incubation temperatures were used in AS and CFU assays and 2) different principles of biomass determination between the two assays.

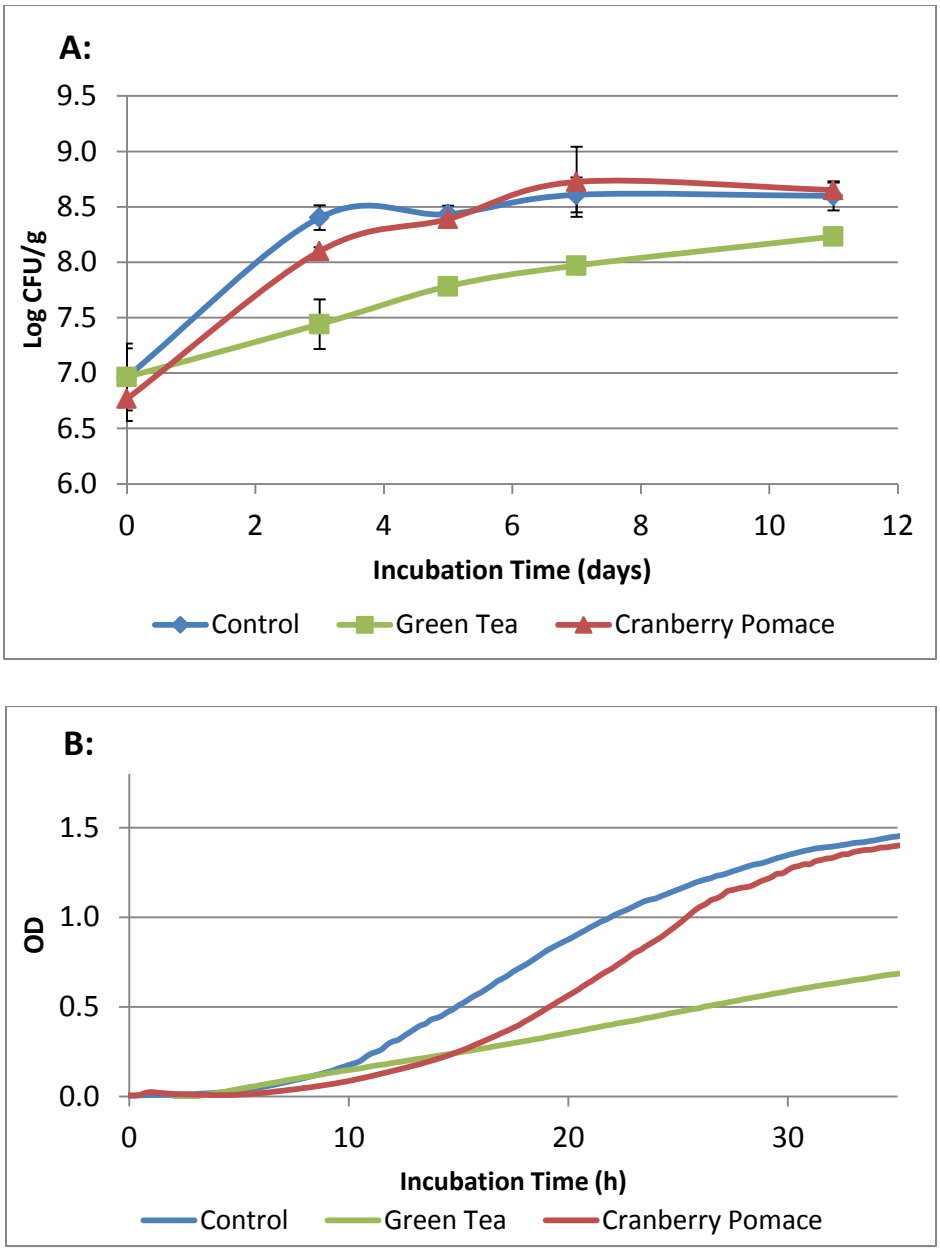


Fig. 3.6. Growth curves of spoilage microbiota in chicken media with addition of 20 g/L green tea extract or 20 g/L cranberry pomace based on CFU (A) and OD (B)

3.3.4. Effectiveness of absorbent pads with addition of selected ingredients

According to the lag phase and μ_{max} of spoilage microbiota obtained from AS assays, the powder of sauerkraut juice at 20 g/L as well as grape seed extract at 10 and 20 g/L showed significant antimicrobial effect. These formula needed to be tested in absorbent pads, to confirm their efficiency in antimicrobial packaging.

Growth of the psychrotrophs in the pads (Fig. 3.7) differed from that in the test-tubes (Fig. 3.1). During the first 72-hour incubation, the mean μ_{avg} of spoilage microbiota from raw exudate obtained in tubes (Fig. 3.1) and the one obtained in mason jars (Fig. 3.7) were 0.074 (h^{-1}) and 0.114 (h^{-1}), respectively. An unpaired t-test suggested the difference of the μ_{avg} between the two assays were significant (P = 0.040). Furthermore, the total viable counts reached in the pads were 10 times higher than those in the broths (Figs. 3.6 & 3.7). The higher growth rates of psychrotrophs in the pads could be partly explained by the more "aerobic" condition in pad assays. The growth of *Pseudomonas* could be affected by the surrounding atmosphere, more aerobic condition could remarkably promote the growth rate of *Pseudomonas* (Liao and Blackburn, 2006). Compared with tests carried out in tubes (CFU) or in wells of Bioscreen microplates (AS), assays carried out in pads allowed a greater exposure of the chicken medium to the air, thus favoring growth faster, due to the larger surface area of pads and the larger base area of the Mason jar.

No inhibitory effect on growth was observed in pads containing the grape seed extract at both concentrations, although in AS assays grape seed at either 10 or 20 g/L reduce the growth rates of the spoilage population (Fig. 3.4 & Fig. 3.7A). In the meantime, sauerkraut at 20 g/L only performed up to 1.0 log cycle reduction at the early stage of spoilage in pad assays, while the same concentration applied in AS assays showed complete inhibitory effect (Fig. 3.4 & Fig. 3.7B).

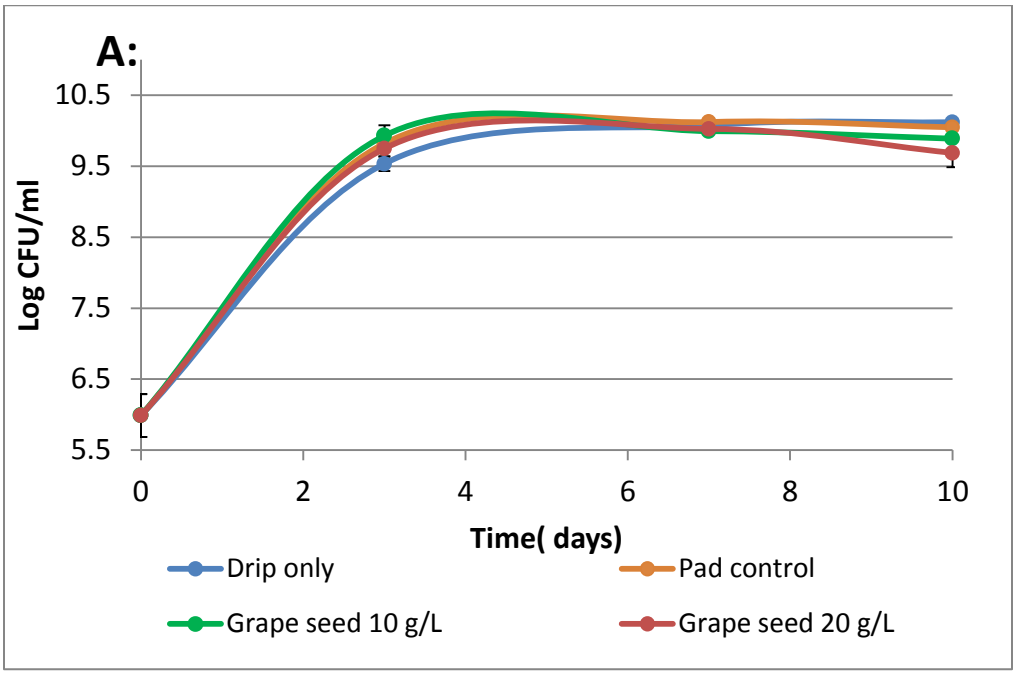
Attempts were made to determine the reasons between the results in pads and those in the AS/CFU studies. It was first examined if the tested ingredients showed weaker effects in pads due to an uneven distribution of the bioactive compounds in pads. Bromocresol purple (BCP), as a pH-indicator between pH level of 5.2 (yellow) and 6.8 (purple), was added in the chicken media added to the pads in order to visually assess the distribution of organic acids from the sauerkraut powder particles based on the color change. The addition of 20 g/L sauerkraut powder to the chicken exudate resulted in a drop of pH to 5.0 (Fig. 3.5B). Thus, the exudate in the pad should initially be yellow (Fig. 3.8G). However, Fig 3.8 shows that the acids from the sauerkraut powder were released gradually in the exudate. Moreover, the exudate never became completely yellow as in the control at pH 5.0. Thus, at the beginning of the incubation, only a fraction of the cells in the exudate were exposed to the acid conditions in the pads. According to

the results from AS assays, the sauerkraut powder only exerted an antimicrobial effect at 20 g/L with an approximate pH level of 5 (Figs. 3.4 & 3.5), suggesting that when the chicken exudate with addition of bromocresol purple was absorbed by the pad, only the area in yellow contained sauerkraut powder of which concentration was high enough to show inhibitory effect. The sauerkraut powder was evenly distributed on the pad and the diameters of sauerkraut particles were acceptably small but big agglomerates still existed (Fig. 3.8A). During the first 6 h, the active compound only dissolved in exudates close to the sauerkraut powder particles (Fig. 3.8. B & C). After 24 h of incubation, the sauerkraut powder gradually dissolved evenly in the exudate based on the more homogenous color of the pads (Fig. 3.8. D & E), however, the overall color of the pads did not appear as complete yellow, suggesting that even at day 3, the actual concentration of sauerkraut powder failed to reach the same level as AS assays (20 g/L).

To ascertain if the uneven distribution of sauerkraut ingredients in pads caused the lesser antimicrobial activity, an additional treatment in pads was carried out by pre-dissolving the sauerkraut powder in chicken exudate which was subsequently added into the absorbent pads. These additional pad assays had the same aerobic condition as those in which the ingredient was inside the pads. A much greater inhibition of the spoilage microbiota was noted when the sauerkraut was pre-dissolved in the exudate (Fig. 3.7B). These observations suggested that the uneven distribution of sauerkraut in the chicken exudate at the beginning of incubation only inhibited the microbiota surrounding the sauerkraut particles, while the bacteria presence in other areas could still grow at a high growth rates and generate alkaline metabolites (Fig. 3.8. D & E & F). Although the organic acid would distribute gradually during incubation, BCP/pH observations suggest that bacteria away from the sauerkraut particles had already generated enough alkaline metabolites to neutralize the organic acids from sauerkraut and reduce the overall antimicrobial effect. A possible solution for pad preparation could be to evenly distribute the sauerkraut juice in the absorbent pads by spraying it on the pads and then drying them at room temperature (Silva et al., 2018). However, it was important to avoid contaminating the pad during dying procedure for the plate count assays.

The effectiveness of antimicrobial absorbent pads could also depend on the volume of exudate lost during storage and the absorbency of the pad. In this study, the pad was selected to absorb the exudate volume but allow its distribution throughout. However, it can be

hypothesized that the exudate would not have spread evenly in the pad if it had a much greater absorbency level. Again, this would result in incomplete dissolution of the ingredients in the pad into the exudate. Studies applying antimicrobial absorbent pads to delay the spoilage of fresh meat and poultry showed remarkable effectiveness at the early stage of storage (Fernández et al., 2010b; Silva et al., 2018; Wang et al., 2013). In this instance it could be hypothesized that, since the drip loss level increases during incubation, if the absorbency of the pads was not strong enough so that the small volume of exudate could diffuse all over the pad, the real concentration of bioactive ingredient would be extremely high at the beginning of storage (Wang et al., 2013). In this case, the population of initial microbiota would be controlled at the early stage, generating less alkaline metabolites during their growth and delay the neutralization of organic acids from sauerkraut. Meanwhile, for grape seed extract, since polyphenols appeared to be more stable in acidic conditions, the delay of the pH increase at the early stage of incubation would maintain the bioactivities of polyphenols from grape seed and enhance their effectiveness (Gutierrez et al., 2009; Yoshino et al., 1999).



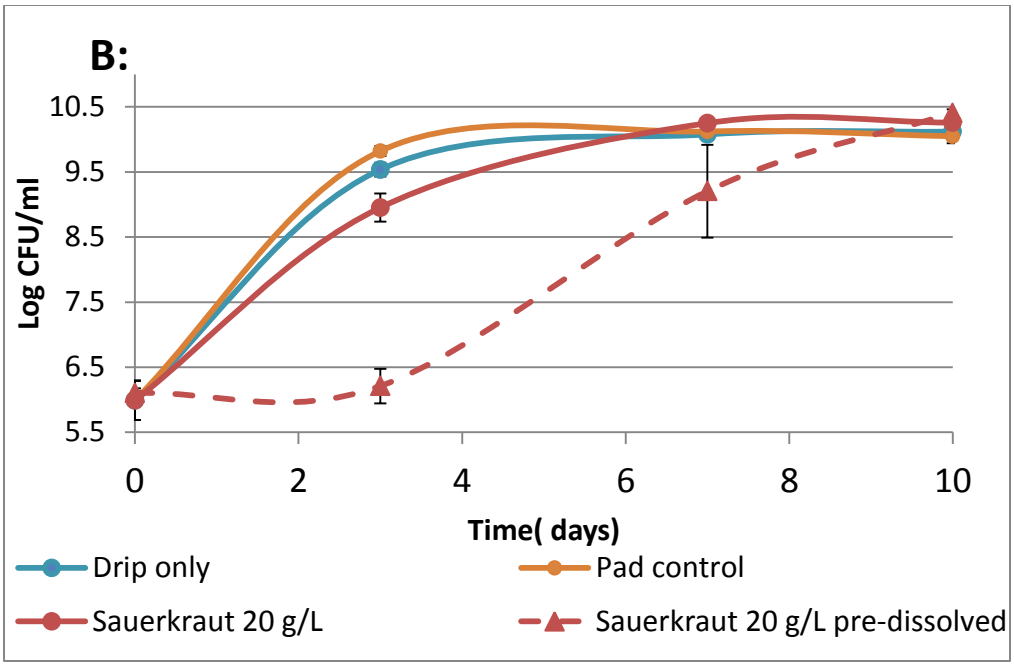


Fig. 3.7. Effect of grape seed extract (A) and sauerkraut juice powder (B) on the spoilage microbiota of chicken exduate in absorbent pads

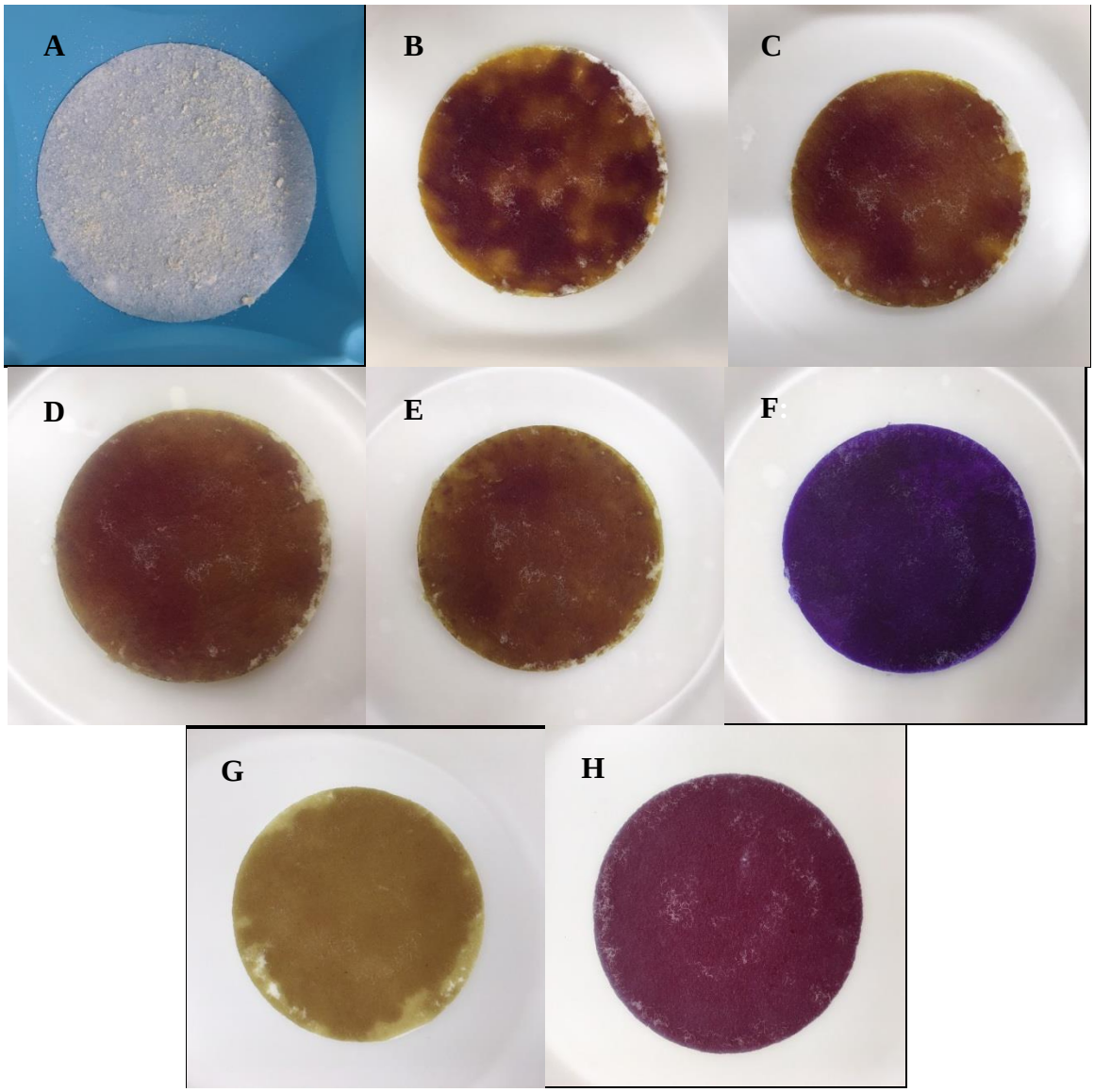


Fig. 3.8. Distribution of sauerkraut powder in chicken exudate on absorbent pads at 0 h before exudate addition (A), with exudate addition at 1 h (B), 6 h (C), 24 h (D), 72 h (E), 120 h (F), as well as two controls, raw exudates with bromcresol purple, with (G and without (H) 20 g/L sauerkraut at 7°C

3.4. Conclusion

This study applied a novel methodology to evaluate water-soluble natural antimicrobial ingredients in the real exudate system under variable concentrations against the initial microbiota from refrigerated chicken. Techniques of preparing representative inoculum of the real spoilage population as well as media based on chicken exudate for automated spectrophotometry (AS) assays were developed. The great correlation between readings of optical density (OD) from AS

assays and the total psychrotroph counts (TPC) was confirmed, suggesting this *in vitro* method carried out in a meat-based system was able to provide reliable biomass data of the spoilage population. The average growth rate (μ_{avg}) during the early storage period was the best growth parameter to ascertain the correlation between the OD reading and the real bacterial counts. In the meantime, the AS assays were able to provide additional parameters such as the lag phase (LagT) and the maximum growth rate (μ_{max}), which were useful for ascertaining the minimum effective concentration of each tested ingredient.

In vitro assays carried out in the exudate-based broth also suggested that the antimicrobial effects of acidic ingredients could be highly reduced by the strong buffering capacity of the chicken exudate. These observations also confirmed the importance of applying representative media based on the chicken exudate instead of using standard laboratory medium in order to avoid over-estimating the antimicrobial effects against meat spoilage.

Finally, the effectiveness of ingredients as well as their concentrations recommended by *in vitro* assays was re-examined by enumerating the microbial loads in absorbent pads. However, formula selected based on data from *in vitro* assays showed reduced antimicrobial effect when being applied *in situ*, which could be explained by 1) the more aerobic condition above the absorbent pads than in the tubes; 2) the slow and uneven distribution of active ingredients in the exudate absorbed by the pads. Thus, industrial tests were highly required by enumerating the microbial loads in the real packed chicken applying absorbent pads with addition of each ingredient under the effective concentration. Meanwhile, to ensure the active ingredient could distribute evenly in the pads, it might be interesting to improve the pad preparation technique by injecting the pad with the solution of selected antimicrobial agent at a certain concentration, followed by drying at room temperature.

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CONNECTING TEXT 2

The previous chapter selected the appropriate antimicrobial agents and ascertained their minimum effective concentration against the spoilage microbiota of chicken exudates under refrigeration. The same potential activities carried out for spoiled sausage would be discussed in the following chapter.

CHAPTER 4. ANTIMICROBIAL ACTIVITIES OF FOOD BY-PRODUCTS AND PLANT EXTRACTS AGAINST SPOILAGE MICROBIOTA FROM FRESH ITALIAN SAUSAGE

Abstract

Extracts of green tea, grape seed and blueberry, as well as by-products from food manufacture (cranberry pomace, powder of sauerkraut juice) were used as natural food preservatives against spoilage microflora of Italian sausage stored under modified atmosphere packages (MAP). The evaluation of their antimicrobial effects was carried out *in vitro* by inoculating the initial spoilage microflora to a media based on sausage homogenates supplied with each antimicrobial ingredient up to 20 g/L, followed by frequent measurements of optical density (OD) using an automated spectrophotometer (AS). The efficacy of the natural ingredients was identified on the basis of growth parameters of the spoilage population including the lag time (LagT) and the maximum growth rate (μ_{max}). Plate count assays were only carried out for samples with 20 g/L of each ingredient, in order to confirm the reliability of the OD data. Based on the data of LagT and μ_{max}, extracts of grape seed and blueberry were the most effective ingredients, the antimicrobial effects of which appeared to start from 5 g/L. Meanwhile, the minimum effective concentrations of green tea extract, powder of sauerkraut juice and cranberry pomace were 10, 10, and 20 g/L, respectively.

4.1. Introduction

Fresh Italian pork sausage is highly perishable due to its high water activity, high protein content combined with a non-inhibit pH value against spoilage microorganisms. Refrigeration coupled with modified atmosphere packaging (MAP) is widely applied to delay the growth of and nature of bacterial species of the spoilage microbiota (Gill, 2003; Torrieri et al., 2011). Some facultative anaerobic or microaerophilic gram-positive bacteria such as psychrotrophic lactic acid bacteria (LAB) and *Brochothrix thermospacta*, which are more tolerant to not only the high CO_2 content in MAP, but also the high salt content in sausage, would overgrow the strictly aerobic species such as pseudomonads during spoilage (Ercolini et al., 2006; Gill, 2003; Stanborough et al., 2017a). Research on meat stored under MAP with high O_2 and high CO_2 contents stimulated the dominance of *B. thermospacta* as well as LABs such as *Lactobacillus sakei, Lactobacillus curvatus, Leuconostoc gelidum* and *Carnobacterium* spp. as spoilage

indicators leading to off-odors (Ercolini et al., 2006; Gill and Harrison, 1989; Hammes and Hertel, 2006; Vihavainen and Björkroth, 2007).

To extend the shelf life of refrigerated Italian sausage under MAP, the addition of natural antimicrobial ingredients was ascertained. Two reasons justified this study. First, the use of natural ingredients is preferred by consumers as clean-label food preservatives (Fernández et al., 2018; Grant and Parveen, 2017). Second, direct addition of those ingredients to the minced meat during manufacture could inhibit bacteria not only from the exterior surfaces, but also from the inside of the product. The direct addition of natural antimicrobials has proved effective in several meat products including fresh pork sausages, overwrapped minced beef and pork patties (Formanek et al., 2003; Kumar et al., 2015; Martínez et al., 2006). However, little is known of the potential by-products from food manufacture as well as some plant extracts as antimicrobial agents to be applied in sausage stored under MAP.

In this project, the powder-form extracts of green tea, grape seed and blueberry were chosen due to their abundant phenolic compounds (Das et al., 2017; Perumalla and Hettiarachchy, 2011). The inhibitory effects of these three plant extracts against *Lactobacillus* spp. vary as a function of strain and methodology (Ankolekar et al., 2011; Chung et al., 1998; Horiba et al., 1991; Moradi et al., 2011; Tahir and Moeen, 2011), while the antimicrobial effects of grape seed and berry extracts against *B. thermospacta* seems established (Alakomi et al., 2017; Corrales et al., 2009). All of the plant extracts tested in this study showed notable inhibitory effects against major Gram positive pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (Lacombe et al., 2013; Mbata et al., 2008; Radji et al., 2013). Thus, it is hoped that Gram positive spoilage organisms, are still sensitive to the membrane damage caused by phenolic compounds even though they have a lower content of lipopolysaccharides (LPS) in their outer membrane compared with Gram negative bacteria (Burt, 2004; Ikigai et al., 1993).

Cranberry pomace and powder of sauerkraut juice, as food by-products, were also tested due to their abundant organic acids (Hong and Wrolstad, 1986; Hutkins, 2006b; Van Immerseel et al., 2006). Gram positive meatborne bacteria such as *B. thermosphacta, Carnobacterium piscicola* were sensitive to several organic acids, while lactic acid bacteria appear to be more resistant to low pH conditions, but could still be affected when the acid reached a critical concentration (Ouattara et al., 1997).

The goal of this study was to test the antimicrobial effects of the various ingredients against a "representative" spoilage microbiota, in order to ascertain their potential to increase storage time. It was decided to inoculate the initial microbiota extracted from spoiled sausage samples to a clarified meat-based media to make the best represent of the spoilage population, rather than inoculate the media with pure cultures of psychrotrophic bacteria. The effective concentration of each antimicrobial agent was ascertained based on overall growth parameters calculated from optical density (OD) data.

4.2. Materials and methods

4.2.1. Antimicrobial ingredients

The antimicrobial agents tested in this study were powder of cranberry pomace (ATOKA Cranberries Inc., Manseau, QC, Canada), powder of green tea extract (New Directions Aromatics Inc., Mississauga, ON, Canada), powder of grape seed extract (New Directions Aromatics Inc.), powder of blueberry extract (Dianafood, Champlain, QC, Canada) and the freeze dried powder of sauerkraut juice (Caldwell Bio Fermentation Canada Inc., Ste-Edwidge, QC, Canada).

The lacto fermented sauerkraut juice (Caldwell Bio-fermentation) was poured into a stainless steel pan placed in a freeze-dryer (FTS Systems Dura-Dry, SP Scientific, Warminster, PA, USA), with the tablet temperature pre-set at -40°C. Two freezing steps were applied as: 300 min holding time at -40ºC, followed by 180 min at -30°C. The drying process was then followed by the parameters shown in Table 4.1. The lyophilized cake from the drying process was processed into a powder and kept in conical tube under vacuum at 4°C.

Table 4.1. Parameters of freeze-drying as the function of the drying time

Temperature raised (°C/min)	Duration (h)	Tablet Temperature (°C)	Vacuum applied (mTorr)
2	48	-30	150
2	16	-15	150
2	16	0	75
2	16	15	75
2	24	20	75

4.2.2. Analysis of bioactive compounds from powder of sauerkraut juice

The freeze dried powder of sauerkraut juice was first diluted at a concentration of 31.4 g/L in the mobile phase, passed on a SepPak C18 plus cartridge (Waters) for the cleaning process and then filtered with a 0.45 µm filter before injected into a HPLC unit. Injection (20 µL) was carried out with automatic injectors (Waters 717 plus, Waters, Milford, MA, USA). The organic acids from sauerkraut juice were analysed by HPLC on an ION-300 column (Concise separations, San Jose, California, USA) coupled with a pre-column of the same type, heated at 65°C, with 0.0085N sulphuric acid at 0.4 mL/min as mobile phase. Detection of organic acids was carried out by a UV detector (Waters 2489, Waters) at 208 nm. Empower 2 software (Waters) was used for integration.

4.2.3. Medium preparation for automated spectrophotometry

The initial microbiota of the sausage was of 4×10^4 CFU/mL on the average and, as error bars of SEM show, it was surprisingly constant within the 3 repetitions (Fig. 4.1). This initial microbiota was representative of the bacterial contamination of the sausage, but not necessarily of the spoilage microbiota of the refrigerated product. To obtain this representative psychrotrophic spoilage microbiota, it was therefore decided to incubate the sausage at 7°C to generate the inoculum for the AS assays. A population of 1×10^9 CFU/g was obtained between 9 and 14 days of incubation at 7°C (Fig. 4.1). Ideally, the composition in bacterial species of these inocula would have been ascertained, and this should be done in further studies.

In order to carry out the AS study in a "representative condition" of microbial sausage spoilage, the medium used in AS assays were obtained from sausage homogenates. Since sausage samples barely have "drip-lost" during storage, it was therefore decided to blend the Italian sausage sample (Olymel, Boucherville, QC, Canada) with a small quantity of water and generate a liquid meat suspension. This sausage homogenate was prepared by mixing the fresh sausage sample with cold deionized water at a ratio of 4:3 by weight in a Stomacher strainer bag, equipped with a filter (Fisher Scientific, Canada), and to homogenize in a Seward 400 circulator (Worthing, West Sussex, UK) at 230 rpm for 60 s (Chaillou et al., 2014). The sausage homogenate was then collected and centrifuged at 30 000 $\times g$ for 20 min at 25°C (Beckman centrifuge model J-20 XPI, Rotor JA-18, Palo Alto, CA, USA). The pH of sausage homogenate

supernatant was first adjusted to 4.3 by 40% lactic acid. The acidified sausage homogenate was then centrifuged at 30 000 ×*g* for 20 min at 25°C (Beckman centrifuge model J-20 XPI, Rotor JA-18, Palo Alto, CA, USA) to remove chemical precipitates formed during acidification. The supernatants were then neutralized with 2N KOH to adjust its pH back to that of original sausage homogenate (pH=5.9 ± 0.1). Since the alkalization would also result in precipitation, one more centrifugation was carried out with the same parameter at 30 000 ×*g* for 20 min at 25°C.

After obtaining the clarified sausage homogenate, the powder of antimicrobial ingredients of interests were dissolved in the homogenate to obtain serial concentrations of 5, 10 or 20 g/L, respectively. An extra tube of sausage homogenate without addition of any ingredients was prepared as the control group. The sausage homogenate supplied with these ingredients was then vortexed for 5 s at 2500 rpm by a digital vortex mixer (Fisher, USA). Afterwards, all samples including the control were kept under 4°C for more than 12 h while being agitated via a Labnet Labroller (H5100, Woodbridge, NJ, USA) at 25 rpm to ensure the complete dissolution of the water-soluble active components from the powder. The sausage homogenate was then centrifuged again at 30 000 ×*g* for 20 min at 25°C (Beckman centrifuge model J-20 XPI, Rotor JA-18, Palo Alto, CA, USA) to remove not only the insoluble part of the antimicrobial ingredients but also most microorganisms in the media. The supernatants from the last centrifugation were finally filter-sterilized via 0.45 µm pore-size membranes.

4.2.4. Inoculum preparation

Sausages were kept in their original commercial MAP units, having high contents of O_2 and CO_2 (proprietary concentrations) at 7°C for 10-14 days. The viable counts eventually reached 10^9 CFU/g (Fig. 4.1). As previously stated, sausage samples barely produced exudates. Inoculum preparation therefore required to prepare a spoiled sausage microbiota homogenate by adding 25 g of spoiled sausage to 225 mL sterile 1 g/L peptone water (Becton, Dikinson and Company Sparks, USA) in a Stomacher strainer bag, and then homogenizing via a Stomacher unit at 230 rpm for 60 s (Chaillou et al., 2014). The sausage homogenate was finally centrifuged at 500 ×*g* for 10 min at 5°C (Beckman centrifuge model GS-6R, Palo Alto, CA, USA) followed by filtration via 10 µm filters for the clearance required in automate spectrometry (AS) assays. Preliminary data showed that the 500 ×*g* centrifugation and 10 µm filtration steps enabled partial clarification of the inoculum while not affecting the CFU count.

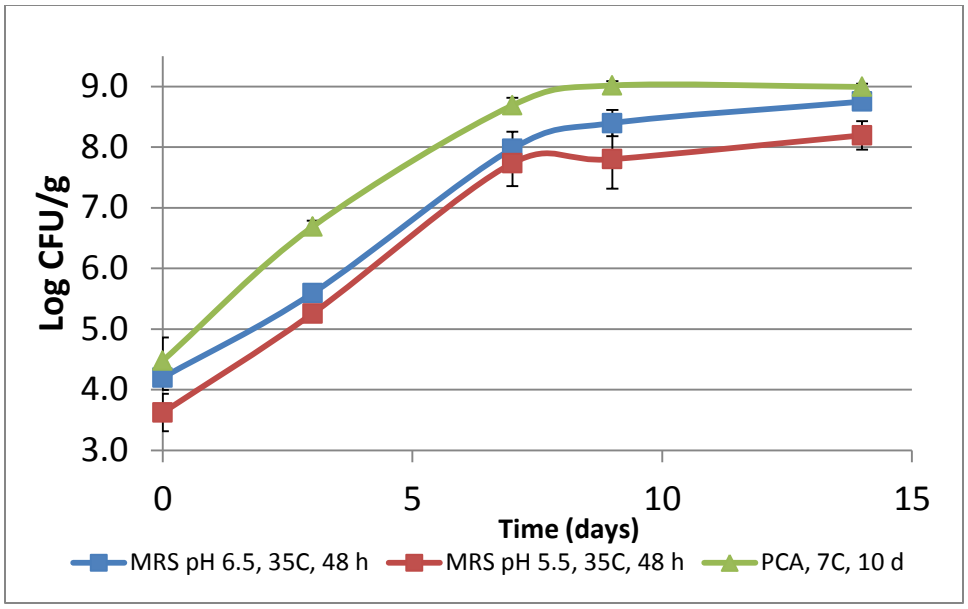

Fig. 4.1. Growth curve of sausage microbiota incubated at 7°C, enumerated by 3 different plate count methods: spread plate on 1) PCA, incubated at 7°C for 10 days; 2) regular MRS agar (pH 6.5), incubated at 35°C for 48 h; 3) acidified MRS agar (pH 5.5), incubated at 35°C for 48 h

4.2.5. Screening of antimicrobial effects *in vitro* by automated spectrophotometry method (AS)

Clarified and sterilized sausage homogenates referred as the "sausage media" with addition of antimicrobial agents was injected into a Bioscreen microplate (Honeycomb, Labsystems, Helsinki, Finland) with a volume of 180 µL. For assays testing antimicrobial effects of cranberry pomace, powder of sauerkraut juice and green tea extract, each well was then inoculated with 20 µL of sausage inoculum containing approximately 5×10^7 CFU/g of spoilage microbe, in order to yield approximately 5×10^6 CFU/g spoilage population at the beginning of incubation. For assays testing grape seed extract and blueberry extract, since their interaction with proteins presented in the inoculum resulted in serious chemical precipitation, inoculum prepared for these assays were further diluted 10 folds with sterilized 8.5 g/L NaCl solution before inoculation. Additionally, non-inoculated filter-sterilized sausage media supplied with tested ingredients was also placed into the microplate wells with a volume of 200 µL each well as negative controls, while inoculated MRS broth was used as the positive control to ensure the inoculum was viable. The microplate was incubated in a Bioscreen C unit (Thermo Labsystems, Espoo, Finland) at 17°C for 42 h (cranberry, sauerkraut and green tea) or 60 h (grape seed and

blueberry), as an accelerated approach to simulate the growth condition required for psychtrophilic spoilage species. Optical density (OD) was measured at 600 nm every 15 min after shaking the microplate at "extra intensive" level during the whole incubation. Screening of inhibitory effect of each ingredient through AS method was carried out five replicates at least.

4.2.6. Assays based on traditional plate counts

Total viable counts (TVC) of the spoilage microbiota from sausage were achieved by plate count method, to evaluate the correlations between OD and bacterial biomass. These plate count assays were carried out applying tested ingredients at the highest concentration (20 g/L) at 7°C, in order to ensure the antimicrobial effects reported from AS assays and the applicability of the antimicrobials under real refrigeration. . The refrigeration temperature was chosen as 7ºC rather than 4ºC because the previous one could simulate the realistic thermal abuse throughout the cold chain (Raimondi et al., 2018). For the plate count assays, each 27 mL of sterile sausage media filtered by 0.45 µm filter but without adjusting pH in advance, was supplied with each antimicrobial agent at the highest tested concentration as well as the control (media without addition of any antimicrobial agents) and then inoculated with 3 mL of clarified inoculum and incubated under modified atmosphere (16% CO_2, 1% O_2 and 83% N_2) created by a BD GasPak^{TM} EZ anaerobe container system (Becton, Dickson and Company, MD, USA) at 7°C. Samples for spread plating were collected at the incubation time of 0, 3, 5, 7 and 10 days, followed by subsequent dilution with 1 g/L sterile peptone water. An intensive homogenization was required for all first 10-fold diluted samples via a homogenization probe (Omni International, Marietta, GA, USA) at 27000 rpm for 30 s in order to break down the link between cells. Samples diluted in appropriate folds were then spread on MRS agar, incubated at 35°C for 48 h aerobically. All plate count assays were carried out in triplicates.

4.2.7. pH measurement

pH-time curves were made for the control group (sausage media without addition of antimicrobials) during the incubation in a Bioscreen unit at 17°C for the first 22 h. The pH of the inoculated as well as the non-inoculated chicken media was measured by a pH meter (Fisher Scientific, Singapore) at 0, 14, 16, 20 and 22, respectively. The pH meter was equipped with the pH MicroProbe (Accumet) for small volumes.

4.2.8. Kinetic analysis for spoilage microbiota

The mathematical analysis of the growth parameters were carried out by Microsoft Excel 2010. In AS assays, recalculated OD data, which were OD values of the inoculated media minus the ODs of the blank media at each OD measuring time, were used for the calculation of growth parameters.

The lag phase (LagT) was defined as the period from time 0 to the ΔOD ($OD_{at\ real\ time}$-$OD_{t=0}$) exceeded 0.075 during incubation, while the maximum growth rate (μ_{max}) was collected as the maximum growth rate during the early exponential growth, which was the period from the end of the lag phase to the time when OD reached 0.5. The reason of defining LagT and μ_{max} this way was that, the AS assays was carried out in meat-based media inoculated with a complex bacterial population from the spoiled product. The contents of meat media were complicated and uncertain, which might degrade or precipitate and lead to the fluctuation of OD during incubation. Some previous studies also considered the lag time as the beginning of incubation to the OD reached a reasonable level such as 0.05 (Champagne et al, 2014; Schirmer and Langsrud, 2010). Calculation of μ_{max} was carried out based on OD data at the early stage of log phase in order to avoid the interference precipitation of meat media mainly caused by bacterial metabolism. Specifically, lactic acid bacteria, as one of the dominant spoilage species of sausage samples applied in this project, would decrease the pH, destabilize and precipitate the proteins presence in the sausage media. These protein precipitates also had absorbency at 600 nm and affect the OD readings. To avoid this, studies had evaluated the μ_{max} it reached before the early stage of exponential phase to describe the growth of bacteria (Champagne et al., 2014; Champagne et al., 2009).

The average growth rate (μ_{avg}) was calculated for both AS assays and plate count assays to analyse the correlation between OD and CFU. μ_{avg} during the first 3 days of incubation was calculated for all plate count assays. In the meantime, μ_{avg} from the end of the lag phase to the end of exponential phase was calculated to have comparable data with the one from plate counts.

4.2.9. Microbiological analyses

The standard method of evaluating the psychrotrophic microbiota (Vasavada and Critzer, 2015), Plate Count Agar at 7°C for 10 d, was used for the evaluation of the total microbiota. For this analysis, the spread plate methodology was used. When a more rapid methodology was

required, a 48-hour incubation at 35°C on MRS agar was carried out. In some instances, the pH of MRS was adjusted to pH 5.5 for the enumeration of total lactic acid bacteria (Njongmeta et al., 2015). For the MRS-based analyses, the pour plate method was used.

4.2.10. Statistical analysis

Statistical analysis was carried out using Sigma Plot 13.0 (Systat Software, San Jose, CA, USA). One-way ANOVA based on the Student-Newman-Keuls multiple comparison tests, was carried out by ingredients based on data of lag phase and μ_{max}, to ascertain the minimum effective concentration of each ingredient. Plus, for assays using extracts of grape seed and blueberry, the inoculum was more diluted to prevent precipitation due to proteins in the inoculum itself. Lag times from these assays were therefore different from the ones testing cranberry, sauerkraut and green tea. Since the microbiota of the inocula varied, the AS assays carried out for different ingredients were not done simultaneously, and because of the lower inoculation level of some treatments: 1) at least 5 independent assays were carried out, 2) controls were made for each assay, 3) ANOVAs were performed individually for each ingredient. For the relationship between data from AS and plate count assays, regression analyses were applied on the μ_{avg} obtained from OD readings and the traditional CFU-based growth curves. For both ANOVA and regression analysis, α was chosen as 0.05.

4.3. Results and Discussion

4.3.1. Growth of the spoilage microbiota in Italian sausage during refrigeration

Major bacterial species involved in the spoilage of Italian sausages stored under MAP are believed to be lactic acid bacteria (LAB) and *Brochothrix thermosphacta*, due to their tolerance to 1) high CO_2 contents in the atmosphere, and 2) high salt content in the product itself (Batt, 2014; Farber, 1991; Njongmeta et al., 2015; Raimondi et al., 2018; Stanborough et al., 2017a). Thus, the enumeration of total spoilage bacteria presented in sausage stored under MAP became a challenge. Plate count agar (PCA) was commonly used as the growth medium to enumerate total aerobic counts of spoiled meat samples (Tomplin et al., 2015; Vasavada and Critzer, 2015). Since a large proportion of the spoilage population was LAB, these bacteria have high nutritional requirements and MRS was tested for some analyses.

Using PCA as the plating medium, the time-demanding incubation (7ºC for 10 d) generated high counts, but only pin-point colonies appeared at 15ºC for 48 h. Thus, when results were needed a 48 h, plating on MRS was carried out. The growth curve obtained from official method (7°C - 10 d - PCA) showed that the sausage microflora grew during the first 9 days of storage up to 9.02 log CFU/g (Fig. 4.1). However, plating on PCA with higher incubation temperature (15-35ºC) and shorter incubation time (48-72 h) aerobically and anaerobically, failed to provide precise colony counts due to the presence of pin points (data not shown). These results suggested that the PCA medium was not optimal for the spoilage microbiota of sausage stored in MAP to form colonies in these rapid approaches. Thus, to plate the sample on MRS agar would be a reasonable alternative. Different from PCA, the meat extract in MRS would improve the growth of LAB as a source of viable fermentable carbohydrate, and the mixture of sugar (glucose, arabinose, sucrose) instead of glucose alone in PCA could also promote the heterofermentative LAB (De Man et al., 1960). Although MRS contains abundant growth factors of lactobacilli, it is not selective medium, and species else than lactobacilli might also grow on it. This made MRS agar even more suitable for obtaining the total bacterial count of spoiled sausage. In this study, samples plated on MRS and incubated at 35ºC resulted in easily readable colony counts, while those incubated at lower temperature (15ºC) failed because of the presence of pin points. The trend of the growth curves obtained from MRS plates was similar from that from PCA at 7ºC (Fig. 4.1), but CFUs on MRS were systematically lower. Compared with the total counts obtained from PCA plates, plate counts from MRS had a maximum reduction of 1.1 log cycle on day 3, afterwards the gap between data from the two enumeration method gradually decreased and finally reduced to 0.24 log CFU (Fig. 4.1). Thus, as the storage period increased, the MRS enumeration at 35°C for 48 h improved as method to ascertain the total psychrotrophs in sausages under MAP. When comparing original MRS (pH 6.5) and MRS at pH 5.5 data ("total LAB"), the growth curves were similar. Although the MRS-pH 5.5 values appeared slightly lower, the reduction was not considered to be significantly different ($P = 0.152$). When comparing with PCA data, the fraction of LAB thus seemed to represent 10% of the total psychrotrophic population at the beginning of storage but increased to 50% after 9-day incubation. These observations agreed with a previous study investigating the bacterial community of Italian sausage stored under MAP with 30% CO_2 and 70% O_2, which also claimed the dominance of lactic acid bacteria in this product through either traditional plate count or 16S

rRNA gene profiling method (Raimondi et al., 2018). The study showed the total LAB counts obtained from MRS exceeded 10^8 CFU/g under 7ºC refrigeration for 12 days. However, even though the LAB was dominant, the sum of proportion of *Lactobacillus* spp., *Leuconostoc* spp. and *Carnobacterium* spp. in the isolates of colonies on PCA was only slightly below 50%.

All these data provided valuable information on the methodology to be used in the Automated Spectrophotometry (AS) method. For inoculum preparation, sausage samples were required to be incubated at 7ºC under their original package for 9-14 days with total counts of about 5×10^8 CFU/g. The homogenization during inoculum preparation and the inoculation level would dilute the bacteria 100 times with a final concentration of 5×10^6 CFU/mL approximately. This level of bacterial cell concentration was commonly used in previous *in vitro* assays ascertaining the antimicrobial effect of chemical and natural ingredients based on turbidity (Bukvicki et al., 2015; Ghabraie et al., 2016; Schirmer and Langsrud, 2010; Turgis et al., 2012). This bacteria concentration was appropriate because of the detection threshold of AS method. The optical density (OD) of inoculated broth would remain stable when the cell exceeded 10^7 CFU/mL, and the lag time was defined as the period with the "stable" OD instead of the period without bacterial growth. To start the AS assays with cell concentration around 5×10^6 CFU/mL could have appropriate length of lag times for the investigation of antimicrobial effects.

Since the CFU data also pointed towards a high content in acidifying bacteria, and a drop in pH was indeed noticed during preliminary assays in the AS microplates (Fig. 4.2), consequently, the potential effect of acidification of the medium on the AS methodology was examined.

4.3.2. Effect of pH change on OD reading of sausage media during incubation

The AS method is reliable if the medium itself remains stable during incubation, and if the rise of OD are solely linked to the increase of bacterial biomass (Champagne et al., 2014). Since the major bacterial species from spoiled sausage was believed to be LAB, it was examined if organic acids generated by these LAB would affect the turbidity of the media based on sausage homogenate and interfere the OD reading during incubation (Batt, 2014; Champagne et al., 2014; Pothakos et al., 2015; Wang et al., 2014). Indeed, there are instances where OD measurements were affected by the precipitation of proteins or minerals due to the reduction of pH (Champagne

et al., 2014). Proteins in the meat media could reach their lowest solubility at their pIs during the pH decrease and then precipitate (Paker et al., 2015).

Preliminary tests were carried out to evaluate 1) the pH change of inoculated and blank sausage media during incubation; and 2) the effect of acidity on the OD of clarified sausage homogenate (Fig. 4.2 & Fig. 4.3A). Organic acids generated from homo- and heterfermatative metabolite pathways of the spoilage LAB indeed resulted in the pH dropping from 5.75 to 5.09 during the first 22 h incubation at 17ºC in a Bioscreen unit. This was in agreement with a previous study that observed a pH decrease of Italian sausage stored under high CO_2 atmosphere, although the pH reduction level varied among different batches of sausage (Raimondi et al., 2018). However, the pH reduction from 5.75 to 5.09 led to an unacceptable OD increase around 1.3 (Fig. 4.3A), exceeding the detection threshold of AS method where there was a linear correlation between OD and bacterial biomass. Therefore, an additional treatment of the sausage extract, was required before the final clarification (centrifugation and filtration), in order to remove the compounds in the sausage extracts precipitated due to pH changes. Thus, as the final methodology shows (*4.2.3. medium preparation for automated spectrophotometry method*), the sausage homogenates was adjusted to pH 4.3, clarified at that pH, brought back at pH 5.8 and submitted to the final clarification. The clarification step at pH 4.3 was successful in keeping the OD stable during a subsequent gradual acidification from pH 5.8 to 5.0 (Fig. 4.3B). It was a concern that the pH 4.3 clarification step would influence negatively the subsequent growth of spoilage microbiota, since some nutritional contents, probably proteins, precipitated during pH adjustment. Fortunately, bacterial acidification in the pH-adjusted media was similar to that of the original sausage extracts (Fig. 4.2). This suggested that both media supported the growth of the spoilage population from sausage. In addition, plate count tests were carried out on both medium during incubations, and no significant difference was observed between the CFUs of the two sausage media by running a paired t-test ($P = 0.096$), which means the pH 4.3-clarified sausage homogenate could still be considered representative of the clarified sausage extract.

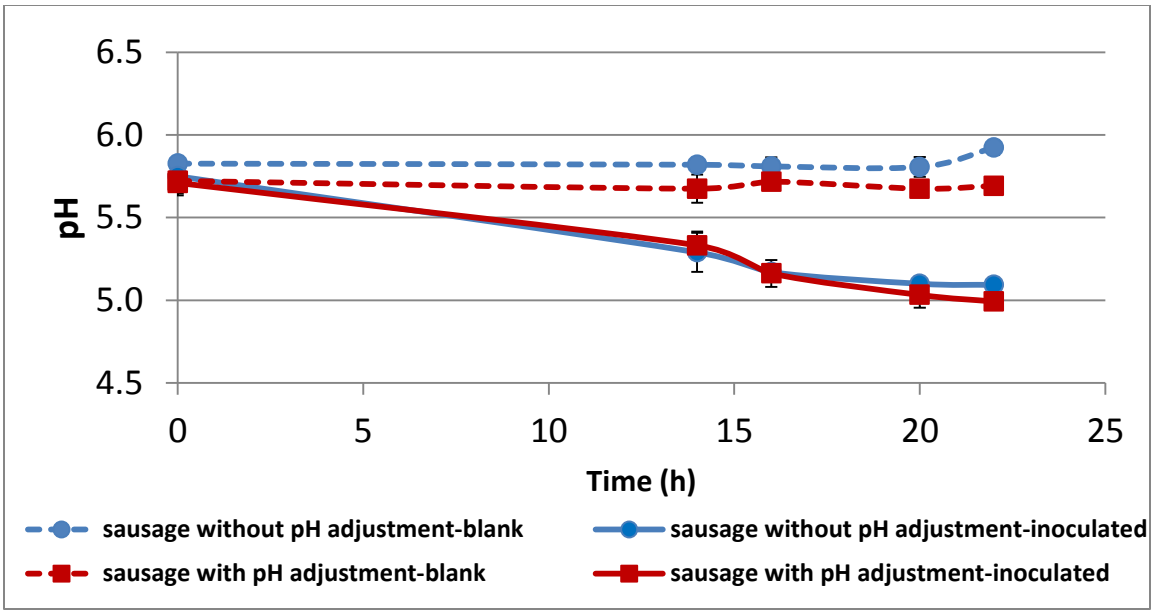

Fig. 4.2. Effect of bacterial growth on the pH of inoculated chicken media during incubation in a Bioscreen unit

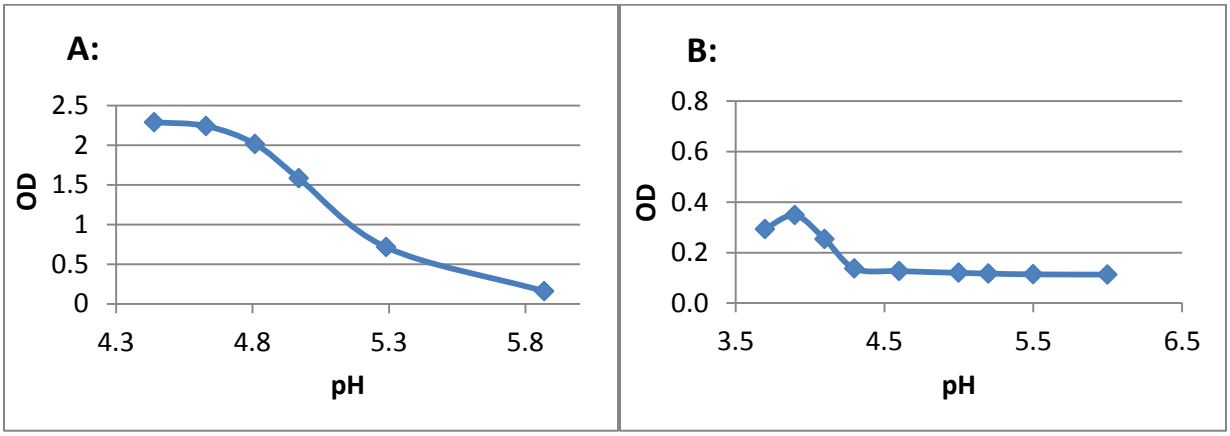

Fig. 4.3. Effect of pH on the O.D. reading of sterile sausage media without (A) and with (B) pH adjustment

4.3.3. Correlation between optical density (OD) and total bacterial counts on MRS

The antimicrobial effectiveness of several natural could be ascertained by AS assays as an accelerated but indirect approach. Plate count assays were usually required as a direct method, to follow spoilage (Hilgarth et al., 2018). Thus, it was necessary to confirm if reduced OD readings were indeed associated with the decrease of viable bacterial counts under the treatment

of these antimicrobial ingredients (Skandamis et al., 2001). Thus, plate count assays during spoilage at 7°C were carried out in sausage extracts supplied with ingredients at the highest concentrations (20 g/L), in order to analyze the correlation between date of the rapid method (AS at 17°C) and the "control" method (CFUs at 7°C spoilage).

The growth curves of sausage microbiota supplemented by 20 g/L of sauerkraut juice powder, cranberry pomace, grape seed extract as well as the control group, are shown in Fig 4.4. The trends of these growth curves matched the AS data at 20 g/L (Fig. 4.5), which indicate that: 20 g/L cranberry had limited antimicrobial effect on sausage microbiota., 20 g/L sauerkraut significantly delayed bacterial growth at the early stage of storage, 20 g/L grape seed extract was highly inhibitory.

A regression analysis was carried out between the average growth rates (μ_{avg}) calculated from AS assays and those of plate count assays, respectively. The linear relation between μ_{avg} obtained from OD and CFU was significant ($P = 0.032$; $R^2 = 0.46$). However, these μ_{avg} data did not fit the linear regression line perfectly with a R^2 of 0.46. Two reasons could explain the relatively low R^2 of the linear regression between OD and CFU in the μ_{avg} results: 1) different working principles of AS and plate count assays; 2) different incubation conditions (temperature, atmosphere) applied in the two assays.

The different incubation parameters applied in AS and plate count assays were probably the main reason affecting the R^2 of linear regression. In the CFU assays, sausage homogenates were incubated at 7ºC which is a widely used storage temperature for spoilage by psychrotrophs. However in AS assays, a 17ºC incubation was selected since it is a temperature often recommended to selectively accelerate the growth of psychrotrophic spoilage microbes within a complex and variable microbiota (Vasavada and Critzer, 2015). The switch of incubation temperature may influence the affinities between polyphenols and the peptides or proteins from the sausage matrix, change the actual concentration of polyphenols in the samples and then affect the antimicrobial properties (Charlton et al., 2002; Ramos-Villarroel et al., 2011; Von Staszewski et al., 2011). The higher temperature applied in AS assays may also promote the growth of some sub species of mesophilic bacteria, which might not generate visible colonies in plate count assays at refrigeration temperature (Vasavada and Critzer, 2015). As for the atmosphere, in plate count assays, samples in tubes were incubated under atmosphere with a relatively high CO_2

content, while in AS assays, the atmosphere in the microplate could not be modified and the samples were incubated aerobically. The limited air from the headspace of microplates may allow the survived aerobic species to grow faster than it could in plate count assays, such difference of bacterial populations between two assays could also result in negative effects on the correlation. Although there were several parameters affecting the fitness of the regression line, this linear relation between the μ_{avg} from AS assays and plate count assay was still significant, meaning that AS assays in this study were able to evaluate the bacterial biomass appropriately by inoculating initial sausage microflora to a media prepared from sausage homogenates.

The lag times (LagT) or the maximum growth rate (μ_{max}) data available from the AS curves could not be used for the regression analysis, because the bacterial enumeration in plate count assays was too time-demanding to be carried out as frequent as the OD measurements in AS assays to obtain accurate LagT and μ_{max}. Furthermore, as opposed to the values obtained from plate count, the LagT obtained from OD was not the "real" lag time with no cell increase, instead, it was the period required for the bacteria in the broth to exceed a population of approximately 10^7 cells per ml (Begot et al., 1996; Métris et al., 2006).

The ideal situation with perfect correlation between AS data and the total viable counts would require that the cells be evenly distributed during OD measurements, and be all culturable (Begot et al., 1996; Kell and Young, 2000). In this study, the distribution of cells was secured by shaking the microplate in an "extra intensive" level right before each OD reading. However, in AS assays, it could not be guaranteed that all cells contributed to the OD increase were viable, injured or VBNC (viable-but-not-culturable) cells could had absorbency as the healthy cells but failed to form colonies on agar plates (Champagne et al., 2014; Kell and Young, 2000).

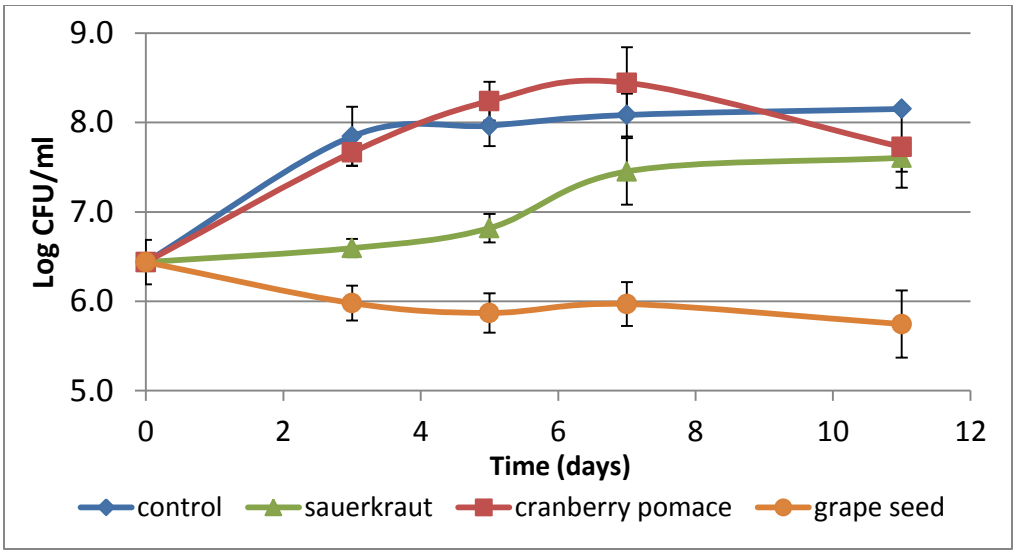

Fig. 4.4. Growth curves of spoilage microbiota treated by 20 g/L sauerkraut juice powder, 20 g/L cranberry pomace and 20 g/L grape seed extract incubated at 7ºC for 11 days. Enumeration of the microbiota were carried out by spread plating on MRS agar (regular pH), and all petri dishes were incubated at 35ºC for 48 h

4.3.4. Antimicrobial effect of tested ingredients against sausage spoilage microbiota

Ideally, inocula used in each repetition would have been characterized for the bacterial composition, as well as the microbiota of products submitted to the antimicrobials. This would have enabled the identification of the species affected by the antimicrobials. Unfortunately this was not possible with the resources available for the project and in the required timeframe. Further studies are required on this aspect. As a result, assumptions on bacterial species involved must be made based on the literature. Nevertheless, the data on MRS provide useful information on the bacterial groups involved.

The spoilage of food products becomes typically detectable when the population spoilage microbiota reaches a certain CFU level, which varies between 10^7 and 10^8 CFU/mL (Vihavainen and Björkroth, 2010). Either the extension of the lag times (LagT) or the decrease of maximum growth rate (μ_{max}) could cause a longer time required for the bacterial population to reach a level indicating the spoilage. Therefore, growth kinetic of sausage microbiota under different treatments was characterized by these two growth parameters calculated from OD data.

The lag times of control groups among assays testing different ingredients were significantly different, due to the fact that inoculum used in samples treated by extract of grape seed and blueberry was 10 more times diluted than the one used for assays testing cranberry pomace, powder of sauerkraut juice and green tea extract. The 10-fold dilution led to an extension of approximately 15 h of lag time (Fig. 4.5). No significant differences in LagT values were observed between control groups in the experimental series of assays with cranberry, sauerkraut and green tea ($P = 0.669$), suggesting that the microbiota extracted from spoiled sausage was active with relatively similar bacterial population between assays and repetitions.

ANOVA carried out separately for each ingredient showed that all antimicrobial agents at their highest concentration (20 g/L) increased LagT and reduced μ_{max}. Furthermore, sauerkraut and green tea were also effective at 10 g/L, while grape seed and blueberry remained inhibitory at 5 g/L (Fig. 4.5A & B). No significant differences in growth parameters appeared between the negative control groups and positive controls (MRS) (Fig. 4.5A & B), which indicated that the inoculum applied for each trial was active, the change of growth parameters were completely caused by the ingredients applied, and the media obtained from homogenized and clarified sausage were able to support the growth of spoilage microbiota as well as a laboratory medium recognized for its ability to support the growth of lactic acid bacteria.

Linear regression was run between the ΔLagT (the real LagT of each test group minus the LagT of the control group) and μ_{max} of all treatments and a negative correlation was obtained ($P < 0.001$, $R^2 = 0.80$). Thus, increased lag times were accompanied by a growth rate reduction, regardless of the antimicrobial agent applied.

Additional tests adjusting the pH of samples treated by 20 g/L cranberry or sauerkraut to that of the original pH level of fresh sausage homogenate were carried out, in order to determine if their effects were related to their acidity. No significant antimicrobial effect appeared after the alkalization for both ingredients (Fig. 4.5A & B). It was not a surprise that sauerkraut lost its antibacterial activity after pH adjustment since its major active compounds were organic acids. Cranberry, however, contains bioactive ingredients other than organic acids, mainly phenolic compounds such as proanthocyanidins (PAC), which could have contributed to its inhibitory activity. The absence of an effect of cranberry by-product against sausage spoilage microbiota, when its pH is neutralized, would be in line with the CFU observations that the LAB are a

significant fraction of the microbiota. Indeed, PAC might not be highly effective against lactic acid bacteria, because one of their antibacterial effects is to act as iron chelators, which limit iron availability from the meat media and then affect the heme enzyme formation as well as ATP synthesis of bacterial cells (Neilands, 1995). Lactobacilli will not be affected in such iron competition since they do not generate heme enzyme for their metabolism (Chung et al., 1998; Neilands, 1995; Santos‐Buelga and Scalbert, 2000). Such metal chelation could still happen at neutral pH, suggesting that the pH adjustment of sausage media with 20 g/L cranberry pomace before inoculation may not reduce their interaction with iron (Kylli et al., 2011; Santos‐Buelga and Scalbert, 2000). Previous studies noticed PAC fraction from cranberry had stronger effects against other Gram positive bacteria such as *Staph. aureus* and *L. monocytogenes* than of *Lactobacillus* spp., (Kylli et al., 2011; Lacombe et al., 2013).

Data from the literature suggest that LAB are more resistant to some plant extracts (green tea, grape seed and blueberry) than some Gram positive foodborne pathogens (Ankolekar et al., 2011; Biswas et al., 2012; Horiba et al., 1991; Moradi et al., 2011; Silva et al., 2013; Tajik et al., 2014),. However, these three ingredients rich in phenolic compounds had strong antibacterial effects on the general spoilage population of sausage (Fig. 4.5). Two hypotheses may explain this observation: 1) the psychrotrophic species of LAB present in the sausage microbiota might behave differently from the mesophilic LAB strains used in previous studies; 2) the ingredients mainly affected other bacterial species than the LAB such as *Brochothrix*, which made up of up to 50% of the whole population even at latter stage of spoilage (Fig. 4.1). Further study identifying the species of psychrothrophic lactic acid bacteria of spoiled sausage, as well as evaluation of each ingredients based on phenolic compounds against each bacterial strain would be required to confirm these hypotheses.

As for ingredients with high acidity, the antimicrobial effects were relatively lower than the plant extracts in general, probably because as acid-producer, lactic acid bacteria was expected to have resistant to low pH in some extents (Hsiao and Siebert, 1999). Cranberry pomace and powder of sauerkraut juice started to significantly inhibit sausage microflora from 20 and 10 g/L, respectively. These food by-products showed partial inhibitory effect, probably due to the presence of other bacterial species which were more vulnerable to high acidity. HPLC carried out for the 6.17% (w/v) water solution of the sauerkraut juice powder used in this study

contained 1.85% lactic acid, which means sausage media with addition of 1% and 2% should theoretically contain 0.3% and 0.6% of lactic acid, respectively. This result corresponded to a previous study claiming that lactic acid started to inhibit the growth of *B. thermospacta* and *Lactobacillus* spp. from 0.1% and 0.3%, respectively, and the inhibitory effect gradually enhanced as the concentration increased (Ouattara et al., 1997). The addition of 10 g/L sauerkraut probably had enough effect on inhibiting bacterial species which were not acid-producers. Such inhibition was notable enough to cause a significant increase of lag time or a decrease of the overall growth rate. As the concentration increased to 20 g/L, it began to inhibit some of the lactic acid bacteria remained in the population and performed stronger antimicrobial effect than that of 10 g/L.

The efficiency of the acidity might also be reduced due to the buffering capacity of the sausage-based media. Due to the presence of proteins and peptides such as anserine and carnosine in meat homogenates, as well as blood components (phosphates, citrate), the pH reduction due to the addition of either cranberry pomace or powder of sauerkraut juice was remarkably lower than if they were added to water (Fig. 4.6). Both ingredients at 5 g/L could easily reduce the pH to levels below 4 in water, while they could only acidify sausage media with pH value from 5.8 to approximately 5.0 at 20 g/L (Fig. 4.6). However, as acid producers, lactic acid bacteria are naturally more resistant to low pH than many psychrotrophic spoilage bacteria. As a result, many LAB are able to grow at pH < 5 (Batt, 2014; Ouattara et al., 1997; Tsakalidou and Papadimitriou, 2011). The pH level of sausage media could only drop below 5.0 with the addition of 20 g/L cranberry pomace or 10 g/L sauerkraut juice powder, which corresponded to the observation that the significant antimicrobial effects only started to appear at 20 g/L and 10 g/L for cranberry and sauerkraut, respectively (Fig. 4.5 & Fig. 4.6).

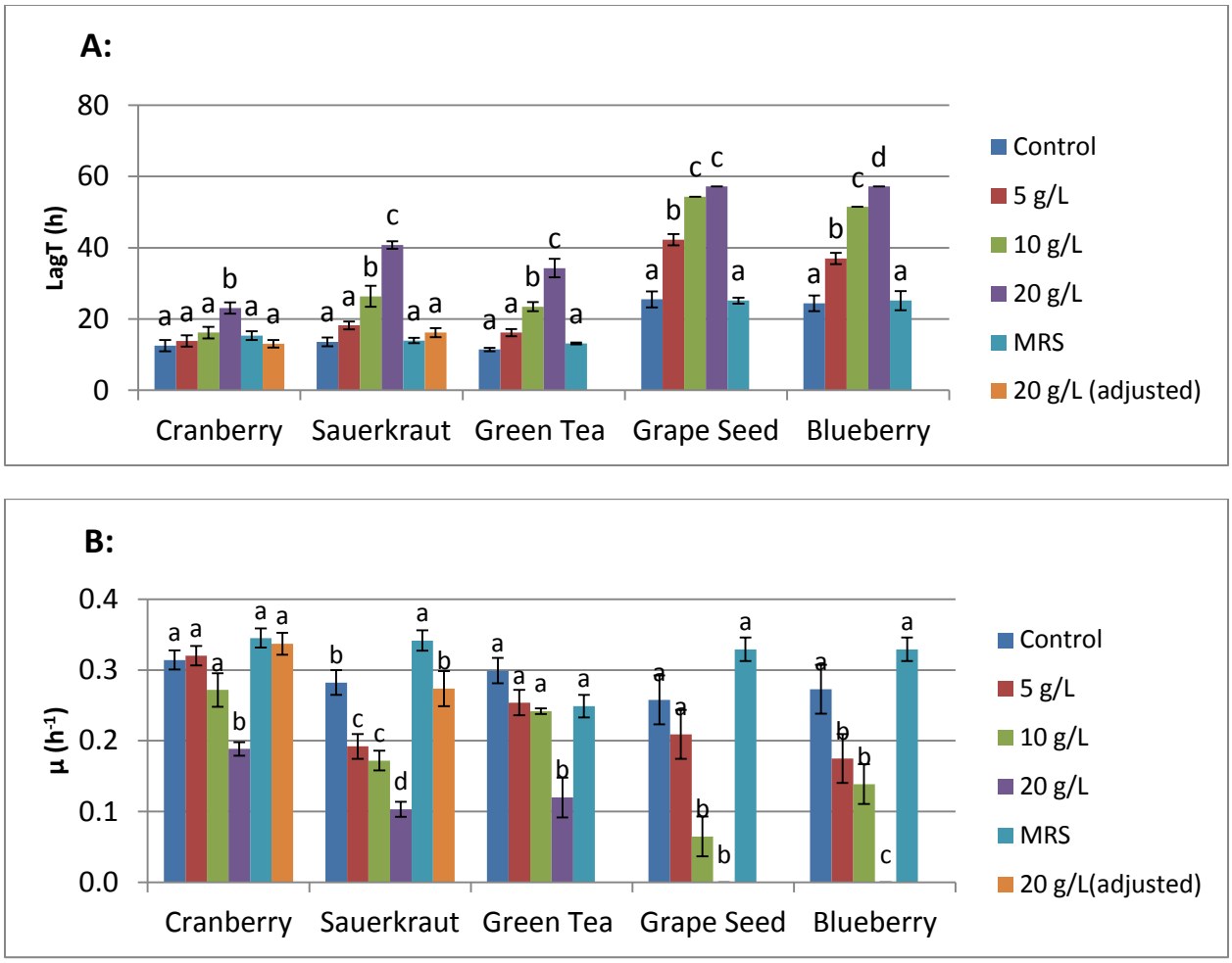

Fig. 4.5. Effect of the addition of the powder of cranberry pomace, sauerkraut juice, green tea and grape seed to chicken media on the lag time (A) and the maximum growth rate (B) of the spoilage microbiota from sausage extracts

With a given antimicrobial ingredient, columns headed with the same letters had values that were not significantly different ($P < 0.05$);

The "20 g/L (adjusted)" treatments (only applicable with sauerkraut and cranberry) represent samples where the pH was adjusted to the level of original media after the addition of 20 g/L ingredients); The "LagT" data represent lag time before an increase in 0.075 on OD; The μ_{max} data represent the maximum growth rate.

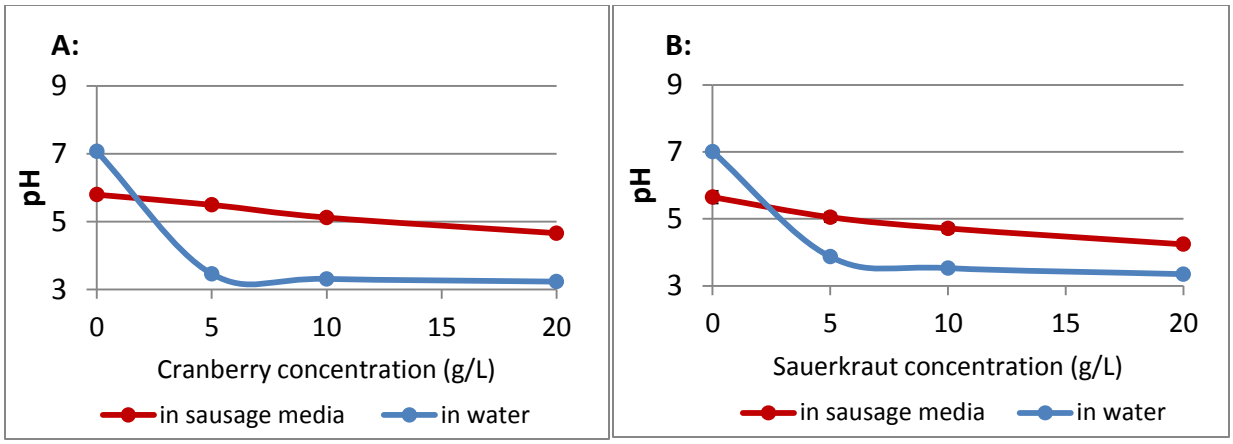

Fig. 4.6. Effect of the addition of cranberry pomace (A) and sauerkraut (B) in sausage extract media and in water

4.4. Conclusion

Although bacterial species identification were not carried out in this study, correlations between plate count data obtained from 1) plate count agar (PCA) under 7ºC incubation for 10 days; and 2) MRS agar (pH 6.5 & 5.5) under 35ºC incubation for 48 h suggested a high level of LAB in the spoilage population of sausage under MAP. In an attempt to carry out the experimentation under a "real situation", with respect to the antimicrobial effect of food-grade preservatives on spoilage microbiota, this study developed a novel methodology, applying a representative meat media based on sausage homogenates inoculated with the initial microflora extracted from spoiled samples to the obtain growth kinetic of sausage spoilage microbiota through automated spectrometry (AS) method. The growth kinetics of the microbiota were characterized by the lag time (LagT) and the maximum growth rate (μ_{max}) calculated, which could be determined with precision thanks to the frequent OD measurements. Plate count assays were also carried out confirming that the increase of OD readings in AS was associated with the actual increase of bacterial cells. There was indeed a significant linear relation between the average growth rate (μ_{avg}) obtained from both assays, meaning that the growth parameters obtained from AS assays were reliable.

According to the LagT and μ_{max} calculated from OD data, all ingredients showed antimicrobial activities at the highest tested concentration (20 g/L). The extract of grape seed and blueberry started to exert their inhibitory effect from 5 g/L, while green tea extract and the powder of sauerkraut juice began to delay the growth of spoilage microbes at 10 g/L.

In the goal of delaying microbial spoilage of sausage, this study provides data as to the potentially effective concentrations of the "natural" ingredients to be added in the sausage formulation. However, numerous other studies now need to be carried out. First, *in situ* plate count assays are required to confirm the AS observations. Secondly, the effects of the ingredients on sensory attributes of the sausage are also now required. Ingredients with strong acidity may lead to color deterioration of the sausage product, and it remains to be confirmed in the slower microbial growth also correlates with delayed off-odors. Finally, it would be good to identify the microbial species involved in sausage spoilage that were affected by the antimicrobial agents. The bacterial identification may provide useful information for the better selection of natural antimicrobial ingredients.

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CHAPTER 5. GENERAL CONCLUSION AND FUTURE WORK

This study developed a novel methodology to evaluate the antimicrobial effects of water-soluble food-grade preservatives on meat spoilage microbiota, by applying representative meat-based media prepared from chicken exudate or sausage homogenates inoculated with initial microflora extracted from spoiled chicken exudates or sausage samples. The inhibitory effect of each antimicrobial agent was ascertained based on two growth parameters (LagT: lag time; μ_{max}: maximum growth rate) of spoilage microbiota of each product calculated from frequent OD measurements by automated spectrometry (AS) method. The total viable counts of the two spoiled products were also evaluated, in order to confirm the increase of OD in AS method was linked to an actual increase of bacterial populations based on the average growth rate (μ_{avg}) obtained from both OD and CFU data.

The LagT extension was associated with μ_{max} reduction of initial spoilage microbiota of both spoiled samples treated by each antimicrobial ingredient in AS assays. For ingredients based on acidity, the powder of sauerkraut juice showed greater inhibitory effect than cranberry pomace against the microbiota from either chicken exudates or sausage homogenates. Also, the chicken spoilage microbiota appeared to be more resistant to organic acids from sauerkraut, compared with sausage spoilage microbes, as evidenced by the higher minimum effective concentration of sauerkraut powder to be effective against the former. It was worth noticing that the effect of both food by-products with high acidity was reduced by the buffering capacity of chicken exudate and sausage homogenates. This partly proved the importance to apply representative growth medium based on meat matrix and to consider the probable interaction between food contents and antimicrobial ingredients, in order to avoid the over-estimation of antimicrobial effect.

Meanwhile, all plant extracts rich in phenolic compounds delayed the growth of spoilage microbiota of both products in different extent. Grape seed extract was more effective than green tea extract in both matrix of chicken exudates and sausage homogenates. Blueberry extract also showed similar antimicrobial effect with grape seed extract when being supplied in the sausage-based media. In general, due to the difference of major bacterial species involved in the spoilage of two samples, microbiota of chicken exudates was more sensitive to food by-products with

high acidity while that of sausage homogenates was more vulnerable to plant extracts which were believed to have high contents of phenolic compounds.

Effective formulations of antimicrobial agents (sauerkraut powder at 20 g/L, grape seed extract at 10 and 20 g/L) were also applied in absorbent pads for chicken exudates, and their effectiveness was further examined through traditional plate counts. However, these selected ingredients were less effective in pads assays, which was probably due to 1) slow and uneven distribution of effective components in chicken exudates absorbed by the pads; 2) more aerobic condition above the pads than in the tubes or above the wells in AS microplates which promoted the growth of aerobic spoilage species.

This study provides useful methodologies and information for the application of natural antimicrobial ingredients in food packaging or as food preservatives. Industrial tests as well as further basic research are required in the future.

Industrial tests:

1) Evaluation of the real microbial loads of antimicrobial absorbent pads with sauerkraut powder at 20 g/L, grape seed extract at 10 and 20 g/L, applied in real packed chicken;
2) Improvement of pad preparation techniques allowing the antimicrobial ingredients to distribute evenly in the chicken exudates absorbed by the pads;
3) Confirmation of antimicrobial formula recommended by AS data through *in situ* plate count assays for sausage;
4) Confirmation of correlations between slower growth of spoilage microbiota and the delay of off-odor generation for both products;
5) Evaluation of whether the antimicrobial agents affect the sensory properties when being applied in sausage contents

Basic research:

1) Identification of microbial species involved in the spoilage of chicken exudates and sausage, respectively;
2) Ascertain if specific species are affected by the antimicrobials and identify those that have the most effect on improved storage stability of both products;

3) Better selection of antimicrobial agents based on the major bacterial species linked mostly to the bacterial spoilage for both products, respectively.

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