# Development and application of a high-throughput luminescent prophage induction assay for the identification of temperate bacteriophage-inducing food-grade compounds

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**Abstract**

In response to consumer trends, conventional synthetic food additives used to control the growth of foodborne bacterial pathogens are being replaced with natural alternatives. Bacteriophages (phages) are viruses of bacteria that have been successfully applied in the control of pathogenic bacteria in food. Cocktails of virulent phages have been designed for exogenous application to specifically target individual bacterial species that are of public health concern. One disadvantage to this approach is the fact that multiple bacterial species can cause foodborne illnesses or food spoilage, and phage cocktails are unable to control multiple bacterial species. An alternative phage-based method that involves the induction of bacterial chromosome-embedded phages (prophages) has recently been proposed, as it exhibits several advantages over the phage cocktail approach. Since the induction of prophages across multiple bacterial genera can be triggered by the addition of a single bioactive compound, the issue of narrow host range is resolved.

Beginning with a comprehensive review of the clean label trend, natural antimicrobials, and the application of phages in the context of food safety, the focus of the current work was to develop, validate and apply a high-throughput luminescent prophage induction assay that identifies bioactive compounds as prophage-inducing agents. Several natural compounds were screened in the assay to identify prophage-inducing agents that satisfy both food safety and clean label standards.

A luminescent prophage induction assay was developed using the indicator strain, *Escherichia coli* BR513, which carries a *lacZ*-prophage λ gene fusion. Since the *lacZ*

gene is fused to a cryptic lambdoid phage in *E. coli* BR513, the production of β-galactosidase occurs upon the cleavage of the lambdoid repressor, CI, which triggers prophage λ induction. β-galactosidase may thus be measured and taken as an indication of prophage λ induction. To develop the assay, three well-known phage-inducing antibiotics (mitomycin C, streptonigrin and norfloxacin) were incubated with *E. coli* BR513, and the production of β-galactosidase was measured luminometrically and compared to that produced by the negative control, wildtype *E. coli* K-12. Statistical analysis revealed that each antibiotic significantly increased β-galactosidase production in the indicator strain. Based on the success of the antibiotic screen, assay parameters were finalized and natural compounds were tested. Gallic acid, rosemary, pH-neutralized cranberry juice, coffee, and a variety of green and black teas were identified as effective prophage-inducing agents.

The assay was then modified for high-throughput in order to screen 3747 compounds from 4 bioactive libraries. Six additional natural compounds were identified as prophage-inducing agents. Dose response experiments were conducted but were not successful in determining optimal concentrations required for induction since the assay could not differentiate prophage induction from other cell death mechanisms.

Overall, this work demonstrated that *E. coli* BR513 can be used as an effective indicator strain for the induction of prophages. The natural compounds identified in this work should be confirmed for their capacity to induce prophages in tests involving the PCR amplification of phage integrase genes, since the presence of increased copies of integrase genes would be indicative of prophages being induced.

**Résumé**

En réponse aux tendances du marché, les additifs alimentaires synthétiques conventionnels utilisés pour contrôler le développement de pathogènes d'origine alimentaire sont remplacés avec des ingrédients naturels. Les bactériophages (phages) sont des virus de bactéries utilisés pour contrôler les bactéries pathogènes dans la nourriture. Les cocktails de phages virulents sont conçus pour des applications exogènes et visent spécifiquement une gamme étroite d'espèces bactériennes dangereuses pour la santé publique. Un des désavantages de cette approche est que la plage cible des cocktails de phages ne s'étend pas aux autres espèces de bactéries pathogènes ou aux bactéries pouvant entrainer la décomposition qui pourraient être présentes. Une méthode alternative basée sur les phages qui comprend l'induction de phages intégrés aux chromosomes bactériens (prophages) à récemment été proposée et possède plusieurs avantages sur l'approche des cocktails de phages. Comme l'induction de prophages à travers différents genres bactériens peut potentiellement être déclenchée par l'ajout d'un seul composé bioactif, le problème de l'étroitesse de la gamme de l'hôte est résolu.

En commençant avec une revue critique de la tendance de l'étiquetage « clean label », des antimicrobiens naturels et de l'application des phages dans l'industrie alimentaire, le focus de cette dissertation est de développer, valider et appliquer un test luminescent à haut débit d'induction de prophage pouvant identifier des composés bioactifs qui agissent comme agents inducteurs de prophages. Plusieurs composés naturels ont été examinés afin d'identifier des agents inducteurs de prophages pouvant satisfaire à la fois aux standards de la salubrité alimentaire et aux standards de « clean label ».

Un test luminescent à haut débit d'induction de prophage a été développé en utilisant la souche indicatrice *Escherichia coli* BR513 qui porte une fusion de gène *lacZ*-prophage λ. Comme le gène *lacZ* est fusionné à un phage lambdoïde cryptique, la production de β-galactosidase survient lors de la séparation du répresseur lambdoïde, CI, qui provoque une induction de prophage λ. β-galactosidase peut donc être mesuré et pris comme une indication de l'induction de prophage λ. Pour développer le test, trois antibiotiques reconnus pour leur capacité à induire les phages (streptonigrine, mitomycine C et norfloxacine) ont été incubés avec *E. coli* BR513, et la production de β-galactosidase a été mesurée avec la luminescence et comparée avec celle produite par *E. coli* K-12 sauvage (utilisé comme contrôle négatif). Les analyses statistiques ont révélé que chaque antibiotique augmente de manière significative la production de β-galactosidase dans la souche indicatrice. En se basant sur le succès de cet examen, les paramètres du test ont été finalisés et les composés naturels ont été testés. L'acide gallique, le romarin, le jus de canneberge au pH neutre, le café et différents thés verts et noirs ont été identifiés comme des agents inducteurs de prophages efficaces.

Le test a été adapté pour un système robotique automatisé afin d'examiner 3747 composés venant de quatre bibliothèques bioactives. Six composés naturels additionnels ont été identifiés comme agents inducteurs de prophages. Des évaluations dose-effet ont été effectuées mais n'ont pas réussies à déterminer la concentration optimale requise pour l'induction comme ce test n'a pas pu différencier l'induction de prophage et les autres mécanismes qui causent la mort des cellules.

Cette dissertation démontre que *E. coli* BR513 peut être utilisé comme une souche indicatrice efficace d'induction de prophages. Les composés naturels identifiés pouvant être responsables de l'induction de prophages devraient voir leur capacité d'induire les prophages confirmée dans des tests d'amplification PCR de gènes intégrases de phages, comme la présence de plusieurs copies de ces gènes serait indicative de l'induction des prophages.

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## Contribution of authors

The work presented in this thesis was completed at the Department of Food Science and Agricultural Chemistry at Macdonald Campus of McGill University in Sainte-Anne-de-Bellevue, Quebec, Canada. The execution of experiments, data analysis and manuscript preparation were carried out by Master of Science Candidate, Elizabeth Tompkins. The research was conducted in the Diagnostic, Enrichment, Testing and Characterization (D.E.Te.CT.) Laboratory under the supervision of Dr. Lawrence D. Goodridge, who oversaw the entirety of the research and the preparation of manuscripts. Dr. Brigitte Cadieux provided guidance in the execution of laboratory experiments.

Whole genome sequencing (WGS) and bioinformatics analyses were carried out at the Institut de Biologie Integrative et des Systèmes (IBIS) at Laval University in Quebec, under the supervision of Dr. Roger Levesque. The high-throughput luminescent prophage induction assay was completed at the Centre for Microbial Chemical Biology (CMCB) at the Michael G. DeGroote Institute for Infectious Disease Research at McMaster University in Hamilton, Ontario, lead by Dr. Tracey Campbell. Dr. Ari Belenkiy (Vancouver, B.C.) kindly provided guidance in the statistical analysis in Chapters 3.

The manuscripts written for publication in the scientific literature in this thesis include:

1. **Tompkins, Elizabeth**; Goodridge, Lawrence. (2019). A review of the clean label trend in the context of food safety: Considerations for the replacement of conventionally used antimicrobials with natural compounds *(Draft prepared).*

2. **Tompkins, Elizabeth**; Cadieux, Brigitte; Amitrano, Margot; Emond-Rheault, Jean-Guillaume; Hamel, Jérémie; Levesque, Roger; Goodridge, Lawrence. (2019). Development of a high-throughput luminescent prophage induction assay for the identification of *prophage* $\lambda$ inducing compounds *(Draft prepared).*

3. **Tompkins, Elizabeth**; Cadieux, Brigitte; Goodridge, Lawrence. (2019). Use of the high-throughput luminescent prophage induction assay to evaluate bioactive compounds for identification of natural prophage inducers *(Draft prepared)*

Work from this thesis has also been presented at the following scientific conferences:

1. **Tompkins, Elizabeth**; Cadieux, Brigitte; Goodridge, Lawrence. (2018). "Development and evaluation of a novel assay to identify prophage inducers as a new class of antimicrobials in foods". International Association for Food Protection (IAFP) Annual Meeting, Salt Lake City, Utah, USA, July 11, 2018.

2. **Tompkins, Elizabeth**; Cadieux, Brigitte; Goodridge, Lawrence, D. (2017). "Development and evaluation of a novel assay to identify prophage inducers as a new class of antimicrobials in foods". SystOMICS Project Meeting, Laval University, Quebec City, June 2017.

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**List of abbreviations**

| | |
|---|---|
| D.E.Te.Ct | Diagnostic, Enrichment, Testing and Characterization |
| WGS | Whole Genome Sequencing |
| IBIS | Institut de Biologie Intégrative et des Systèmes |
| HTS | High-Throughput Screening |
| CMCB | Centre for Microbial Chemical Biology |
| IAFP | International Association for Food Protection |
| CFIA | Canadian Food Inspection Agency |
| Phage | Bacteriophage |
| BHA | Butylated hydroxyanisole |
| MIC | Minimum Inhibitory Concentration |
| ss | Single-stranded |
| ds | Double-stranded |
| kb | Kilobase |
| FDA | Food and Drug Administration |
| STEC | Shiga Toxin-producing *Escherichia coli* |
| ACHIM | Anaerobically Cultivated Human Intestinal Microflora |
| HGT | Horizontal Gene Transfer |
| ARG | Antibiotic Resistance Gene |
| USDA | United States Department of Agriculture |
| IMH | Israel Ministry of Health |
| EPA | Environmental Protection Agency |
| FSANZ | Food Standards Australia New Zealand |

| | |
|---|---|
| Swiss BAG | Swiss Federal Office of Public Health |
| $OD_{600}$ | Optical Density, wavelength = 600 nm |
| EGCG | Epigallocatechin Gallate |
| GFF | Gene Feature Format |
| TSA | Trypticase Soy Agar |
| TSB | Trypticase Soy Broth |
| TSB 0.2 M glucose | Trypticase Soy Broth with 0.2 M glucose |
| RLU | Relative Light Units |
| log[RLU/$OD_{2\mu g/ml}$] | log-transformed RLU/$OD_{600}$ from antibiotic-treated bacteria |
| log[RLU/$OD_{0\mu g/ml}$] | log-transformed RLU/$OD_{600}$ from non-treated bacteria |
| ONPG | Ortho-Nitrophenyl-β-Galactoside |
| MOA | Mechanism of Action |
| DMSO | Dimethyl Sulfoxide |
| BHT | Butylated Hydroxytoluene |
| THC | Tetrahydrocannabinol |
| CBD | Cannabidiol |

## Chapter 1: Introduction

### 1.1 General introduction

Despite advances in food industry practices to produce safe foods, bacterial pathogens continue to cause foodborne-related illnesses and deaths around the world (Kirk et al., 2015). In Canada, foodborne illnesses affect approximately 4 million people annually, leading to 11,600 hospitalizations and 238 deaths. The top bacterial foodborne pathogens involved in these illnesses include nontyphoidal *Salmonella* spp., *Campylobacter* spp., verotoxin-producing *Escherichia coli* O157 and *Listeria monocytogenes* (Thomas et al., 2015). Foodborne pathogens result in significant economic losses, costing the US approximately $15.5 billion per annum, according to a 2015 estimate (Hoffman et al., 2015).

Canadian salmonellosis outbreaks linked to the consumption of frozen raw breaded chicken products have been persistent over recent years. Since May 2017, and as of March 22, 2019, there have been seventeen national outbreaks linked to frozen raw breaded chicken products, which were caused by *Salmonella* Enteritidis, *Salmonella* Heidelberg and *Salmonella* Braenderup[1]. As of March 1, 2019, *Salmonella*-contaminated raw chicken, including frozen raw breaded chicken products were responsible for 555 cases of salmonellosis across Canada, as confirmed by whole genome sequencing (WGS)[1]. To reduce the risk of *Salmonella* levels in frozen raw breaded chicken products,

[1] https://www.canada.ca/en/public-health/services/public-health-notices/2018/outbreaks-salmonella-infections-linked-raw-chicken-including-frozen-raw-breaded-chicken-products.html#a2

the Canadian Food Inspection Agency (CFIA) is enforcing the implementation of manufacturing and processing procedures that aim to mitigate the problem[2]. The CFIA's response to this current food safety issue demonstrates a need for novel and effective food safety solutions.

In line with modern clean label trends, the use of natural compounds as antimicrobials, as opposed to synthetic additives, is garnering attention from the food industry. There is no generally accepted definition for clean label or natural foods, as consumer interpretations may be subjective (Baines, 2012), yet natural claims on food labels are often associated with better quality. One study showed that natural claims gave consumers an impression of improved taste, nutrition, safety and animal welfare (Dominick et al., 2018). Although, these attributes are by no means automatically inherent of natural products, it is still in the best interest of food producers to consider consumer preferences, so as to ensure repeated purchase of products (Meneses et al., 2014).

Bacteriophages (phages) are viruses of bacteria that are increasingly being used as natural antimicrobial agents in the food industry. This approach to food safety has emerged as an effective method to control the growth of bacterial foodborne pathogens and it relies on the use of virulent phages that enter bacterial hosts to cause cell lysis. An alternative phage-based approach that involves the activation or induction of bacterial chromosome-embedded phages (prophages) using DNA-damaging agents, has recently been proposed as a suitable means of bacterial pathogen control on foods (Cadieux et

[2] https://www.canada.ca/en/public-health/services/public-health-notices/2018/outbreaks-salmonella-infections-linked-raw-chicken-including-frozen-raw-breaded-chicken-products.html#a2

al., 2018). This approach has important advantages over the conventional virulent phage-based antimicrobials, as well as the use of antibiotics and synthetic food additives. These topics are explored throughout the chapters of this dissertation. The work of Cadieux et al. (2018) establishes the premise of the study at hand, which aims to identify natural compounds that induce prophages, and kill bacterial pathogens that persistently contaminate food.

## 1.2 Research objectives

### 1.2.1 Overall objective

The aim of this work is to identify food-grade compounds that may be added to foods to lyse and kill bacterial foodborne pathogens by means of triggering the induction of prophages that are naturally present within bacterial chromosomes. Compounds that are identified may be considered for application in the food industry as novel food-grade antimicrobials.

### 1.2.2 Specific objectives

**Objective 1:** To develop a high-throughput luminescent prophage induction assay for use in identifying food-grade prophage inducers.

**Objective 2:** To use the high-throughput luminescent prophage induction assay to screen libraries of bioactive compounds to identify food-grade prophage inducers.

**1.3 References**.

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Dominick, S.R., Fullerton, C., Widmar, N.J.O., and Wang, H. (2018). Consumer associations with the "all natural" food label. *Journal of Food Products Marketing* 24(3)**,** 249-262. doi: 10.1080/10454446.2017.1285262.

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## Chapter 2: A review of the clean label trend in the context of food safety: Considerations for the replacement of conventionally used antimicrobials with natural compounds

### 2.1 Abstract

The clean label movement has prompted food manufacturers to rethink conventional ingredients that have long been in use to produce safe food (Asioli et al., 2017). As such, it is important to explore new ingredients and processing methods with the best interest of consumers in mind. A variety of natural antimicrobials have been shown to effectively eliminate bacterial foodborne pathogens, however the compounds that replace conventional, synthetically produced antimicrobials should be chosen carefully, so that food safety is not compromised. The use of bacteriophages (phages) in the form of phage cocktails represents an emerging natural approach to food safety. An alternative phage-based approach that involves the activation or induction of bacterial chromosome-embedded phages (prophages) using DNA-damaging agents is a new approach to control foodborne pathogens. This approach has important advantages over conventional virulent phage-based methods, as conventional phage cocktails are very specific, limiting the range of pathogenic serovars they target. This comprehensive review describes the use of phages as antimicrobials by the food industry, its practicality and the reported risks and benefits for use in the context of food safety.

## 2.2 Introduction

Preservatives and additives play an indispensable role in providing safe food to consumers (Emerton and Choi, 2008). In recent years, consumer preferences have shifted toward the use of natural or non-synthetic ingredients (Garcia et al., 2008). Consumer demand for clean label products has prompted food manufacturers to rethink conventional ingredients that have long been in use to produce safe food (Asioli et al., 2017). As such, it is important to explore new ingredients and processing methods with the best interest of consumers in mind.

It is especially important to consider how a shift to non-synthetic, or all-natural ingredients might affect food safety, as the food industry replaces tried and tested antimicrobial agents. Studies from the literature provide evidence of natural compounds that exhibit antimicrobial properties that may be used to control bacterial foodborne pathogens. For example, the use of bacteriophages (phages) as natural antimicrobials in the control of bacterial foodborne pathogens continues to gain traction in the food industry (Moye et al., 2018).

To fully understand the scope of phages as agents in the control of bacterial foodborne pathogens, a thorough understanding of bacteriophage lifestyle is imperative. This review will focus on the use of phages as antimicrobials to control foodborne bacterial pathogens, with an emphasis on the practicality of their applications, along with reported risks and benefits for use in the food industry in the context of food safety.

## 2.2 Drivers of the clean label trend

The value of a nutritious diet for healthy living has become increasingly apparent to consumers in industrialized societies. With the notion of 'food as medicine', there is a focus on the consumption of minimally processed, natural foods to reduce the risk of diet-related diseases (Garcia et al., 2011; Asioli et al., 2017). Consumers have also become more interested in details regarding production methods of the foods they purchase (e.g. organic versus conventional agriculture), as they are concerned about environmental and sustainability issues (Garcia et al., 2011; Asioli et al., 2017). Among consumers, the acceptance of foods made with natural ingredients is much higher than it is for those that contain artificial additives or unfamiliar ingredients, and processed foods are generally poorly received (Bearth et al., 2014).

Consumer skepticism towards processed foods is not unwarranted, as the literature points to evidence linking diets high in ultra-processed foods with health risks. Several studies have shown that overall quality of diets and health decreases when the consumption of ready-to-eat foods is higher (Louzada et al., 2015; Moubarac et al., 2012; Alkerwi et al., 2015). This fact is generally attributed to processed foods being high in sugars, salt and fats, and low in dietary fibre. The same foods often also contain preservatives, colourants and artificial sweeteners that consumers have come to distrust.

Many consumers are aware that some artificial preservatives have detrimental effects on human health. Butylated hydroxyanisol (Mangat et al.), for example, is an antioxidant added to foods to inhibit fat breakdown, but research has shown that it can cause cancer (Anand and Sati, 2013). Ultimately, an underlying lack of information and understanding

regarding artificial food additives leads consumers to believe that in general, all artificial food additives are not safe or healthy, which has lowered interest in purchasing products that contain them (Shim et al., 2011).

The term ‘clean label’ does not have an officially recognized definition, and its interpretation may be subjective (Meneses et al., 2014; Asioli et al., 2017). The term is generally applied to food products or household products which contain easily recognized natural ingredients that are understood and trusted by the average consumer. While one product may be considered clean label based on the ingredients it contains, it may equally qualify as clean label by what it does not contain. The use of ingredients that are not natural, or even those that are presented on a label by their chemical name, may be interpreted negatively by consumers whether they are natural in origin or not (Meneses et al., 2014; Asioli et al., 2017).

### 2.2.1 Clean label implications for food safety

The food industry must consider the factors that drive the clean label trend. The fact remains that today’s consumers live fast-paced lifestyles and they seek processed foods out of convenience (Asioli et al., 2017). It is therefore crucial for food manufacturers to comply with consumer demands to provide convenient foods that do not contain artificial or synthetic ingredients (Delves-Broughton, 2012). Several food manufacturers have taken it upon themselves to use labelling that readily informs their clientele of the clean characteristics of their products. Claims that support the clean label trend may deal with environmental and/or social sustainability, ingredients that are present or absent, as well as processing methods that were used (Asioli et al., 2017).

As companies are challenged with reformulating their products by introducing new ingredients as a consequence of removing others, the replacement of ingredients that act as antimicrobials could potentially compromise food safety. Food additives have played an important role in the global food supply, and the benefits they provide ultimately outweigh the risks (Emerton and Choi, 2008). The demand for food products to be free of synthetic and artificial additives creates a challenge for food manufacturers. To stay in competitive business and provide food for a growing population, the food industry has begun to move away from the use of certain ingredients and additives that were accepted in the past. While it is important to make products that consumers want to purchase, it is also imperative that food safety is not compromised.

Many consumers falsely associate natural ingredients with food safety, however, the origin of an additive is not indicative of its inherent ability to protect against harmful microorganisms (Bearth et al., 2014), and newly proposed natural preservatives must still undergo full toxicological assessments before they may be legally used in product formulations (Delves-Broughton, 2012). The only exception to this applies to previously legislated food ingredients that have already gained recognition in terms of safe use in foods (Delves-Broughton, 2012).

Food additives such as colourants and sweeteners may be more readily replaced with natural compounds with little concern for food safety (Emerton and Choi, 2008), but it is more challenging to replace additives that prevent the growth of pathogenic microorganisms. Food additives with antimicrobial effects prevent foodborne illnesses, whereas other additives such as colourants and sweeteners play a more minor role for most individuals (Emerton and Choi, 2008; Bearth et al., 2014).

## 2.3 Natural compounds as antimicrobials for the control of bacterial foodborne pathogens

Research on natural antimicrobials has provided evidence that a number of compounds may be added to foods to increase safety (Tiwari et al., 2009). Moreover, before the advent of artificial food additives, natural ingredients like salt, vinegar and spices were traditionally used as effective food preservatives (Anand and Sati, 2013). Compounds with antimicrobial properties may be derived from microorganisms, animals and plants (Delves-Broughton, 2012), as well as algae and fungi (Pisoschi et al., 2018). Examples of naturally derived antimicrobial compounds that have been successfully used in the food industry include bacteriocins, microbial fermentates, compounds derived from milk (e.g. lactoperoxidases), chitin and chitosan from crustaceans and mushrooms, and essential oils extracted from plants (Delves-Broughton, 2012).

Consumer acceptance of food additives may depend on the source of the compound. Some compounds, although natural, may not be accepted by certain consumers (Asioli et al., 2017). For example, religious or socio-cultural beliefs in many cases could limit the acceptance of certain food additives, such as those that are products of microbial fermentation that require growth medium that contains animal-based ingredients that are not acceptable for kosher or halal production (Al-Mazeedi et al., 2013). Compounds extracted from plants, algae and fungi may therefore have a broader range of consumer acceptance.

Many studies have considered the antimicrobial effects of plant extracts (Tassou et al., 1995; Tiwari et al., 2009). Extracts from leaves, flower buds, bulbs, seeds, rhizomes, and

fruits have been shown to exhibit antimicrobial properties and demonstrate potential for the control of foodborne pathogens. Specific plant compounds that are known to have antimicrobial properties include peptides such as thionins, plant defensins, and lipid transfer proteins, as well as phytochemicals, including simple phenolic compounds, flavonoids, quinones, tannins, coumarins, essential oils, and alkaloids (Hintz et al., 2015).

A recent study investigated the effect of a variety of essential oils on the growth of *Salmonella* Enteritidis and *Listeria monocytogenes* on fresh Atlantic salmon. After decontamination of filets, enumerated cultures of both bacterial strains were used to inoculate the exterior of the fish samples. A mixture of 0.125 ml of 1% coriander, garlic and orange peel and 0.037 ml of 0.3% rosemary oil were applied to both sides of the salmon pieces. After 96 hours at 2˚C, bacterial enumerations of 25 g samples stomached in 0.1% peptone water showed statistically significant reductions in cell counts of both bacterial strains as compared to a control group. This work indicated that essential oils of plants may be applied to fresh fish to control the growth of pathogenic bacteria (Yasemin et al., 2018).

Another study showed promise of algal extracts for the control of bacterial foodborne pathogen growth. Varying concentrations of nine algal extracts were added to liquid cultures ($10^6$ CFU/ml) of five foodborne pathogenic bacteria, including *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* Typhimurium, and *Listeria monocytogenes*. Minimum inhibitory concentrations (MICs) were determined after 24 hours of incubation at 37˚C by measuring culture turbidity (optical density at 540 nm), and by the addition of an iodonitrote-trazolium chloride alcoholic solution, which indicated by colour change the occurrence of bacterial growth. Of note, an organic extract from the

algal genus, *Padina* inhibited growth of *B. cereus* and *S. aureus* with MIC values of 63 and 130 µg/ml, respectively. An organic extract of *Ulva* sp. also inhibited growth of these organisms with MIC values of 130 µg/ml, as well as *L. monocytogenes* with a more moderate MIC of 250 µg/ml. Given the history of human consumption of these marine plants, the authors suggest that their extracts may prove useful as antimicrobial agents in food systems (Dussault et al., 2016).

### 2.4 Bacteriophages as natural antimicrobials

An alternative natural approach to eliminating pathogenic bacteria in foods is the employment of bacterial viruses, known as bacteriophages (phages) (Salmond and Fineran, 2015). Phages are natural entities that specifically infect bacterial cells, leaving eukaryotic cells unharmed, which makes them useful tools in food safety applications (Garcia et al., 2011).

Phages were co-discovered in 1915 by British pathologist, Frederick Twort, and in 1917 by French-Canadian microbiologist, Félix d'Herelle (Twort, 1915; d'Herelle, 1917). Phages are present in all environments where bacteria thrive (Clokie et al., 2011), and there is an estimated $10^{31}$ phage particles on earth (McNair et al., 2012). Each phage family has different morphological properties. A phage may consist of a head containing either single-stranded (ss) or double-stranded (ds) DNA, a tail which may be long and flexible, contractile, or short, tail fibres, and a core (Garcia et al., 2008). Morphology of head, tail, and tail fibres varies depending on phage family (Coffey et al., 2010). Most discovered phages belong to three families; *Myoviridae, Siphoviridae*, and *Podoviridae,* of the order *Caudovirales*, which are non-filamentous, tailed dsDNA phages (Ackermann,

2007). Bacteriophage taxonomy may also be based on host specificity, genome, replication strategy, pathogenicity, and even geographic location (Mathews, 2001).

### 2.4.1 Bacteriophage infection

Infection of a bacterial cell begins with adsorption to the host's exterior, in which phage tail fibres bind to receptors present on the bacterial cell wall (Garcia et al., 2011). Affinity of a phage for a particular bacterial host is highly specific, as it depends on attachment sites on both the phage and the bacterium (Feiner et al., 2015). Outer membrane proteins, oligosaccharides and lipopolysaccharides in Gram negative bacteria, or murein in Gram positive bacteria may be involved in phage attachment (Ceyssens and Lavigne, 2010). Peptidoglycan hydrolases produced by phages degrade the bacterial host cell wall before a phage introduces genetic material through its tail (Garcia et al., 2011).

Replication of progeny phages takes place at the expense of the bacterial cell, which synthesizes proteins for the structural components of the phage (Feiner et al., 2015). The assembly begins with the phage head, or procapsid. The attachment region for the tail, a pore complex, forms at the procapsid vertex. A terminase directs the translocation of the phage genome into the procapsid. Finally, phage DNA is packaged and the tail attaches to the head to form a mature virion (Ceyssens and Lavigne, 2010).

Phages may release progeny from bacterial cells either by continuous extrusion without killing the host, or by causing lysis of the host cell (Garcia et al., 2011). Filamentous phages exhibit the former characteristic while non-filamentous phages exhibit the latter. Non-filamentous phages may lyse bacterial host cells by inhibiting peptidoglycan synthesis or by cleaving the peptidoglycan layer using enzymes (Garcia et al., 2011).

Cleavage of the peptidoglycan layer begins with holin— a small hydrophobic protein capable of permeabilizing the bacterial cell wall's inner membrane. The pores formed by holin allow for the entry of phage endolysins which degrade peptidoglycan. Endolysins cause lysis from within the host cell by breaking covalent bonds in the bacterial cell wall, causing the host cell to burst due to its inability to contain internal osmotic pressure (Young et al., 2000). A variety of endolysins exist, and while most are specific to one phage species, some are known to be present in multiple phages (Salmond and Fineran, 2015). Upon lysis of the bacterial cell wall, newly assembled phage progenies are liberated and ready to infect new hosts (Garcia et al., 2011).

### 2.4.2 Bacteriophage life cycles

Two distinct life cycles exist amongst phages; the lytic cycle and the lysogenic cycle (Salmond and Fineran, 2015). In both cases, upon encountering bacterial cells, phages attach to the bacterial host's cell wall, introduce their DNA into the cell and replicate through transcription and translation, taking advantage of the host's replication machinery. Once new phages are assembled, they lyse the host cell wall, and may then move on to infect surrounding bacterial cells (Coffey et al., 2010). The main difference between the two life cycles lies within the onset of viral replication in the host.

#### 2.4.2.1 The lytic cycle: Virulent bacteriophages

In the lytic cycle, phage replication occurs as described above, immediately after the viral DNA enters the host cell. Phages that portray such behavior are referred to as virulent phages (Salmond and Fineran, 2015). The most well studied virulent phage is phage T4.

It has a large (169 kb) genome and infects strains of *Escherichia coli* and *Shigella* and belongs to the family *Myoviridae* (Kurzepa et al., 2009).

Morphologically, phage T4 has a prolate icosahedral head, a contractile tail with fibres, and a whiskered collar. Tail fibres aid in host recognition and attachment to bacterial cell surface while a baseplate at the end of the tail determines host specificity, as it binds to receptors on the host's cell wall. DNA insertion works like a syringe; the phage genome is injected through the tail core to the inside of the bacterial cell as the tail sheath contracts (Kurzepa et al., 2009; Mathews, 2010).

Phage T4 was one of seven virulent phages isolated from a sewage system by Demerec and Fano in the 1940s (Demerec and Fano, 1945). They found that the group of *E. coli* infecting phages had ideal properties for laboratory use. T4 is one of the 'T-even' phages; a group that also contains phages T2 and T6, all of which are very closely related. Phage T4 was recommended for use as a model organism for biological systems because it was especially easy to isolate and store, and retained viability as a stock solution. Phage T4 is now the focus of many bacteriophage studies and its genome is well understood. It has played an integral role in the understanding of molecular biology, serving as a choice model in studies in DNA replication, transcription mechanisms and mutagenesis (Demerec and Fano, 1945; Mathews, 2001).

#### 2.4.2.2 The lysogenic cycle: Temperate bacteriophages

In the lysogenic cycle, phage DNA is introduced into a bacterial cell and immediately integrates into the host chromosome (as opposed to the lytic cycle), while repressing genes that are responsible for initiating the lytic cycle. Phages that undergo the lysogenic

cycle are known as temperate phages, and they remain as a prophage within the bacterial chromosome, unless host DNA damage triggers the onset of the lytic cycle. When DNA damage triggers the excision of the phage genome, lytic genes are no longer repressed, and are thus expressed, initiating new phage progeny assembly and ultimately the lysis of the bacterial host cell. Spontaneous induction of prophages may also occur, in cases where an apparent stressor is not present (Feiner et al., 2015). As long as prophage DNA is not excised from the bacterial chromosome, it is replicated along with host DNA and passed onto subsequent bacterial generations (Salmond and Fineran, 2015). Bacterial strains possessing prophages are termed lysogenic bacteria (Garcia et al., 2011).

The role of the temperate phage lambda (phage λ) in research is analogous to that of phage T4. Early studies in France by Esther Lederberg revealed it as the first temperate bacteriophage, as the lysogenic cycle had not yet been previously observed (Mathews, 2001). Like T4, phage λ also infects *E. coli* and has had significant impact on molecular genetics research as a model organism. The phage belongs to a group known as the lambdoid phages and is a member of the *Siphoviridae* family. Its dsDNA chromosome is smaller than that of phage T4, at 48 kb (Casjens and Hendrix, 2001).

Phage λ has an icosahedral head as well as a non-contractile tail with a tapered end and four side-tail fibres (Casjens and Hendrix, 2001). The tail binds to receptors on the bacterial cell wall, and the side-tail fibres are ‘non-essential’ in the sense that they are not used in host attachment. The life cycle that phage λ undergoes is determined at the molecular level. The *cI* gene encodes a repressor protein, CI, that binds to the phage λ

operon in the operator regions, $O_L$ and $O_R$. Consequently, CI blocks the transcription of the two earliest promoters of the operon, $P_L$ and $P_R$. Transcription of the promoters is thus prevented, lytic genes are silenced, and phage λ remains in lysogeny (Ptashne et al., 1980).

The onset of the lytic cycle in phage λ can occur due to exposure to ultraviolet light or DNA-damaging antibiotics such as mitomycin C (Casjens and Hendrix, 2001). Induction begins with damage to host DNA, triggering the SOS response, which results in high levels of protein RecA synthesis. RecA binds to and inactivates the CI protein repressor, allowing RNA polymerase to act on the operon and begin transcription. Temperate phage DNA must be excised from the host's genome and become circularized for new phage progeny to form. This is achieved by the production of high levels of integrases (integrating enzymes) and the Xis protein, which work together to reverse integration, and initiate excision. After successful excision and recircularization of prophage DNA, new phage progeny are formed and released when the bacterial cell lyses (Casjens and Hendrix, 2001).

## 2.5 Bacteriophage applications in the food industry

### 2.5.1 Phage cocktails

Virulent phage cocktails have become available for use in commercial food production and are used to eliminate bacterial foodborne pathogens of public health concern (Moye et al., 2018). Phages in these cocktails are carefully selected with the practical application and target bacteria in mind. Typically, several isolated phages are combined so that the likelihood of killing the closely related target pathogens (including its prominent or likely

present serovars in a processing plant) is maximized (Gill and Hyman, 2010). The first phage cocktail to be regulated by the Food and Drug Administration (FDA) in the United States was a blend called LMP-102. It consists of six virulent phages that are specific for *L. monocytogenes* strains and was developed for use on the surface of prepackaged ready-to-eat meat and poultry products (Bren, 2007). Applications for phage cocktails exist in the control of environmental contamination in food production plants, elimination of pathogens on raw foods or carcasses, and may be incorporated into feed in order to treat animal infections (Garcia et al., 2011). Table 2.1 shows commercially available phage products.

Phage cocktails have the advantage that they can be designed to reliably eliminate specific groups of pathogenic bacteria (Kazi and Annapure, 2016). A food manufacturer will select a phage cocktail based on the risk microorganisms that are commonly present in the foods that they produce. However, the host range is typically narrow and limited to one bacterial species, or serovars of highest public health concern within a single species, which is a disadvantage when control of multiple pathogens is needed (Moye et al., 2018).

### 2.5.2 Prophage induction for the control of bacterial foodborne pathogens

The prophage induction approach for control of bacteria relies on the premise that prophages are ubiquitous within bacterial DNA. One recent bioinformatics study examined the prevalence of prophages within bacterial genomes (Kang et al., 2017). Of the almost 12,000 bacterial genomes acquired from an online database, about 10,000 (83%) had at least one prophage present. A number of compounds have been experimentally shown to induce prophages in the laboratory. For example, Kosugi et al.

(1983) demonstrated that roasted coffee beans could be used to induce λ phages in a strain of *E. coli* K12.

To date, there has only been one study that considered temperate phage induction as a food safety measure to control the growth of bacterial foodborne pathogens (Cadieux et al., 2018). In this work, it was demonstrated that prophage-inducing compounds could effectively control the growth of *Salmonella enterica* and shiga toxin-producing *E. coli* (STEC) strains in both *in vitro* and food system models.

In the *in vitro* model, varying concentrations of mitomycin C and streptonigrin were added to liquid cultures of *S. enterica* and STEC strains at the mid logarithmic growth stage. Cell growth determined by optical density (600 nm) and plate counts showed that both agents effectively reduced growth of the pathogens. PCR amplification of phage integrase genes from viral DNA in the lysates confirmed that the reduction in cell number was caused by the induction of prophages, showing that a single inducer can induce multiple prophages across different bacterial genera (Cadieux et al., 2018). Therefore, this approach has the advantage over the use of phage cocktails because multiple bacterial species could be controlled using prophage induction, in contrast to phage cocktails in which only a single species is targeted. In a food system model, fresh greenhouse tomatoes and spinach were inoculated with the pathogens, and mitomycin C (6 µg/ml) was sprayed on the surface of the produce. After overnight storage at 4˚C, 1-1.5-log and 2-log reductions in cell concentration of *S. enterica* were observed on tomatoes and spinach, respectively. Treatment with mitomycin C on STEC-contaminated produce resulted in 3-log and 1-log reductions in growth on tomatoes and spinach, respectively (Cadieux et al., 2018).

It is important to consider that the modern consumer is increasingly interested in foods that contain only natural ingredients. Although phages are natural entities, phage cocktails that are added to foods may not be widely accepted as clean label ingredients or processing aids, due to a lack of scientific understanding of phages by the average consumer (Garcia et al., 2011). Prophage-inducing natural compounds may have socio-cultural advantages as antimicrobial food additives when compared to the addition of exogenous virulent phages.

Furthermore, since the approval for a new food additive or ingredient, for example a new phage cocktail, can be a lengthy process, the use of natural compounds that have already been approved by food governing agencies would save time and money. In Canada, new food additive legislation falls under the Food and Drug Act and Regulations. A company that wishes to use a new food additive must submit an application which includes a complete and thorough safety assessment. A pre-market evaluation by Health Canada's Food Directorate is then conducted to further verify the safety of the new food additive[3]. With this in mind, it is beneficial to use a phage inducing compound that has already been regulated as an approved additive or ingredient.

### 2.5.3 Safety considerations of bacteriophage applications

Phage treatment has been safely used to treat human infections for many years in Eastern Europe and the former Soviet Union (Garcia et al., 2011). Since phages cannot

[3] https://www.canada.ca/en/health-canada/services/food-nutrition/public-involvement-partnerships/food-directorate-market-submission-management-process-food-additives-infant-formulas-novel-foods.html

bind to eukaryotic cells, they cannot infect them, and are thus harmless to human and animal cells (Garcia et al., 2011). This notion is supported by a study in which humans ingested up to $10^5$ PFU/ml of T4 phage without experiencing any associated health issues (Bruttin and Brüssow, 2005).

The specific and narrow host range of phages means they have a negligible effect on beneficial microbes, such as those in the human gut. Recently, a study by Hu et al. (2018) compared the use of phages versus antibiotics in the treatment of *Salmonella* infection in an *in vitro* human gut microbiota model consisting of anaerobically cultivated human intestinal microflora (ACHIM). The authors reported that the *Salmonella* phage cocktail, SalmoFresh had less impact on the non-target ACHIM bacteria than the antibiotic, azithromycin, based on sequence data after treatment. These findings position phage therapy as an effective and ostensibly safe alternative to the treatment of bacterial infections without negative consequences on the established healthy microbiota (Hu et al., 2018).

Another study that considered the effect of phages and antibiotics on the gut microbiome involved the oral administration of a *Salmonella* phage cocktail and an antibiotic (ASP250) to piglets (Schmidt, 2016). Based on 16S sequencing analyses from cecum, ileum and fecal content samples, the authors determined that there was no significant difference between the gut microbiome of phage-treated versus control group piglets. The microbiome of the antibiotic-treated piglets, was however, altered significantly, compared to that of phage-treated and control groups (Schmidt, 2016).

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The relationship between phages and their bacterial hosts sometimes exhibits a mutually beneficial symbiosis (Feiner et al., 2015). While the bacterial host supports the latent survival of a temperate phage, it, in turn may acquire beneficial genes from the phage genome, thus increasing its fitness. Lysogenic conversion refers to a phenomenon in which bacterial hosts express traits of prophages in the chromosome. Alternatively, a bacterial host can acquire genes from temperate phages through horizontal gene transfer (HGT) which in certain cases, ultimately allows the host to gain pathogenic properties. HGT from a phage to a bacterium is called transduction (Kelly et al., 2009). In generalized

transduction, bacterial DNA is packaged into a temperate phage capsid and may be transferred to another host. In specialized transduction, phage DNA, along with neighbouring bacterial host DNA is packaged by a temperate phage (Thierauf et al., 2009). Botulinum toxin, shiga toxins, and cholera toxin are examples of virulence attributes gained by bacteria from phages through horizontal gene transfer (Feiner et al., 2015).

Horizontal gene transfer is a major culprit in the acquisition of antibiotic resistance genes (ARGs) in clinical pathogens, and ultimately contributes to the increasing pandemic of antimicrobial resistance (von Wintersdorff et al., 2016). Since phages can confer antibacterial resistance and virulence genes to their hosts by transfer of ARGs, there is concern that the use of temperate phages in the food industry may create stronger pathogenic bacterial strains. The risk of ARG transfer by prophages in foodborne pathogens is a well-studied topic in the literature (Colavecchio et al., 2017).

However, the rates of transduction in the environment as well as its role in the evolution of bacteria in phage therapy is not well understood, and therefore. the role of phage-mediated gene transfer of ARGs, as well as the conditions required for it, should be further studied (Colavecchio et al., 2017; Touchon et al., 2017). For example, while laboratory studies have demonstrated the ability of temperate phages to transduce ARGs to susceptible bacteria (Marinus and Poteete, 2013; Shousha et al., 2015), Kelly et al. (2009) explains that there are several barriers against the successful uptake of new genes in bacteria by phage-mediated transduction and that the nature of the phage-host interaction in transduction is very specific and serves as a barrier for horizontal gene transfer in and of itself.

This statement is supported by *in vivo* studies that have examined the ability of temperate phages to transduce ARGs. A 2011 study on phage-mediated gene transfer examined the *in vivo* prophage inducing effects of antibiotics in the gut (Allen et al., 2011). Using a metagenomics approach, the authors monitored fecal phage and bacterial communities of swine that were fed antibiotics (carbadox and ASP250) and compared results to a control group of swine that were on a non-antibiotic diet. In the carbadox group, shifts in bacterial diversity were noted after 16S rRNA sequencing, and there was an increase in phage integrase genes, suggesting that prophage induction had occurred. However, since no changes in the relative number of ARGs that these prophages carried were observed, the authors concluded that phage-mediated gene transfer did not occur (Allen et al., 2011).

Another study reconsidered the bioinformatics approaches that are conventionally used in identifying ARGs in phage genomes (Enault et al., 2017). After investigating the ARGs present in online database sequences with a conservative approach, the authors suggested that the prevalence of ARGs in phage genomes is overestimated. Typical parameters in ARG searches can result in hits that have low homologies to actual ARGs. The authors propose that ARGs in phages are uncommon, and that the majority of previously identified ARGs in viromes do not actually confer antibiotic resistance.

## 2.6 Conclusion

This review presents a rationale for the use of phages in the context of food safety. The natural entities may be employed in controlling the growth of bacterial foodborne pathogens, and they have sociological advantages over synthetically produced

antimicrobial food additives. The application of prophage induction in bacterial foodborne pathogens presents as a noteworthy food safety measure to investigate. Further research should begin with screening natural compounds for their potential to damage bacterial DNA and trigger the induction of prophages.

## 2.7 Tables

| Company | Phage Product | Target Organism(s) | Regulatory Approval |
|---|---|---|---|
| FINK TEC GmbH (Hamm, Germany) | Secure Shield E1 | *E. coli* | FDA (Food and Drug Administration) pending as of 19 March, 2018 |
| Intralytix, Inc. (Baltimore, Maryland, USA) | Ecolide | *E. coli* O157:H7 | USDA (United States Department of Agriculture) |
| | EcoShield | *E. coli* O157:H7 | FDA, Israel Ministry of Health (IMH), Health Canada (HC) |
| | ListShield | *L. monocytogenes* | FDA, EPA (Environmental Protection Agency), IMH, HC |
| | SalmoFresh | *Salmonella* spp. | FDA, USDA, IMH, HC |
| | ShigaShield, ShigActive | *Shigella* spp. | FDA |
| Micreos Food Safety (Wageningen, Netherlands) | PhageGuard Listex | *L. monocytogenes* | FDA, Food Standards Australia New Zealand (FSANZ), European Food Safety Authority (EFSA), Swiss Federal Office of Public Health (Swiss BAG), IMH, HC |
| | PhageGuard S | *Salmonella* spp. | FDA, FSANZ, Swiss BAG, IMH, HC |
| | PhageGuard E | *E. coli* O157:H7 | FDA Pending as of 19 March, 2018 |
| Passport Food Safety Solutions (West Des Moines, Iowa, USA) | Finalyse | *E. coli* O157:H7 | USDA, HC |
| OmniLytics (Sandy, Utah, USA) | AgriPhage | *Xanthomonas campestris* pv. *Vesicatoria* *Pseudomonas syringae* pv. *tomato* | EPA |
| | AgriPhage CMM | *Clavibacter michiganensis* pv. *michiganensis* | EPA, HC |
| | SalmoPro | *Salmonella* spp. | FDA |

**Table 2.1**. Commercially available phage products for control of foodborne bacteria.

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## Connecting text

A review of the scientific literature revealed various factors that influence the food industry to choose clean label alternatives, such as natural compounds as antimicrobials instead of those that are produced synthetically. Bacteriophage (phage) cocktails represent a unique and natural approach to food safety. They are currently employed to control the presence of pathogenic bacteria in foods, however a fundamental disadvantage lies in the fact that host range is limited by which phages are selected for a given cocktail. Since phages are highly specific in terms of host affinity, phage cocktails risk allowing rare serovars of bacterial pathogens to survive in the foods to which they are added. The induction of prophages using DNA-damaging agents represents an alternative phage-based approach for the control of foodborne pathogenic bacteria. This approach has an advantage over current phage approaches because it may address the issue of narrow host range, as a single prophage-inducing compound could induce prophages across multiple bacterial genera. The proceeding chapter considers prophage induction as a noteworthy approach in the control of foodborne bacterial pathogens and focuses on the development and application of a high-throughput assay to detect compounds that could prove useful as food-grade inducers of prophages in a model *Escherichia coli* organism.

## Chapter 3: Development of a high-throughput luminescent prophage induction assay for the identification of *prophage λ* inducing compounds

### 3.1 Abstract

Prophage induction and subsequent bacterial lysis represents an emerging approach to bacterial control in foods. In this work, an assay was developed to facilitate high throughput screening (HTS) of compounds that induce prophages. *Escherichia coli* BR513 is a genetically engineered K-12 derivative that carries a *lacZ*-prophage λ gene fusion and produces β-galactosidase upon induction of prophage λ. To develop the assay, 3 antibiotics (mitomycin C, streptonigrin and norfloxacin), which are well known prophage inducers, were used as positive controls to assess the ability of *E. coli* BR513 to identify prophage induction. The antibiotics were incubated with *E. coli* BR513 and wildtype *E. coli* K-12 (as a control) at subinhibitory concentrations, for 24 hours at 37°C, followed by measurement of β-galactosidase production using a luminescent substrate. A multivariate regression analysis revealed that each antibiotic significantly increased β-galactosidase production in the *E. coli* BR513 strain. The log-log model ($R^2$ = 0.999) showed a robust power law relation between the ratio of relative light units (RLU) to optical density at a wavelength of 600 nm ($OD_{600}$) after 24 hours of incubation of the non-treated cultures and the same ratio from cultures that were treated with antibiotics. Statistical analysis showed that streptonigrin was the most potent prophage-inducing compound, followed by mitomycin C and norfloxacin. Based on the results with the antibiotics, assay parameters (incubation time, luminescent substrate concentration) were finalized, and the completed assay was used to screen several natural compounds and foods for prophage inducing activity.

The results indicated that gallic acid, rosemary, pH-neutralized cranberry juice, coffee, and a variety of green and black tea effectively induced prophages in *E. coli* BR513. The assay developed in this study validated the effectiveness of using *E. coli* BR513 as a biological indicator for the detection of prophage inducing compounds.

## 3.2 Introduction

The use of bacteriophages (phages) as biocontrol agents is an emerging method for reducing the level of bacterial pathogens in foods (Touchon et al., 2017; Moye et al., 2018). Phage cocktails, comprised of mixtures of virulent phages, are invaluable tools in the food industry because specific phages can be used to infect and lyse bacterial pathogens, leaving other, perhaps beneficial bacteria, unharmed (Sulakvelidze et al., 2001). There are many examples of commercially available phage cocktails used as antimicrobials in food safety applications (Table 2.1) to control the growth of foodborne bacterial pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* (Boyacioglu et al., 2013; Silva et al., 2014; Soffer et al., 2016).

With respect to phage life cycle, two types of phages exist, including virulent phages and temperate phages. Virulent phages are exclusively used in food safety applications, because they replicate according to the lytic cycle in which viral replication occurs following immediate introduction of viral DNA into the bacterial host cells, causing subsequent bacterial cell lysis. Temperate phages represent a separate phage class that may either grow lytically, resulting in lysis bacterial host cells, or phage DNA may integrate into the bacterial host cell's chromosome and enter into a dormant state (Salmond and Fineran, 2015). The tendency of temperate phages to integrate into the host cell chromosome explains why their use in phage cocktails has been opposed (Gill and Hyman, 2010; Kazi and Annapure, 2016). However, recent work demonstrated that temperate phages may be used as antimicrobials by taking advantage of the fact that they are already inside bacterial cells in the form of prophages. Prophages are carried by the majority of bacteria (Kang et al., 2017 Bailey et al., 2017).

Prophage induction as an antimicrobial approach differs from traditional phage-based biocontrol of bacterial pathogens in that the traditional approach employs cocktails of phages that are added exogenously to infect and lyse bacterial cells (Sulakvelidze et al., 2001). To induce prophages, compounds may be added exogenously, to trigger the SOS response, leading to lytic growth of temperate phages and ultimate bacterial cell lysis (Campoy et al., 2006). One potential advantage of the prophage induction approach as opposed to the traditional phage-based biocontrol, is the fact that only single bacterial species are typically targeted in traditional phage biocontrol since it would be impractical to include the large number of phages required to control multiple bacterial species. In prophage induction, compounds used to induce prophages can induce multiple prophages from multiple bacterial species, indicating that prophage induction could be used to control multiple foodborne pathogens simultaneously.

Recently, Cadieux et al. (2018) conducted a model study in which fresh greenhouse tomatoes and spinach were inoculated with *Salmonella enterica* and Shiga toxin-producing *E. coli* (STEC). The produce was sprayed with mitomycin C (6 µg/ml) and was then stored at 4˚C overnight. Based on plate counts, the resulting cell concentrations of *S. enterica* and STEC were significantly reduced (Cadieux et al., 2018). In an accompanying *in vitro* model, it was demonstrated that mitomycin C was capable of inducing prophages at a concentration of 2 µg/ml, as the presence of phage integrase genes in the viral DNA lysates was confirmed by PCR. Cadieux et al. stressed that prophage induction stimulated by the addition of certain compounds could be a useful food safety measure, but that the use of antibiotics to control growth of pathogenic bacteria on food is not a practical option, especially since this could contribute to

increases in antibiotic resistance. Thus, the identification of non-antibiotic prophage inducers is needed. Ideally these compounds would be naturally occurring, given current consumer trends of rejecting synthetic compounds in foods (Asioli et al., 2017).

Previous studies have described several antibiotics as being efficient prophage inducers, notably the DNA-damaging agents mitomycin C, streptonigrin, and norfloxacin (Levine and Borthwick, 1963; Raya and H'Bert E, 2009; McDonald et al., 2010). However, only a few natural compounds have been identified to be prophage inducers. The work of Hossain et al. (2013) did not specifically focus on the induction of prophages, but the authors found that compounds in tea and coffee were potent DNA-damaging agents. It was determined that pyrogallol-like polyphenols from tea and coffee cause breaks in DNA. Specific examples of DNA-damaging compounds include gallic acid, epigallocatechin gallate (EGCG), and pyrogallol (Hossain et al., 2013). Since prophage induction can begin with the initiation of the SOS response caused by DNA damage (Campoy et al., 2006), the above mentioned compounds as well as the foods in which they are found may serve as effective prophage-inducing agents that could be used to reduce the growth of bacterial pathogens in food.

In this study, a high-throughput luminescent prophage induction assay for the identification of prophage-inducing compounds was developed and validated using known antibiotic prophage inducers. The assay was subsequently used to screen food-grade compounds for prophage inducing activity that could lead to lysis of pathogenic bacteria. Compounds identified by the assay may be considered for use as novel and natural food-grade antimicrobials in the future.

## 3.3 Materials and methods

### 3.3.1 Bacterial strains

Two bacterial strains were used in this work: *E. coli* BR513 (ATCC 33312) and *E. coli* K-12 (ATCC 33694). *E. coli* BR513, previously used by Elespuru and Yarmolinksy (1979), in the development of a colorimetric assay to measure prophage induction, is a genetically engineered bacterial strain that is useful for identifying prophage-inducing compounds. *E. coli* BR513 is a K-12 derivative that carries a *lacZ*-prophage λ gene fusion. Prophage-inducing mechanisms such as DNA damage, triggers the cleavage of the lambdoid phage repressor, CI, resulting in the synthesis of β-galactosidase (Elespuru and Yarmolinsky, 1979). Thus, *E. coli* BR513 synthesizes β-galactosidase when induction of the temperate phage λ occurs. Wildtype *E. coli* K-12 was selected as a control in the assay since it does not carry the *lacZ*-prophage λ gene fusion. Instead, it contains a wildtype *lacZ* operon under the control of the *lac* promoter.

### 3.3.2 Whole genome sequencing (WGS) of *Escherichia coli* BR513

A library of *E. coli* BR513 DNA was constructed using the NEBNext Ultra II DNA library prep kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) according to manufacturer's instructions. Whole genome sequencing (WGS) was performed at the Genomics Analysis Platform[4] (part of the Institute of Integrative Biology and Systems (IBIS) at Laval University, Quebec City, Quebec, Canada) on an Illumina® MiSeq sequencer that generated 300-bp paired-end reads with 40X coverage. Resulting reads

[4] http://www.ibis.ulaval.ca/en/services-2/genomic-analysis-platform/

were assembled *de novo* using the A5 pipeline (Tritt et al., 2012). The *E. coli* BR513 genome was also sequenced using the Oxford Nanopore Technology MinION and the associated SQK-LSK108 library preparation kit and a R9.4 flowcell. Hybrid assembly of the MinION data was performed using Unicycler (v0.4.0) with the MiSeq and base called Oxford Nanopore reads (Wick et al., 2017).

The hybrid-assembled *E. coli* BR513 genome was annotated using Prokka 1.13.3 (Seemann, 2014). The resulting Gene Feature Format (GFF) file was visualized using DNAPlotter (Carver et al., 2009) and Artemis[5] (Carver, 2012). Location of the cryptic lambdoid phage and β-galactosidase fusion was determined based on BLASTn alignment of the wildtype lambda phage (GenBank accession number J02459.1) and *E. coli* BR513.

### 3.3.3 Development of the high-throughput luminescent prophage induction assay

Cultures of *E. coli* BR513 and *E. coli* K-12 (stored in 20% glycerol at -80°C) were streaked onto tryptic soy agar (TSA) (Becton, Dickinson and Company, Sparks, Maryland, USA) followed by incubation at 37°C for 18-24 hours. Triplicate liquid cultures of each strain were prepared by inoculating trypticase soy broth (TSB; Becton, Dickinson and Company) supplemented with 0.2 M glucose (TSB 0.2 M glucose) with 4-6 well isolated colonies. Glucose was added to TSB to reduce the expression of β-galactosidase via catabolite repression in the wildtype *E. coli* K-12 strain, since its *lacZ* gene is regulated by the *lac* promoter and repressor. The liquid cultures were incubated at 37°C, with orbital shaking at 250 rpm for 16 hours.

[5] https://www.sanger.ac.uk/science/tools/artemis

Similar to the *in vitro* experiment conducted by Cadieux et al. (2018), overnight cultures of *E. coli* BR513 and *E. coli* K-12 were diluted to an optical density (wavelength = 600 nm) ($OD_{600}$) of 0.1 as determined by an Ultrospec 100 Pro spectrophotometer (Biochrom Ltd, Cambridge, England). Cultures were subsequently grown to mid-logarithmic phase ($OD_{600}$ of 0.5). The following antibiotics were added to a final concentration of 2 µg/ml: mitomycin C (Sigma, St. Louis, Missouri, USA), streptonigrin (Sigma), and norfloxacin (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cultures were incubated at 37°C, with orbital shaking at 250 rpm for 24 hours.

The relative light units (RLU) of each culture were measured after the 24-hour incubation period, using the luminescent substrate, Beta-Glo® (Promega, Madison, Wisconsin, USA) in a luminometer plate reader (HTX Synergy Multi Mode Reader, Bio Tek Instruments, Winooski, Vermont, USA). The luminescent reagent contains a lysis buffer and the substrate D-luciferin-*o*-β-galactopyranoside. β-galactosidase cleaves the D-luciferin-*o*-β-galactopyranoside releasing luciferin, which is then catalyzed by luciferase to produce light. The measurement of RLU is therefore indicative of the amount of β-galactosidase (Hannah, 2003).

To determine whether the induction of prophage λ resulted in bacterial cell lysis and/or inhibition of growth, the $OD_{600}$ of antibiotic-treated and non-treated cultures was measured at 0, 2, 4, 6, 8 and 24 hours after incubation ($OD_{600}$ was used as an indication of cell concentration). Significant differences between antibiotic-treated and non-treated cultures at 24 hours were determined by a two-sample t-test, assuming unequal variances ($\alpha = 0.05$).

RLU values from both *E. coli* strains were normalized to $OD_{600}$ to reduce the effect of cell concentration on background β-galactosidase levels and to allow for the two *E. coli* strains to be compared on a per cell basis. RLU values were normalized by dividing each replicate's RLU value by its corresponding $OD_{600}$ value taken after 24 hours of incubation.

#### 3.3.3.1 Data analysis

The ability of antibiotics to enhance the production of β-galactosidase (which is indicative of prophage induction) in *E. coli* BR513 was assessed by performing a multivariate regression analysis, using the SPSS Version 24 statistical analysis system (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.) The log-transformed $OD_{600}$-normalized RLU values from the antibiotic-treated (2 µg/ml) samples ($\log[RLU/OD_{2\mu g/ml}]$) was considered to be the dependent variable. The independent variables were the $OD_{600}$-normalized RLU values from the non-treated (0 µg/ml) samples ($\log[RLU/OD_{0\mu g/ml}]$), the antibiotics, and the strain types.

The reference category was mitomycin C-treated *E. coli* K-12. The constant variable was set as zero. The null hypothesis was that each antibiotic had an equal effect on $\log(RLU/OD_{2\mu g/ml})$ and that the addition of antibiotics did not affect $\log(RLU/OD_{2\mu g/ml})$ for either *E. coli* strain.

### 3.3.4 Screening of food-grade compounds

Twelve food-grade compounds (Table 3.2) were tested in the high-throughput luminescent prophage induction assay. Tea, coffee, and rosemary were prepared by

steeping dried leaves or ground coffee beans in 100°C water for 15 minutes, followed by filtration through cheesecloth and sterilization using 0.2 µm syringe filters.

The food-grade compounds were screened using the methods that were developed for the high-throughput luminescent prophage induction assay (section 3.3.3), with certain exceptions: the $OD_{600}$ was not measured over time; thus, RLU was not normalized to $OD_{600}$ and non-treated cultures were not included in the screen. These alterations of the HTS protocol were necessary since natural pigments in the foods resulted in inaccurately high $OD_{600}$ readings of liquid cultures (data not shown). Similarly, the intensity of the natural pigments drastically depressed luminescent signals, which resulted in generation of RLU levels that were lower than their non-treated counterparts. Non-treated cultures were therefore not as useful for interpreting results as they were in the initially developed assay using antibiotics.

#### 3.3.4.1 Data analysis

Since RLU could not be normalized to $OD_{600}$ and non-treated cultures were not included in the screen, modifications were made to the analysis for the food-grade compounds screen, as compared to the antibiotics screen. β-galactosidase levels, as measured by luminescence both immediately and 24 hours after the addition of food-grade compounds, were compared to determine whether prophage induction occurred. A statistically significant increase in RLU over time in the indicator *E. coli* BR513 strain, determined via paired two-tailed t-tests, inferred prophage induction. To identify the strongest prophage inducers, each increase in RLU over time ($RLU_{24\ hours} - RLU_{0\ hours}$) for each compound

that resulted in a significant increase in RLU over time was compared. These results were compared to one another via unpaired, two-tailed t-tests.

## 3.4 Results

### 3.4.1 Bioinformatics analysis confirms presence of cryptic phage λ in *E. coli* BR513

The MinION produced 97,308 reads with a median length of 7,774 nucleotides, for a total of 794,600,212 sequenced bases. MiSeq Illumina sequencing yielded 79 contigs with an estimated genome size of 4,547,678 bp. The Oxford Nanopore MinION combined hybrid assembly yielded two circular contigs: a chromosome of 4,512,014 bp and a plasmid of 82,939 bp. BLASTn analyses with the nt/nr NCBI database of the BR513 plasmid revealed 99% identity over 100% of its sequence with the *E. coli* K-12 F plasmid (GenBank accession number AP001918.1). Annotation of the of *E. coli* BR513 genome confirmed the presence of the *lacZ*-prophage gene fusion, as illustrated in Figure 3.1. Bioinformatic analysis revealed that this region is 37,243 bp in length, with the $P_L$ promoter positioned 5,732 bp downstream of *lacZ*. Since *lacZ* is fused to the cryptic phage and the *lac* promoter is absent, the production of β-galactosidase is regulated by $P_L$. Prophage induction, triggered by cleavage of the CI repressor that allows transcription of *lacZ* to initiate, results in the production of β-galactosidase, as described by Elespuru and Yarmolinsky (1979).

### 3.4.2 Multivariate regression analysis characterizes change in relative light units (RLU) in antibiotic-treated cultures

The average RLU produced by both *E. coli* strains with and without antibiotics is shown in Figure 3.2. Multivariate regression analysis of these values revealed that each antibiotic significantly increased β-galactosidase production in the *E. coli* BR513 strain. The log-log model ($R^2$ = 0.999) showed a robust power law relation between the $RLU/OD_{600}$ ratio of non-treated cultures ($RLU/OD_{0\mu g/ml}$) after 24 hours of incubation and that of antibiotic-treated cultures ($RLU/OD_{2\mu g/ml}$).

$$\log\left(\frac{RLU}{OD_{600}}\right)_{2\mu g/ml} = \beta_1 \log\left(\frac{RLU}{OD_{600}}\right)_{0\mu g/ml} + \beta_2 D_S \log\left(\frac{RLU}{OD_{600}}\right)_{0\mu g/ml} + \beta_3 D_N \log\left(\frac{RLU}{OD_{600}}\right)_{0\mu g/ml} + \beta_4 D_E \log\left(\frac{RLU}{OD_{600}}\right)_{0\mu g/ml}$$

Where;

$D_S$ is a dummy variable for the difference between streptonigrin and mitomycin C

$D_N$ is a dummy variable for the difference between norfloxacin and mitomycin C

$D_E$ is a dummy variable for the difference between *E. coli* BR513 and *E. coli* K-12

The multivariate regression equation may be simplified as:

$$\left(\frac{RLU}{OD_{600}}\right)_{2\mu g/ml} = \left(\frac{RLU}{OD_{600}}\right)_{0\mu g/ml}^{k}$$

Where k coefficients were calculated by adding the corresponding β coefficients (listed in Table 3.1) for each antibiotic and strain combination.

While the exponents for the control strain were statistically indistinguishable from 1 (see Table 3.1 for $\beta_1$ with $\beta_2$ and with $\beta_3$, or k coefficients calculated for *E. coli* K-12), the exponents for *E. coli* BR513 were statistically above 1 at a 1% significance level (see

Table 3.1 for $\beta_4$ with $\beta_1$, with $\beta_2$ and with $\beta_3$, or k coefficients calculated for *E. coli* BR513). Regression analysis therefore confirmed that *E. coli* BR513 behaved differently than the wildtype *E. coli* K-12 strain. *E. coli* BR513 produced significantly higher levels of β-galactosidase as a result of exposure to antibiotics, whereas the wildtype *E. coli* K-12 strain did not. Since *E. coli* K-12 is a wildtype strain, it was not expected that DNA-damaging antibiotics would cause a significant change in β-galactosidase production and thus $RLU/OD_{2\mu g/ml}$. Overall, the regression analysis demonstrated that the genetically engineered strain, *E. coli* BR513, can be successfully used to detect prophage induction. Regression analysis also identified streptonigrin as the strongest prophage-inducing antibiotic, followed by mitomycin C and norfloxacin, with k coefficients of 1.282, 1.213, and 1.143, respectively. The null hypothesis that the effect of each antibiotic on $\log(RLU/OD_{2\mu g/ml})$ is equal was therefore rejected.

### 3.4.3 Antibiotics reduce the cell concentration of *E. coli* BR513 and *E. coli* K-12

The average $OD_{600}$ measurements at 0, 2, 4, 6, 8 and 24 hours after the addition of antibiotics showed that mitomycin C, streptonigrin, and norfloxacin all resulted in statistically significantly lower cell concentrations of both *E. coli* BR513 and *E. coli* K-12. This is illustrated by differences in $OD_{600}$ measurements between average antibiotic-treated (2 µg/ml) and non-treated (0 µg/ml) cultures after 24 hours of incubation, as shown in Figure 3.3.

### 3.4.4 Food-grade compounds induce prophage λ in *Escherichia coli* BR513

The paired two-tailed t-tests comparing RLU values at 0 versus 24 hours after the addition of food-grade compounds in *E. coli* BR513 showed that 10 of 12 compounds resulted in significantly increased RLU values over time, as illustrated in Figure 3.4. All compounds except for sodium citrate and chai tea caused a significant increase in RLU in the indicator strain. The average RLU in the wildtype *E. coli* K-12 strain decreased significantly in response to all compounds except for sodium citrate and chai tea (data not shown). The average RLU of *E. coli* K-12 cultures treated with sodium citrate significantly increased, whereas that of chai tea decreased, but not significantly.

The change in average RLU of the wildtype *E. coli* K-12 strain supports the observations made with the indicator *E. coli* BR513 strain. Since the *lacZ* operon in *E. coli* K-12 is not controlled by phage genes, β-galactosidase production should increase with increasing cell concentration and it should decrease with decreasing cell concentration. When a putative prophage-inducing compound is added, the cell concentration is expected to decrease due to cell lysis or inhibition of bacterial growth, thus resulting in decreased production of β-galactosidase over time. In the case of *E. coli* BR513, average RLU is expected to increase when prophage λ is induced due to its *lacZ*-prophage gene fusion. Since sodium citrate and chai tea did not exhibit a significant increase in average RLU in the indicator strain, nor was there a significant decrease in average RLU in the wildtype strain, it was concluded that neither of the two compounds are prophage inducers.

The prophage-inducing strength of each compound was compared via unpaired, two-tailed t-tests. The average increase in RLU from 0 to 24 hours for *E. coli* BR513 that resulted from the addition of each compound was compared. Figure 3.5 ranks the average increase of RLU for each compound, which shows significant differences between them. The strongest compounds identified were gallic acid, followed by kukicha and iron goddess green teas. These two teas had statistically equal average increases in RLU. The strongest antibiotic inducer, streptonigrin, which was included in the comparison for reference, was the third strongest prophage-inducing agent in this analysis. The inducing strength of iron goddess green tea was not significantly different than that of streptonigrin or kukicha green tea; however, the effect of streptonigrin and kukicha on prophage induction were significantly different.

### 3.5 Discussion

The goal of this work was to develop a luminescence-based HTS assay for the identification of prophage-inducing agents using known antibiotic prophage inducers. This work is based on the work of Elespuru and Yarmolinsky (1979), in which a genetically engineered bacterial strain, *E. coli* BR513, was used. This strain's *lacZ*-prophage λ gene fusion makes it useful for identifying prophage-inducing agents, as indicated by the production of β-galactosidase. The authors relied on colorimetric methods to detect β-galactosidase, using the chromogenic substrates ortho-nitrophenyl-β-galactoside (ONPG), X-gal, and Fast Blue. The elementary principle behind colorimetric detection of β-galactosidase is based on the enzyme's ability to act upon substrates to produce a visual colour change (Smale, 2010).

In this work, several improvements were made to the original prophage induction assay developed by Elespuru and Yarmolinsky (1979). For example, the luciferase-dependent luminescent substrate, β-Glo®, was used to detect β-galactosidase production by the two *E. coli* strains. HTS assays are commonly based on luciferase reporters, since they are convenient to use and highly sensitive (Inglese et al., 2007). One study showed that a luminescence-based assay was 4-fold and 1,000-fold more sensitive as compared to fluorometric- and colorimetric-based assays, respectively (Van Poucke and Nelis, 1995). Thus, luminescence was selected as the detection strategy for quantifying β-galactosidase levels in the current high-throughput luminescent prophage induction assay.

In addition, the incubation period of induction was increased to 24 hours. Elespuru and Yarmolinsky (1979) used a shorter induction incubation period of 2-3 hours to minimize the effects of growth differences between treated and non-treated *E. coli* BR513 cultures. Since inducing agents can inhibit bacterial growth or cause bacterial cell lysis, an extended incubation time can generate highly inaccurate basal β-galactosidase levels, which corresponds to the basal level produced by non-treated *E. coli* BR513 that was taken into account for calculating induction activity of compounds (Elespuru and Yarmolinsky, 1979). For extended incubation periods of 5-6 hours, Elespuru and Yarmolinsky used ampicillin-supplemented culture media to inhibit growth of the non-treated cultures.

As demonstrated by the $OD_{600}$ values in Figure 3.3 B and C, significant growth differences between antibiotic-treated and non-treated cultures can be observed in as

little as 2 hours. This shows that the limited induction incubation time used by Elespuru and Yarmolinsky (1979) may not have effectively addressed the issue of discrepancies in β-galactosidase levels between treated and non-treated cultures. Furthermore, the incubation period of prophage induction should ideally be longer than 5-6 hours in case food-grade compounds require more time to cause prophage induction. In the work of Cadieux et al. (2018), mitomycin C and streptonigrin were added to lysogenic bacterial cultures and incubated for 20 hours.

To account for the differences in growth between treated and non-treated cultures and the effect of cell concentration on basal levels of β-galactosidase, the RLU values generated were normalized to their corresponding $OD_{600}$ values. Normalization of RLU allowed for an increased incubation period of 24 hours so that there was a longer exposure time to the compounds tested, resulting in maximal β-galactosidase production in *E. coli* BR513. The $OD_{600}$ values also indicated that a reduction in cell concentration over time occurred with the antibiotic-treated *E. coli* BR513 and *E. coli* K-12 cultures, supporting the hypothesis that mitomycin C, streptonigrin, and norfloxacin induce prophages and consequently cause cell lysis and/or inhibition of bacterial growth.

The multivariate regression analysis not only revealed that each antibiotic significantly increased $RLU/OD_{2\mu g/ml}$ (β-galactosidase production) in *E. coli* BR513, but also showed that streptonigrin was a stronger prophage-inducing agent as compared to mitomycin C and norfloxacin. Both streptonigrin and mitomycin C are used as anti-tumor drugs and are known for their ability to induce lambdoid prophages in *E. coli*

(Heinemann and Howard, 1964; Cadieux et al., 2018). However, mitomycin C historically is considered to be the standard compound used to induce the lytic cycle of temperate phages (Raya and H'Bert E, 2009). The findings of this study suggest that streptonigrin should be the first choice for effective induction of lambdoid phages, instead of mitomycin C.

Food-grade compounds tested in this work (coffee, tea, and their shared chemical constituent, gallic acid) were selected based on previous research that identified them as DNA-damaging agents (Hossain et al., 2014). Rosemary and cranberry juice were selected based on previous studies that showed that these natural compounds possess antimicrobial activity (Côté et al., 2011; Nieto et al., 2018). Sodium citrate is a naturally derived, commonly used preservative that also has antimicrobial properties (Sallam, 2007). Rosemary, cranberry juice, and sodium citrate were included in the screen to determine whether their mode of action (MOA) could be in part explained by their ability to induce prophages.

The food-grade screen determined that gallic acid, tea, rosemary, cranberry juice, and coffee induced prophages, as indicated by significant increases in RLU over time (see Figures 3.4 and 3.5). These results also suggest that the MOA of the compounds listed above depends at least partially on prophage induction. For example, rosemary is a well-known antimicrobial agent that is used in food to inhibit bacterial pathogens, but it was not previously described in the literature as being a prophage-inducing agent. Compounds contained in rosemary that were previously reported to have antimicrobial effect include phenolic compounds such as carnosic acid, carnosol, and rosmarinic acid (Nieto et al., 2018). Interestingly, water-soluble phenolic compounds from pH-

neutralized cranberry juice were reported to inhibit foodborne pathogens, such as *E. coli* O157:H7, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Côté et al., 2011). Côté et al. concluded that the antimicrobial effect of cranberries was not exclusively due to low pH. The significant increase in RLU over time in the assay supports this hypothesis and suggests that the antimicrobial effect of cranberries can also be explained by the induction of lambdoid phages present in *E. coli*.

The prophage induction capacity of tea and coffee may be attributed to their pyrogallol-like polyphenol content, which includes gallic acid and epigallocatechin gallate (EGCG). These compounds, found in both tea and coffee, cause DNA damage (Hossain et al., 2013), which ostensibly triggers the SOS response that induces the lytic cycle of prophages.

The fact that gallic acid was identified as being the strongest prophage-inducing agent in the screen of food-grade compounds supports the hypothesis that gallic acid is a DNA-damaging agent. However, the chemical composition of tea varies with the degree of fermentation (Zuo et al., 2002). Green tea contains lower concentrations of gallic acid as compared to tea that is subjected to additional fermentation, such as black tea and pu-erh tea (Kongpichitchoke et al., 2016) that were the weakest prophage-inducing teas in the screen of food-grade compounds. Since the strongest prophage-inducing tea identified in this work was green tea (kukicha, iron goddess, and phoenix oolong), the EGCG content likely had greater impact on prophage induction than gallic acid, as green tea is subjected to less fermentation and thus contains higher levels of EGCG (Zuo et al., 2002; Zhang et al., 2011). Further evidence supporting the hypothesis that coffee may induce prophages comes from Kosugi et al. (1983), who showed that lyophilized

coffee preparations induced lambdoid prophages in an *E. coli* K-12 strain. The authors concluded that compounds generated in the coffee bean roasting process caused prophage induction, since green coffee beans included in the same study had no prophage-inducing activity (Kosugi et al., 1983).

In addition to the alterations made to the HTS method and analysis due to dark colours present in some of the compound preparations, the food-grade screen was also limited because it was difficult to determine concentrations of tea, coffee, and rosemary, since they were prepared as infusions. The HTS screen therefore could not determine the precise concentration at which these compounds induced prophages. Furthermore, the reduction of the luminescent signal may have had unforeseen effects on the overall increase in RLU over time, as it was observed that the weakest inducers (coffee, black tea and pu-erh tea) were also visually the darkest. Nonetheless, the highest prophage-inducing tea (iron goddess and kukicha) were darker in colour than streptonigrin, which was confirmed to have lower prophage-inducing activity as compared to the two green teas.

### 3.6 Conclusion

In conclusion, this work demonstrates the utility of an HTS assay for detection of induction of the cryptic prophage λ in *E. coli* BR513. The ability of three known antibiotics to enhance prophage induction were compared, which showed that streptonigrin was a more potent inducer of lambdoid phages as compared to the more commonly used mitomycin C. Future studies of lambdoid phage induction should consider this finding. Additionally, the assay identified nine common plant-based foods and gallic acid as being

prophage inducers. The results also indicated that the method used to process tea influences the prophage-induction capability of an infused preparation, as variations in prophage induction were observed between different types of tea (green, black, pu-erh).

Prophage-inducing compounds may have a broader target range during microbial control as compared to phage cocktails, as prophages are found ubiquitously throughout bacterial genera (Kang et al., 2017). A single compound may thus induce several prophages across different bacterial strains and genera, as demonstrated by Cadieux et. al (2018), whereas phage cocktails are designed to eliminate specific limited groups of pathogenic bacteria (Kazi and Annapure, 2016). Since a single prophage-inducing compound could have a broader target range than a phage cocktail, a single compound could eliminate not only pathogenic bacteria, but spoilage bacteria as well, serving as a robust approach for industrial food safety and quality.

Future work should focus on the screening of additional food-grade antimicrobial compounds with other types of temperate phages, as the current study focused only on the effect of a small number of compounds on lambdoid phages. The concentration of prophage inducers required to inhibit growth of bacterial foodborne pathogens, such as *Salmonella* spp., *Listeria monocytogenes,* and *E. coli* should be determined.

### 3.7 Tables and figures

**Model Summary**

| | |
|---|---|
| R | 0.999 |
| $R^2$ | 0.999 |
| Adjusted $R^2$ | 0.998 |
| Standard error of the estimate | 0.226 |

**ANOVA**

| Model | Sum of Squares | df | Mean Square | F | P-value |
|---|---|---|---|---|---|
| Regression | 598.142 | 4 | 149.535 | 2919.776 | 0.000 |
| Residual | 0.717 | 14 | 0.051 | | |
| Total | 598.859 | 18 | | | |

**Unstandardized β coefficients**

| Variable | β | Standard error | P-value |
|---|---|---|---|
| $\log(\frac{\mathrm{RLU}}{\mathrm{OD}_{600}})_{0\mu g/ml}$ | $\beta_1$ = 0.999 | 0.021 | 0.000 |
| $D_S\log(\frac{\mathrm{RLU}}{\mathrm{OD}_{600}})_{0\mu g/ml}$ | $\beta_2$ = 0.069 | 0.025 | 0.015 |
| $D_N\log(\frac{\mathrm{RLU}}{\mathrm{OD}_{600}})_{0\mu g/ml}$ | $\beta_3$ = -0.07 | 0.022 | 0.000 |
| $D_E\log(\frac{\mathrm{RLU}}{\mathrm{OD}_{600}})_{0\mu g/ml}$ | $\beta_4$ = 0.214 | 0.021 | 0.007 |

**k coefficients**

| Variable | k coefficient computation | *E. coli* BR513 | *E. coli* K-12 |
|---|---|---|---|
| Mitomycin C | $k_1 = \beta_1\ (+\beta_4)$ | 1.213 ± 0.077 | 0.999 ± 0.054 |
| Streptonigrin | $k_2 = \beta_1\ + \beta_2\ (+\beta_4)$ | 1.282 ± 0.100 | 1.068 ± 0.084 |
| Norfloxacin | $k_3 = \beta_1\ + \beta_3\ (+\beta_4)$ | 1.143 ± 0.095 | 0.929 ± 0.079 |

**Table 3.1.** Results from the multivariate regression analysis, where mitomycin C-treated wildtype *E. coli* K-12 ($\beta_1$) is the reference category. The 99% confidence intervals for each k coefficient, where the summation of β was involved, were calculated as the square root of squares of the corresponding confidence intervals for the individual β coefficients. $(+\beta_4)$ in the k coefficient computation applies to the calculation of k for *E. coli* BR513.

| Compound | Company/Brand | Final concentration |
|---|---|---|
| Japanese Kukicha green tea | Cabane à Thé, Montreal, Quebec | 0.17 g/ml in $dH_20$ |
| Chinese Phoenix Oolong tea | | 0.17 g/ml in $dH_20$ |
| Chinese Pu-Erh tea | | 0.17 g/ml in $dH_20$ |
| Chinese Iron Goddess tea | | 0.17 g/ml in $dH_20$ |
| Irish Breakfast tea | Twinings, Andover, United Kingdom | 0.17 g/ml in $dH_20$ |
| Green tea | | 0.17 g/ml in $dH_20$ |
| Black/green chai tea blend | | 0.17 g/ml in $dH_20$ |
| Dried rosemary leaves | Les Aliments G Dion, Saint-Jerome, Quebec, Canada | 0.17 g/ml in $dH_20$ |
| Colombian coffee | Café Cimo Inc., Saint-Leonard, Quebec, Canada | 0.375 g/ml in $dH_20$ |
| Gallic acid | Bio Basic Inc., Markham, Ontario, Canada | 10 µg/ml in $dH_20$ |
| Sodium citrate | Fisher Scientific Company, Toronto, Ontario, Canada | 30 mg/ml in $dH_20$ |
| Cranberry juice (pH adjusted to 6.5 by addition of concentrated NaOH) | Nutra-Fruit Inc., Quebec City, Quebec, Canada | Full strength used as 1:4 juice:culture |

**Table 3.2.** Compounds tested in the high-throughput luminescent prophage induction assay.

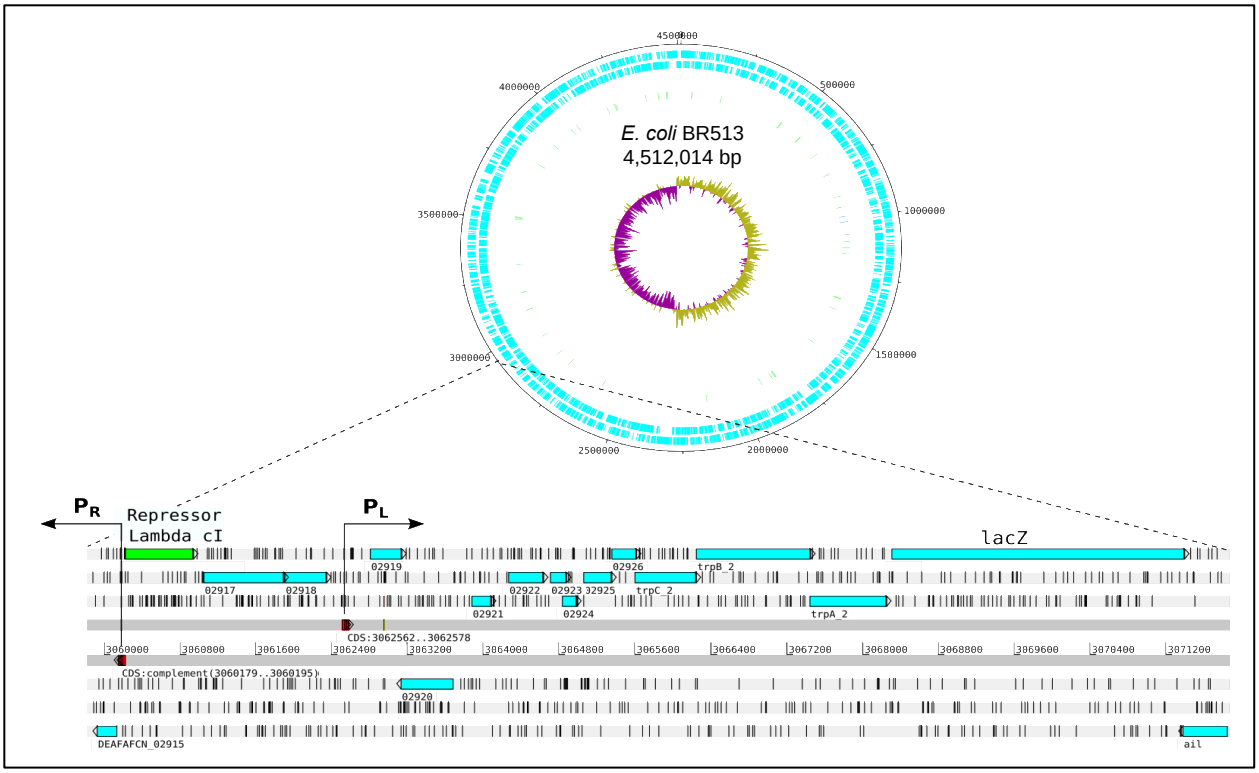


**Figure 3.1.** The genetically engineered indicator strain, *Escherichia coli* BR513 carries a cryptic lambdoid phage fused to the *lacZ* operon. The region displayed above shows the 37 kb gene fusion, with the phage promoter, $P_L$, positioned 5.7 kb downstream from *lacZ*. Due to the gene fusion, the production of β-galactosidase is regulated by $P_L$.

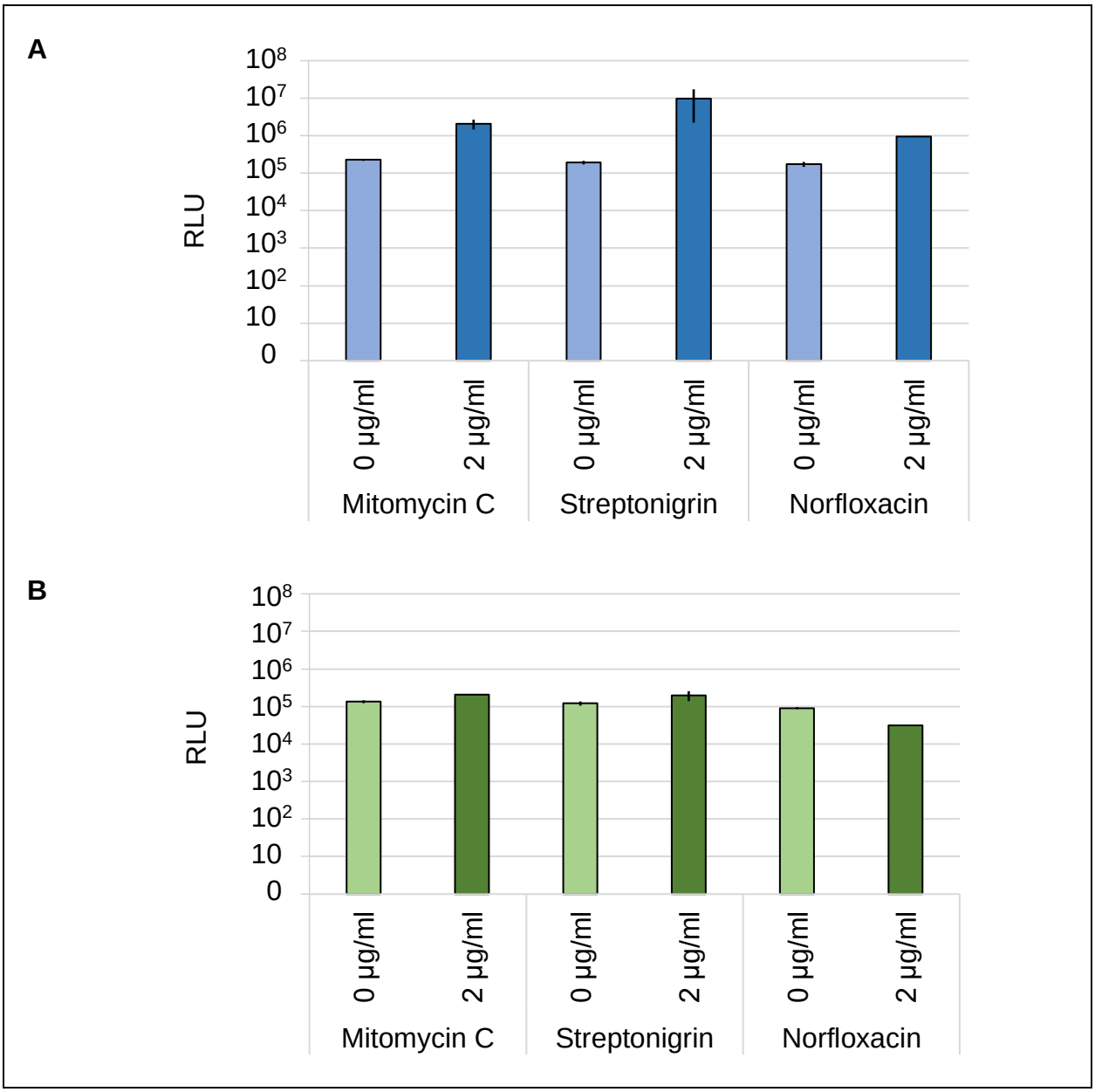


**Figure 3.2.** Average $OD_{600}$-normalized RLUs of triplicate cultures of A) *Escherchia coli* BR513 and B) *Escherchia coli* K-12 after 24 hours of incubation with 0 µg/ml and 2 µg/ml mitomycin C, streptonigrin, and norfloxacin. Incubation with the DNA-damaging antibiotics resulted in increased production of β-galactosidase in *E. coli* BR513, due to its *lacZ* prophage λ gene fusion.

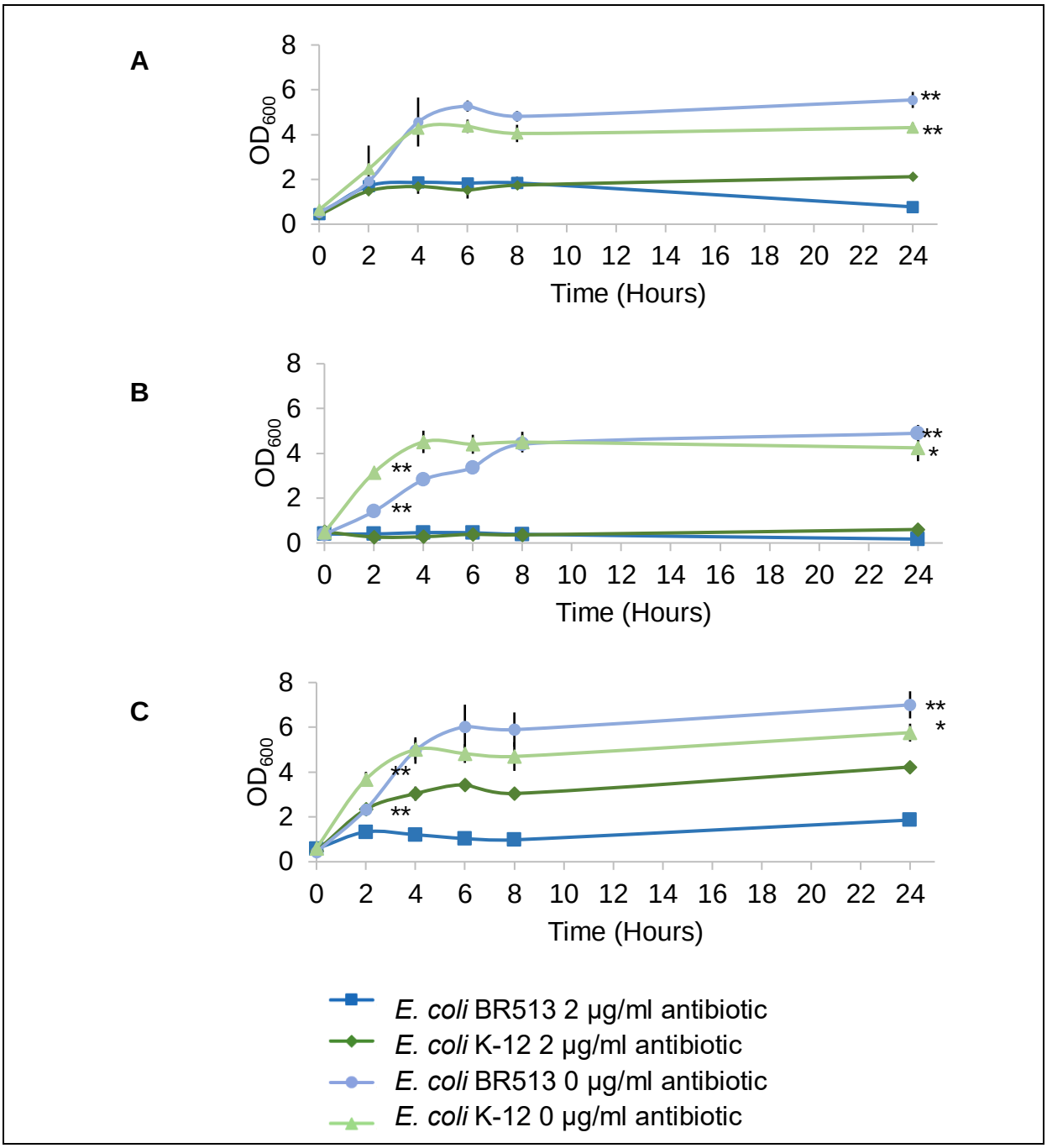


**Figure 3.3.** Average $OD_{600}$ over time of triplicate cultures of *Escherchia coli* BR513 and *Escherchia coli* K-12 treated with 0 µg/ml and 2 µg/ml of A) mitomycin C, B) streptonigrin, and C) norfloxacin. Statistically significant differences between treated and non-treated cultures at 2 and 24 hours are marked by asterisks, where $P<0.005$ and $P<0.05$ are represented by ** and *, respectively.

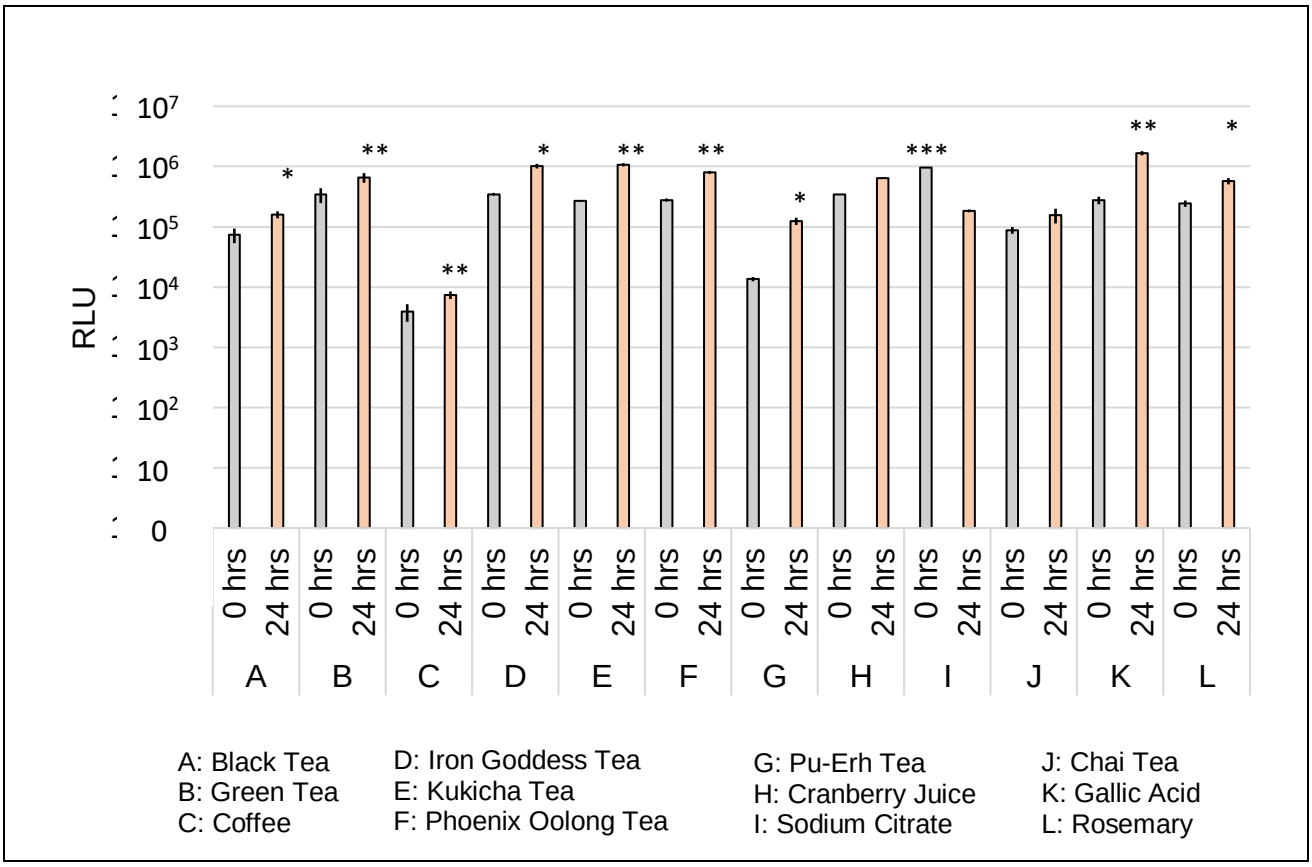


**Figure 3.4.** RLU of *Escherchia coli* BR513 at 0 and 24 hours following the addition of each compound tested in the food-grade screen. Compounds that resulted in a significant increase in RLU between 0 and 24 hours, determined by a paired t-test, were considered positive hits for prophage induction. $P<0.001$, $P<0.005$, and $P<0.05$ are represented by ***, **, and *, respectively.

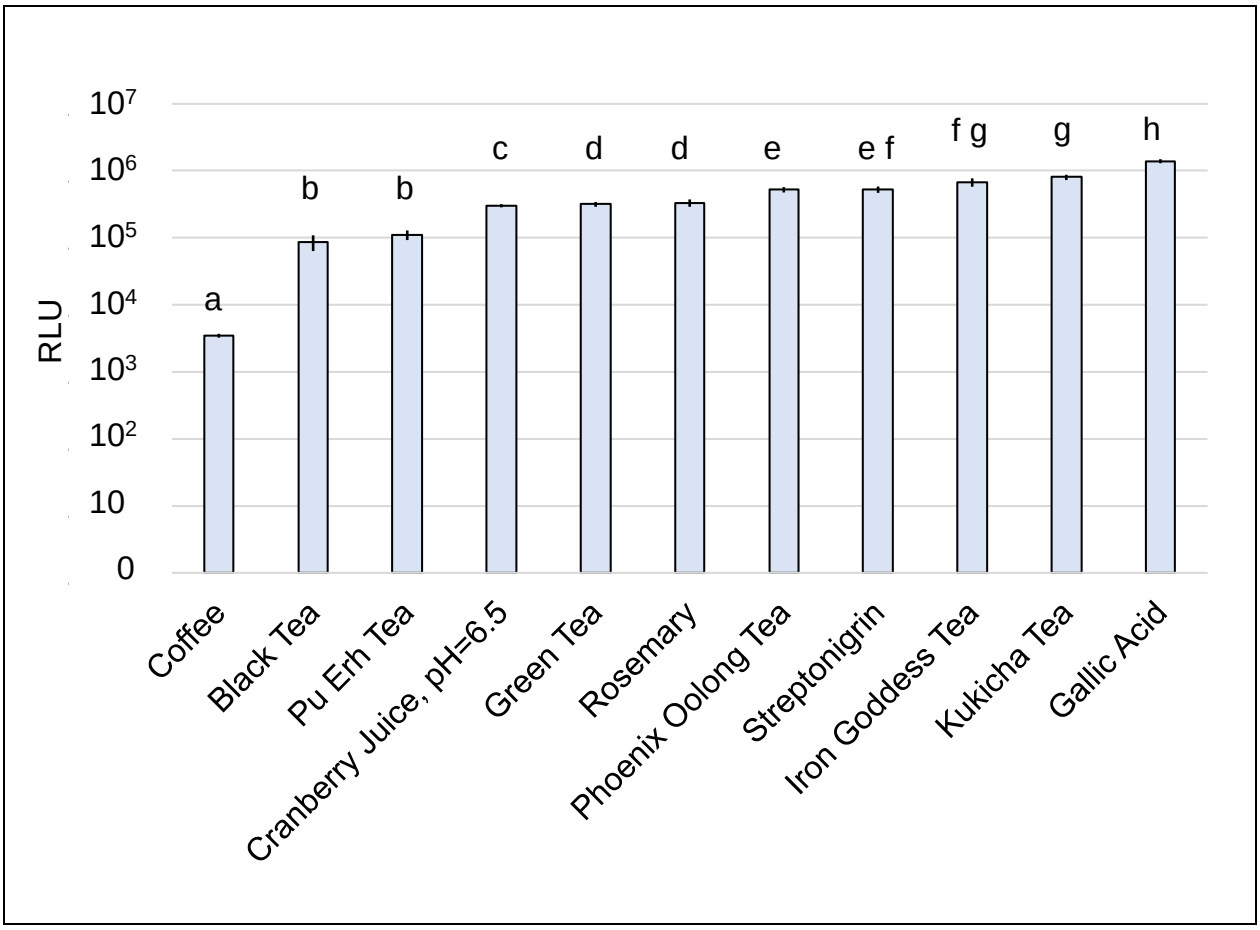


**Figure 3.5.** Increase in RLU in *Escherichia coli* BR513, calculated as the difference between RLU at 0 hours and RLU at 24 hours following the addition of food-grade compounds and streptonigrin. Bars marked with different letters are statistically significantly different ($P<0.05$), whereas those marked with the same letter are not.

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## Connecting text

The results from the food-grade high-throughput luminescent prophage induction assay showed that the mechanism of action of certain known antimicrobial agents could be explained by the induction of prophage λ in *E. coli* BR513. Additional compounds with known bioactive properties therefore serve as a promising starting point in the identification of additional compounds that could be used to induce prophages in pathogenic bacteria in foods. In the following chapter, the high-throughput luminescent prophage induction assay was used in a screen of several bioactive compounds to further identify natural prophage inducers.

## Chapter 4: Use of the high-throughput luminescent prophage induction assay to evaluate bioactive compounds for identification of natural prophage inducers

### 4.1 Abstract

Given the prevalence of clean label trends among today's consumers, natural approaches for controlling pathogenic bacteria that commonly contaminate food are gaining significance. A recently proposed phage-based food safety approach involves the induction of prophages found within pathogenic bacteria to control bacterial growth on food. This study conducted an extensive screen to identify compounds that may serve as prophage-inducing agents. The high-throughput luminescent prophage induction assay developed in Chapter 3 was optimized for use with an automated robotic system that facilitated preparation of duplicate cultures of *Escherichia coli* BR513 in 384-well plates that were incubated with bioactive compounds from four chemical libraries (final concentration 10 μM) for 24 hours at 37ºC. *E. coli* BR513 is a genetically engineered strain that produces β-galactosidase as a result of prophage λ induction, due to the fact that it carries a *lacZ*-prophage λ gene fusion. The prophage induction capacity of each compound was assessed by using a luminescent substrate to quantify β-galactosidase levels. Relative light units (RLU) were normalized to cell concentration (as measured by optical density at a wavelength of 600 nm ($OD_{600}$)) and to the interquartile mean of each respective 384-well plate. The cut-off of normalized RLU values that was used as an indicator of prophage induction for each replicate was set at 2.25 standard deviations above the mean of the data set. A positive hit for prophage induction was defined as a compound in which both replicate cultures generated normalized RLU values above their

respective cut-off. Dose response experiments confirmed that four naturally-derived compounds for which only one replicate generated a normalized RLU value above the cut-off, were positive for prophage induction. These compounds, along with rosemary and gallic acid, which were identified as prophage inducers in Chapter 3, as well as a selection of positive hits from the current assay, were included in the dose response experiments in an attempt to determine ideal concentrations for prophage induction. The dose response results showed that the assay cannot differentiate whether cell death occurred due to prophage induction or if the bacteria was killed by some other mechanism, and thus optimal concentrations for prophage induction could not be determined. The assay should nevertheless be considered a useful tool for the preliminary screening of bioactive compounds that may have the capacity to induce prophages.

## 4.2 Introduction

In developed countries, consumers are increasingly demanding that foods only contain natural and easily recognizable ingredients (Asioli et al., 2017). This consumer demand, known as the clean label trend is largely driven by an increasing awareness of the potential toxicity of certain synthetic food additives (Carocho et al., 2014). As such, consumers tend to choose additive-free foods or foods containing natural additives over products that contain synthetic additives (Carocho et al., 2014). The food industry is thus shifting toward the use of naturally-sourced antimicrobials as replacements for commonly-used synthetic compounds, such as benzoates, sorbates, propionates, nitrites, and parabens (Carocho et al., 2015) that have traditionally been used to control the growth of foodborne bacterial pathogens (Emerton and Choi, 2008; Garcia et al., 2008).

The food industry's transition to the strict use of naturally-sourced additives should not compromise food safety and care should be taken when choosing replacements for synthetic antimicrobials. Natural compounds from plants, animals, and microorganisms are known to have antimicrobial properties (Carocho et al., 2015). Varying mechanisms for the antimicrobial action of natural compounds have been proposed, which may involve interactions with proteins, enzymes, or membrane function (Fan et al., 2018). Bacteriophages (phages) have also emerged as effective natural antimicrobials (Table 2.1) that can be added to foods to eliminate foodborne bacterial pathogens. Virulent phages introduce their DNA into cells of the target bacterial pathogens to produce progeny phage that ultimately lyse the host cells, in what is termed the lytic cycle (Salmond and Fineran, 2015; Moye et al., 2018).

Induction of temperate phages is an alternative phage-based antimicrobial mechanism of action (MOA) that was recently proposed for controlling bacterial pathogens on foods (Cadieux et al., 2018). Temperate phages are viruses of bacteria that infect their hosts by integrating their DNA into the bacterial host chromosome and immediately enter a lysogenic life cycle in which no harm is inflicted onto host cell (Feiner et al., 2015). Various environmental stresses trigger the chromosomally-embedded phage (prophage) to enter into the lytic cycle, in which the phage DNA excises from the bacterial DNA and forms progeny that eventually lyse the host cell (Salmond and Fineran, 2015). Activation of the lytic cycle, known as prophage induction, can be initiated by the addition of bioactive compounds, which could be used as an approach for eliminating bacterial pathogens in food (Cadieux et al., 2018), since the majority of bacteria possess prophages (Kang et al., 2017).

It is well known that certain antibiotics induce prophages of foodborne bacterial pathogens (Cone et al., 1976; Raya and H'Bert E, 2009; McDonald et al., 2010); however, fewer natural compounds have been shown to induce prophages. In Chapter 3, the high-throughput luminescent prophage induction assay demonstrated that various teas, coffee, gallic acid, rosemary and cranberry juice are inducers of prophage λ in *Escherichia coli* BR513. These natural compounds have been described in the literature as having antimicrobial activity against bacterial pathogens (Côté et al., 2011; Nieto et al., 2018) however, the potential of gallic acid, rosemary and cranberry juice as prophage inducers, which may represent an undiscovered MOA, has not been explored.

In Chapter 3, the high-throughput assay was used to detect prophage inducing activity of compounds within 24 hours. In this work, the assay was modified in order to render it

amenable to automated systems so that thousands of known bioactive compounds from chemical libraries could be screened. The high-throughput luminescent prophage induction assay serves as a preliminary screen of compounds that could be used to induce prophages of foodborne bacterial pathogens.

## 4.3 Materials and methods

### 4.3.1 Bacterial strain

The assay used in this work, as in Chapter 3, relies on the use of the genetically engineered *Escherichia coli* BR513 (ATCC 33312). Since *E. coli* BR513 carries a *lacZ*-prophage λ gene fusion, cleavage of the lambdoid phage repressor, CI, results in the synthesis of β-galactosidase (Elespuru and Yarmolinsky, 1979). The cleavage of the CI repressor and thus prophage induction may be caused by UV irradiation, other treatments that cause DNA damage, or it may occur spontaneously. Prophage λ induction can also be caused by overexpression of genes that regulate capsular polysaccharide synthesis, rcsA and dsrA (Rozanov et al., 1998). β-galactosidase produced by *E. coli* BR513 may be measured and taken as an indication of prophage λ induction.

*E. coli* BR513 was stored at -80ºC in 20% glycerol and cells were revived by streaking a loopful of frozen stock onto tryptic soy agar (TSA) (Becton, Dickinson and Company, Sparks, Maryland, USA). Plates were incubated at 37°C for 18-24 hours.

### 4.3.2 High-throughput luminescent prophage induction assay

The high-throughput luminescent prophage induction assay established in Chapter 3 was adapted for use with an automated robotic system at the Centre for Microbial Chemical Biology (CMCB), which is part of the Michael G. DeGroote Institute for Infectious Disease Research at McMaster University (Hamilton, Ontario, Canada).

To begin the assay, duplicate cultures of *E. coli* BR513 were grown overnight in trypticase soy broth (TSB; Becton, Dickinson and Company, Sparks, Maryland, USA) supplemented with 0.2 M glucose (TSB 0.2 M glucose). The next morning, cells were diluted in fresh TSB 0.2 M glucose to an optical density of 0.1 at a wavelength of 600 nanometres ($OD_{600}$), which was measured using a Spectramax spectrophotometer (Molecular Devices, San Jose, California, USA). The cultures were grown at 37°C with shaking at 250 RPM until the $OD_{600}$ reached 0.5. A Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to add 50 µl of culture to the wells of 384-well plates.

Next, 0.5 µl of each test compound from the CMCB bioactives library (Table 4.1), having been dissolved in dimethyl sulfoxide (DMSO), were added to the cultures using a Biomek $FX^P$ liquid handler (Beckman Coulter, Indianapolis, Indiana, USA) to achieve a final concentration of 10 µM. Plates were incubated at 37ºC for 24 hours. Positive and negative controls were included in each 384-well plate. Streptonigrin (final concentration of 2 µg/ml) was added to appropriate wells as a positive control and DMSO was used as the negative control.

The $OD_{600}$ was measured following the 24-hour incubation period, followed by measurement of luminescence. For this, 50 µl of Gal-Screen β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific) were added to each well. This assay system includes a lysis buffer and the β-galactosidase substrate, Galacton-Star®, which are used in a single-step reaction that emits light proportional to the amount of the lactose-degrading enzyme. Luminescence readings were measured five minutes after the addition of the Gal-Screen β-Galactosidase Reporter Gene Assay System using an Envision plate reader (Perkin Elmer, Waltham, Massachusetts, USA).

#### 4.3.2.1 Compounds tested in the assay

Compounds from the CMCB bioactives collection were screened for their ability to induce prophage λ in the high-throughput luminescent prophage induction assay. The CMCB bioactives collection (Table 4.1) contains 3,747 compounds from four vendor libraries. The libraries contain Food and Drug Administration (FDA)-approved drugs, off-patent drugs, natural products, and other compounds with demonstrated biological activity (Miller et al., 2009; Davenport et al., 2014; Torres et al., 2016).

#### 4.3.2.2 Data analysis

As in Chapter 3 (Section 3.3.3), the RLU generated by the treated *E. coli* BR513 cultures were normalized to $OD_{600}$ to account for any effects of cell concentration on β-galactosidase production. Additionally, the $OD_{600}$-normalized RLU results were further normalized to the interquartile mean to account for variation between plates. For this, the $OD_{600}$-normalized RLU from each well were divided by the interquartile mean of the corresponding 384-well plate (excluding controls). The interquartile mean is defined as

the mean of the middle 50% of the rank-ordered data (Mangat et al., 2014). All bioactive compounds for which both replicates had a value greater than 2.25 standard deviations from the mean of treated *E. coli* BR513 samples were considered positive for prophage induction, as described by the following equation:

$$\frac{RLU/_{OD}}{\mu_{iq}} > 2.25\ standard\ deviations_{mean}$$

Where;

$RLU/OD$ is the $OD_{600}$-normalized RLU

$\mu_{iq}$ is the interquartile mean

### 4.3.3 Dose response

Dose response experiments were performed to investigate the effect of compound concentration on prophage induction capacity and to verify whether naturally-derived compounds with inconclusive results were indeed prophage-inducing agents. Rosemary and gallic acid were also included in the dose response experiments because they were previously identified as being prophage inducers in Chapter 3 (section 3.4.4). Since they are natural, food-grade compounds, they were included in the dose response screen so they could be directly compared to the compounds selected from the high-throughput luminescent prophage induction assay.

The response to varying doses of selected compounds was evaluated by incubating duplicate cultures of *E. coli* BR513 with 11 concentrations of each compound following the methods for the high-throughput luminescent prophage induction assay. The highest

tested concentration of each compound varied, between 2 and 100 µg/ml (Table 4.4). Each consecutive concentration of antibiotic was prepared as a two-fold dilution. All concentrations of the remaining compounds were half-log dilutions of the starting concentration. Streptonigrin, ciprofloxacin, chloramphenicol, and ampicillin dissolved in DMSO were also included in the dose response experiments as positive controls since they have been described in the literature as being prophage-inducing antibiotics (Levine and Borthwick, 1963; Shinagawa et al., 1977; Goerke et al., 2006; Maiques et al., 2006).

## 4.4 Results

### 4.4.1 The High-throughput luminescent prophage induction assay identified 61 prophage-inducing agents

The entire CMCB bioactives collection, consisting of 3747 compounds was screened in the high-throughput luminescent prophage induction assay to identify natural compounds that could be used in the induction of prophages in bacterial pathogens that contaminate foods. The hit cut-offs, calculated as 2.25 standard deviations from the mean, were 2.7 and 2.6 for replicates 1 and 2, respectively. Compounds were considered positive hits for prophage induction when both replicate signals were greater than the respective cut-offs. Several structurally-related compounds were identified (Table 4.2; Figure 4.1), indicating that certain chemical classes contribute to prophage induction activity.

Sixty-one bioactive compounds were identified as potential prophage-inducing agents. Of the 61 positive hits, there were 22 antibiotics, 9 antifungals, 5 antiparasitics, 3 antipsychotics, 2 antibacterials, 2 antiseptics, and 18 miscellaneous compounds, two of which were of natural origin (α-mangostin and tschimganidin). In addition to the positive

hits, there were 22 compounds that were inconclusive, or non-replicating hits (Table 4.3), which were compounds for which only one replicate signal surpassed its cut-off. The non-replicating hits consisted of 5 antibiotics, 2 antifungals, 1 antiparasitic, 3 antibacterials, 1 antiseptic, and 10 miscellaneous compounds. Six of the non-replicating hits were natural compounds that are derived from plants and lichens, but osthol, roccellic acid, galangine, and sclareol had higher normalized RLU signals than leoidin and xanthone (data not shown).

Several compounds identified by the assay could be useful in the induction of the lytic cycle of prophage λ; however, not all of them should be considered for food safety applications. The results suggest that similarities in chemical structure amongst the identified compounds may nevertheless indicate which chemical moieties may be responsible for prophage λ induction.

### 4.4.2 Dose response

A subset of positive hits from the high-throughput assay was selected for evaluation in dose response experiments. The selection included compounds with structural similarities as well as compounds that were natural in origin, since they could have potential for use in clean label food applications. For example, two tyrphostins and CGP-7930 all contain a di-tert-butyl phenol group and were thus evaluated and compared. Four of the inconclusive hits that were compounds of natural origin (osthol, roccellic acid, galangine, and sclareol) were also included, to confirm whether they were capable of inducing prophage λ and to investigate whether their concentration had an effect on prophage induction capacity. Tschimganidin and α-mangostin were selected from the

positive hits since they are naturally-derived compounds, and streptonigrin, ciprofloxacin, chloramphenicol and ampicillin were used as positive controls since these antibiotics have all been previously described as agents that can induce prophages (Levine and Borthwick, 1963; Shinagawa et al., 1977; Goerke et al., 2006; Maiques et al., 2006).

The dose response curves (Figure 4.2) confirmed the four natural compound inconclusive hits as being prophage λ-inducing agents, since the 10 μM concentration included in the dose response (the same concentration used in the initial screen that resulted in non-replicating hits), generated a normalized RLU response greater than the 2.25 standard deviation cut-off for both replicates. For most of the compounds included in the dose response experiments, the $OD_{600}$-normalized RLU increased and the $OD_{600}$ decreased as the concentration of bioactive compounds increased. This was the case for streptonigrin, indicating that a higher concentration resulted in higher production of β-galactosidase due to the induction of prophage λ.

The only compound that did not generate a dose response in RLU/$OD_{600}$ was rosemary, which was included in the dose response experiments because it is a known antimicrobial compound and was shown to induce prophage λ in Chapter 3 (Section 3.4.4). Gallic acid, an organic acid naturally found in teas, was the only other compound that did not cause a significant response in $OD_{600}$, suggesting that a higher concentration may be required to cause cell death.

## 4.5 Discussion

The high-throughput luminescent prophage induction assay was developed to expand on the work from Chapter 3 that identified compounds capable of inducing prophage λ in *E. coli* BR513 (Section 3.4). In this study, automated equipment at the CMCB improved the efficiency of the assay that allowed for the screening of thousands of bioactive compounds. To simplify the assay developed in Chapter 3, the control wild-type strain, *E. coli* K-12, was eliminated from the experiment and duplicate rather than triplicate cultures were used. The interpretation of results was also adapted and simplified. The compounds that resulted in the highest normalized luminescent signals were considered positive hits for prophage induction. Data analysis involved normalization to the interquartile mean to reduce variation between plates, as systematic error is inherent in high-throughput screening (HTS) data (Mangat et al., 2014). Luminescence was measured using the Gal-Screen β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific) instead of β-Glo® (Promega), (used for luminescence detection in Chapter 3), since the Gal-Screen β-Galactosidase Reporter Gene Assay System was specifically designed for HTS applications. The dose response experiments were conducted to confirm the prophage inducing capacity of inconclusive hits as well as two natural compounds (gallic acid and rosemary) that were selected based on the results of Chapter 3 (Section 3.4).

Most of the compounds identified by the assay are not suitable for use in foods, however some natural compounds were positive hits. The most abundant class of compounds identified by the assay were antibiotics, but antiseptics, antifungals, antiparasitics, antipsychotics, antibacterials, antiseptics and miscellaneous drugs were also identified

as prophage inducers. Although not all the bioactive compounds identified in the screen may be used in foods, the results may indicate which types of compounds and/or which chemical moieties may possess prophage inducing characteristics.

The notion that chemical structure may be correlated with ability to induce prophages is supported by the fact that three compounds identified as positive hits in the current screen, tyrphostin A9, tyrphostin AG 879, and CGP-7930, contain a common chemical moiety: a di-tert-butyl phenol group. In total, twenty tyrphostin analogues were screened in the assay as part of the Lopac library, but the only two tyrphostins (A9 and AG879) identified as positive hits contain di-tert-butyl phenol groups. Although these positive hits are not natural compounds, the fact that they were positive hits suggest that naturally-derived compounds with similar chemical structure may be useful as suitable food additives for the control of the growth of bacterial pathogens by means of prophage induction. For example, 2,4-di-tert-butylphenol is an antioxidant that may be extracted from seeds, fruits and sweet potato and can be produced as a fermentation product of certain lactic acid bacteria (Choi et al., 2013; Varsha et al., 2015). It has been shown to exhibit antioxidant and antifungal activity, thus demonstrating its potential as a natural food additive. (Varsha et al., 2015).

The widely-used synthetic phenolic antioxidant, butylated hydroxytoluene (BHT), also contains a di-tert-butyl phenol group. BHT is used as a preservative in foods and in food packaging and acts as an antioxidant as well as an antimicrobial (Ayaz et al., 1980), but its antimicrobial activity has not yet been linked to prophage induction. BHT was included in the MicroSource library in the current screen, but it was not a positive hit for prophage

induction. It could, nevertheless be capable of inducing prophages, as the interpretation of results relied on identifying the compounds with the highest normalized RLU of the compounds in the libraries tested and does not qualify compounds as positive or negative results, per se.

The synthetic cannabinoid, CP-55940, was also identified as a positive hit in the high-throughput luminescent prophage induction assay. CP-55940 was developed by Pfizer, Inc. (New York City, New York, USA) in the 1970s but was never marketed. CP-55940 shares similar structural features and mimics the euphoric effects of the naturally occurring cannabinoid, tetrahydrocannabinol (THC) (Debruyne and Le Boisselier, 2015). Since the two compounds exhibit similar toxicological effects and are structurally similar, the naturally-occurring cannabinoids, THC and cannabidiol (CBD), could be screened for their ability to induce prophages. Considering the recent assent of the Cannabis Act in June 2018 in Canada (Cannabis Act, 2018) and the prospects for the drug to be permitted in foods no later than October 17, 2019, the naturally-occurring cannabinoids that are structurally similar to CP-55940 may serve an additional purpose: the reduction of bacterial foodborne pathogens in cannabis food products.

Two natural compounds that originate from plants within the *Umbelliferae* or *Apiaceae* family were identified as positive hits. A terpenoid, tschimganidin was identified in the assay, and a coumarin derivative, osthol, was an inconclusive hit that was confirmed as a prophage-inducing agent in the dose response experiment. Tschimganidin and osthol may be extracted from the roots of *Ferula tschimganica* and *Ferula campestris*, respectively (Kadyrov et al., 1972; Basile et al., 2009). Both compounds are also found in

other umbellifers, but have a common source: root resin of *Ferula persica* (Kerminov, 1992). The plant may therefore represent an important source for the extraction of effective natural prophage-inducing compounds.

Osthol has been shown to possess antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae,* and Methicillin-sensitive *Staphylococcus aureus,* all at MICs of 125 µg/ml (Tan et al., 2017). Interestingly, green teas contain coumarins (Yang et al., 2009), which could elucidate why green teas were identified as the most potent inducers of prophage λ in Chapter 3 (Section 3.4.4). Since gallic acid, another component of green tea, was also identified in Chapter 3 as a prophage-inducing agent, green teas represent an important group of food-grade ingredients that should be the focus of future prophage induction studies.

Gallic acid was identified as the strongest natural prophage inducer in Chapter 3 (Section 3.4.4), but the gallic acid included in the Spectrum Collection in the current screen was not identified as a positive hit, likely because the concentration was lower than that tested in Chapter 3. The 10 µM concentration used in the current assay is equivalent to 1.7 µg/ml, but the concentration used in Chapter 3 was 10 µg/ml. These results suggest that the prophage induction activity of gallic acid may be dose-dependent, and gallic acid was thus included in the dose response experiment, in which an increase in RLU/OD was observed with increasing concentration. Increased concentration of gallic acid did not however, significantly affect the $OD_{600}$. Higher concentrations should be tested to confirm whether gallic acid is able to cause cell death.

The fact that gallic acid is a known antimicrobial (Borges et al., 2013) with DNA-damaging properties (Hossain et al., 2013) elucidates, at least partially, its effectiveness as a prophage inducer, as DNA damage is the first step in the predominant mechanism of prophage induction. DNA damage, whether caused by exposure to UV irradiation or the treatment with a specific compound, triggers the SOS response in bacterial cells, thus initiating the production of the RecA protein, in turn causing autoproteolytic cleavage of prophage repressors that are responsible for the phage's lysogenic state. The cleavage of phage repressors thus allows the prophage to excise the bacterial host chromosome and enter the lytic cycle (Sassanfar and Roberts, 1990; Campoy et al., 2006). Mitomycin C, streptonigrin and norfloxacin are antibiotics that are known to cause this recA-dependent pathway to prophage induction (Levine and Borthwick, 1963; Raya and H'Bert E, 2009; McDonald et al., 2010).

These three well-known prophage-inducing compounds that were included in the current screen in the BIOMOL 2865 Natural Products Library and the Prestwick Chemical Library were not, however, identified as positive hits. The normalized RLU for streptonigrin, mitomycin C and norfloxacin did not surpass the required cut-offs to be considered positive hits, but they were previously confirmed as being prophage-inducing compounds in Chapter 3 (Section 3.4.2). The concentration used in Chapter 3 was 2 µg/ml for each antibiotic, and in the current screen, the concentrations used were 5.1 µg/ml, 3.6 µg/ml, and 4.4 µg/ml for streptonigrin, mitomycin C and norfloxacin, respectively.

Since the concentrations of the three antibiotics used in the current screen were higher than those used in Chapter 3, false negative results may be attributed to the age of the chemical stocks used in the bioactives library, as freezing and thawing as well as

hydration of compounds stored in DMSO over time may diminish compound concentration, solubility, and potency (Kozikowski et al., 2003). This is supported by the fact that the streptonigrin used as a positive control and in the dose response experiment was prepared as a fresh stock and resulted as a positive hit for prophage induction.

Overall, the MOA in regard to prophage induction of the compounds tested was not confirmed by the dose response experiments. As reported by Elespuru and Yarmolinsky (1979) the inducing treatments inhibited growth of *E. coli* BR513. Since the RLU was normalized to $OD_{600}$, the responses in normalized RLU could have been caused by cell death as a result of other direct mechanisms. Although certain compounds at sublethal concentrations may induce prophages and lyse the host cell from the inside, additional mechanisms could also cause cell death when a given compound is used at higher concentrations. For example, chloramphenicol, which was identified as a positive hit, is known to known to generally inhibit protein synthesis (Shinagawa et al., 1977), suggesting it could inhibit the production of repressor proteins that keep prophages in their lysogenic state, thus causing induction of the lytic cycle. This may be the case when used at sublethal levels, but at higher concentrations, chloramphenicol could kill bacterial cells by another mechanism; the inhibition of protein synthesis required for cell wall formation, which is an additional pathway to cell death that does not involve phages (Schwarz et al., 2016). The differing MOAs of compounds can therefore not be distinguished by observing the dose response results, thus an optimal concentration for prophage induction of the compounds tested cannot be determined using the dose response experiment as it is currently developed.

To overcome the issue of the assay's inability to distinguish prophage induction from other mechanisms that cause cell death, a negative control strain should be used. The assay used in Chapter 3 relied on wildtype *E. coli* K-12 (Section 3.3.1) because it does not produce excess β-galactosidase as a reaction to prophage induction. It was therefore helpful in assessing whether test compounds were truly prophage-inducing agents. The wildtype strain was not used in the current study since it would have doubled the cost of the high-throughput assay, as each compound would be tested twice. The purpose of the current study was to conduct a preliminary screen of a large number of compounds to narrow down a selection that should later be confirmed for capacity as prophage inducers.

Overall, the naturally-derived compounds that were included in the dose response experiments did not exhibit as marked of a response as the synthetic compounds, which parallels the results from the initial screen. Sclareol, roccellic acid, osthol, and galangin were all inconclusive hits in the high-throughput luminescent prophage induction assay, thus their prophage-inducing capacity was expected to be less potent than the synthetically-derived compounds included in the screen. Indeed, these compounds were confirmed to be positive for inducing prophage λ in the dose response experiments, but they should be considered as hits of lower intensity, since their normalized RLU values were closer to the hit cut-offs (data not shown). Since these plant-derived and lichen-derived (roccellic acid) bioactive compounds generally demonstrated the expected profile and are known to exhibit antimicrobial properties (Cushnie et al., 2003; Hayet et al., 2007; Sweidan et al., 2017; Tan et al., 2017), they should be considered for use in future prophage induction screens.

The dose response curves for osthol, α-mangostin, and CP-55940 reached a plateau in both RLU/$OD_{600}$ and $OD_{600}$ signals, suggesting that these compounds elicited a maximum response, or that higher concentrations would have no significant effect on inhibition of cell growth or ability to induce prophages. The dose response for rosemary was contrary to the expected result, as signals were likely skewed due to the colour of the extract powder used for its preparation. The $OD_{600}$ and the RLU/$OD_{600}$ results in the dose response experiments for rosemary should thus not be considered accurate, as an increased concentration of the pigmented solution could have caused an increase in $OD_{600}$ as well as a decrease in the luminescent signal.

### 4.6 Conclusion

In conclusion, a vast library of bioactive compounds was screened for capacity to induce prophage λ, as indicated by the production of β-galactosidase in the model organism *E. coli* BR513. This work identified several compounds that could be used to induce prophages in foodborne bacterial pathogens. The naturally-derived prophage-inducing compounds identified in this work, as well as other naturally-derived compounds with chemical structure similar to that of the synthetically-derived positive hits should be further tested for their ability to induce prophages.

### 4.7 Tables and figures

| Library | Source | Number of compounds | Average molecular weight (g/mol) |
|---|---|---|---|
| Prestwick Chemical Library | Prestwick Chemical, Illkirch-Graffenstaden, France | 1120 | 372 |
| BIOMOL2865 Natural Products Library | Enzo Life Sciences Inc., Farmingdale, New York, USA | 502 | 401 |
| Lopac1280 (International Version) | Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada | 1280 | 337 |
| Spectrum Collection | MicroSource Discovery Systems Inc., Gaylordsville, Connecticut, USA. | 2000 | 354 |
| | **Total*** | 3747 | |

**Table 4.1.** The CMCB bioactives collection consisting of four vendor libraries was used in the high-throughput luminescent prophage induction assay. *Total not including duplicate compounds.

| Antibiotics |
|---|
| Chlortetracycline hydrochloride |
| Demeclocycline hydrochloride |
| Doxycycline hyclate |
| Doxycycline hydrochloride |
| Meclocycline sulfosalicylate |
| Minocycline hydrochloride |
| Minocycline hydrochloride |
| Oxytetracycline |
| Tetracycline hydrochloride |
| Chloramphenicol |
| Florfenicol |
| Thiamphenicol |
| Colistimethate sodium |
| Colistin sulfate |
| Polymyxin B sulfate |
| Fusidic acid sodium |
| Lasalocid sodium |
| Lasalocid sodium salt |
| Monensin |
| Mupirocin |
| Nigericin |
| Tyrothricin |

| Antifungals |
|---|
| Bithionate sodium |
| Dichlorophene |
| Econazole nitrate |
| Miconazole nitrate |
| Niclosamide |
| Pentachlorophenol |
| Phenylmercuric acetate |
| Salinomycin sodium |
| Sulconazole nitrate |

| Antiparasitics |
|---|
| Bithionate sodium |
| Dichlorophene |
| Niclosamide |
| Ronnel |
| Triclabendazole |

| Antipsychotics |
|---|
| Fluspirilene |
| Pimozide |
| Penfluridol |

| Antibacterials |
|---|
| Chloroxine |
| Triclosan |

| Antiseptics |
|---|
| Alexidine hydrochloride |
| Hexachlorophene |

| Miscellaneous compounds |
|---|
| *α-mangostin |
| Biochanin A |
| Bromoacetyl alprenolol menthane |
| Calmidazolium chloride |
| CGP-7930 |
| CP-55940 |
| Enoxolone |
| Fiduxosin hydrochloride |
| GBR-12909 dihydrochloride |
| L-162,313 |
| NNC 55-0396 |
| Ononetin |
| PQ-401 |
| *Tschimganidin |
| Tyramine |
| Tyrphostin A9 |
| Tyrphostin Ag 879 |
| 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1h-pyrazole |

**Table 4.2.** Prophage λ inducers, based on results from the high-throughput luminescent prophage induction assay. *Naturally-derived compounds.

| Antibiotics |
|---|
| Calcymicin |
| Chloramphenicol hemisuccinate |
| Monensin sodium |
| Nonactin |
| Diphenyleneiodonium chloride |

| Antifungal |
|---|
| Butoconazole nitrate |
| Miconazole |

| Antiparasitic |
|---|
| Ivermectin |

| Antibacterial |
|---|
| Narasin |
| *Osthole |
| Salinomycin |

| Antiseptics |
|---|
| Methylbenzethonium chloride |

| Miscellaneous compounds |
|---|
| *Galangine |
| *Leoidin |
| Lynestrenol |
| Perhexiline maleate |
| *Roccellic acid |
| *Sclareol |
| Tamoxifen citrate |
| Thapsgargin |
| *Xanthone |
| 7-Cyclopentyl-5-(4-phenoxy)phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine |

**Table 4.3.** Compounds identified as inconclusive hits in the high-throughput luminescent prophage induction assay. *Naturally-derived compounds.

| Compound | Maximum concentration | Source |
|---|---|---|
| Streptonigrin | 2 µg/ml | CMCB collection |
| Ciprofloxacin | 10 µg/ml | CMCB collection |
| Chloramphenicol | 32 µg/ml | CMCB collection |
| Ampicillin | 100 µg/ml | CMCB collection |
| Tyrphostin A9 | 14 µg/ml | CMCB collection |
| Tyrphostin AG 879 | 16 µg/ml | CMCB collection |
| CGP-7930 | 15 µg/ml | CMCB collection |
| CP55940 | 19 µg/ml | CMCB collection |
| *Osthol | 12 µg/ml | CMCB collection |
| *Tschimganidin | 19 µg/ml | CMCB collection |
| *Sclareol | 15 µg/ml | CMCB collection |
| *Galangine | 13 µg/ml | CMCB collection |
| *α-mangostin | 21 µg/ml | CMCB collection |
| *Roccellic acid | 15 µg/ml | CMCB collection |
| *Gallic acid | 10 µg/ml | Bio Basic Inc., Markham, Ontario, Canada |
| *Rosemary extract | 0.042 % (w/v) | Z Natural Foods, West Palm Beach, Florida, USA |

**Table 4.4.** Maximum concentrations of compounds used in the dose response screen. *Naturally-derived compounds.

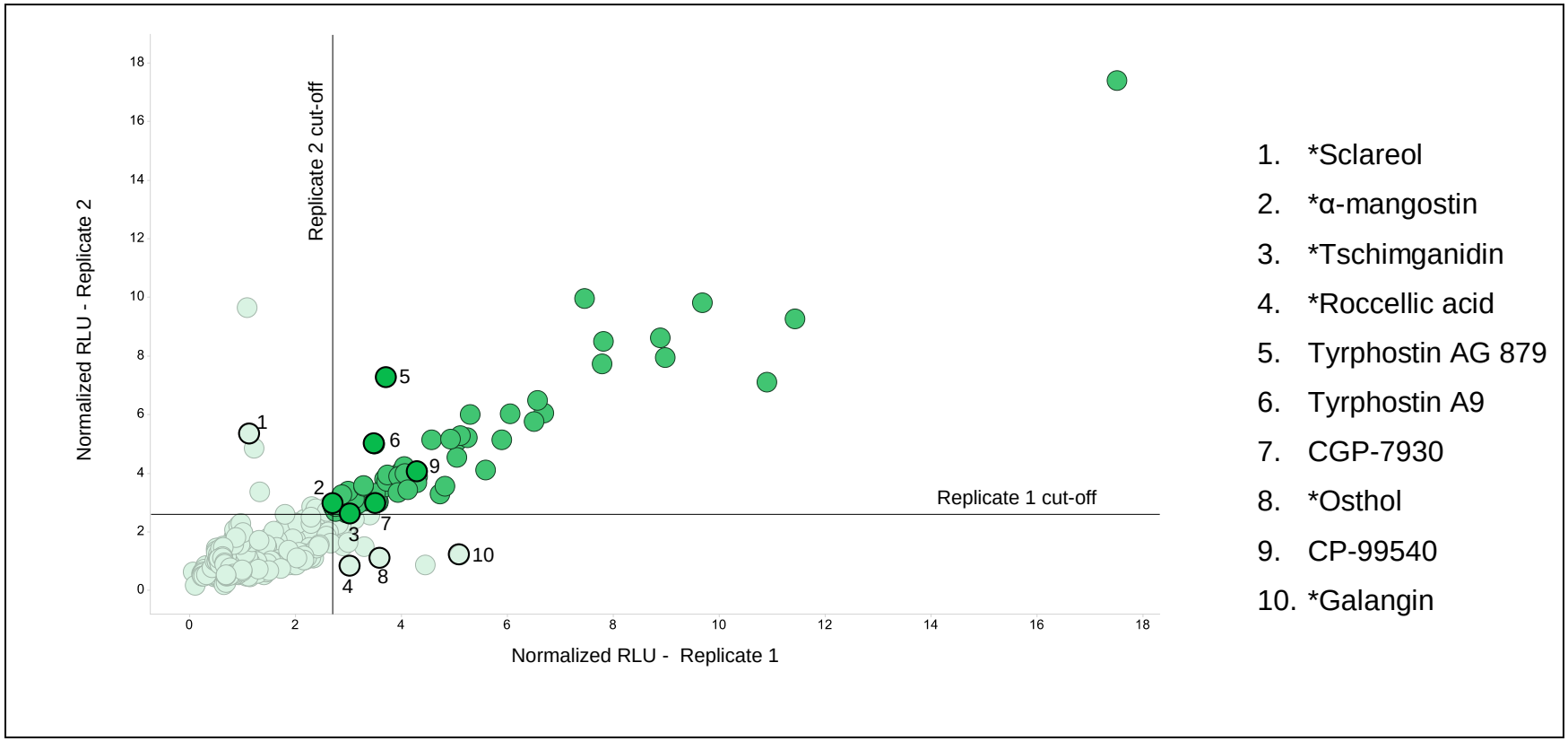


**Figure 4.1.** Replicate plot showing the normalized RLU signal in *E. coli* BR513 resulting from the addition of each compound tested in the high-throughput luminescent prophage induction assay, where the x-axis represents the signal from replicate 1 and the y-axis represents that of replicate 2. RLU values were normalized to $OD_{600}$ and the interquartile mean, and those in solid green represent positive hits, or compounds that had normalized RLU values greater than 2.25 standard deviations of the mean of each replicate. The numbered points outlined in solid black highlight positive and inconclusive hits that were further tested in the dose response experiments. *Naturally-derived compounds.

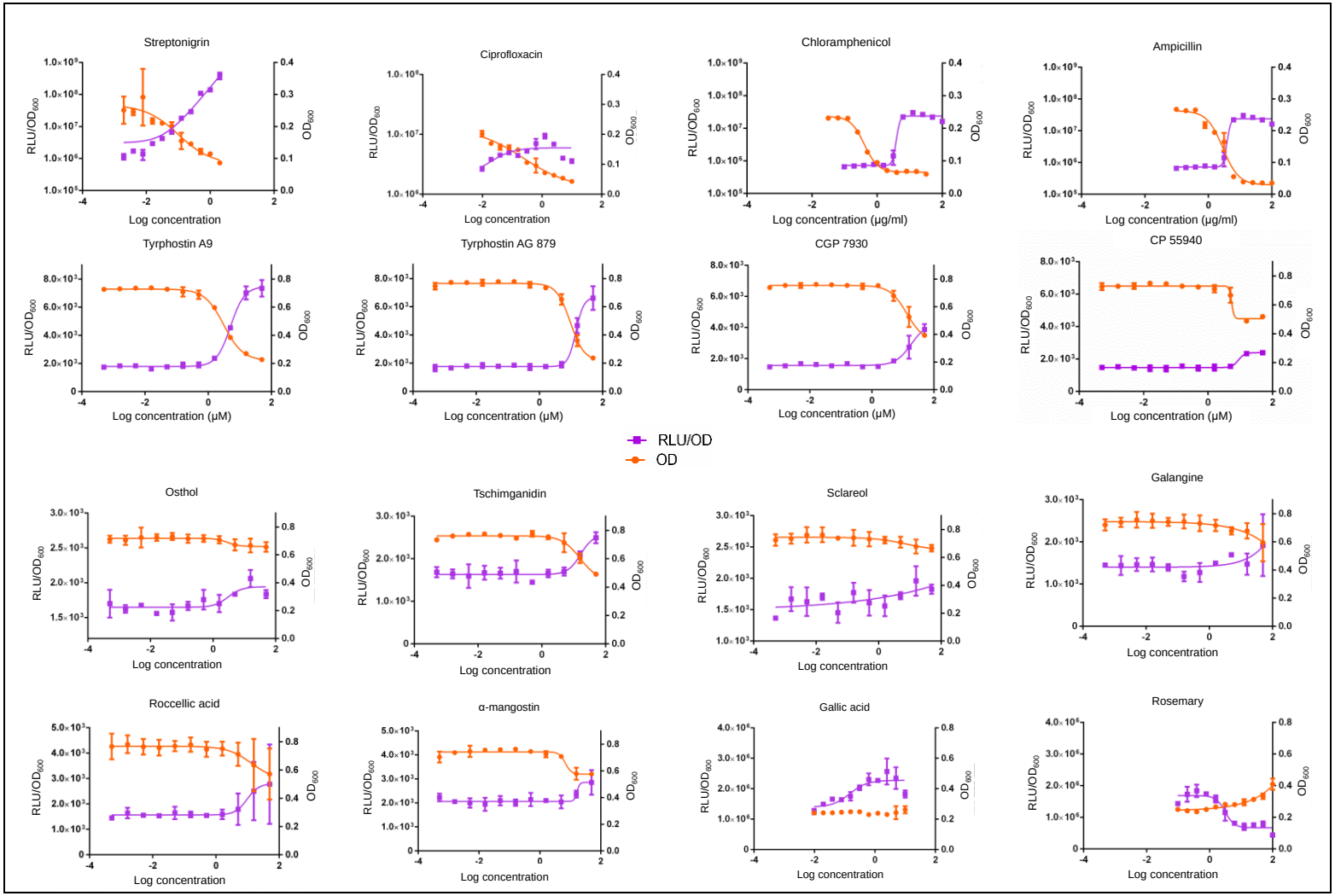


**Figure 4.2.** See caption on next page.

**Figure 4.2.** Average response in RLU/$OD_{600}$ and in $OD_{600}$ in *Escherichia coli* BR513 after 24-hour exposure to varying concentrations of bioactive compounds from the CMCB library, four antibiotics that were used as positive controls (top row) and two compounds that were positive for prophage induction in Chapter 3; gallic acid and rosemary. Violet squares represent values that correspond to RLU/$OD_{600}$ whereas orange points represent values that correspond to $OD_{600}$. Error bars represent standard deviations of the duplicate cultures that were incubated in the dose response experiments. Compounds in the bottom two rows are naturally-derived.

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# Chapter 5: Conclusion

## 5.1 General conclusion

The induction of temperate bacteriophages (prophages) within bacterial chromosomes, triggered by the addition of bioactive compounds represents a novel approach for the control of pathogenic bacteria that commonly contaminate foods. The current study was conducted to identify food-grade compounds that may be used to that effect, since the majority of bacteria contain endogenous prophages. Due to an increased consumer demand for the removal of synthetic additives from foods, natural antimicrobial compounds were the focus of the high-throughput luminescent prophage induction assay.

The work presented here demonstrated that *Escherichia coli* BR513 could be used as an effective indicator strain for the induction of lambdoid phages. Since the genetically engineered strain carries a *lacZ*-prophage λ gene fusion, production of β-galactosidase increases when prophage λ is induced. The multivariate regression analysis in Chapter 3 revealed that prophage induction took place due to the addition of known prophage-inducing antibiotics. After the development and validation of the high-throughput luminescent prophage induction assay, several common foods were tested and identified as prophage-inducing agents. These foods included green and black teas, coffee, rosemary and pH-adjusted cranberry juice. Gallic acid, which is found in teas was shown to be the strongest prophage-inducing agent, which could elucidate the effectiveness of teas as prophage inducers.

The findings from the high-throughput luminescent prophage induction assay linked chemical structure to the ability of certain compounds to trigger the lytic cycle in prophage λ. The dose response results showed that the assay could not differentiate whether cell death occurred due to prophage induction or if the bacteria was killed by some other mechanism, and thus optimal concentrations for prophage induction could not be determined. Although these results indicated that the assay risked identifying false positive results, it should still be regarded as a useful tool in the initial screening of chemical libraries to identify potential prophage inducers that may be later validated through confirmatory tests.

### 5.2 Contributions to knowledge

The present work contributed novel scientific findings:

1. Through validating the usefulness of *Escherichia coli* BR513 as an appropriate model organism in the prophage induction assay using known prophage-inducing antibiotics it was shown that streptonigrin was a more potent prophage λ-inducing agent than mitomycin C and norfloxacin. This finding is relevant because mitomycin C is widely known as the conventional antibiotic used to induce prophages in lambdoid induction studies.
2. Based on the results of the food-grade high-throughput luminescent prophage induction assay, the ability of tea and coffee to induce prophages may be explained by their gallic acid content.

3. The fact that the current work demonstrated that several foods exhibit the capacity to induce prophages, indicates that common foods consumed by humans could influence bacterial communities in the gut and may thus have implications on the state of the microbiome.
4. The results of the high-throughput luminescent prophage induction assay suggest that the antimicrobial mechanism of action of rosemary and cranberry juice may be explained, at least partially, by their capacity to induce prophages within bacterial chromosomes.
5. Natural compounds that contain di-tert-butyl phenol moieties represent an important class of chemicals that should be tested for their capacity to induce prophages.

### 5.3 Future research

The findings presented here suggest that studies in which prophage λ must be induced should use of streptonigrin as an inducing agent, rather than mitomycin C. The food-grade results from the high-throughput luminescent prophage induction assay should be confirmed as agents that trigger prophage induction in foodborne pathogens and spoilage bacteria before they are tested in food applications. The efficacy of the putative prophage-inducing agents should be confirmed by assessing the presence of phage integrase genes in culture lysates, which should be detected using PCR, as the presence of increased copies of integrase genes would indicate that prophages entered the lytic cycle and caused host cell lysis.

Once appropriate prophage-inducing agents are identified, research on practical food industry applications should be conducted. These studies should focus on the cell reduction capacity of prophage-inducing compounds as well as the concentrations required to control bacterial growth on foods or within food matrices. Prophage-inducing agents could be added directly to foods as ingredients, applied to crops or carcasses, or even added to the feed of food-producing animals to reduce the growth of pathogens that cause foodborne illnesses. The proper experiments, which will vary depending on the specific application, must be conducted to determine the efficacy of a given prophage-inducing agent before it may be applied as an effective food safety measure.

Furthermore, the dose response assay used in Chapter 4 (Section 4.4.2) should be improved by including *E. coli* K-12 as a negative control strain, as was done in Chapter 3, since its normalized RLU and $OD_{600}$ responses are useful in the discerning the discrepancy between prophage induction versus other non-phage related mechanisms of action that could cause lyse bacterial cells.

A need for novel, robust approaches in industrial food safety is demonstrated by persistent outbreaks and product recalls linked to *Salmonella*-contaminated frozen breaded chicken products in Canada. The food-grade compounds identified in the present work should therefore be tested for efficacy in controlling the growth of *Salmonella* Enteritidis. Additionally, the induction of prophages within other bacterial pathogens such as Shiga toxin-producing *Escherichia coli* and *Listeria monocytogenes* should be assessed using the same compounds.

Finally, the findings of this research suggest that common foods such as tea and coffee might affect gut microbiota in humans by means of prophage induction. Thus far, the effect of prophage induction within the human gut as a result of ingesting prophage-inducing foods has not been extensively studied. The extent to which certain foods alter the microbiome by means of prophage induction remains to be determined.

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