UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

THE USE OF BACTERIOPHAGES TO PREVENT BACTERIAL SPOILAGE OF BEER

A Manuscript Style Seminar Paper Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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May, 2019

THE USE OF BACTERIOPHAGES TO PREVENT

BACTERIAL SPOILAGE OF BEER

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We recommend acceptance of this seminar paper in partial fulfillment of the candidate's requirements for the degree of Master of Science in Microbiology

The candidate has completed the oral defense of the seminar paper.

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ABSTRACT

Mollinger, J. M. The use of bacteriophages to prevent bacterial spoilage of beer. MS in Microbiology, May 2019, 109pp. (M. Rott)

In the United States, several FDA and USDA-approved phage cocktails are regularly used to limit or eliminate bacterial pathogens such as *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in foods. Though no phage cocktails have been produced for beer, phages that infect beer spoilage bacteria are readily found in the environment. A phage cocktail could easily be developed through plaque assays, purification, propagation, developed through field testing, then in incorporated into brewing processes. All aspects regarding phage biocontrol in brewing will be reviewed by examining the chemical properties of beer, beer spoilage bacteria, bacteriophage basics, phage-host interactions, and current methods of phage application in the food industry. Furthermore, the advantages and disadvantages of the phage biocontrol strategy will be reviewed. Ideal characteristics for bacteriophages for biocontrol in beer will be discussed along with proposed methods for its implementation into the brewing process.

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INTRODUCTION

Unwanted microorganisms cause spoilage in beer, adversely affect quality, and inflict financial consequences in the brewing industry. Over 90% of the American beer market is controlled by 11 major companies possessing higher-end processing equipment and well-established, timely distribution to prevent microbial spoilage (National Beer Wholesalers Association, 2018). Microbreweries, which produce less than 15,000 barrels per year, in the U.S. have proliferated dramatically over the last 50 years. Of 6,372 breweries in the United States, 6,064 are microbreweries and brewpubs, which are much more prone to spoilage incidents because they lack the financial means to acquire higher-end technology (National Beer Wholesalers Association, 2018). Cheap and available spoilage prevention measures are needed for smaller breweries to increase their product shelf-life, promote competitiveness throughout the entire American consumer market, and diversify beer varieties on store shelves.

In brewing, any organism other than brewing yeasts are generally considered contaminants and spoilage organisms can adversely affect beer by imparting undesirable flavors, odors, or appearance. Although beer is a microbiologically stable product, unwanted microorganisms contaminate beer with potentially detrimental financial consequences to the brewer (Bokulich & Bamforth, 2013; Vaughan, O'Sullivan, & Van Sinderen, 2005). The financial impact of beer spoilage in the brewing industry is difficult to determine because statistical data is fragmented. Few official reports have been

released and most published studies focus on a specific organism, facility, country, or region. Quantifying economic loss becomes difficult since no official records exist, and many companies do not release data to the public for the sake of commercial interests.

Consequences of contamination can be catastrophic for the brewer and may result in complete loss of a brew, product recalls, and financial loss (Vaughan et al., 2005). Any means that reduces or controls microbial contamination is of great economic interest to the brewing industry. Breweries which cannot afford expensive control processes would benefit the most from cheap and available methods. Current prevention methods, such as pasteurization and high-end filtration, are expensive to install and maintain.

Phage biocontrol of spoilage bacteria in beer is a viable, cost-effective tool for breweries lacking advanced technology and expensive spoilage prevention measures. Such treatment would greatly reduce production costs, significantly increase shelf life, and preserve final product quality for smaller breweries. A stable, effective phage cocktail could have significant economic implications by giving smaller breweries a competitive boost and would bring balance to the current American beer market.

CAUSES OF CONTAMINATION

Minimizing contamination is best achieved by eliminating the sources. Even under best practices, occasional contamination occurs because complete eradication of spoilage organisms is not possible (Hammond, Brennan, & Price, 1999). Spoilage is caused by the production of undesirable metabolites from contaminating microorganisms, resulting in undesired changes in flavor, aroma, turbidity, or viscosity (Kelly et al., 2011). Many organisms involved in beer spoilage are associated with plant matter, humans, insects, or other environments. The entry of contaminants into the brewery is inevitable and they are widely dispersed through malt dust, aerosols, and equipment (Bokulich & Bamforth, 2013). Thus, beer may become contaminated from a variety of sources. Primary contaminants originate from raw materials and brewhouse vessels. Secondary contaminants are introduced during downstream processes (Storgårds, 2000). The consequences of primary contamination tend to be more catastrophic and account for roughly half of documented spoilage incidents (Back, 1997; Vaughan et al., 2005).

Many stages of the brewing process reduce the likelihood of contamination and discourage the growth of bacteria (Hammond et al., 1999). Mashing, cooling, and fermentation are inherent to brewing since beer cannot be made without them. Each step imparts physical and chemical changes upon the liquid environment which ultimately transform wort into beer while killing bacteria or inhibiting their growth.

Mashing temperatures generally range between 40-72°C for 1-2 hours (Kunze, 2004; Rabin & Forget, 1998). After mashing, the wort is boiled for 45-90 minutes with the addition of hops in a brew kettle (Lewis & Bamforth, 2006). This process serves to "sterilize" the wort, enhance flavor, and cease enzymatic activity (Bamforth, 2011; Denny, 2009). The heat applied during mashing and boiling kills the vast majority of bacteria, yeasts, and molds, however certain LAB and spore-forming bacilli may still survive (Suzuki, 2011; Vriesekoop et al., 2012). After boiling, wort is rapidly cooled to allow for yeast addition. Yeast cells are added in high densities, usually between 5.0 x 10^6 and 2.0 x 10^7 cells per mL ("Commercial Pitch Rates," 2018), which impede the growth of contaminating bacteria. In addition to depleting nutrients, yeast fermentation rapidly brings extreme changes in the chemical environment such as acidity and inhibitory concentrations of ethanol and carbon dioxide (Deasy et al., 2011; Sakamoto & Konings, 2003; Suzuki, 2011).

Primary Contamination

Primary contamination is derived from brewhouse vessels, pipework, and raw materials such as barley, malts, water, brewing yeast, and packaging components. Each raw material carries its own microbiota which drives the composition of microbial communities throughout breweries (Bokulich, Bergsveinson, Ziola, & Mills, 2015; O'Sullivan, Walsh, O'Mahony, Fitzgerald, & Sinderen, 1999). With barley and malts, bacterial proliferation occurs during steeping when water triggers the germination of barley (Vaughan et al., 2005). The use of water can also introduce large subsets of microorganisms into breweries depending on the source and treatment prior to entering tanks and brew kettles (Ault, 1965). Contaminated yeast cultures are another source of

contamination as they can frequently house spoilage bacteria. Most pitching yeasts in the U.S., whether re-pitched or freshly propagated cultures, are often contaminated even when stringent cleaning and sterilization measures are used upon pipes and vessels (Ault, 1965; Vaughan et al., 2005).

Secondary Contamination

Though most contaminants originate from raw materials as primary contaminants, secondary contamination results from beer coming into unintended contact with surfaces other than raw materials (Bokulich et al., 2015). Secondary contamination is commonly referred to as cross-contamination and is a result of exposing product to unintended microorganisms. Though microorganisms that grow in beer are restricted to a relatively small selection of species, wort, fermenting beer, and beer can be exposed to numerous sources of contamination throughout the brewing process, (Ingledew, 1979; Mamvura, Iyuke, Cluett, & Paterson, 2011; Matoulkova, Kosar, Slaby, & Sigler, 2012; Storgårds, Tapani, Hartwall, Saleva, & Suihko, 2006; Timke, Wang-Lieu, Altendorf, & Lipski, 2005, 2008; Vaughan et al., 2005). Once introduced into the brewery, microorganisms exhibit diverse dispersal patterns throughout the facility. Non-production surfaces that encounter beer or waste streams accumulate substrates that support microbial survival and growth. Aerosols and splashes also enable microbial spread from surfaces onto other surfaces while human and insect traffic further contribute to dispersal (Bokulich et al., 2015). Physical partitions inhibit passage of microorganisms but do not prohibit them completely.

Airborne microorganisms present high risk to brewing operations. Such contaminants can contaminate beer during filling operations, contaminate empty

containers, and occupy open fermentation and storage vessels (Storgårds, 2000). Increased humidity can exacerbate this problem as it fosters greater amounts of airborne contaminants (Oriet & Pfenninger, 1998). Biofilms are problematic in breweries because they spread contamination and provide environments for niche microbial communities. Filling equipment is particularly prone to biofilm formation due to large volumes of water used during bottle rinsing which creates ideal conditions for microbial attachment and accumulation on surfaces (Back, 1994; Storgårds, 2000). Biofilms also shield microorganisms during cleaning and disinfection, can degrade equipment surfaces, and negatively affect processes when formed on filtration units or heat exchangers (Storgårds et al., 2006; Vaughan et al., 2005).

Stagnate areas and standing liquids house unique arrays of microbes and can contact beer through splashing and carryover from insects, humans, and equipment. Acetic acid bacteria and enterobacteria proliferate in open puddles of beer and will eventually form slimes that protect accompanying bacteria from disinfectants and dehydration. If product residues remain for extended periods of time, yeasts will proliferate alongside to produce metabolites that create ideal conditions for Lactic Acid Bacteria (LAB). In turn, the lactic acid produced by LAB can be metabolized to propionic acid by anaerobic species such as *Pectinatus frisingensis* (Tholozan, Membré, & Grivet, 1997). This disastrous sequence can pose significant harm to beer if within the vicinity of open product or unsealed containers (Vaughan et al., 2005).

PREVENTION OF CONTAMINATION AND SPOILAGE

Innate Qualities of Beer

Beer intrinsically presents a hostile environment for microbial growth due to a number of factors (Suzuki, 2011; Suzuki, Iijima, Sakamoto, Sami, & Yamashita, 2006; Vriesekoop, Krahl, Hucker, & Menz, 2012). Beer is inhospitable due to the presence of hop bitter acids (HBAs), ethanol, carbon dioxide, low oxygen, low pH, and trace nutrient availability (Deasy, Mahony, Neve, Heller, & van Sinderen, 2011; Sakamoto & Konings, 2003). There is wide assumption that foodborne pathogenic bacteria cannot survive in beer, and studies show that survival of pathogens in beer is generally poor (Hammond et al., 1999; Menz, Aldred, & Vriesekoop, 2011; Suzuki, 2011; Vriesekoop et al., 2012).

Beer is a poor growth medium for most bacteria because nutrients are depleted by yeast during fermentation (Deasy et al., 2011; Sakamoto & Konings, 2003). Beers vary in their ability to resist bacterial growth given the wide variety of beer styles and brewing processes. As such, there are large variations in the susceptibilities of beers to microbial spoilage. Beers with low acidity, alcohol, hop content, carbon dioxide, and those with added sugars are naturally more prone to spoilage than others (Dolezil & Kirsop, 1980; Vaughan et al., 2005). Though the microbial stability of beer cannot be attributed to a single trait or set of traits, it is likely that combinations of many factors impart different levels of spoilage resistance to any particular style or brand of beer.

Antimicrobial Compounds

Ethanol is a natural byproduct of yeast fermentation and inhibits or kills most bacteria. Many bacteria, including pathogens, are inactivated by ethanol in beer. For instance, *E. coli* O157:H7 and *Salmonella typhimurium* were unable to initiate growth in beers ranging from 2.7 to 5.0% (v/v) ethanol. Inactivation times in these concentrations are reduced as temperature increases (Menz et al., 2011). Beers typically contain 3.5 to 5.0% (v/v) ethanol with some as high as 12% which constitute a negligible risk of pathogen transmission. However, a small risk remains in draught beers with lower alcohol content (Menz et al., 2011).

The antimicrobial properties of hops and hop bitter acids (HBAs) are well characterized and have been studied for decades. Hops are best known for the bittering flavor they impart upon beers due to the α-acids humulone, cohumulone, and adhumulone (Rigby & Bethune, 1952). During wort boiling, α-acids become isomerized into iso-α-acids which are more soluble, bitter, and possess antibacterial activity against gram-positive bacteria (Sakamoto & Konings, 2003; Vaughan et al., 2005). Gram-negative bacteria are generally insensitive to HBAs due to the hydrophobic outer membrane (Sakamoto & Konings, 2003). HBAs act as ionophores, dissipating the proton-motive force across cell membranes through oxidative stress (Behr & Vogel, 2010; Bokulich et al., 2015; Simpson & Fernandez, 1992). The low pH of beer further enhances the antimicrobial activity of HBAs. An increase of 0.2 pH may decrease the antibacterial effects of HBAs by up to 50% (Vaughan et al., 2005). The terminal effects of HBAs cause subsequent inhibition of respiration, disruption of protein, DNA, and RNA synthesis, and impairment of leucine uptake (Behr & Vogel, 2010).

pH

The acidification of beer is a natural result of yeast fermentation and most beers range between pH 3.9 to 4.4 (Suzuki, 2011). Organic acids excreted and absorbed by yeast have substantial effects upon pH. The dissolution of carbon dioxide into carbonic acid and absorption of primary phosphates by yeast lower pH to a smaller extent (Coote & Kirsop, 1976; Suzuki, 2011).

Low pH inhibits cell membrane functions and can lead to death for many microorganisms. Low pH can destroy enzyme systems, interfere with nutrient uptake, and alter the proton-motive force (Beales, 2004; Ingram, 1986; Meyer-Rosberg, Scott, Rex, Melchers, & Sachs, 1996). The ability of a cell to maintain intracellular pH varies between species and strains and is primarily done through ATP-driven movement of cations across the membrane. If a cell's mechanisms of passive and active pH homeostasis becomes overwhelmed, it leads to starvation and eventual death (Vriesekoop et al., 2012).

The extent of damage also depends on the types of acids responsible for the reduced pH. Juven (1976) demonstrated that growth inhibition of *Lactobacillus brevis* occurred at pH 3.6 with lactic acid, while acetic acid induced inhibition between pH 3.7 and 4.0. Furthermore, Chung and Goepfert (1970) demonstrated that *Salmonella* could grow at pH 4.05 with citric acid as the acidulant, but was inhibited at pH 5.50 with propionic acid. In beer fermentation, yeast produce pyruvate, lactate, succinate, α-ketoglutarate, α-hydroxyglutarate, and malate as metabolic byproducts (Coote & Kirsop,

1976), each of which may have varying degrees of adverse effects upon different bacteria.

Low oxygen content

Beer contains elevated carbon dioxide as a result of yeast fermentation. The production of carbon dioxide creates anaerobic conditions, lowers pH due to the formation of carbonic acid, affects enzyme activity and cell membrane functions, and influences carboxylation and decarboxylation reactions (Suzuki, 2011). While low levels of carbon dioxide (< 3 g/L) appear to have little effect upon LAB and may even enhance growth, higher levels are inhibitory. The concentration of carbon dioxide in packaged beer generally ranges between 4 to 7 g/L and contributes to beer's resistance to spoilage by LAB (Hammond et al., 1999).

Current Strategies for Spoilage Prevention

Many brewers subject beer to specific processes to increase its quality and reduce the likelihood of spoilage. Examples of such processes are filtration, bottle conditioning, and pasteurization. Though these processes are commonly used to impart specific characteristics upon beer, they are not strictly required to produce beer.

Bottle-conditioned beers are subjected to an additional fermentation in the bottle to yield natural carbonation. Bottle-conditioning is accomplished by bottling beer with a viable yeast population present, additional wort may be added if no residual fermentable sugars remain (Boulton & Quain, 2001). Bottle conditioning makes beer less prone to spoilage as the fermenting yeast produce additional carbon dioxide and reduce the oxygen content of the headspace (Vriesekoop et al., 2012). Dolezil & Kirsop (1980) found that bottle conditioning was a factor in contamination-resistant beers.

Pasteurization eliminates microorganisms and extends shelf life through heating. Heat tolerance varies between bacteria, but most beer spoilage bacteria are killed by pasteurization. However, pasteurization does not completely eliminate vegetative bacteria and bacterial endospores (Tewari & Juneja, 2007). Tunnel and flash pasteurization are the two methods used in breweries (Vaughan et al., 2005). Flash pasteurization is a rapid, bulk process where beer passes through a heat exchange system, is rapidly cooled, and filled directly into the container. This process is commonly used on large containers, such as kegs, which cannot be effectively pasteurized with a tunnel system (Vaughan et al., 2005).

Despite the appeal of pasteurization to reduce spoilage, it often causes deterioration in flavor and many brewers choose not to use it (Vaughan et al., 2005). Heat treatment causes protein denaturation, formation of tannin-protein complexes, turbidity enhancement, alteration of color, and formation of undesirable flavors (Stewart, 2006). The pasteurization process promotes negative sensory qualities relating to oxidation and staling resulting in off-flavors resembling paper and cardboard (Franchi, Tribst, & Cristianini, 2011; Zufall & Wackerbauer, 2000). Lastly, pasteurization is a costly process which not all brewers can afford. Beyond initial equipment and installation, enormous cost is required for water and electrical utilities, equipment maintenance, and employment of certified mechanics and electricians to maintain systems and equipment.

Filtration serves to remove yeast and solids from beer and imparts a polished shine and brilliance. Filtration prevents spoilage by removing microorganisms, the degree of prevention is dependent upon the pore size of the filter(s) and thoroughness of

the process. Filtered beer is considered stabilized since contact with yeast has ceased or been reduced dramatically. Not all beer is filtered, and filters may be rough, fine, or sterile. Rough filtration allows for some cloudiness in the form of residual yeast and solid particles to remain, while fine filters remove the majority of yeast, some smaller microorganisms, and cloudiness. High-end filtration, sometimes referred to as "sterile" filtration removes most microorganisms including bacteria (Esslinger, 2009). However, many spoilage bacteria can pass through filters and persist into the final, packaged product, allowing them metabolize remaining nutrients with relatively no other competition (Esslinger, 2009; Storgårds et al., 2006). Like pasteurization, filtration can be a costly process to implement and is therefore less commonly used in smaller breweries.

Sanitation and Physical Safeguards

The most efficient means for preventing spoiling and contamination are good production hygiene, rational running of process lines, and effective use of biocides and disinfectants (Maukonen et al., 2003). However, bacteria are ubiquitous and are frequently found in finished products and product contact surfaces even when hygienic practices are used (Holah, Taylor, Dawson, & Hall, 2002). All equipment that directly contacts wort or beer must be kept in prime condition and cleaned regularly with approved detergents. Proper sanitation of the filler is critical and high standards of filler hygiene will minimize likelihood of contamination (Vaughan et al., 2005). Suitable equipment and materials, and elimination or minimization of rough surfaces and dead spaces, correct construction, process layout and automation are imperative to reduce the risk of contamination (Back, 1994; Holah et al., 2002; Kumar & Anand, 1998).

Despite high levels of purity and quality of brewing and washing waters, bacteria such as *Enterobacter* are often found in such waters despite clean reads on presumptive tests (Ault, 1965). The microbial composition is inconsequential prior to boiling, but subsequent water additions must be treated to prevent contamination (Ault, 1965).

Brewhouse vessels and pipework are prime sources for microbial contamination due to their complex design and contents. Further contamination occurs if vessels and pipes are not properly cleaned or maintained (Vaughan et al., 2005). Biofilm formation, dead spaces, bends, and irregular surfaces within pipes further complicate the sanitation of brewing vessels and equipment. Many breweries use automated hot caustic systems, chlorine solutions, or industrial grade biocides or disinfectants to clean tanks, vessels, and piping. The frequency, timing, and effectiveness of such systems are critical to minimizing contamination (Holah et al., 2002; Kumar & Anand, 1998; Maukonen et al., 2003; Vaughan et al., 2005).

Acid washing is a common practice for eliminating contaminating bacteria from collected yeast slurries. Acid washing typically brings yeast slurries to a low pH for 1-2 hours immediately prior to pitching. Phosphoric or sulfuric acids are used to bring yeast slurries to pH 2.0-2.1 at <5°C, higher temperatures and lower pH is avoided to prevent loss of yeast viability (Simpson & Hammond, 1989).

Packaging materials must also be considered. Special precautions must be taken with primary containers such as cans, bottles, caps, and kegs since they come into direct contact with beer and are also a source of primary contamination. Kegs are the most likely to harbor contaminants because they are returned from trade, sit for undetermined amounts of time, and contain residual beer product which can promote microbial growth

(Vaughan et al., 2005). Kegs undergo rigorous washing and sterilization cycles prior to filling, and filling failures undoubtedly lead to contaminated product. All facilities in the United States that fill cans and bottles are required to reduce the risk of physical and biological hazards in primary containers to comply with the FDA Hazard Analysis Critical Control Point (HACCP) regulation (HACCP Systems, 2018). HACCP requires rinsing of primary containers moments before the filling operation. The rinsing process reduces residual contamination from primary containers, though the end result is dependent upon the quality of water being used (Ault, 1965; Vaughan et al., 2005).

Geographical safeguards are equally critical as airborne contamination is a significant problem in breweries without pasteurization technology (Back, 1994). The first step in controlling brewery microbiota is sourcing raw materials with low microbial loads. Raw materials must be processed with safeguards in place and be kept physically separated from downstream processes. The majority of bacteria found in breweries originate from raw materials which spread and occupy different areas over time. (Bokulich et al., 2015). Furthermore, facilities must be hygienically designed to eliminate cross-contamination between tanks, pipelines, joints and accessories (Storgårds, 2000).

PROMINENT SPOILAGE ORGANISMS

Spoilage organisms are not deliberately introduced but can survive and proliferate in wort, fermenting wort, in-process beer, or packaged beer. Many bacteria that persist in beer for extended periods of times without proliferating or imparting adverse effects are not considered beer spoilage organisms (Lawrence, 1988). Generally, the array of organisms capable of growing in finished beer are limited to a few genera (Lee, Mabee, & Jangaard, 1978). The most common bacterial spoilers are LAB, particularly *Lactobacillus* and *Pediococcus* (Jespersen & Jakobsen, 1996). Forty years ago, acetic acid bacteria such as *Acetobacter* and *Gluconobacter* were much more problematic (Sakamoto & Konings, 2003). Other genera, such as *Pectinatus* and *Megasphaera*, are not as prominent but cause the most severe, unpleasant spoilage ever encountered (Back, 2005; Haikara, 1992; Lee et al., 1978).

Lactic Acid Bacteria

LAB occur naturally as barley microflora and are more prolific in malts with higher nitrogen content (Ault, 1965; Vaughan et al., 2005). These bacteria persist through malting and mashing and are found in all stages of the brewing process as a result (Hollerová & Kubizniaková, 2001; O'Sullivan et al., 1999; Vaughan et al., 2005). LAB are Gram-positive, coccoid or rod-shaped, non-sporulating, catalase-negative, aerotolerant, acid tolerant, nutritionally fastidious, non-respiring prokaryotes which

produce lactic acid as a major end-product from carbohydrate metabolism (Axelson, 1998; Klaenhammer, Barrangou, Buck, Azcarate-Peril, & Altermann, 2005). LAB generally grow well in the presence of oxygen though its presence may partially or completely inhibit some species. (Condon, 1987). In the presence of oxygen, the formation of ethanol is reduced in favor of acetate, but some LAB will produce CO_2 plus acetate, acetoin, or diacetyl in the presence of oxidants (Condon, 1987; Hammes, Weiss, & Holzapfel, 1992; Kandler, 1983). Homofermentative species produce lactic acid from carbohydrates through the glycolytic pathway. Heterofermentative species produce lactic acid, CO_2, and ethanol through the phosphoketolase pathway (Hammes et al., 1992; Kandler, 1983; Okano et al., 2009). Homofermentative strains that commonly spoil beer ferment maltose, the most abundant sugar in wort, and will grow poorly on glucose without an arginine supplement (Hammes et al., 1992; He et al., 2014; Rainbow, 1975).

Though LAB are commonly associated with food fermentation and preservation, they are generally regarded as the most hazardous of beer spoilage organisms (Back, 1994; Jespersen & Jakobsen, 1996). Their acid-tolerant and anaerobic nature makes LAB well suited to withstand the onslaught of beer conditions, though only select species and strains are genuinely capable of spoiling beer (Bergsveinson, Baecker, Pittet, & Ziola, 2015). LAB spoilage is a result of their growth and ability to produce organic acids, exopolysaccharides, and other metabolites (Gindreau, Walling, & Lonvaud-Funel, 2001).

LAB are by far the most common culprits in beer spoilage incidents (Suzuki, 2011). Among them, the genera *Lactobacillus* and *Pediococcus* are the most commonly found in modern breweries and responsible for up to 70% of all beer spoilage incidents (Back, 1994; Jespersen & Jakobsen, 1996; Suzuki, Asano, Iijima, Kuriyama, &

Kitagawa, 2008). *Lactobacillus brevis* and *L. lindneri* are reported as the most frequent lactobacilli causing spoilage and *P. damnosus* most frequent of the pediococci (Jespersen & Jakobsen, 1996; Satokari, Mattila-Sandholm, & Suihko, 2000). LAB beer spoilers have adapted to the extremes of the brewery environment. They tolerate low pH values (3.8-4.3), demonstrate HBA-resistance, and ferment a narrow range of carbohydrates with complex nutritional requirements (Hammes et al., 1992; Richards & Macrae, 1964).

LAB can spoil beer through acidification, haze formation, diacetyl formation, super-attenuation, and/or slime or rope formation (Bokulich & Bamforth, 2013; Hammes et al., 1992; Lawrence, 1988; Vaughan et al., 2005). Sensory defects are a result of metabolites such as lactic acid, which gives beer a sour milk or yogurt like flavor, or diacetyl, which imparts a buttery off-flavor (Bokulich & Bamforth, 2013; Hammes et al., 1992; Williamson, 1959). Strains which generate haze, rope, or slime do so as a result of glucan and dextran production (Sharpe, Garvie, & Tilbury, 1972; Walling, Gindreau, & Lonvaud-Funel, 2005; Williamson, 1959). Super-attenuation is the lowering of specific gravity past the designated limit due to over-production of alcohol (Andrews & Gilliland, 1952). Super-attenuation is undesirable because it can affect the flavor, body, and alcohol content in ways the brewer did not intend. LAB strains that cause spoilage are generally resistant to HBAs and may grow continuously throughout the fermentation process without yeast restriction in the early stages (Ault, 1965; Bokulich & Bamforth, 2013; Suzuki et al., 2006).

Lactobacillus brevis

Lactobacillus brevis was first described by Louis Pasteur in 1876 and isolated in 1892 by Van Laer. *Lactobacillus brevis* was originally named *L. pastorianus* in honor of Pasteur, but later renamed. As such, it was the first beer spoilage *Lactobacillus* discovered (Priest, 1981; Suzuki, Shimokawa, Yako, & Yamagishi, 2017). As an obligate heterofermentative bacteria, *L. brevis* grows optimally at 30°C, pH 4-6, and produces CO_2, lactic acid, and ethanol during fermentation (Kandler, 1983; Sakamoto & Konings, 2003; Vaughan et al., 2005). *Lactobacillus brevis* is used to ferment foods such as sauerkraut and is normal microbiota of the human intestinal tract and vagina (Pavlova et al., 2002).

Lactobacillus brevis is reported as the most frequent beer spoiler, with some sources naming it responsible for more than half of spoilage incidents. As a result, *L. brevis* is the most commonly studied spoilage organism (Back, 1994; Back, Breu, & Weigand, 1988; Hollerová & Kubizniaková, 2001; Suzuki et al., 2006). There are approximately 16 known strains that spoil beer and their spoilage ability varies widely. (Pavlova et al., 2002). Contaminations from *L. brevis* result in off-flavors, turbidity, and super-attenuation. Off-flavors are caused by the production of lactic acid. Super-attenuation is due to the organism's ability to metabolize residual starch and dextrins in the wort, which contribute to further ethanol production (Lawrence, 1988; Vaughan et al., 2005).

Other *Lactobacillus* species

Other beer spoiling lactobacilli are *L. lindneri, L. buchneri, L. casei, L. collinoides, L. coryneformis*, and *L. plantarum*, though none are as common in spoiled beer as *L. brevis* (Deasy et al., 2011; Suzuki, Koyanagi., & Yamashita, 2004). *Lactobacillus lindneri* is the most common beer spoiler next to *L. brevis*. Reports vary in *L. lindneri's* frequency in beer spoilage, with it being implicated between 5 and 25% of spoilage incidents (Back, 1994, 2005; Back et al., 1988; Suzuki, 2011). *Lactobacillus lindneri* spoils beer by causing turbidity, sedimentation, and acidification. Natural brewery habitats include fermentation rooms, storage cellars, and yeast stores (Back, Bohak, Ehrmann, Ludwig, & Schleifer, 1996).

Pediococcus damnosus

Pediococcus damnosus is a facultatively anaerobic, homofermentative coccus which grows in beer as single cells, pairs, and tetrads and grows optimally at 21-25°C (Ault, 1965; Suzuki et al., 2006; Vaughan et al., 2005). Several other pediococci species have been detected in beer but *P. damnosus* is the most prolific spoiler of its genus.

Pediococcus damnosus is often prevalent in lager breweries with strain-dependent preferences for glucose, galactose, fructose, maltose and sucrose (Ault, 1965). *Pediococcus damnosus* is most frequently encountered in brewing yeast and beer but has also been implicated in the spoilage of wines, ciders, and juices (Brodmann et al., 2005; F. Priest, 1996). Though *P. damnosus* is almost always considered a contaminant of beer and wine, its presence is desired in certain beer styles such as Lambic, Berliner Weisse, and sour beers.

The type of spoilage caused by *P. damnosus* is sometimes referred to as "sarcina sickness." This term was derived roughly 100 years ago due to pediococci's morphological similarities to the genus *Sarcina*, and is therefore, a misnomer (Shimwell & Kirkpatrick, 1939). Sarcina sickness is characterized by visible turbidity, granular sediment, acidity due to lactic acid, and a buttery off-flavor due to diacetyl (Ault, 1965; Back, 2005; Suzuki, 2011). *Pediococcus damnosus* is also capable of causing ropiness and slime depending on the presence of residual sugars in the beer after fermentation, and typically appears when the bacterial population reaches 10^5 CFU/mL (Gindreau et al., 2001; Shimwell & Kirkpatrick, 1939; Vaughan et al., 2005). Production of ropiness is due to glucan synthesis from a plasmid-associated gene though this characteristic is beneficial in some dairy fermentations (Gindreau et al., 2001).

Pectinatus and *Megasphaera*

Pectinatus and *Megasphaera* were first isolated in the 1970s and are notorious for producing extremely unpleasant odors and off-tastes (Haikara, 1992). The only species known to spoil beer are *Pectinatus cerevisiiphilus* and *Megasphaera frisingensis* (Tholozan et al., 1997). Both species are gram-negative, strictly anaerobic and more sensitive to ethanol and pH than most LAB but extremely tolerant to hops (Back, 1981; Haikara, 1984, 1992; Haikara, Penttilä, Enari, & Lounatmaa, 1981).

In 1981, *Pectinatus* and *Megasphaera* accounted for 2% of brewery contaminations but rose to 7% in 1987 (Back, 1981; Back et al., 1988; Haikara, 1992). Both species have been found to only exist in beer and brewing environments (Haikara, 1992). Their nearly simultaneous discovery is well explained by their anaerobic nature, since the dissolved oxygen content of beer and headspace in containers dramatically

reduced with development of advanced filling technology (Back, 1981; Chelack & Ingledew, 1987; Haikara, 1992).

Pectinatus cerevisiiphilus

Pectinatus cerevisiiphilus grows under anaerobic conditions between 15 and 40°C with optimal growth at 32°C (Chelack & Ingledew, 1987; Vaughan et al., 2005). Given these preferences, *P. cerevisiiphilus* can flourish after packaging if stored in non-refrigerated conditions. Ethanol tolerance tests have shown *P. cerevisiiphilus* to withstand up to 10% (v/v) ethanol in nutrient broth (Haikara et al., 1981; Hettinga & Reinhold, 1972), but no growth has been observed in beers exceeding 5.2% (v/v) ethanol. *Pectinatus cerevisiiphilus* is capable of growth in pH 4.5-8.5 with the optimum at pH 6.0-7.0 (Haikara et al., 1981). In beer, pH 4.4 did not restrict growth while pH 4.0 considerably reduced growth and pH 3.1 completely restricted growth (Haikara, 1992). Another hallmark of *P. cerevisiiphilus* is the production of propionic acid and sulfur compounds in beer (Haikara et al., 1981; Lee et al., 1978; Schisler, Mabee, & Hahn, 1979). Other compounds include acetic, succinic, and lactic acids and acetoin, as well as an array of sulfur compounds such as hydrogen sulfide, methyl mercaptan, and dimethyl trisulfide (Haikara, 1992; Haikara et al., 1981; Hettinga & Reinhold, 1972; Lee, Mabee, Jangaard, & Horiuchi, 1980).

Pectinatus causes extensive turbidity and an unpleasant rotten egg smell due to hydrogen sulfide and methyl mercaptan. It also imparts a body odor-like aroma from the production of propionic acid (Haikara et al., 1981; Lee et al., 1978, 1980). Besides finished beer, *P. cerevisiiphilus* has been found in equipment lubrication oils mixed with beer, drainage and water pipe systems, in the air of breweries, condensed water on

ceilings, chain lubricants, and steeping water. Based upon these locations, water is the most likely source of contamination and *P. cerevisiiphilus* is capable of survival in aerosols despite its anaerobic nature (Back et al., 1988; Haikara, 1992; Lee et al., 1980). The presence of LAB can enhance the growth of *P. cerevisiiphilus* because it can metabolize lactate (Haikara, 1992).

Megasphaera frisingensis

Megasphaera frisingensis are strictly anaerobic, gram-negative cocci inhibited by pH values below 4.1 and ethanol concentrations above 2.8% (w/v) (Lawrence, 1988; Vaughan et al., 2005). *Megasphaera frisingensis* is more sensitive to lower pH in beer than *Pectinatus* spp. An increase of pH in beer from 4.1 to 4.7 accelerates the growth of *Megasphaera* while no growth occurs at pH 4.1 (Haikara, 1992; Seidel, Back, & Weiss, 1979). Ethanol is arguably the most important factor in inhibiting the growth of *M. frisingensis*. Growth did not occur in experiments in commercial beers with 4.3-6.5% (w/v) ethanol (Haikara, 1992; Seidel et al., 1979).

Metabolic end products of *M. frisingensis* are butyric, valeric, acetic, propionic, caproic acids, acetoin, and hydrogen sulfide (Engelmann & Weiss, 1985; Lee, 1994; Vaughan et al., 2005). The ability to ferment carbohydrates and produce volatile fatty acids are characteristic of *M. frisingensis*. The fatty acids produced are dependent upon the available carbon source. In beer, the predominant end products are caproic and butyric acids, but *M. frisingensis* will produce valeric acid in the presence of lactate (Engelmann & Weiss, 1985; Haikara, 1992). Like *Pectinatus*, the presence of LAB can enhance the growth of *M. frisingensis* due to the ability to metabolize lactate (Haikara, 1992).

The mixture of fatty acids and hydrogen sulfide makes beer contaminated by *M. frisingensis* quite unpleasant (Haikara, 1992). Spoilage results in slight haze and severe off-flavors through considerable quantities of putrid-smelling compounds such as butyric acid with lesser amounts of acetic, caproic, and valeric acids and hydrogen sulfide. The formation of such compounds makes beer undrinkable (Back, 2005; Haikara, 1992; Lee, 1994; Suzuki, 2011; Vaughan et al., 2005).

Other Bacterial Spoilers

Several other species of bacteria cause beer spoilage to a lesser frequency than LAB or *Pectinatus* and *Megasphaera*. Many of the miscellaneous spoilers were problematic in the past but are less relevant with the advent of new technologies. These various organisms are still detected in modern brewing environments, though their likelihood of causing spoilage has decreased. Yet as brewing technology and processes have changed over the years, so has the microbiome of the breweries that use them (Bokulich & Bamforth, 2013; Sakamoto & Konings, 2003). Home brewers and less advanced breweries, however, are likely to encounter problems from other spoilage bacteria more frequently.

The Genus *Bacillus*

Bacillus spp. are readily found in barley malt. They are gram-positive, aerobic, endospore-forming bacteria well known to persist through the mashing and boiling processes. However most species are unable to germinate during later stages of brewing due to the low pH of fermenting wort and sensitivity to HBAs (Smith & Smith, 1993; Vaughan et al., 2005). *Bacillaceae* are generally incapable of spoiling beer, but some species have been isolated from spoiled, home-brewed beer which contained hop-

resistance genes and were capable of growth when reinoculated into beer (Haakensen & Ziola, 2008).

With *Bacillus coagulans,* the production of n-nitroso compounds are of particular concern since nitrate is reduced to nitrite in the absence of oxygen resulting in the formation of nitrosamine. N-nitroso compounds are highly toxic, carcinogenic, and the American Society of Brewing Chemists' recommended limit is only 20 µg/L (Bokulich & Bamforth, 2013; Delaware Health and Social Services, 2015; N. A. Smith, Smith, & Woodruff, 1992; US Department of Health and Human Services, 2016). *Bacillus coagulans* contaminations result in elevated N-nitroso levels far higher than the recommended limit at prolonged mash temperatures (55-70°C). The organism further raises apparent total N-nitroso compound levels when persisting through fermentation, where it reduces nitrate in the absence of oxygen (Calderbank & Hammond, 1989; Massey, Key, McWeeny, & Knowles, 1987; N. A. Smith & Smith, 1993; Vaughan et al., 2005). In addition, *B. coagulans* is known to produce large amounts of lactic acid under the same conditions.

Acetic Acid Bacteria

Acetic acid bacteria are aerobic, gram-negative, acid-tolerant, hop-resistant, rod-shaped bacteria which oxidize sugars and/or ethanol to produce acetic acid (Ault, 1965; Bokulich & Bamforth, 2013; Raspor & Goranovic, 2008). *Acetobacter aceti*, *A. pasteurianus,* and *Gluconobacter oxydans* are known to spoil beer (Bokulich & Bamforth, 2013). Contrary to popular belief, no acetic acid is produced when acetic acid bacteria grow in wort. Instead gluconic acid is produced from glucose (Ault, 1965). During and after fermentation, acetic acid bacteria produce acetic acid from ethanol if

oxygen is present, essentially turning beer into vinegar (Ault, 1965). Many years ago when beer was aged in barrels, acetic acid bacteria were much more prevalent in beer spoilage, but are still found in barrel-aged beers (Bokulich, Bamforth, & Mills, 2012). Modern brewing technology has essentially rendered these bacteria irrelevant in most breweries. Conical steel fermenters, tank pressurization systems, and controlled headspace keep oxygen levels low during fermentation and storage while advanced filling technology reduces the oxygen content of packaged beer (Ault, 1965; Bokulich & Bamforth, 2013; Sakamoto & Konings, 2003; Vaughan et al., 2005). Regardless, acetic acid bacteria are ubiquitous air contaminants and contamination is nearly inevitable in breweries (Ault, 1965). Beer spoilage can certainly occur in the event of tank failures, packaging defects, and improper filling operations. Though kegged beers are generally free from contaminants, acetic acid bacteria are known to be prevalent in unsanitary draught dispensing systems and can cause haze and surface film in beer (Harper, 1981; Vaughan et al., 2005).

Zymomonas mobilis

Zymomonas mobilis is the only species found in this genus. It is a gram-negative, motile, facultatively anaerobic, rod-shaped bacteria that produces high levels of acetaldehyde and hydrogen sulfide (Bokulich & Bamforth, 2013; Dadds, Macpherson, & Sinclair, 1971; Dadds & Martin, 1973; Vaughan et al., 2005). The natural habitats of *Z. mobilis* are the sugary liquid from the flower spikes of *Agave* spp., the sugar palm (*Aregna saccharifera*), and the Gomuti palm (*Aregna pinnata*). Furthermore, *Z. mobilis* is unable to ferment maltose or maltotriose in wort and beer and therefore is typically only problematic in beers primed with adjunct sugars (Bokulich & Bamforth, 2013;

Dadds & Martin, 1973). If introduced to its ideal beer environment, *Z. mobilis* is a formidable spoiler. *Zymomonas mobilis* can grow in pH as low as 3.4 with ethanol content up to 10% (w/v) and can cause considerable damage within 48 hours in warm weather. Beer spoiled by *Z. mobilis* has considerable turbidity with an unpleasant odor resembling rotten apples due to acetaldehyde and hydrogen sulfide (Dadds & Martin, 1973).

SURVIVAL MECHANISMS OF SPOILAGE BACTERIA

Lack of Nutrients

Wort only exists in the early stages of brewing and is a complex medium rich in sugars that can support the growth of many spoilage organisms. As wort is fermented into beer by brewing yeast, it is rapidly depleted of nutrients. In this aspect, the keys to survival and growth are the ability to utilize energy sources that brewing yeast cannot use, or to target specific sugars and energy sources before they are metabolized by brewing yeast. During initial stages of fermentation, brewing yeast prefer maltose and will deplete it to significant levels before turning to glucose, fructose, sucrose, or maltotriose (He et al., 2014). Organisms adapted to beer spoilage may prioritize the latter sugars before they are consumed by brewing yeast. The second strategy utilizing energy sources unavailable to brewing yeast. Though sugars levels drop quickly, LAB present in fermenting wort can metabolize citrate, pyruvate, malate, and arginine to generate ATP (Suzuki, 2011; Suzuki, Iijima, Ozaki, & Yamashita, 2005). Furthermore, the ability to metabolize starches and dextrins with enzymes is displayed by numerous LAB (Jespersen & Jakobsen, 1996; Lawrence, 1988). The time between production and sale provide opportunities for spoilage organisms to flourish in packaged beer.

Ethanol and Acid Tolerance

In order to withstand acid and ethanol stress, bacteria may adjust the composition of cell membranes to negate the effects of acid and ethanol since the cell membrane is the primary point of contact between a cell and its environment. Changes in the lipid and protein composition of the membrane are induced in response to numerous forms of stress such as temperature, pH, solvents, and toxins (Dombek & Ingram, 1984). In response to alcohols, a common strategy is modification of the fatty acid and phospholipid composition of the cell membrane (Grandvalet et al., 2008). For example, *Clostridium acetobutylicum* is known to utilize a cis-trans isomerase upon the fatty acids of its cell membrane during acetone-butanol fermentations. When the double bond is reconfigured into the trans- form, the shape of the molecule changes to increase membrane rigidity (Lepage, Fayolle, Hermann, & Vandecasteele, 1987). *Bacillus subtilis*, *Escherichia coli* and *Oenococcus oeni* use a similar method, but instead convert monounsaturated fatty acids to cyclopropane fatty acids which reduce membrane fluidity to prevent undesirable molecules from entering the cell (Chang & Cronan, 1999; Grandvalet et al., 2008; Rigomier, Bohin, & Lubochinsky, 1980). *Oenococcus oeni*, a LAB associated with wine fermentation and spoilage, is known to upregulate heat shock proteins and cyclopropane fatty acids to reduce membrane permeability to protect the cell from ethanol (Grandvalet et al., 2008; Suzuki, 2011). *Lactobacillus fructivorans*, a sake spoilage LAB, produces fatty acids of over 24 carbons in response to high ethanol concentrations (Ingram, 1986). Though the adjustment of membrane composition in response to ethanol has not been well-characterized in beer spoilage organisms, it is highly likely that similar strategies are used to tolerate the harsh conditions of beer.

Bacteria employ similar strategies of altering membrane composition in response to low pH. *Escherichia coli*, *S. typhimurium*, and *C. acetobutylicum* have been observed to increase membrane cyclopropane fatty acid content in response to acid stress. These species became susceptible to acids under induced cyclopropane fatty acid deficiencies (Chang & Cronan, 1999; Grandvalet et al., 2008; Kim et al., 2005).

Hop Resistance

Resistance to HBAs, is a critical requirement for spoilage organisms to survive and grow in beer (Behr, Gänzle, & Vogel, 2006). HBAs operate as mobile-carrier ionophores and cause complete dissipation of the transmembrane pH gradient of sensitive cells (Vaughan et al., 2005). Several resistance mechanisms have been characterized such as the hop transporters HorA and HorC and the divalent cation transporter HitA (Hayashi, Ito, Horiike, & Taguchi, 2001; Iijima, Suzuki, Ozaki, & Yamashita, 2006; Sami et al., 1997; Suzuki et al., 2006; Suzuki, Sami, Ozaki, & Yamashita, 2005). All of these proteins are encoded by genes located on plasmids, transferable via horizontal gene transfer, upregulated during growth in beer, and prevalent in LAB isolated from spoiled beer (Bergsveinson et al., 2015; Pittet, Phister, & Ziola, 2013; Suzuki et al., 2006; Suzuki, Sami, et al., 2005). The *horA*, *horC*, and *hitA* hop-resistance genes have been well characterized in common LAB spoiler organisms. The extent to which these genes are actively transferred in breweries is the subject of much speculation, but it is hypothesized that gene transfer occurs in biofilms containing LAB where the close proximity of cells would be conducive to plasmid transfer. In some cases, HBA resistance genes cannot be cured from cells, indicating that the plasmid also carries genes

necessary for survival under the conditions of growth in studies (Bergsveinson et al., 2015; Bokulich et al., 2015).

HorA and HorC are pumps which dispel HBAs using ATP, thus preventing HBAs from transporting divalent cations outside the cell (Iijima et al., 2006; Sakamoto, Van Veen, Saito, Kobayashi, & Konings, 2002; Simpson, 1993). H*orC* was the most abundant hop-resistance gene found in Bokulich's 2015 study and has been proposed to serve as the primary genetic marker to detect beer spoilage LAB (Bokulich et al., 2015; Iijima, Suzuki, Asano, Kuriyama, & Kitagawa, 2007). H*itA* is another hop resistance gene which encodes the non-ATP binding transporter HitA involved in manganese transport thus counteracting the effects of HBAs (Hayashi et al., 2001; Sakamoto & Konings, 2003).

LAB are also known to increase the content of large lipoteichoic acids in their cell envelopes in response to hop compounds. Lipoteichoic acids reduce the intrusion of HBAs past the cell wall and are hypothesized to act as reservoirs for divalent cations thus competing with HBAs for them (Behr et al., 2006; Suzuki, 2011; Vogel, Preissler, & Behr, 2010).

THE BACTERIOPHAGE

Bacteriophages, commonly referred to as phages, are viruses which infect bacteria. Phages are natural predators of bacteria with diverse life cycles and lethal effects upon their hosts (Abuladze et al., 2008; Garcia, Martínez, Obeso, & Rodríguez, 2008; Salmond & Fineran, 2015). Phages are ubiquitous, found wherever bacteria exist. Bacteriophages are abundant in in saltwater, freshwater, soil, plants and animals. It is estimated there are over 10^{31} phages on Earth, making them the most abundant biological entity on the planet and more numerous than all other organisms on Earth combined (Bergh, Børsheim, Bratbak, & Heldal, 1989; Hoyland-Kroghsbo, Mærkedahl, & Svenningsen, 2013; LaFee & Buschman, 2017). Considering that 10^{6} to 10^{8} bacteriophages exist in each milliliter of natural water, it is further estimated that phages outnumber bacteria 10-fold in most natural environments (Brussow & Hendrix, 2002).

Bacteriophages are also numerous and measurable components in foods at every stage from farm to point of consumption. Phages are extremely stable and easily recovered from soil, sewage, water, farm, and factory effluents (Greer, 2005). As the dominant constituent of the human virome, phages are described as living symbiotically with bacteria in the human digestive tract, saliva, respiratory tract, and skin (De Paepe, Leclerc, Tinsley, & Petit, 2014). In the dairy industry, phages are infamous contaminants, often ruining fermentations, destroying starter cultures, or deteriorating

product quality (Hammes et al., 1992; Mahony, Bottacini, van Sinderen, & Fitzgerald, 2014; Marcó, Josiane. Garneau, Tremblay, Quiberoni, & Moineau, 2012; Nes & Sørheim, 1984).

Life Cycle of Bacteriophages

The term "life cycle" is a misnomer because bacteriophages, like all viruses, are not living organisms and are metabolically inert. However, phage infections cause subsequent actions that result in the production of more phages (Goodridge & Abedon, 2003). The first step in the phage life cycle is adsorption, when the phage targets a specific receptor on the host cell and injects its nucleic acids (Bertin, de Frutos, & Letellier, 2011; Moldovan, Chapman-McQuiston, & Wu, 2007; Samson, Magadán, Sabri, & Moineau, 2013). Upon entry into the host cell, the virus will seize control of many host proteins to execute of one two distinct life cycles: (i) the lytic cycle, which is an active infection in which virion components are produced and assembled into virions, or (ii) lysogeny, where the phage genome integrates into the host as a prophage. Lytic phages are ideal for phage biocontrol and bioprocessing, while lysogenic, or temperate phages, are useful for cloning (Goodridge & Abedon, 2003; O'Flaherty et al., 2004).

The ability of a phage to bind to its host is key to its survival and proliferation. Phages initiate infection by binding specific sites on the host surface. Phage specificity ultimately dictates host range as they can only infect cells which display receptors it can bind (Bertin et al., 2011; Gabashvili, Khan, Hayes, & Serwer, 1997). Phages rely on chance to randomly collide with hosts and cannot actively move toward their prey. Naturally, the chances of infection increase with greater affinity between receptors and greater host cell densities (De Paepe et al., 2014).

Caudovirales, the most numerous and well-known order of bacteriophages, use a distinct, proteinaceous, tube-like structure (a tail) to pass the viral genome through the cell membrane and into the cytoplasm (European Food Safety Authority (EFSA), 2009). The tail is the driving force behind *Caudovirales'* ability to infect their hosts (McGrath & Van Sinderen, 2007). The tail is a central tube that allows for the passage of the viral genome and tail fibers contain the receptor-binding proteins. The tail is attached to the capsid by a protein structure called a collar, which seals the capsid after genome packaging and opens upon infection (Cuervo & Carrascosa, 2001). On the other hand, *Myoviridae* use a syringe-like motion to inject their genome into the host. (Cuervo & Carrascosa, 2001). *Podoviridae*, short-tailed phages, use small, tooth-like tails fibers to degrade a portion of the cell envelope and insert their material while others penetrate via enzymatic degradation (Aksyuk et al., 2012; Cornelissen et al., 2012).

After entering the host, phages must replicate their genomes, produce proteins, and assemble and package the components. Proteins and nucleic acids assemble into well-defined three-dimensional objects (Aksyuk & Rossmann, 2011). Upon capsid assembly, DNA enters the capsid and tightly packs into concentric patterns until genome packaging is complete. After packaging, the structure is sealed, tail proteins attach, and mature progeny phage are produced. (Aksyuk & Rossmann, 2011).

The Lytic Cycle

The lytic cycle involves the destruction of the bacterial cell resulting in cell lysis allowing phage progeny to be released into the environment to infect other host cells (Abedon, Herschler, & Stopar, 2001). Virulent phages, such as coliphage T4, only replicate through the lytic cycle. After assembly of mature phage progeny, the phage will utilize endolysins to rupture the host cell to release the newly assembled phages into the environment (Schmelcher, Donovan, & Loessner, 2012). By contrast, *Inoviridae* (filamentous phages) release through extrusion, which involves the exiting of phage progeny without causing cell death (Goodridge & Abedon, 2003; McGrath & Van Sinderen, 2007). Some lytic phages undergo a phenomenon known as lysis inhibition, where progeny will not be released from the cell if extracellular phage concentration is high. Lysis inhibition is a reproduction strategy and is not the same as dormant lysogenic phage (Abedon, 1992).

The Lysogenic Cycle (Temperate Phages)

The lysogenic cycle does not immediately result in lysis of the host cell. Phages that undergo the lysogenic cycle are referred to as lysogenic or temperate phages (EFSA, 2009). Temperate phages have the ability to either lay dormant or cause an active infection (Goodridge & Abedon, 2003). During lysogeny, the viral genome integrates into the host DNA and remains dormant until some sort of factor, usually an environmental condition, induces the phage to activate. This activation process is known as induction. Once induced, the phage will initiate its reproductive cycle, produce phage progeny, and lyse the host (Mason, Losos, Singer, Raven, & Johnson, 2011; Ptashne, 2004).

Once inside the host, viral genomes can integrate directly into host genome or exist in a plasmid-like state. Either state is referred to as a prophage, which can replicate harmlessly along with the host (Salmond & Fineran, 2015). The prevalence of prophage among some LAB is astounding. Genome sequencing has revealed that many dairy lactococci and lactobacilli contain prophages (Canchaya, Proux, Fournous, Bruttin, & Brüssow, 2003; Marcó et al., 2012; Sechaud, Cluzel, Rousseau, Baumgartner, & Accolas, 1988). Other sequenced bacterial genomes reveal that prophages constitute 3-10% of the host chromosomal content (Brussow & Hendrix, 2002; Desiere, Lucchini, Canchaya, Ventura, & Brüssow, 2002).

Though the bacterial host is metabolically burdened by replicating the prophage DNA, coupled with the disadvantage that the prophage will potentially kill it, the prophage may compensate the host by providing genes beneficial to its survival (Brussow & Hendrix, 2002; Desiere et al., 2002). This phenomenon is called lysogenic conversion. The most commonly conferred traits are immunity and super-infection, which protect the host, now called a lysogen, against further phage infection. Other examples of prophage genes which benefit the lysogen are those encoding diphtheria toxin, streptococcal erythrogenic toxin A, or verocytotoxin (EFSA, 2009).

Prophages exit the lysogenic cycle and become lytic through induction. Stochastic processes, intercellular signaling, or cellular stress can induce a prophage from its lysogenic state into an active infection. Once induced, the bacteriophage will turn on its lytic machinery to produce more virions and destroy the host (Goodridge & Abedon, 2003; Pouillot et al., 2012; Salmond & Fineran, 2015). Lysogeny is a complicated, yet

fascinating phenomenon and prophages have often been referred to as “molecular time bombs.”

Desired Traits of Effective Phages for Biocontrol

Obligately Lytic

Lytic phages are ideal for biocontrol because they can rapidly reduce bacterial populations by lysing cells and are a relatively harmless antibacterial approach (B. Coffey, Mills, Coffey, McAuliffe, & Ross, 2010; Hagens & Loessner, 2010; Kelly et al., 2011). Any phage used for the control of beer spoilage must be obligately lytic with no chance for lysogeny or lysis inhibition. Lysogenic phages have been well-characterized to transform harmless commensal bacteria into pathogens through transduction and horizontal gene transfer (HGT) (Lerminiaux & Cameron, 2019; Wagner & Waldor, 2002; Waldor & Mekalanos, 1996). However, in the case of beer application, the risk is the ability to transfer beer spoilage or hop resistance genes. Though lytic phages are capable of HGT in rare cases, the concern is dramatically reduced by selecting phages that lack the ability to lysogenize their host (Goodridge & Abedon, 2003; Keen et al., 2017; Rokyta, Burch, Caudle, & Wichman, 2006).

Use of lytic phages dramatically reduce the likelihood of HGT, which is a primary concern in phage application for any product involving human consumption (Goodridge & Abedon, 2003; Rokyta et al., 2006). Fortunately with beer, pathogenic phenotypes and antibiotic resistance are not involved with spoilage organisms. However, the risk of spreading beer spoilage traits is a possibility. Generalized transduction, where bacterial DNA is mispackaged during phage replication and then transduced into nearby cells is a possible route of transfer (Salmond & Fineran, 2015; Zinder & Lederberg, 1952).

Furthermore, phage-induced lysis promotes the release of bacterial DNA, which can be acquired by adjacent competent cells in rare cases (Salmond & Fineran, 2015). Though transfer of genes through either method is possible, they are unlikely to pose risk because of their infrequency. Furthermore, phages applied for biocontrol would quickly kill nearby hosts. Regardless, any phage used in industry application must have its genome fully characterized to ensure that it is solely lytic and free from beer spoilage or hop-resistance genes (Kelly et al., 2012).

Retain Activity in Processing and Storage Environments

Phages used in brewing should remain stable from early fermentation through remaining brewing stages and persist for several months in the container up to the point of consumption. Phages must retain activity throughout the entire duration in order to prevent spoilage bacteria from growing. Important physiochemical conditions include pH, temperature, ionic conditions, and others (Hudson, Billington, Carey-Smith, & Greening, 2005; Kelly et al., 2011). While some bacteriophages may degrade easily, it is impossible to generalize their survivability outside or away from the host bacterium. Therefore, stability throughout the brewing process and in finished beer must be characterized for each individual phage. Since phages are metabolically inert, their inactivation likely follows first order kinetics and is further influenced by environmental factors (EFSA, 2009).

Phage screening and selection should be biased towards phages with the ability to persist longer than their hosts. Some phages are extremely persistent, as seen in *Pseudomonas* phages, and remain stable in liquid for 1 year at 48°C or on solid tissue in vacuum-sealed environments at 28°C for 6 months (Greer, 2005; Greer & Dilts, 1990).

Since phages generally show a greater level of stability than their hosts, LAB phages should remain stable in beer longer than their hosts, which can survive in spoiled beer for months.

Low pH can inhibit proteins involved in adhesion such as receptor-binding proteins and capsule-degrading enzymes. Though phages tend to exist in the same environments as their hosts, phage stability in acidic environments varies considerably. For instance, *Salmonella* phages effectively prevented host growth on melons (pH 5.77) but not on apple slices (pH 4.37) and dropped below detectable levels within 24 hours (Leverentz et al., 2003). For brewing application, many LAB phages are known to be stable at typical beer pH values from 3.8 to 4.7 (EFSA, 2009). In addition, phages of LAB are known to frequently propagate and lyse acidic dairy starter cultures. Given the range of pH stability, phages that lyse spoilage LAB are unlikely to be compromised by the acidity of beer environments (Kelly et al., 2011).

For successful phage application in brewing, phages must remain stable and retain activity in any temperature they are exposed to. The thermotolerance of bacteriophages is generally greater than that of their hosts, indicating that they may persist after their host is killed. Bacteriophage activity, however, peaks when host growth temperature and conditions are favorable. Since phage replication requires metabolic processes associated with cell growth, phage activity at refrigeration temperatures is generally reduced while phage persistence is enhanced (EFSA, 2009; Greer, 2005; O'Flynn, Ross, Fitzgerald, & Coffey, 2004).

Phage activity at certain temperatures varies widely but generally coincides with the preferences of the host. Coliphages have been reported to eliminate their hosts from

30-37°C but not at 12°C (Modi, Hirvi, Hill, & Griffiths, 2001). On the other hand, *Salmonella* phages have retained activity from 5-20°C (Greer, 2005; Leverentz et al., 2001). Regardless, phage activity at temperatures that do not promote host growth may be insignificant (Hudson et al., 2005). It is still beneficial if phages activate once the beer is brought to room temperature, a common condition during warehouse storage, shipping, and on store shelves.

For many years, it was widely assumed that divalent ions such as calcium are required for phage infections, but numerous studies have demonstrated otherwise (Deasy et al., 2011; Sciara et al., 2010; Watanabe & Takesue, 1972). During years past, commonly studied phages were native to calcium-rich environments as in the dairy industry (Mahony et al., 2014; Sciara et al., 2010). In addition, Lactococcal phages display varying requirements for divalent cations (Mahony et al., 2014; Veesler et al., 2012). It is likely that phage infections in beer spoilage bacteria are independent of high divalent cation concentrations since divalent cations are not present in brewing environments. The *L. brevis* phage isolated by Deasy et al. (2011) infected and killed its host efficiently regardless of divalent cation concentration.

In brewing, selected phages should demonstrate adequate virulence in liquid environments at all stages since the specific gravity and viscosity of beer changes throughout fermentation and processing. Since phages rely on chance to randomly collide with hosts, the physical matrix of the environment is an important consideration. Fortunately, the beer environment is fluid and can allow greater chance for virions to collide with their hosts than in more viscous media such as milk or yogurt.

Broad Host Range

Due to the high specificity of receptor-binding proteins, bacteriophages commonly display a narrow host range usually restricted to one genus, but more frequently restricted to a limited number of species within a genus or limited number of strains within a species (Jarvis et al., 1991; O'Flaherty, Coffey, Meaney, Fitzgerald, & Ross, 2005). The ideal phage for application in beer is a polyvalent phage with a broad host range. Even though such phages are not common, polyvalent phages have been found in numerous studies. Phages SH6 and SH7 were found to infect dozens of *Shigella flexneri*, while *Listeria* phage A511 can infect the entire genus (Hagens & Loessner, 2007; Hamdi et al., 2017). Most impressive, phages isolated from natural lake water were shown to infect bacteria across several phyla (Malki et al., 2015).

Low Host Density and MOI Requirement

The ratio of bacteriophages to host cells, or multiplicity of infection (MOI), is a critical factor for successful infection (Bigwood, Hudson, & Billington, 2009; EFSA, 2009). Phage concentration and incubation time ultimately determine the number of infections with higher levels of infection occurring amongst higher MOI (Bigwood et al., 2009; Goode, Allen, & Barrow, 2003).

In many studies, phage efficacy is tested in media with commercially unrealistic levels of bacteria (10^3-10^6 CFU/mL), which leads to the misconception that a high host density is required to produce a sufficient number of infections (Ellis, Whitman, & Marshall, 1973; Greer, 2005). However, bacteriophages in natural ecosystems show exceptions, as most bacteria in natural environments are in a stagnant physiological state (Bergh et al., 1989; EFSA, 2009). Some studies have shown that phages required a

minimum of 10^4 CFU/mL and others as low as 46 CFU/g (EFSA, 2009; Greer, 1988; Wiggins & Alexander, 1985). Since phages rely on chance to collide with their hosts and cause infection, higher host concentrations increase the chances for infection.

A great deal of variability exists in phages, given their high diversity and abundance on the planet. As such, the efficacy of any phage must be determined on a case-by-case basis. In practice, the concentration of host cells in a food will not be known (Bigwood et al., 2009). When developing or screening phages for brewing application, efficacy at low host concentrations must be determined. In most situations contaminating bacteria will be initially low. Phages that can eliminate their hosts at low concentrations are preferable. For any application, including brewing, an ideal phage is one which has a low host density requirement but can also exterminate their hosts at high concentrations as well. Such a phage would excel at eliminating target hosts before growing into concentrations that damage beer.

At low cell concentrations, the phage concentration will determine the level of infected cells (Bigwood et al., 2009). Low concentrations of phage and host generally produce little effect because the two are less likely to meet. Once a certain threshold of phage is met (i.e., enough to cover the entire matrix), host cell density is irrelevant and all host cells will become infected (Hagens & Loessner, 2007). In addition, a high enough MOI may also achieve lysis from without and cause death of bacteriophage-insensitive mutants that do not possess anti-adhesion defenses (Abedon, 2011). Lysis from without occurs when a high number of phages adsorb to a cell, and the sheer number of penetrations cause the cell to lyse. In essence, lysis from without kills bacteria without causing infection (Abedon, 2011).

ADVANTAGES OF PHAGE APPLICATION

The key advantages to phage application in any product for human consumption are that phages are safe for human consumption as they do not infect mammalian cells and their narrow host range (Garcia et al., 2008; Park et al., 2011). In most styles of beer, quality deteriorates over time which makes phage application an appealing strategy to increase the stability of beer and protect it from bacterial spoilage. Arguably, post-production is the most critical period as brewers lose control of their product and it can be exposed to higher temperatures, unsanitary draught outlets, or other adverse physical conditions (Harper, 1981; Vaughan et al., 2005). There is a clear demand for longer shelf-life of consumables yet a preference for minimal processing and fewer chemical preservatives. Given this predicament, phage application is a viable solution to meet the desires of the modern consumers while extending the shelf life of beer (Ross, Morgan, & Hill, 2002). Timing of sale and storage conditions varies between brands and labels. Large and regional breweries tend to have better marketing, distribution systems, and storage facilities than microbreweries, resulting in quicker sales and are less prone to spoilage conditions. Microbreweries, which lack many of these abilities, could potentially benefit the most from phage application.

Narrow Host Range

Host specificity is a characteristic of phage infections and poses a major advantage to phage application in fermented foods, allowing phages to leave remaining microbiota untouched (Sillankorva, Oliveira, & Azeredo, 2012). The nature of interactions between phages and hosts are very specific. Even with phage mutations, phages rarely expand further than the species or genus levels. Consequently, bacteriophages are unable to infect eukaryotic cells and would have virtually no effect upon fermentation (EFSA, 2009). These traits allow phages to attack target organisms without causing imbalances in the environmental microflora (Atterbury et al., 2007; Sillankorva et al., 2012).

Safe to Consume

It is widely known that ingestion of bacteriophages is harmless to humans since they do not infect mammalian cells (Garcia et al., 2008; Park et al., 2011). As measurable components of many foods, phages have been recovered from many food products with counts as high as 104 phages per gram (Greer, 2005; Hagens & Loessner, 2010). Many reports have been released enumerating the numbers of phages present in foods. One report showed that 38 different phages were recovered from 22 of 45 refrigerated foods purchased from stores in the US. Phages have also been isolated from cucumbers, salami, lettuce, kimchi, crab meat, pork, oysters, mussels, mushrooms, pies, biscuit dough, roast turkey, chicken, cheese, yogurt, buttermilk, sauerkraut, and beef (Hagens & Loessner, 2010; Hudson et al., 2005). Safety studies involving the *Listeria* phage P100, a component of FDA-approved LISTEX P100, showed no measurable effects when fed to rats in large quantities (Carlton, Noordman, Biswas, de Meester, &

Loessner, 2005). Another study with *E. coli* phages showed no adverse effects upon humans with minimal effects upon the gut microbiota (Bruttin & Brüssow, 2005).

CHALLENGES OF PHAGE APPLICATION IN BREWING

Though phage application certainly has benefits, difficulties and challenges must be addressed. Ironically, some of the greatest benefits of phage application are also, in some ways, its limitations. The greatest challenges facing phage treatments are horizontal gene transfer, narrow host range, variable stability, and others.

Horizontal gene transfer

Horizontal gene transfer is undoubtedly the greatest concern for the use of phages in the food and medicine industries. HGT is the movement of genetic material between cells other than by the vertical transmission of DNA from parent to offspring, and phages are excellent vehicles for facilitating this (Ochman, Lawrence, & Groisman, 2000). HGT is known to occur in lysogenic phages and prophages and results in the transmission of traits from one organism to another (Hagens & Loessner, 2010). Despite the perpetual state of war between phage and host, HGT can be beneficial to both sides in natural environments. In the case of human pathogens and microflora, HGT can also result in transfer of unwelcome characteristics, such as genes for virulence factors or toxins, which offer selective advantages to the host while ensuring the longevity of the phage. HGT is widely known to convert harmless strains of bacteria into dangerous human pathogens through prophages. This phenomenon is also known as lysogenic conversion (Goodridge & Abedon, 2003). Though the occurrence of lysogenic conversion has been well

documented in animal and plant pathogens, there is no evidence that it has occurred in foodborne pathogens. However, it is presumptuous to assume that foodborne or beer-borne bacteria are unable to harbor temperate phages (Ackermann, Greer, & Rocourt, 1988; Greer, 2005).

HGT is a relatively common occurrence due to the rapidly evolving nature of phages and bacteria. It was generally assumed that phage-mediated HGT only occurred within a genus. However, phages with broad host ranges may facilitate larger taxonomic leaps. In fact, intergenus transfer was observed in a 2009 study where spontaneous prophage induction arose when toxin-encoding pathogenicity islands were transferred from *S. aureus* to *L. monocytogenes* (Chen & Novick, 2009). There are numerous examples of virulence factors originating from HGT that are known to be encoded by lysogenic phages. Such virulence factors and have been well characterized in *Shigella, P. aeruginosa, C. botulinum, S. aureus,* and *S. pyogenes* (Desiere et al., 2002; EFSA, 2009). Other known virulence factors acquired via HGT allow the bacteria to evade host immune systems, adhere to cells, display antibiotic resistance, and survive inside eukaryotic cells (Boyd, 2012; Lerminiaux & Cameron, 2019). For beer spoilage organisms, a foremost concern would be the transfer of hop-resistance genes. This could largely be mitigated through the use of lytic phages instead of lysogenic, but some phages are known to shift from lytic, virulent types into temperate phages (Greer, 2005). It is therefore imperative to conduct complete genome sequencing of any phage used to prevent beer spoilage to ensure that no HGT factors are present.

Narrow host range

Though host specificity is often touted as a primary benefit of phage application, it also poses limitations. Host specificity is most often confined to the strain or species levels and rarely to the genus level (Hagens & Loessner, 2010). For instance, research on phages infecting pseudomonads and *Brochothrix thermosphacta* showed severely restricted host ranges (Greer, 1982, 2005). Phages isolated from spoiled meats could only infect their homologous hosts and were unable to prevent spoilage of naturally contaminated beef (Greer, 1982, 2005; Greer & Dilts, 1990). In another study, four *C. jejuni* phages were isolated from three successive flocks raised from the same farm. Though the phages were similar in morphology genome size, and host range, *C. jejuni* cells from the third flock were resistant to all four phages. This study demonstrates how rapidly bacteriophage-insensitive mutants can develop to evade the phage (Connerton et al., 2004). Deasy et al. (2011) showed that the most successful *L. brevis* phage could only infect 3 of 22 spoilage strains isolated from breweries across Europe. Separate geographic locations will harbor genetically distinct spoilage strains, making it difficult to isolate a phage that could infect all spoilers. Phages, as the most ubiquitous biological entity on Earth, are so variable and numerous that it is probable that numerous ideal candidates exist. Though isolating the perfect phage may be difficult, forcing mutations through successive propagations and exposure to numerous strains could broaden the host range.

Environmental susceptibility and stability

The ability for phages to remain stable and infect hosts depends upon environmental conditions such as pH, temperature, and the physical environment. pH is a critical factor as low pH can denature or cause conformational changes in proteins and thus affect affinities between molecules such as receptors and attachment proteins. Several studies suggest that low pH is the most crucial inactivation factor of bacteriophages (García, Madera, Martínez, & Rodríguez, 2007). The food matrix can physically prevent phages from adhering to hosts as adhesion relies upon chance for them to come in contact. It has been reported that milk whey prevents phages from reaching host surfaces due to the agglutination of *S. aureus* cells upon contact with raw whey (Gill, Sabour, Leslie, & Griffiths, 2006; O'Flaherty, Coffey, et al., 2005).

Temperature also plays an important role in phage stability and virulence. Phages generally replicate best at the same optimal temperatures as their hosts so it is often assumed that temperature cannot be used to prevent phage replication (Hammes et al., 1992). Regardless, temperature does play a role in the rate of infection for many phages and in some cases, refrigeration temperatures halt phage activity completely (Bach, McAllister, Veira, Gannon, & Holley, 2002; Greer, 2005; Hudson et al., 2005; Modi et al., 2001). Though phages show remarkable versatility in a variety of conditions, all environmental factors must be considered when determining if a phage is appropriate for use in food processing and preservation.

PHAGE RESISTANCE

The development of bacteriophage-insensitive mutants is a significant hurdle in developing effective phage application systems and must be discussed. As victims of predatory viruses, bacteria have evolved many anti-phage mechanisms to ensure their survival (Dy, Richter, Salmond, & Fineran, 2014). The frequency of bacteriophage-insensitive mutants and consequences of their mutations vary according to the phage, host, and environmental conditions (EFSA, 2009).

The presence of bacteriophages, intentional or accidental, in an industrial fermentation or process will ultimately result in a unique ecosystem specific to that environment. Therefore, understanding the broader effects of phage-host relationships is critical to determining how the two will exist together in an industrial environment. Predation from viruses is a key determining factor of the size, composition, structure, and development of microbial communities. Naturally, bacteria have developed a plethora of defenses to withstand the onslaught. As obligate parasites, lytic phages depend on destroying their hosts and could, in theory, cause their own extinction by completely annihilating their hosts within closed systems. The coexistence of phages with their hosts pose an interesting paradox which is deconflicted through two different models of evolution: (i) the arms race model and (ii) the fluctuating selection model (Avrani, Wurtzel, Sharon, Sorek, & Lindell, 2011).

In the arms race model, mutations occur on both sides. Mutations in the host result in viral-resistance and grow into bacteriophage-insensitive populations. Subsequently, mutations occur in the virus which restore the ability to infect its host and spread amongst a now sensitive bacterial population (Avrani et al., 2011). The result of the rapid coevolution is a perpetual molecular arms race (Samson & Moineau, 2013; Seed, 2015).

Despite the undeniability of the arms race model, many experimental studies indicate that the molecular arms race does not continue indefinitely due to limitations of both host and phage (Avrani et al., 2011; Bohannan & Lenski, 2000; Hall, Scanlan, Morgan, & Buckling, 2011; Middelboe et al., 2001). Since phages are genetically constrained due to the size of the capsid, a host mutation can emerge with no subsequent phage mutation to overcome it (Avrani et al., 2011; Cuervo & Carrascosa, 2001). In such a situation, bacteria would dominate the population and drive the phage into extinction. (Avrani et al., 2011; Hall et al., 2011; Middelboe et al., 2001). For the host, a phage-resistance mutation often comes at a price. In the instance of a nutrient-acquisition protein which also serves as the viral receptor, mutating it to become phage-resistant often impairs the organism's ability to uptake nutrients, hence becoming less fit for survival. Compromises such as this occur frequently in bacteriophage-insensitive mutants (Avrani et al., 2011; Bohannan & Lenski, 2000; Hall et al., 2011; Lennon, Khatana, Marston, & Martiny, 2007; Winter, Bouvier, Weinbauer, & Thingstad, 2010). Since maintaining such defenses are costly to the host, bacteria have evolved separate tactics to compensate.

The fluctuating selection model explains the antagonistic coexistence based upon host density. Unlike the arms race model, mutations do not need to be continuously produced but the result is *different*, rather than *greater* levels of resistance and infectivity. The result is that the proportion of bacteriophage-insensitive mutants, susceptible hosts, and bacteriophages oscillate in a community over time due to alternating selection pressures – viral selection for bacteriophage-insensitive mutants and competitive selection for faster growing hosts (Avrani et al., 2011; Woolhouse, Webster, Domingo, Charlesworth, & Levin, 2002).

Without more data, it is difficult to determine which model occurs more frequently in nature. Given the diversity of environments, bacteria, phages, and population densities, it is likely that both mechanisms dynamically occur depending on the conditions. If applying phage to beer, developers must aim for a one-sided arms race model scenario using a cocktail of lytic phages to quickly destroy contaminating populations and thus deny any likelihood for the development of persistent bacteriophage-insensitive mutants.

Phage-Resistance Mechanisms of Bacteria

Bacteria have acquired a remarkable array of natural defense systems to phages such as adsorption inhibition, restriction modification systems, clustered regularly interspaced short palindromic repeats-Cas (CRISPR-Cas), and abortive infection (Abi) systems (Chopin, Chopin, & Bidnenko, 2005; Deveau et al., 2008; Josephsen & Neve, 2004; Labrie, Samson, & Moineau, 2010). Phage-resistance mechanisms have been well characterized in *E. coli* and *Lactococcus lactis*, in which over 50 separate systems have been described (Haaber, Rousseau, Hammer, & Moineau, 2009; Moineau & Levesque,

2004). Many resistance genes are encoded on plasmids which are advantageous in allowing conjugative dissemination (Forde, Daly, & Fitzgerald, 1999).

Adsorption inhibition

Bacteria inhibit adsorption or alter receptors in order to prevent phage infection. Adsorption inhibition is accomplished by mutating, masking, and producing an extracellular matrix to obstruct receptors, or exploiting competitive receptor inhibitors (Dy, Richter, et al., 2014; Hoyland-Kroghsbo et al., 2013).

Genes that encode for phage receptors can be mutated or deleted in order to evade phage attachment. Unfortunately for the host, often times these receptors conduct important cellular functions. In some cases, surface proteins are subject to reversible or temporal expression, known as phage variation. Phase variation is a heritable and reversable type of regulation, similar to bet-hedging, that results in complete expression or repression of a gene. The result is interchangeable heterogeneity within the bacterial population. When utilized, some members of the population will be susceptible while others immune (Dy, Richter, et al., 2014). Multiple studies showed that phase variation occurred in low frequency and did not confer long-term phage resistance within environmental bacterial populations, which would revert to sensitivity (Connerton et al., 2004; Greer, 2005; O'Flynn et al., 2004).

Restriction Modification Systems

Many bacteria use restriction endonucleases to cleave phage nucleic acids at specific points into fragments which are subject to degradation by other endonucleases. It seems clear that the primary function of restriction modification systems is to protect against phage infection (Bickle & Krüger, 1993). Restriction modification systems

destroy foreign phage DNA to prevent infection after adsorption and injection. Select strains of *E. coli* have a restriction modification system specific for T4 infection (Bickle & Krüger, 1993; Jabbar & Snyder, 1984; Levitz et al., 1990). Roughly one-fourth of bacteria possess restriction modification systems. Among these, half possess multiple systems. For instance, *L. lactis* subsp. *cremoris* has three distinct restriction modification systems encoded by plasmids (Josephsen & Vogensen, 1989).

CRISPR-Cas Systems

CRISPR-Cas systems are very common in prokaryotes and provide adaptive immunity from phages and other mobile genetic elements. CRISPR sequences are essentially memories from past infections and are based upon previous invading genomes. Resistance is granted when small CRISPR RNAs (crRNAs) bind to complementary sequences of the invading phage genome and subject it to subsequent degradation by the Cas9 protein (Dy, Richter, et al., 2014). Cas 9, or CRISPR-associated 9, is an enzyme guided by the CRISPR sequence that cleaves DNA accordingly (Barrangou, 2015).

Bacteria acquire resistance with CRISPR-Cas through three basic steps: (i) adaptation, in which new genetic sequences are incorporated into CRISPR spacer as a result of infection, (ii) crRNA generation, where the transcript is processed into short interfering fragments, and (iii) interference, where the crRNA and Cas9 cooperatively recognize and destroy the phage target (Dy, Richter, et al., 2014). CRISPR-Cas systems are phenomenal defense weapons for bacteria against phage infection. *S. thermophilus*, which has CRISPR-Cas systems, generates bacteriophage-insensitive mutants at a much greater frequency than *Lactococcus*, which does not (Mahony et al., 2014).

Abortive Infection

Abortive infection (Abi) systems are commonly described as altruistic death systems activated after phage infection. By committing suicide, the host cell limits viral replication and essentially protects the rest of the bacterial population from subsequent infection. There are over 20 characterized Abi systems, most of which are plasmid-based lactococcal systems, and the molecular basis for phage resistance for most is unclear (Chopin et al., 2005). Abi systems are considered to be the most efficient of phage defense mechanisms since they are capable of disruption at any stage of the infection (Haaber et al., 2009).

Toxin-antitoxin (TAT) systems are similar to Abi systems in the respect that they are suicidal mechanisms. Unlike Abi systems, TAT systems are always regulated and their disruption (not activation) leads to bacteriostasis or cell death. TAT systems encode a toxin gene preceded by an antitoxin gene transcribed from the same promoter (Gerdes, Christensen, & Løbner-Olesen, 2005; Schuster & Bertram, 2013). Toxins interfere with a variety of essential processes such as translation, replication, or others. Antitoxins neutralize their corresponding toxins and often regulate the TAT operon. Antitoxins have shorter half-lives than their toxins and must be continually produced to prevent the toxin from exerting its detrimental effects. Certain stimuli will degrade antitoxins resulting in bacteriostasis or cell death (Dy, Przybilski, Semeijn, Salmond, & Fineran, 2014; Dy, Richter, et al., 2014; Fineran et al., 2009). It is hypothesized that degradation of host DNA or shutdown of transcription limits antitoxin synthesis, resulting in free, unhindered toxin available to induce cell death and disrupt phage multiplication (Dy, Przybilski, et al., 2014; Dy, Richter, et al., 2014; Fineran et al., 2009; Pecota & Wood, 1996).

Defense Mechanisms of Bacteriophages

Though bacteria have many well-known, well-characterized systems to circumvent or defeat phage infection, phages in return have developed tactics to bypass host defenses such as adsorption adaptation, disruption of restriction modification systems, anti-CRISPR-Cas systems, and Anti-abortive infection systems (Dy, Richter, et al., 2014; Sillankorva et al., 2012). Bacteria often succumb to phage infection regardless of multiple defense mechanisms. Due to the genomic versatility and rapid multiplication rates, phages have evolved equally diverse mechanisms to thrive amongst their hosts. Given the abundance of phages on the planet, there are near infinite opportunities for them to win or lose a battle with bacteria (Samson et al., 2013). These skirmishes leave behind evolved phages, fit for survival, that will press forward to infect another host. In the event that phages are unable to overcome host defenses, bacteriophage-insensitive mutants commonly revert to sensitivity to negate the disadvantages that often coincide with immunity (Atterbury et al., 2007; O'Flynn et al., 2004; Scott et al., 2007; Sillankorva et al., 2012).

Adsorption Adaptation

While bacteria may mutate or mask receptors to prevent adsorption, phages can overcome this by adapting to new receptors, "digging for receptors", or stochastic expression (Dy, Richter, et al., 2014; Hoyland-Kroghsbo et al., 2013; Samson et al., 2013). While phages use mutate their receptor-binding proteins in response to bacterial receptor mutations, they are also a means to expand or change their host range.

One method to overcome host cell receptor mutations is by adapting to new receptors, in which phages modify their tail fibers, or receptor-binding proteins, to adhere

to the newly altered cell receptor or to bind a completely different receptor (Dy, Richter, et al., 2014). If a bacterial receptor is masked by capsule or compound, phages can "dig" for these receptors by hydrolyzing the barrier. Numerous coliphages degrade the exopolysaccharide capsule with endosialidases and glycosidases to gain access to receptors, and some *S. pyogenes* phages possess hyaluronan lyases to break the hyaluronic acid barrier (Baker, Dong, & Pritchard, 2002; Cornelissen et al., 2012; Samson et al., 2013; Scholl, Adhya, & Merril, 2005). Such enzymes may be translated from the phage genome inside the host cytoplasm and released upon host lysis, released by the phage directly, or associated with the phage tail protein (Baker et al., 2002; Samson et al., 2013; Scholl et al., 2005).

Stochastic expression is the ability of a phage population to randomly express multiple receptor-binding proteins to bind more than one type of receptor. Such phages have been characterized for *Bordetella* spp., *L. lactis*, and *E. coli* where phages can vary their receptor of choice or adsorb hosts in different physiological states (Liu et al., 2002; Samson et al., 2013; Stockdale et al., 2013; Tetart, Repoila, Monod, & Krisch, 1996).

Restriction Modification Disruption

Phages counter host restriction modification systems through passive mechanisms such as removing, modifying, or masking restriction modification sites or by active methods such as disrupting host restriction endonucleases with enzymes or proteins. Restriction sites can be protected in phage DNA through point mutations (Iida, Streiff, Bickle, & Arber, 1987). Phages can also utilize methyl transferase enzymes to mimic the host DNA (Walkinshaw et al., 2002) or use proteins that are structural analogs of

restriction sites that sequester host restriction endonucleases to prevent digestion of the viral genome (Dy, Richter, et al., 2014).

Passive phage methods to evade restriction modification systems involve modification of phage DNA. A simple technique is to reduce the number of restriction sites to gain selective advantage (Bickle & Krüger, 1993; Dy, Richter, et al., 2014; Krüger & Bickle, 1983; Tock & Dryden, 2005). Since restriction endonucleases must recognize sequences in a specific orientation and spacing, reorienting or changing the distance between recognition sites will prevent cleavage of DNA (Samson et al., 2013). Coliphages T3 and T7 are known to use such strategies (Krüger, Barcak, Reuter, & Smith, 1988; Meisel, Bickle, Krüger, & Schroeder, 1992). Some phages will substitute thymine with uracil or integrate modified bases such as hydroxymethyl cytosine, glucosylated hydroxymethyl cytosine, hydroxymethyl uracil, or N6-adenine to protect restriction sites from host restriction endonucleases (Drozdz, Piekarowicz, Bujnicki, & Radlinska, 2012; Krüger & Bickle, 1983; Samson et al., 2013).

Active methods to evade host restriction modification systems involve the use of proteins or enzymes that mask restriction sites or directly interact with host restriction endonucleases (Iida et al., 1987; Krüger & Bickle, 1983; Labrie et al., 2010; Walkinshaw et al., 2002). Such systems are well characterized in phages that infect *E. coli*. Coliphage P1 co-injects two proteins that bind to and mask restriction sites within the phage genome (Iida et al., 1987). Other phage proteins closely resemble restriction sites, as seen in coliphage T7, which have high affinity for and thereby sequester the host restriction endonucleases (Walkinshaw et al., 2002). Ral in coliphage λ hyperactivates the activity of a *Eco*KI methyltransferase, resulting in methylation of phage DNA and

protecting it from the *Eco*KI restriction endonuclease, essentially using host defense mechanism against the host (Loenen & Murray, 1986). Lastly, coliphage T3 is known to remove an essential restriction modification cofactor, therefore retarding its activity (Penner, Morad, Snyder, & Kaufmann, 1995).

Anti-CRISPR-Cas Systems

Though CRISPR-Cas systems are versatile and efficient defense systems, they are frequently circumvented by phages since a deletion or point mutation in the protospacer or protospacer adjacent motif will cause the system to fail (Deveau et al., 2008). However, some bacteria have a positive feedback loop which allows quick uptake of new invading sequences to restore phage resistance and reduce likelihood of future infections (Datsenko et al., 2012; Swarts, Mosterd, Passel, & Brouns, 2012).

A more direct phage response to CRISPR-Cas involves the heat-stable nucleoid structuring (H-NS) protein which is native in many bacteria and known to repress CRISPR-Cas systems. Interestingly, a phage infecting *Candidatus Accumulibacter phosphatis* was found to encode a H-NS homologue and is speculated to be able to use it to repress CRISPR-Cas in its host (Dy, Richter, et al., 2014; Pul et al., 2010; Skennerton et al., 2011). Another method was observed in *Vibrio cholerae* phages which contain a CRISPR-Cas gene. When translated by the host, the phage protein excises an anti-phage gene found on a phage-inducible chromosomal island on the host genome which allows the phage to complete the lytic cycle (Samson et al., 2013; Seed, Lazinski, Calderwood, & Camilli, 2013).

Anti-Abortive Infection System Disruption

Some phages can negate the effects of Abi systems by producing antitoxins or interfering with Abi transcription (Labrie et al., 2010; Samson et al., 2013). Some *E. coli* strains contain the Rex two-component Abi system, in which RexA responds to phage infection by activating RexB, which forms an ion channel in the cell membrane, inhibits growth, and aborts the infection (Snyder, 1995). T4 "escape phages" are known to disrupt the Rex system by redirecting the host RNA polymerase from the Rex promoter (Hinton, 2010). Another Abi evasion mechanism is to include an anti-toxin in the phage genome. After infection, T4 coliphages and phages of *Pectobacterium atrosepticum* will utilize host systems to produce proteins which act as anti-toxins (Blower, Evans, Przybilski, Fineran, & Salmond, 2012; Dy, Richter, et al., 2014; Koga, Otsuka, Lemire, & Yonesaki, 2011; Otsuka & Yonesaki, 2012).

CURRENT PHAGE APPLICATIONS IN THE FOOD INDUSTRY

Though bacteriophages are problematic for the dairy industry, they pose numerous benefits in the fields of food safety and spoilage control (Mahony et al., 2014). Despite developments that minimize transmission of contaminants, new tactics are required to further reduce bacterial contamination and fulfill consumer demands for fewer chemical preservatives (Garcia et al., 2008). Since phages are metabolically inert and prey upon bacteria, lytic phages possess many desirable traits to eradicate problematic bacteria in food processing and brewing (Goodridge & Abedon, 2003). Such treatment results in the elimination of only targeted organisms without compromising other bacteria in their habitat such as in fermented foods. As antimicrobial agents, phages could help maintain product quality, eliminate target pathogens and spoilage organisms, and extend shelf life while posing low health risk to humans (Deasy et al., 2011; Guenther, Huwyler, Richard, & Loessner, 2009; Park et al., 2011).

The stability of phages makes them well suited to withstand the environmental stresses of food processing and physiochemical conditions of food products. Application strategies in the food industry are categorized into four methods: (i) pre-harvest preventative control, (ii) biocontrol, (iii) biosanitation, and (iv) biopreservation (Sillankorva et al., 2012). Furthermore, each application strategy can utilize a lysis from within or lysis from without method. Lysis from within refers to the normal phage

infection, where the phage nucleic acid enters the cell and lyses it from within to kill the cell and complete its life cycle. Lysis from within is an active approach since it involves active infections, while lysis from without is passive.

Pre-Harvest Preventative Control

Pre-harvest preventative control is a pre-harvest application method used on live animals before slaughter or plants and plant products before harvest. In animal products, phages are administered directly to animals to reduce the pathogen load in the gastrointestinal tract and feces in order to minimize environmental carryover into the food supply (Sillankorva et al., 2012). With the passive approach, or lysis from without, phages are added in large quantities to overwhelm the target organisms. Though much higher amounts of phage are needed, lysis from without can eliminate even small populations of bacteria. In lysis from without, intracellular phage resistance mechanisms, such as CRISPR, are ineffective since surface adhesion and penetration are the sole mechanisms contributing to cell death (EFSA, 2009). Active methods, or lysis from within, are self-amplifying and require a smaller dose of phage. Active methods rely upon the phages' ability to cause infection and spread amongst and eliminate the population of target organisms. The timing of the active method is important since hosts cells must exist in sufficient quantities and be in a physiological state which promotes the phage lytic cycle (EFSA, 2009).

Several studies have shown promising results in reducing bacterial pathogenic loads of *E. coli*, *Salmonella*, *Shigella,* and *Staphylococcus* in animals (Alisky, Iczkowski, Rapoport, & Troitsky, 1998; Barrow & Soothill, 1997; Smith & Huggins, 1983; Smith, Huggins, & Shaw, 1987; Sulakvelidze, Alavidze, & Morris, 2001). Furthermore, several

pre-harvest preventative control products have been approved by the FDA. EcoShield™, manufactured by IntraLytix, was approved in 2011 for use on red meat prior to grinding and has been shown to eliminate 95 to 100% of *E. coli* O157:H7. Finalyse™, by Elanco Food Solutions, uses naturally occurring *E. coli* O157:H7 phages sprayed onto cattle prior to entering packing facilities. BacWash™ (OminLytics Inc.) and BIOTECTOR S1™ (CheilJedang Corp.) are specific to *Salmonella*. Bacwash is applied to live animals prior to slaughter while BIOTECTOR S1 is applied to animal feed (Sillankorva et al., 2012).

In regard to plant-based food products, pre-harvest preventative control involves introducing bacteriophage into the ecosystem (the croplands) to reduce harmful bacteria. Many studies refer to this application as "biocontrol," but it is not to be regarded as such in the scope of this review (Goodridge & Abedon, 2003). In crops, this method is used to improve product yields by targeting bacteria harmful to plants rather than human pathogens in animal products. Pre-harvest preventative control has been successful against numerous bacteria that damage peach, cabbage, tobacco, mungbean, potato, apple, and mushroom (*Agaricus bisporus*) crops (Balogh et al., 2003; Goodridge & Abedon, 2003; Greer, 2005; Munsch, Oliver, & Hondeau, 1991; Randhawa & Civerolo, 1986; Schnabel, Fernando, Meyer, Jones, & Jackson, 1999). Since 2005, the US Environmental Protection Agency has approved numerous AgriPhage products manufactured by OmniLytics for use on farmlands which contain between 2.8×10^8 and 4.1×10^9 plaque forming units (PFU)/mL of phage (AgriPhage, 2018).

Biocontrol

Biocontrol is phage application during food processing by applying phages directly onto food surfaces, fresh produce, or mixed into raw liquids such as milk. Biocontrol can significantly reduce target bacteria levels in many products and is a promising alternative to other food safety and preservation measures (Sillankorva et al., 2012). If food processing conditions are not ideal for bacterial growth, then lysis from without using a higher phage concentration is required. Likewise, lysis from within can be applied using a lower phage titer if processing conditions are conducive to bacterial growth (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003a; Goode et al., 2003; Sillankorva et al., 2012; Snyder & Champness, 2007).

Biocontrol is not as well studied as pre-harvest preventative control or biopreservation due to several reasons. Firstly, this method is most effective when applied to food surfaces, as such it makes more sense to apply it as the final step (biopreservation) before packaging or storage. Doing otherwise could potentially result in loss of phage through the physical stressors of downstream processes. Secondly, most studies to date focus upon controlling human pathogens in animal products, the processing conditions of which are typically non-conducive to bacterial growth. Since such conditions are typically non-conducive to bacterial growth, it would require larger amounts of phage to be used resulting in greater expenses to the manufacturer. However, biocontrol is ideal for the control of spoilage organisms in fermented foods. Since fermenting conditions are ideal growth conditions for many spoiler bacteria, low amounts of phage could be a viable means for controlling contaminants through lysis from within.

Biosanitation

Biosanitation is the use of bacteriophages on food contact surfaces and equipment, but not on the food itself. Phages have been proposed as a possible alternative to chemical sanitizers, which are corrosive, toxic, and must be used with caution to treat surfaces that directly contact food or beverages (Deasy et al., 2011). Biofilms are problematic on equipment surfaces in food handling, storage, and processing, particularly in sites which are not easy to clean or sanitize. Phages have shown to inflict significant reductions in pathogenic bacteria upon *in vitro* biofilms. Siringan et al. (2011) showed 1 to 3 log reductions in *Campylobacter* biofilms on glass surfaces. A separate study showed a 4.5 log reduction of *E. coli* O157:H7 on harvesting blades for spinach processing and another eradicated the bacteria after 10 minutes of exposure at 37°C and 1 h at 23°C on various hard surfaces (Patel, Sharma, Millner, Calaway, & Singh, 2011; Viazis, Akhtar, Feirtag, & Diez-Gonzalez, 2011).

Though control of problematic bacteria through phage biosanitation has been proven in laboratory conditions, the efficiency and applicability in food processing or brewing plants is questionable. Firstly, limited studies have been conducted in comparison to pre-harvest preventative control, biocontrol, and biopreservation. Secondly, biosanitation in practice poses significant challenges due to the diversity of bacteria and wide range of conditions within food processing environments (Sillankorva et al., 2012). Similar results are likely seen using standard industrial sanitizers which are cost-effective and readily available. Thirdly, biosanitation has few advantages over industrial sanitizers. Though phages are generally safe for human consumption, the

toxicity of sanitizers are easily mitigated through rinsing. In addition, sanitizers are cost effective and kill a broader spectrum of microorganisms.

Biopreservation

Biopreservation is the application of phages to the final food product to prevent contamination and unwanted bacterial proliferation during food during storage and sale (Sillankorva et al., 2012). The method involves application of phage onto the surface or into the liquid matrix during the end stages of food processing, the desired end state is that the phage is present and can exert its effects upon the final product.

Dozens of studies have shown promising results in animal- and plant-based foods. Successful control has been demonstrated in significantly reducing the loads of *Campylobacter* and *Salmonella* on chicken skin (Goode et al., 2003), *Salmonella enteritidis* in cheese (Modi et al., 2001), *Salmonella* on raw and cooked meat surfaces (Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008), and *E. coli* O157:H7 on meat (O'Flynn et al., 2004). Successful control of *L. monocytogenes* was also demonstrated in various produce and liquid foods (Guenther et al., 2009; Leverentz et al., 2003; Leverentz, Conway, Janisiewicz, & Camp, 2004). The collective results of these studies indicate that phages are excellent vehicles for food preservation. Some are able to lyse their hosts at temperatures as low as 1°C to limit the growth of problematic bacteria during refrigeration, and can further act upon their hosts once the food is taken to room temperature (Bigwood et al., 2008; Greer, 1982, 1988).

The most notable achievements for the use of phages in biopreservation were the FDA approvals of LMP-102 and LISTEX P100 in 2006. Both products are used to reduce risk of *L. monocytogenes* on ready-to-eat meat products and cheese. LMP-102

was the first of its kind and contains a cocktail of six phages effective against 170 strains of *L. monocytogenes* (Abuladze et al., 2008; Bren, 2007; A. Coffey, 2010; Garcia et al., 2008).

PROPOSED METHODS FOR PHAGE BIOCONTROL TO PREVENT MICROBIAL SPOILAGE IN BREWING

The fundamental aspects of phage application in brewing have been reviewed except for the application itself. With this background, methods can be formulated to overcome laboratory challenges and propose protocols for scaling up to an industrial scale. As a basis, several questions must be raised:

1. Is the product at risk from contamination?
2. What are the target organisms?
3. What is a realistic scenario for contamination?
4. What is the most effective method for phage application?

For reasons reviewed earlier, beer is at risk from contamination and risk is higher for smaller breweries who do not possess key spoilage prevention assets such as pasteurization, filtration, cold storage, and rapid distribution and sales. Therefore, the most high-risk beers are produced in microbreweries, brewpubs, and by homebrewing. However, phage application could still be a viable option in lieu of pasteurization or high-end filtration to reduce costs and preserve products for larger breweries.

The best targets for phage biocontrol are LAB, particularly *L. brevis* and *P. damnosus*, because they are the most common contaminants found to spoil beer. Control of these two species in brewing environments would prevent the majority of spoilage

incidents (Kelly et al., 2011; Satokari et al., 2000; Suzuki et al., 2008; Van der Aa Kühle & Jespersen, 1998).

The most realistic scenario for bacterial contamination is during the early fermentation process, when wort is exposed to contaminants. Wort contamination is often due to an unsanitary tank, pipe, or water source. Such contaminants can persist through downstream brewing processes and into the final product with the ability to flourish once conditions become favorable. A phage or phage cocktail should therefore be tested in its ability to eliminate the target organism at low densities (<100 CFU/mL) in wort, immature beer, and finished beer. Furthermore, the phage's ability to cause infection should be studied in environments reflective of the temperature and durations which coincide with the conditions of processing and final storage. Lastly, phage ability to kill the target host should be tested in a variety of beer styles in order to account for different chemical conditions.

The most effective method for phage application is biocontrol, where phages are applied early in the fermentation process along with yeast pitching. Biocontrol will enable phages to control contamination early in the brewing process, persist into the final product, and allow the phages to exert their effects until the point of consumption.

Host Targets

The best target for phage biocontrol in brewing is *L. brevis* followed by *P. damnosus*. The general consensus of studies regarding beer spoilage is that *L. brevis* is the most frequently encountered, accounting for over half of all reported bacterial spoilage incidents, while *P. damnosus* responsible for 12-31% (Back, 2005; Suzuki,

2011; Suzuki et al., 2006). These two species combined are reported to be involved in 60-90% of all spoilage incidents in Europe (Menz et al., 2011; Suzuki, 2011).

Selection of proper test strains is extremely important. Hosts should be isolated directly from brewing environments, preferably spoiled beer. For phage cocktails involving pathogens in food safety, propagation on non-pathogenic strains is required to avoid transduction of virulence-associated genes through phages (Cheetham & Katz, 1995). Virulence genes are of minimal concern in brewing, and spoilage strains should be used for propagation as certain spoilage genes, such as those encoding membrane bound hop-resistance proteins, could provide targets for phage receptor-binding proteins (Hayashi et al., 2001; Sakamoto et al., 2002; Sami et al., 1997; Suzuki et al., 2005). In order to ensure a broad host range, all known spoilage strains should be collected and challenged. It is important to note that continued subculturing of hop resistant strains in media devoid of HBAs leads to decreased hop resistance (Richards & Macrae, 1964; Suzuki, 2011). To mitigate this problem, subculturing should be kept to a minimum while ensuring realistic concentrations of HBAs are present in propagation media. De Man, Rogosa, and Sharpe (MRS) broth media is highly effective for culturing LAB and common spoilage strains. Supplementing growth medium with 4-5% (v/v) ethanol is beneficial for the isolation of wine spoilers, and should be considered for beer spoilers as well (De Man, Rogosa, & Sharpe, 1960; Hammes et al., 1992; Yoshizumi, 1975). Culture media containing ethanol would encourage bacterial cells to express membrane composition similar to that expressed in beer, thus providing more realistic conditions for phage adsorption.

Targeting of specific membrane proteins is an effective strategy in addition to species targeting. Since hop resistance is a requirement for beer spoilers, proteins expressed on the cell surface, such as HitA, HorA, HorB, and HorC, may play a key role in attaining a broad host range (Hayashi et al., 2001; Sakamoto et al., 2002; Sami et al., 1997; Suzuki, Iijima, et al., 2005). Of 51 strains examined by Suzuki et al., 94% expressed HorA and 96% expressed HorC, which are frequently expressed in *L. brevis*, *L. lindneri*, and *P. damnosus* (Suzuki, Iijima, et al., 2005; Suzuki et al., 2006). Though an effective strategy, targeting specific membrane proteins is not a cure-all because not all spoilage organisms express one or all of such genes (Bergsveinson, Pittet, & Ziola, 2012). Regardless, targeting appropriate membrane proteins would make a phage lethal against numerous spoilage strains and perhaps transcend lines between species and genera. Furthermore, doing so would reduce the chance of bacteriophage-insensitive mutant development since such mutants would succumb to the effects of HBAs in hopped beers.

Phage Isolation, Propagation, and Purification

Phages coexist with their hosts in natural and man-made environments. The two maintain a natural, at times mutually beneficial, state of evolutionary equilibrium (Brussow & Hendrix, 2002; Samson & Moineau, 2013). As such, phages are easily recovered from nearly any source. With respect to food spoilers and foodborne pathogens, phages are frequently isolated from pre-production sources, raw materials, the final product, and the environment. For instance, phages active against *Shewanella putrefaciens* and pseudomonads in fish fillets were isolated from pier water (Delisle & Levin, 1969). Isolating phages directly from the food product usually requires a

sufficient host population (10^5 CFU/g) to be present, but *Campylobacter* phages have isolated from raw, but saleable, chicken portions in retail outlets (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003b; Greer, 2005). Raw materials, such as unprocessed milk, are also a reliable source (Patel & Jackman, 1986).

Environmental sources are abundant suppliers of bacteriophages. Phages utilized for bacterial control in foods usually originate from the environment or nonfood sources such as sewage, municipal waste water, soil, farms, and processing facility effluents (Greer, 2005; Pao, Rolph, Westbrook, & Shen, 2004). *Salmonella*, *C. jejuni*, and *S. typhimurium* phages have all been isolated from animal excrement and human sewage (Bao, Zhang, & Wang, 2011; Berchieri, Lovell, & Barrow, 1991; Connerton et al., 2004). Ideally, phages should be isolated from their natural environment, such as spoiled beer or brewing habitats. Since spoiled beer is a relatively short-lived and isolated environment, sufficient phage-host relationships may never arise, and alternative sources of phages should be explored. Phages that infect spoilage strains of *L. brevis* and *P. damnosus* have been isolated from silage, farm slurry pits, and sewage treatment facilities (Deasy et al., 2011; Kelly et al., 2011). To date, no authors have reported phage isolation from spoiled beer or brewing environments, though open vat beer and whiskey fermentations may prove to be a reliable source.

Phages may be isolated by adding raw samples to bacterial cultures and incubating overnight. Cultures may then be centrifuged, filter-sterilized (0.45 μm filter), and subjecting the filtrate to phage plaque assays (Kelly et al., 2011; O'Flaherty, Ross, et al., 2005). Plaques are further purified through successive rounds of culturing, centrifugation, and filtration and challenged against target strains to determine host range.

Propagation of phages is achieved by increasing the volumes of cultures. Large-scale propagation requires greater centrifuge capability. More problematic is the need for virus purification through cesium chloride (CsCl) density gradients, which generally require expensive ultracentrifuges. Fortunately, standard centrifuges have shown nearly identical purification results using longer processing times (40,000 x *g*, 2 h) than ultracentrifugation (100,000 x *g*, 1 h) (Nasukawa et al., 2017). The degree of propagation necessary is dependent upon the required MOI to cause infection and brewing volumes. These factors must be examined to determine the feasibility and cost-effectiveness of scaling up candidate phages.

Use of Phage Cocktails

The simultaneous use of multiple phages, commonly called a phage cocktail, is more effective for eliminating the target host than using a single phage (Bach et al., 2002; Greer, 2005; H. W. Smith et al., 1987). In general, employment of a phage cocktail serves to increase the killing efficiency and counters phage resistance from the moment of administration (Goodridge & Abedon, 2003). However, phage resistance will ultimately appear with the use of a single phage. The time required to develop resistance depends upon host-phage relationship, host-phage densities, and environmental conditions (Guenther, Herzig, Fieseler, Klumpp, & Loessner, 2012). LMP-102 utilizes six different phages to destroy *Listeria* (Bren, 2007). Provided that phage cocktails target different host receptors, use of a cocktail greatly reduces the occurrence of bacteriophage-insensitive mutants since a bacterial cell is unlikely to develop resistance to multiple targets quickly (Bren, 2007; A. Coffey, 2010). Despite the development of resistance, bacteriophage-insensitive mutants often display reduced virulence (Greer,

2005; Randhawa & Civerolo, 1986). In the case of beer spoilage bacteria, a reduced ability to survive in, grow in, and/or spoil beer would likely be observed. Furthermore, the development of resistance to one phage may cause the bacterial cell to become susceptible to others (Avrani et al., 2011).

Timing of Application

The timing of phage application is a critical factor. The most effective strategy is to apply phages as early as possible after the wort has cooled with the goal of maintaining phage activity throughout the remaining brewing processes and enable phage carryover into the final packaged product. Early application mitigates contamination at different stages, thus preventing growth and spoilage up to the final consumption (Sillankorva et al., 2012). Though LABs are prevalent in malts and barley, phages should not be applied to raw materials prior to mashing as they are unlikely to survive the extreme temperatures (Vaughan et al., 2005).

Pre-harvest preventative control has shown to be most effective when administered before or along with infections (Bach et al., 2002; Smith & Huggins, 1982, 1983). The same trend is likely to apply to phage biocontrol as well. If true, then application of phages immediately following wort cooling and alongside yeast pitching would be ideal. At this stage, wort is extremely susceptible to microbial growth due to elevated pH, low ethanol, low CO_2, and a high level of available nutrients (Bach et al., 2002; Fernandez & Simpson, 1995; Vriesekoop et al., 2012). Overall this method would be convenient, economical, and minimally invasive to the brewing process. If applied immediately after wort cooling, phage stability must be tested to ensure that phages can withstand the environmental pressures of fermentation, storage, aging, filtration and

packaging. In addition, a sufficient number of phages must be applied to produce infections and self-amplification cannot be relied upon as LAB, if present, are often in low numbers during early fermentation (Guenther et al., 2009).

CONCLUSION

Though the potential of phage application has been assessed for many food pathogens, there are few studies which examine the potential to enhance the microbial stability of beer with bacteriophages. In one such study, Deasy et al. (2011) showed that *L. brevis* phage SA-C12 annihilated a spoilage strain from 10^6 CFU/mL within 48 hours. In addition, Kelly et al. isolated four *Siphoviridae* phages which infected numerous strains of *L. brevis* and one strain of *L. paraplantarum* (Kelly et al., 2011). Though phages such as these meet initial criteria for brewing application, their stability in beer and brewing processes was not examined. Furthermore, both used strains isolated throughout Europe, a thorough study should also include bacterial spoilage strains isolated from the United States to ensure applicability to geographically distinct breweries.

In addition to the benefits already reviewed, phages present a potential cost-effective solution as well. Since phages are ubiquitous and inexpensive to isolate and propagate, the greatest time and financial investment is screening for highly virulent, broad spectrum, and non-transducing candidates (Kelly et al., 2011; Sillankorva et al., 2012). Once complete, a phage or phage cocktail would be easy and inexpensive to produce. Besides the expense of the phage product itself, phage application could reduce other production costs associated with improving the stability of beer such as high-end

filtration and pasteurization. Consumer demand for unpasteurized beer has increased due to the fresher taste. If successful, phage application could reduce installation, maintenance, and energy costs associated with pasteurization and high-end filtration. Furthermore, avoiding pasteurization would remove the deterioration in flavor associated with thermal processing (Deasy et al., 2011; Vaughan et al., 2005). Since phages are self-replicating and self-limiting, a low dose will multiply amongst sufficient numbers of host cells (Sillankorva et al., 2012).

In order to bring phage biocontrol into regular industrial brewing practices, regulatory requirements must be overcome. Though alcoholic beverages are typically regulated in the United States by the Tax and Trade Bureau, FDA jurisdiction will determine if phages are safe to use as an additive (Zahn, 2019). Complete genome sequencing is essential in obtaining regulatory approval and it must be demonstrated that undesirable genes are not transferred from the bacteriophage to non-target bacteria (Bach et al., 2002; A. Coffey, 2010). Since several phage-based products (with high-risk bacterial hosts) have been FDA approved and given the natural prevalence of phages in foods, the regulatory hurdle can be overcome through proper testing and should not present a significant challenge for the future (Bren, 2007; Brussow, 2005; A. Coffey, 2010; Greer, 2005; Hagens & Loessner, 2010; Hudson et al., 2005).

Overall, the general public is relatively ignorant of the existence and potential of bacteriophages to combat bacteria in any field. In the age of antibiotic resistance, alternatives to antibiotics are continually sought and interest in bacteriophages is steadily gaining traction. As our scientific methods and understanding of phages increase, so will their use in the fields of phage application and food safety. Unfortunately, the world is

not likely to see in phage biocontrol in brewing until it is more prevalent in medical treatments, animal raising, and food safety. However, with consumer preference shifting towards smaller breweries, phage application is an appealing option for microbreweries and home brewers to increase the quality and shelf life of beer.

In theory, phage biocontrol in brewing is a feasible strategy to prevent bacterial spoilage. The use of phages to control spoilage is complicated by many factors, but the ubiquity and diversity of bacteriophages make it likely that ideal candidates exist (Hudson et al., 2005). Phages suitable for controlling beer spoilage should be strictly lytic, easy to scale up for commercial production, free of transducable phenotypes, stable in storage and application, and have a broad host range (Hagens & Loessner, 2007). Though phage application has great potential, it is not a substitute for good brewing practices and high standards of hygiene and maintenance. Phage biocontrol provides an additional hurdle for spoilage organisms to overcome while reducing the need for pasteurization or high-end filtration, potentially decreasing production costs for breweries of all sizes (Vaughan et al., 2005).

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