EFFECTS OF SALT AND GARLIC CONCENTRATION ON THE MICROBIAL SAFETY, BIOCHEMICAL PROPERTIES, AND SENSORY ACCEPTANCE OF SPONTANEOUSLY FERMENTED BEET KVASS

By

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An Abstract of the Thesis Presented
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Fermentation is the process by which primarily lactic acid bacteria (LAB), an environmentally ubiquitous group of organisms, convert carbohydrates into several byproducts, including acid, ethanol and/or gas. Often these resulting byproducts create desirable foods with unique flavor attributes and an increased inherent safety due to acidification. Vegetables such as red beetroot are suitable commodities for fermentation, due to abundant nutrient sources to sustain the LAB population. However, sufficient growth of LAB may lead to the production of harmful biogenic amines, specifically histamine and tyramine which can cause an allergic reaction and poisoning at high levels. Additionally, red beetroot is grown in direct contact with soil, a known fomite for foodborne pathogens. Thus, the objectives of this study are to determine the effects of three different salt (0.5%, 1.5%, and 2.5% NaCl (w/w)) and garlic (0%, 0.5%, and 1.0%(w/w)) concentrations on bacterial pathogen (STEC, *Salmonella,* and *L. monocytogenes*) survival and biochemical properties (biogenic amines, organic acids, sugars, and alcohol), during both spontaneous fermentation and storage of beet kvass. The sensory perception of fermented beet kvass at two different salt

(1.5% and 2.5% NaCl (w/w)) and garlic (0% and 0.5% (w/w)) concentrations and the impact of health-related messaging on product consumer acceptability were also assessed.

Results indicate that neither the salt or garlic concentrations tested had a significant effect on pathogen survival. Although there was a decrease in *Salmonella* and *L. monocytogenes* survival after storage, neither pathogen was completely eradicated in all samples. Therefore, to better ensure consumer safety, it is important for fermenters to maintain cleanliness and avoid cross-contamination during production. Salt and garlic concentration, however had a significant effect on organic acid and fructose content. Specifically, beet kvass produced with garlic had significantly reduced lactic and acetic acid, glucose, and fructose content, but significantly increased ethanol levels, when compared with samples without garlic. This biochemical profile suggests that garlic favors the growth of yeast in beet kvass. These observations suggest that formula development has considerable impacts on microbial diversity in this product. Biogenic amine analysis determined that low accumulation of these compounds in the production and storage of kvass are insufficient to pose obvious safety risks to consumers. Sensory evaluation revealed that salt concentration is a significant deciding factor on overall product acceptance, with consumers generally preferring lower salt levels. Inclusion of information on the potential health benefits of the product increased panelists' interest in consuming the product, indicating that participants may be willing to compromise sensory characteristics for health benefits. Therefore, producers may formulate beet kvass with lower salt and with or without garlic, without adversely effecting product acceptability.

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LITERATURE REVIEW

1.1. History of Fermentation

Fermentation is one of the oldest forms of food preservation, with first evidence of the process dating back to 6,000 B.C. in the Fertile Crescent in the Middle East (Fox, 1993). The process was then investigated by French microbiologist Louis Pasteur (1822-1895) in 1870, when he termed fermentation “La vie sans l’air” (life without air). Lactic acid fermentation is an anaerobic metabolic process by lactic acid bacteria (LAB) that converts carbohydrates to acids, gases, and/or alcohols. Besides preservation of food, food fermentation was found to produce desirable food products for consumers, as it improves sensory properties, extends shelf life, and increases the bioavailability of nutrients (Tamang et al., 2016; Septembre-Malaterre et al., 2018).

1.2. Lactic Acid Bacteria

Lactic acid bacteria (LAB) refers to a broad group of bacteria that produce lactic acid. They are Gram positive, non-respiring, non-spore forming cocci or rods. They are obligate fermentative organisms known to mediate the fermentation of vegetables. LAB are highly diverse and able to withstand extreme conditions. For example, they are capable of growing at temperatures as low as 4℃ or as high as 45℃ (Hamasaki et al., 2003; Marceau et al., 2004; Chen et al., 2013). While the majority of strains grow at pH 4.0 - 4.5, some are active at pH levels as low as 3.2 or as high as 9.6 (Caplice & Fitzgerald, 1999). LAB are considered to be aerotolerant organisms. They lack cytochromes and heme-containing proteins, and do not possess a complete citric acid cycle (TCA) due to the absence of catalase. Therefore, these organisms are unable to undergo energy-linked oxygen metabolism (Higuchi et al., 2000). Although LAB do not require oxygen, oxygen can serve as an electron acceptor due to the

byproducts of lactic acid fermentation, including ethanol, CO_2, and acetic acid (Endo & Dicks, 2014). LAB are desirable microorganisms in many food products.

The success of a lactic acid vegetable fermentation involving liquid brine, such as fermented beet kvass, is dependent on the solute movement from the plant material into the surrounding liquid (Daeschel et al., 1987). Plant materials, such as red beetroot and cabbage, contain high levels of glucose, fructose, and sucrose. The availability of these carbon sources is a factor in sequential microbial growth.

1.2.1. Metabolism of Carbohydrates

The genera of LAB can be grouped by the type of fermentation they perform (Table 1.1).

Table 1.1. Common LAB and their fermentation type

Genus	Species	Fermentation type
Enterococcus	*faecalis*	Homo
Lactobacillus	*acidophilus* *salivarius* *plantarum* [a] *casei* [b]	Homo
	brevis *fermentum* *reuteri* *plantarum* [a] *casei* [b]	Hetero
Lactococcus	*lactis*	Homo
Leuconostoc	*mesenteroides*	Hetero
Pediococcus	*pentocaceus*	Homo
Streptococcus	*thermophilus* *salivarius*	Homo

[ab] *Lb. plantarum* and *Lb. casei* can be both homo- and heterofermentative (facultatively heterofermentative), depending on carbon source availability

The conversion of carbohydrates to lactic acid by LAB is a critical component of food fermentation. The distinction between the fermentation types is dependent on the presence or absence of aldolase or phosphoketolase, which are the key enzymes in glycolysis and the phosphoketolase pathway, respectively. LAB are considered obligately homofermentative when only aldolase is present. However, in the presence of only phosphoketolase, LAB are considered obligately heterofermentative (Kandler, 1983). Facultatively heterofermentative LAB, on the other hand, have enzymes from both metabolic pathways. The two major metabolic pathways of glucose fermentation are the Emden-Meyerhof-Parnas (EMP) pathway (glycolysis) and the phosphoketolase pathway, as shown in Figure 1.1.

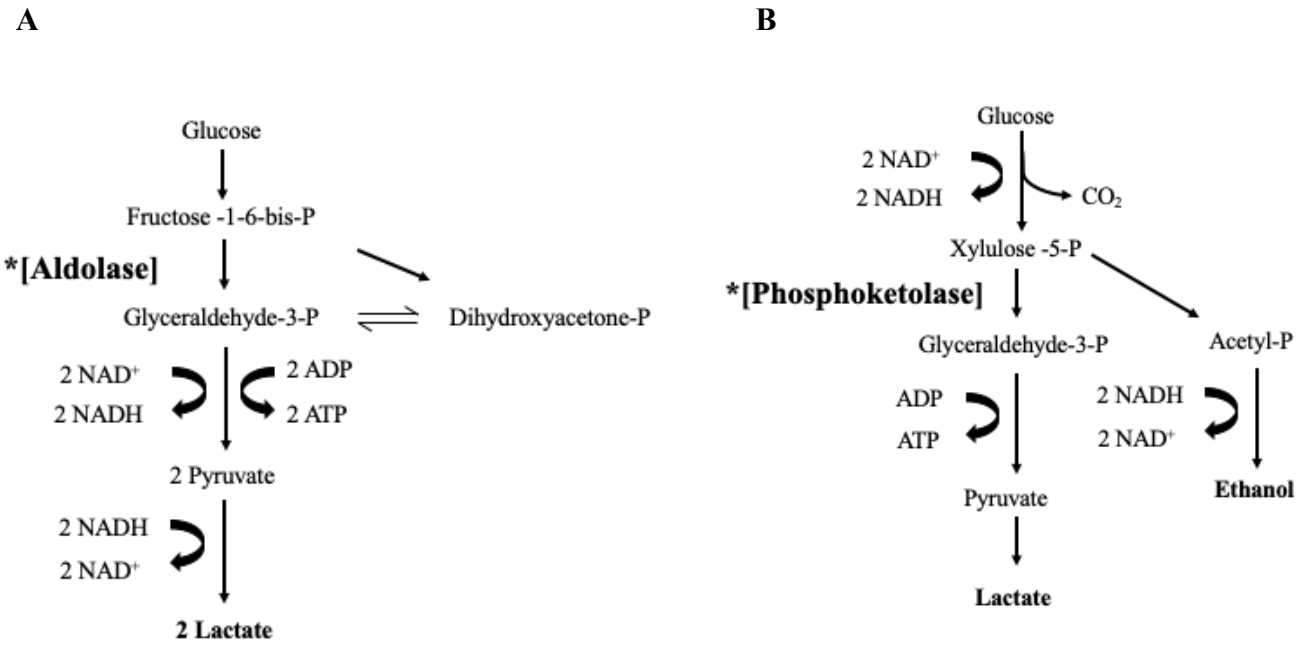

Figure 1.1: The metabolic pathway of glucose. A) Homofermentation; EMP, key enzyme is aldolase B) Heterofermentation; phosphoketolase pathway, key enzyme is phosphoketolase (Adapted and drawn from Gänzle (2015))

The homofermentation of glucose utilizes the EMP pathway and yields 2 molecules of lactate and 2 ATP, while the heterofermentation of glucose utilizes the phosphoketolase pathway yielding 1 molecule of each lactate, ethanol, CO_2, and ATP (Gänzle, 2015).

However, glucose is not the only carbon source for LAB. Sucrose and fructose are also used for energy and are abundant in many fruits and vegetables. Sucrose is a disaccharide and is hydrolyzed by sucrose 6-phosphate hydrolase, yielding glucose 6-phosphate and fructose for metabolism. The heterofermentation of fructose yields different end products. For every three molecules of fructose that are consumed, one lactate and acetate, two mannitol, one CO_2, and two ATP molecules are formed (Endo & Dicks, 2014). Apart from being a substrate, certain strains of LAB utilize fructose as an electron acceptor, specifically when present as the sole carbon source.

As the pH of the environment decreases, and as carbohydrate levels are reduced, LAB fermentation favors the utilization of amino acids. The change in metabolism is a survival mechanism to maintain the pH of the cell, which is discussed in detail in Section 1.4.2. Glutamine, glutamate, and arginine play a major role in pH homeostasis and stationary phase survival of LAB by increasing acid-resistance (Teixeira et al., 2014).

1.2.2. Metabolism of Amino Acids

Amino acid metabolism by lactic acid bacteria has received much attention, as this process gives rise to toxic biogenic amines formed during the fermentation of various foods. Biogenic amines (BA) are the byproducts formed from the breakdown of proteins by microorganisms that possess decarboxylases. They are often found in fish, fish products, alcoholic beverages, and fermented foods. Biogenic amine content has been used as an indicator

of microbial spoilage in non-fermented foods (Lázaro et al., 2015). Besides affecting food quality, BA are also linked to food safety risks.

Safety concerns associated with BA consumption are often linked to fish and fishery products, due to the high levels of BA precursors, decomposition of product, and unhygienic handling of ingredients (Prester, 2011). The most relevant BA in food are histamine, tyramine, putrescine, cadaverine, and phenylethylamine, which are products formed from the decarboxylation of histidine, tyrosine, ornithine, lysine, and phenylalanine, respectively (EFSA, 2011). Putrescine can also be formed through deimination of agmatine. Histamine and tyramine are the most toxic and relevant to food safety due to histamine or scombroid fish poisoning (HFP), as well as histamine and tyramine poisoning (Morrow et al., 1991; Finberg & Gillman, 2011).

LAB are capable of metabolizing all amino acids, however, the ability to degrade amino acids varies greatly among species. Generally, preformed amino acids are required for growth but specific requirements vary within species and across strains (Williams et al., 2001; Liu et al., 2003). Biogenic amines are formed by the decarboxylation of amino acids, and this is a safety concern due to the possible health implications, specifically with histamine and tyramine. Histamine is associated with histamine poisoning, also known as scombroid fish poisoning, which includes symptoms similar to an allergic reaction such as skin rash, diarrhea, flushing, sweating, and headache (Bartholomew et al., 1987; ten Brink et al., 1990).Whereas tyramine is associated with the interaction of monoamine oxidase inhibitors (MAOIs), which are drugs that are often used for treatment of clinical depression (McCabe-Sellers et al., 2006). Histamine and tyramine both have been found in fermented vegetables due to the high microbial activity (Kalač et al., 1999; Tsai et al., 2005).

Histidine decarboxylation (HDC) activity is regulated by the internal pH of the organism (Schelp et al., 2001). The decarboxylation of histidine is a protective mechanism against low pH, and the HDC pathway only becomes activated in a highly acidic environments. Therefore, histamine formation by LAB is often associated with fermented food products due to the highly acidic environment. HDC activity has been found in LAB such as *Lactobacillus parabuchneri, Lactobacillus vaginalis,* and *Lactobacillus reuter* which have been isolated from cheese (del Valle et al., 2018; Diaz, del Rio, et al., 2015; Diaz, Ladero, et al., 2016).

Similar to HDC, tyrosine decarboxylase (TDC) is an enzyme that catalyzes the decarboxylation of tyrosine to tyramine. This activity has been associated with *Enterococcus faecalis* and *Lactobacillus brevis*, LAB that have been isolated in fermented foods (Pessione et al, 2009; Moreno-Arribas & Lonvaud-Funel, 2001). Although these strains are more often associated with fermentation of wine and cheese, there is still a potential risk for fermented vegetable products including beet kvass due to the uncharacterized nature of spontaneous fermentations.

1.2.3. Role in Fermentation

The LAB dynamics in a fermented food system affect the final physicochemical properties of the finished product. Several fermented vegetables, such as sauerkraut, kimchi, and table olives, have been intensely studied to identify the predominant LAB which result in a high quality product. The fermentation pattern and the sequence of LAB growth is highly dependent on environmental factors such as pH level and salt concentration. Heterofermentative LAB such as *Leuconostoc mesenteroides* often serve as essential microbial precursors for the induction of a proper growth sequence (Stamer et al., 1971). Specifically, the production of lactic and acetic acid is required for a rapid decrease in pH levels (Adams & Hall, 1988). As organic acids

accumulate and the pH of the substrate decreases, *L. mesenteroides* is inhibited while more acid-tolerant, homofermentative LAB, such as *Lactobacillus plantarum,* will continue fermenting the remaining carbohydrates throughout the fermentation period (Paramithiotis et al., 2010).

1.3. Fermented Vegetables

Cultures around the world regard fermented vegetables as an important part of their diet. Vegetable fermentation initially was used as a means to preserve food. However, with increased interest in disease treatment and prevention, they are growing in consumer popularity as functional food products (Stanton et al., 2005). The Institute of Medicine's Food and Nutrition Board of the US defined functional foods as “one or more food constituents manipulated to enhance [their] contributions to a healthful diet” (IOM/NAS, 1994). With respect to fermented vegetables, the major health benefits associated with functional food products are due to the probiotic and biogenic effects associated with consumption. Probiotic benefits are attributed to the ingestion of live microorganisms, as well as the biogenic effects due to the presence of microbial metabolites synthesized during fermentation (Gobbetti et al., 2010).

Spontaneous lactic acid fermentation is a process accomplished by naturally occurring or native lactic acid bacteria found in vegetables. The list of LAB found in common spontaneously fermented vegetable products is listed in Table 1.2.

Table 1.2. Overview of LAB found in spontaneously fermented vegetables

Product	Vegetables	LAB	Reference
Sauerkraut	Cabbage	*Strep. faecalis* *Leuc. mesenteroides* *Lb. brevis* *Ped. pentocaceus* *Lb. plantarum*	Pederson & Albury 1969
Kimchi	Napa cabbage Daikon radish Garlic Ginger Green onions	*Leuc. citreum* *Leuc. gasicomitatum* *W. koreensis*	J. Cho et al. 2006
Pickles	Cucumber	*Lb. plantarum* *Leuc. mesenteroides*	Egbe et al. 2017
Fermented Olives	Olives	*Lb. pentosus* *Lb. plantarum* *Lb. casei* *Ent. durans*	Tofalo et al. 2014
Fermented Radish	Radish	*Lb. plantarum* *Ped. pentosaceus*	Pardali et al. 2017
Fermented Curly Kale	Curly Kale	*Lb. plantarum* *Lb. paraplantarum* *Lb. brevis* *Lb. curvatus* *W. hellenica* *W. cibaria* *Ped. pentosaceus*	Michalak et al. 2018

In addition to the suitability of these food matrices to sustain live cultures, health mediated effects have also been studied. *Lb. plantarum* and *Lactobacillus sakei,* for example, were isolated from store-bought white kimchi, and found to reduce the body weight and total weight gain of diet-induced obese mice (Park et al., 2016). The potential for probiotic-containing fermented vegetable products has also previously been investigated in tables olives, kimchi, and beet juice. With consideration of live culture viability, potential probiotic bacterium *Lactobacillus pentosus* was able to survive the processing and packing of table olives (Rodríguez-Gómez et al., 2014). Red beetroot were similarly discovered to be a suitable substrate to sustain lactic cultures (including *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii,* and *Lb. plantarum*) which were capable of rapidly utilizing beet juice for cell synthesis and lactic acid production (Yoon et al., 2005).

While there are many health benefits linked to fermented vegetables, there are also concerning side effects. Sauerkraut was found to possess anti-carcinogenic effects, but side effects such as diarrhea and interaction with monoamine oxidase inhibitors (MAOIs), a class of drug often used to treat depression, were found in the habitual intake of sauerkraut (Raak et al., 2014). Therefore, considerations of metabolite changes should also be studied in the context of fermented vegetable safety.

1.3.1. Salt

Salt is an essential ingredient in fermenting vegetables as it provides a competitive environment for LAB growth. Besides the acidic flavor that results from fermentation, salt level also contributes to the desired taste. Viander et al. (2003) observed that sauerkraut fermented with 0.5% mineral salt (wt/wt), resulting with a final concentration of 0.3% NaCl (wt/wt), had the highest quality rating, from a trained sensory panel, due to the less acidic flavored sauerkraut

juice. Taste is not the only important sensory factor in fermented vegetables. In a different study, reduced salt fermented sauerkraut (0.9% wt/wt) was found to be the least desirable sample by trained sensory panelists due to the softening of cabbage that produced an unappealing texture, even though the product had lower acidity levels compared to samples treated with 1.5% NaCl (wt/wt) (Wolkers-Rooijackers et al., 2013). Similarly, fermented paocai (10% NaCl) had higher texture ratings over mid-salt (4-5% NaCl) and lower-salt (1-3% NaCl) products. However, lower-salt formulations (1-3% NaCl) had overall higher preference ratings based on other sensory attributes such as acidity, taste, flavor, and color (Zhang et al., 2016). Hence, the effect of salt concentration on sensory acceptability of fermented vegetables is dependent on many factors.

In addition to sensory appeal, salt concentration in fermented vegetables also has safety implications. Salt recommendations vary from Sandor Katz's (2012) recommendations of using a pinch per quart to using ~2.0% salt from the National Center for Home Food Preservation's (2016) recommendations. However, salt recommendations are important and can affect consumer health because fermented foods are typically preserved without additional preservation strategies, such as thermal processing. Red beetroot is identified as "rarely consumed raw" by the FDA and is not subjected to the FSMA produce safety rule (FDA, 2015). Therefore, growers may follow less stringent safety-related protocols for maintaining the cleanliness and safety of these crops.

To ensure appropriate fermentation, NaCl levels in fermented vegetables must be well balanced. Prior research has shown that high salt levels can actually delay fermentation. Stamer and team (1971), for example, observed the inhibition of LAB in cabbage sauerkraut produced with 3.5% NaCl. Therefore, it is critical to determine the ideal salt concentration to decrease

potential pathogen safety risks, without compromising the sensory attributes of fermented vegetables.

1.3.2. Garlic

Garlic (*Allium sativum*) is a plant belonging to the allium family. The bulb is often used as a popular spice in many foods. Aside from contributing taste, flavor, and aroma, garlic is known for having many potential health benefits including antimicrobial, anticancer, antioxidant, immune boosting, antidiabetic, hepatoprotective, antifibrinolytic, antiplatelet aggregatory activity, and cardiovascular disease prevention (Santhosha et al., 2013). A wide spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria in various garlic preparations have been reviewed in Ankri and Mirelman's paper (1999).

Garlic holds many different roles in fermented foods. Despite the antimicrobial effect of allicin, fermentation of raw garlic is feasible when inoculated with *Lb. plantarum* as a starter culture (Tocmo et al., 2017). Garlic was found to be a major contributor of native lactic acid bacteria, such as *Leuconostoc* sp. and *Weissella* sp., in the spontaneous fermentation of cabbage kimchi (Lim et al., 2015). In a Thai low-salt fermented fish product, garlic served as a carbohydrate for LAB, while also inhibiting Gram-negative bacteria and yeasts from predominating (Paludan-Müller et al., 1999).

1.3.3. Biogenic Amines

While BA production is most often linked to protein rich foods such as seafood products, fermented food products, including vegetables, fulfill all criteria required for BA production. The production and accumulation of BA in foods is influenced by the availability and concentration of the substrate amino acids, the presence of microorganisms with the appropriate

decarboxylases, and the favorable environmental conditions to support growth and decarboxylation activity (Pinho et al., 2004; Alvarez & Moreno-Arribas, 2014). Sauerkraut has a considerably higher tyramine concentration (4.9 mg/100 g) in comparison to fresh cabbage (0.3 mg/100 g). This increase in biogenic amines in fermented cabbage suggests a potential health risk (Moret et al., 2005). Although it is unlikely that the amount of accumulated tyramine and histamine in fermented vegetables alone will cause adverse health effects, such as histamine and tyramine poisoning, the combination of tyramine with dietary histamine has demonstrated synergistic toxicity (del Rio et al., 2017). Lactic acid bacteria, yeast, and possibly other Gram-positive bacteria are believed to be responsible for the majority of BA formation in fermented products due to the suppression of Gram-negative microorganisms. The formation of biogenic amines by LAB has mainly been associated with fermented foods such as wine and dairy products (Landete et al., 2007; Novella-Rodríguez et al., 2002). *Lb. plantarum,* commonly found in spontaneously fermented vegetables, has also been noted to potentially decarboxylate ornithine into putrescine, a biogenic amine (Arena & Nadra, 2001). Therefore, due to the presence of BA precursors in red beetroot, fermented beet kvass may present a health risk to sensitive individuals, particularly when eaten in large quantities or consumed with other foods that may contain high levels of BA.

1.3.4. Microbial Dynamics in Fermented Vegetables

The ubiquity of LAB in nature offers an advantage in the inoculation of raw materials. The outcome of spontaneous vegetable fermentation is dependent on naturally occurring microbial populations in the raw material as well as environmental conditions such as pH, temperature, and salt concentration. The natural microflora of fresh vegetables consists of mostly Gram-negative aerobic bacteria and yeast, with LAB comprising a small portion of the initial

population (Mundt, 1970). When plant materials are subjected to an anaerobic environment, the small population of lactic acid bacteria becomes the predominate microflora, and vegetables will undergo lactic acid fermentation. The physiochemical properties of the fermented food product will change dependent on the influences of this predominate microbial community.

A successful lactic acid fermentation process is comprised of the succession of two fermentation stages (Paramithiotis et al., 2010; Lavefve et al., 2019). The first stage, also known as the heterofermentative stage, occurs during the first 3-7 days and it is dependent on the substrate. During this stage, native LAB ferment the available glucose into several products such as organic acids, alcohol, and CO_2. The subsequent homofermentative stage results exclusively in lactic acid production. The bacterial composition varies greatly between the two stages due to the chemical composition changes within the substrate.

Besides LAB, yeast also plays an important role in fermentation. Depending on the food system, yeast and LAB can have either synergistic or antagonistic effects. As an example, the combined metabolic activity of both LAB and yeast populations in fermented olives resulted in a synergistic effect, creating unfavorable conditions for the survival of pathogens (Grounta et al., 2013). However, an antagonistic relationship was observed between LAB and yeast in the fermentation of watery kimchi. The growth of *Saccharomyces* around 30 days of fermentation was reflected by free sugar consumption which resulted in the production of glycerol and ethanol. But, after 40 days of fermentation, the *Saccharomyces* population decreased which allowed for bacterial counts to increase (Jeong et al., 2013).

While the increasing population of yeast, such as *Saccharomyces*, *Zygotorulaspora*, and *Pichia*, can create unfavorable environment for pathogens, they are often associated with off-flavors and spoilage in fermented vegetables such as olives, kimchi, and cucumber (Alves et al.,

2012; Franco & Pérez-Díaz, 2012; Moon et al., 2014). Therefore, the presence and growth of yeast should be monitored to maintain the quality of fermented vegetables.

The spontaneous fermentation of vegetables can also be unpredictable at times due to the diversity of naturally occurring microbial populations and environmental conditions. Therefore, the microflora of fermented vegetable products, including beet kvass, should be further investigated to characterize the microbial diversity during the fermentation process.

1.4. Beet Kvass

Beetroot, *Beta vulgaris L.*, is the taproot portion of the beet plant. It can be consumed in both raw and cooked forms. It contains many healthful components such as vitamins, minerals, phenolics, carotenoids, nitrate, ascorbic acids, and betalains (Chhikara et al., 2019). Depending on the variety, beetroot comes in red-purple, golden yellow, or red-white color. Red beetroot is a root vegetable rich in carbohydrates, protein, micronutrients, and several functional constituents that have substantial health-promoting properties. It also contains a considerable amount of both essential and non-essential amino acids, as seen in Table 1.3.

Table 1.3: Amino acids found raw red beetroot (Nemzer et al., 2011)

Amino Acid	g per 100 g of edible portion	Amino Acid	g per 100 g of edible portion
Tryptophan	0.019	Valine	0.056
Isoleucine	0.048	Cystine	0.019
Leucine	0.068	Arginine	0.042
Lysine	0.058	Histidine	0.021
Threonine	0.047	Alanine	0.060
Methionine	0.018	Glutamic Acid	0.428
Phenylalanine	0.046	Glycine	0.031
Tyrosine	0.038	Proline	0.042
Aspartic Acid	0.116	Serine	0.059

Red beetroots are an abundant source of nitrates and are utilized in exercise enhancement supplements (Wruss et al., 2015). Beetroot has also been associated with health benefits such as lowering hypertension, enhancing exercise performances, and benefiting cardiovascular health, due its rich nitrate source (Jones, 2014; Kapil et al., 2014; Kapil et al., 2015).

Beet kvass, a fermented beverage made by infusing red beetroot in salt and water, is gaining popularity among home fermenters (Sarnacki, 2018). Red beetroot contains naturally occurring LAB such as *Lb. plantarum* and *Lactobacillus fermentum,* making this tuber a suitable substrate for spontaneous fermentation (Di Cagno et al., 2013). There is currently limited literature available regarding this product. However, studies on similar food products, such as red beetroot juice suggest that beet kvass has potential as a functional food supplement based on its polyphenols content (Wootton-Beard & Ryan, 2011). The microbial activity and matrix softening during fermentation of red beetroot have been found to release betalains, compounds responsible for strong antioxidant capacity (Sawicki & Wiczkowski, 2018). Given the nutritional

value of red beetroot and the probiotic benefits of lactic acid fermentation, beet kvass should be studied for both the microbial ecosystem diversity and commercial standardization. There are also currently no studies to date assessing the consumer acceptability of this product.

1.5. Factors Affecting Growth and Survival of Pathogens

1.5.1. Acids

The acidity of a solution is expressed by its pH value, which represents the negative logarithm of the hydrogen ion concentration. When the pH of a food is reduced to below the growth threshold, bacterial cells stop growing and viability is also lost, depending on the extent of pH reduction. More specifically, acids are known to have adverse effects on bacterial cells by disrupting the proton motive force within the bacterial cell which causes cell damage. However, this effect is not universal to all acids. Organic acids, such as acetic acid, lactic acid, and citric acid, are more effective at deactivating cells than inorganic acids such as hydrochloric, sulfuric, and nitric acids. While inorganic acids dissociate in aqueous solution, organic acids remain in equilibrium, in both dissociated and undissociated forms. The undissociated compounds enter bacterial cells and dissociate in the cell interior due to its higher pH level (when compared to the exterior of the cell).

Acidification of the cell interior can cause changes to the cell structural proteins, enzymes, nucleic acids, and phospholipids, which may lead to cell death (Davidson & Taylor, 2007). The inhibitory effect of organic acids however, is acid dependent. Acetic acid, for example, has been found to be the most effective at deactivating cells due to its low molecular weight and high liposolubility, compared to lactic and citric acids, which makes it more penetrable in bacterial membranes. Acetic acid also has a higher dissociation constant (pK) compared to lactic acid (with pK values of 4.8 and 3.8, respectively). At the same pH, acetic acid

has more undissociated molecules than lactic acid, which increases its antimicrobial effects (Ray & Bhunia, 2014).

1.5.2. Acid Adaptation

Some bacterial strains can become more acid resistant after exposure to a mildly acidic environment (Foster & Hall, 1990; Davis et al., 1996; Cheng et al., 2003). When cells are exposed to a mild acidic environment (pH 5.0 - 5.8) for a short period of time, acid tolerance response (ATR) is initiated, which allows cells to survive subsequent exposure to more acidic environments (pH 2.4 - 4.0). In contrast, when cells are exposed to mild acidic environments (pH 5.0 - 5.8) for an extended period of time, acid adaptation of cell will be amplified, facilitating survival in subsequent exposure to even more acidic environments (pH < 2.5). This acidity resistance among some bacterial strains has created concerns surrounding the perceived safety, preservation, and processing of certain low pH foods such as fermented vegetable products.

1.5.3. Salt

Common table salt, also known as sodium chloride (NaCl), is regularly used in food preparation for taste and preservative purposes. As a preservation strategy, NaCl can lower the water activity (a_w) of a food matrix, reduce oxygen solubility in water, and causes bacteria to accumulate certain amino acids (Hua et al. 1982; Lück and Jager, 1997). These effects result in less favorable microbial growth conditions (Bae & Lee, 2010). When there is an imbalance in the concentration of water on the interior and exterior of the microbial cell, water will diffuse through the gradient. Low a_w causes the loss of turgor and cell shrinkage, which results in bacterial growth inhibition (Dodd et al., 1997).

Besides lowering watering activity (a_w), NaCl is capable of interfering with substrate utilization, which ceases normal microbial cell functions (Csonka, 1989). The effects of NaCl are

concentration dependent. Although salt by itself may not be sufficient to totally eliminate the growth of all microorganisms, previous studies have shown the synergic effect of salt when combined with other environmental stresses such as acid, are more effective in controlling pathogenic bacterial growth, and will be discussed in later sections.

1.5.4. Antimicrobial Effect of Garlic and Other Herbs

In addition to organic acids, salt, and bacteriocins, plant derived compounds such as thymol, carvacrol, and allicin have been found to possess antimicrobial effects. Allicin (C_3H_5 -S-S(O)-C_3H_5) or diallyl thiosulphinate, is the principal bioactive compound released from crushed garlic. After garlic is crushed, the allinase enzyme is released, which acts on alliin (present in intact garlic) to produce allicin (Banerjee et al., 2003). The antimicrobial properties of allicin are variable but include inhibition of -SH group enzymes, acetyl-CoA synthetase in fatty acid biosynthesis in yeasts, bacterial acetate kinase, and phosphotransacetylase enzyme systems. Additionally, the compound also affects fatty acids, lipid biosynthesis, and RNA synthesis in microorganisms. Allicin is reversibly bonded to enzymes forming non-covalent bonds, and its antimicrobial activity is dose dependent (Ceylan & Fung, 2014). Han et al. (1995) reported that the antibiotic activity of 1 mg of allicin is equivalent to15 IU of penicillin. Beyond allicin, other compounds in garlic also possess antimicrobial activities. It was suggested that these other compounds, 3-vinyl-1,2-dithiacyclohex-5-ene and 3-vinyl-1,2-dithiacyclohex-4-ene, in garlic extracts have been associated with bacterial cell death through the destruction of structural integrity of their cell membranes (Chen et al., 2018).

In a low-salt, fermented fish product, the addition of garlic extract (1.5%) was found to prevent the growth of *Vibrio* strains, while having only a slight effect on *Salmonella* when treated with 2% garlic extract (Bernbom et al., 2009). The antimicrobial activity of garlic may be

pathogen-specific and limited to extracts. However, Bernbom and others (2009) hypothesized that the garlic effect was attributed to the increase of acid production in fermented foods, not the antimicrobial effects of allicin. Hence, there is may be untapped potential for the use of garlic in fermented vegetables to control pathogen growth and ensure the safety of fermented vegetables.

1.6. Foodborne Pathogens

1.6.1. *Escherichia coli*

Escherichia coli is a non-spore forming, Gram-negative, and rod-shaped bacteria. It is a facultative anaerobe commonly associated with fecal matter due to its omnipresence in the lower intestines of warm-blooded organisms (Schaechter, 2009). Although not all strains of *E.coli* are pathogenic, Shiga toxin-producing strains of *E. coli* (STEC) are a major human health concern. Shiga toxins are often associated with hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenia purpura (TTP). These compounds were found to inactivate ribosomes in target cells, which inhibits protein synthesis in hosts and leads to apoptosis by inducing cell signaling (Bergan et al., 2012). The seven *E. coli* serotypes that are most often associated with foodborne illness are O26:H11, O45:H2, O103:H2, O111:H8, O121: H19, O145:H28, and O157:H7 (Delannoy et al., 2013).

In 2013, the Economic Research Service (ERS) of the U.S. Department of Agriculture (USDA) estimated the total annual cost of foodborne illnesses related to *E.coli* O157 contamination was $271 million dollars, while non-O157 Shiga toxin-producing incidents cost $27 million dollars. Even though no foodborne illnesses have yet been reported from the consumption of fermented vegetables in the U.S., there have been incidents where *E.coli* in fruit juices (which have similar pH levels) have caused foodborne illnesses (Besser et al., 1993; Centers for Disease Control and Prevention, 1996; 1999). Fresh vegetables and other types of

produce have also been implicated in outbreaks attributed to enterohemorrhagic *E. coli* (EHEC) (Lynch et al., 2009). There have been several outbreaks of enterotoxigenic *E. coli* associated with kimchi reported in Korea (S. Cho et al., 2014; Shin et al., 2016). However, the kimchi that caused the reported illnesses was either unfermented or at the early stages of fermentation. Therefore, this pathogen poses a potential threat to the safety of fermented vegetable products, especially when the products are not adequately prepared or properly acidified through the natural fermentation process.

1.6.1.1. Acid

E.coli is able to grow at pH levels as low as 4.4 (Ray & Bhunia, 2014). Exposure to a lower pH level can stop growth and even eliminate some cells. However, as previously mentioned, studies have shown *E. coli* is capable of adapting to acidic environments and possibly increasing its acid resistance to low pH levels over time (Foster, 2004; Leyer et al., 1995; Lin et al. 1996). This adaptation can occur when *E. coli* is exposed to moderately acidic environments for an extended period of time (Spyropoulou et al., 2001; Cheng et al., 2003). Hence, there is a possibility for an increased survival rate of this pathogen after being habituated in fermented vegetables or in the human stomach, despite the low pH levels, which may lead to infection. Therefore, it is imperative to prevent the contamination of fermented foods with pathogens, such as *E. coli.*

Among non-habituated cultures, *E. coli* O157:H7 was found to be the most acid resistant foodborne pathogen, followed by *Listeria monocytogenes*, and then *Salmonella enterica* ser. Typhimurium when tested in laboratory media (Koutsoumanis & Sofos, 2004). Enhanced survival of *E. coli* O157:H7 due to acid adaptation has also been observed in acidic food matrices, such as fruit juices, as previously mentioned. However, Cheng and Chou (2001) found

that despite the enhanced acid adaptation, *E. coli* O157:H7 survival was reduced in fermented milk products, but not in commercial fruit juices. The researchers hypothesized that while acid adaptation of *E. coli* O157:H7 increased resistance to subsequent acid stress, it might have also increased the susceptibility to antimicrobial substances produced by LAB in these fermented milk products. Similarly, *E. coli* did not survive in fermented cabbage kimchi and radish kimchi products with added raw pork meat (G. Cho et al., 2011). Even though the gradual decrease in pH and high titratable acidity produced by LAB during fermentation could induce acid adaptation, it was not observed in this study. The authors hypothesized that the bacteriocins produced by the LAB population, and the presence of spices, may have decreased the *E.coli* population in the fermented cabbage kimchi, although this specific deactivation mechanism was not explored in this study.

1.6.1.2. Salt

As previously mentioned, acid and salt are commonly used in the food industry as food preservation methods to control bacterial pathogen growth. The combination of two or more of these inhibitory agents is known as hurdle technology, in which the effect of the combined treatments are more effective in pathogen eradication than application of an individual stressor. However, prior experiments conducted on both laboratory media and food systems have challenged this strategy. Acid tolerance can be initiated by salt presence. Casey & Codon (2002), for example, found *E. coli* treated with salt (4%) in a enriched medium broth exhibited an increased acid tolerance. Although this was not reported in a specific food medium, these findings suggest that *E. coli* might be able to survive in acidic product formulations that contain salt, such as fermented vegetables. However, certain studies have found that *E. coli* survival can be negatively affected by salt. For example, *E. coli* exposed to 0.3 M and 0.8 M NaCl

experienced altered gene expression, enzyme activity, and cofactor levels. The increase in NaCl resulted in higher energy requirements needed to maintain cellular homeostasis (Arense et al., 2010). In addition to delaying and slowing the metabolism of *E. coli*, pre-exposure to NaCl at a 10% concentration was also found to decrease *E. coli* O157:H7's resistance to acetic acid. However, when *E. coli* was exposed to 1% NaCl prior to the acid treatment, there were no significant affects to this pathogen's survival in laboratory media (Bae & Lee, 2010). Therefore, the concentration of salt was found to play a role in moderating the survival of this pathogen in an acidic environment.

Moreover, Lee and Kang (2016) also found the addition of salt in fact increased acid resistance in *E. coli* O157:H7 grown in laboratory media. *E. coli* O157:H7 showed an antagonistic effect in a combined treatment of salt and acetic acid. The cytoplasmic pH of *E. coli* O157:H7 was increased after the addition of salt to help balance the cytoplasm pH following exposure to organic acids. *E. coli* O157:H7 displayed the same antagonistic effect of acetic acid and salt in pickled cucumber puree (Lee et al., 2010). However, in cabbage sauerkraut, no associations were found between salt concentration and *E. coli* O157:H7 survival. Instead, there was an association between salt concentration and the isolation of acid tolerant *E. coli* O157:H7 in whole head cabbage sauerkraut, with more isolates found in the 2.25% salt concentration treated samples, rather than 1.8% or 3%. The researchers suggested that the lower salt concentration allowed competitive bacteria to inhibit *E. coli*, while the higher salt concentration inhibited *E. coli* after 19 days of fermentation (Niksic et al., 2005). The differences in the two food systems could be attributed to the type of acid, as lactic acid was more abundant in cabbage sauerkraut than pickled cucumber puree. Hence, it is a possible that the antagonistic effect of acid and salt is acid dependent. The combined treatment of lactic acid (pH 4.2) and salt (4%) on

E. coli O157:H45 was observed to have a higher decimal reduction time (136 mins) than the treatment of lactic acid alone (24.94 mins) (Casey & Codon, 2002). Therefore, based on these studies, organic acid treatment alone appeared to have a higher bactericidal effect on *E. coli*, than the combination of organic acid and salt, with lactic acid being more effective than acetic acid when combined with salt (Cheng et al, 2003; Casey & Codon, 2002; Bae & Lee, 2015).

Besides salt and acid, the attachment of pathogens plays a role in survival. Complete elimination of *E. coli* O157:H7 was observed in naturally fermented black olives (pH 3.95; NaCl 6.05%). Grounta et al. (2013) concluded that the elimination of pathogens was caused by the physiochemical characteristics of fermented black olives, such as its slippery surface, that may have prevented the attachment of pathogens.

1.6.2. *Salmonella*

Salmonella is a non-spore forming, Gram-negative rod-shaped bacteria (Cox & Pavic, 2014). It is a facultative anaerobe and enteric bacterium commonly associated with poultry and reptiles. There are only two species of *Salmonella*; *Salmonella enterica* and *Salmonella bongori. S. enterica* is mainly found in warm-blooded animals, while *S. bongori* is commonly associated with cold-blooded animals. Salmonellosis is one of the main causes of bacterial foodborne illnesses worldwide. In 2013, the USDA ERS estimated a total annual cost of $3.7 billion dollars from *Salmonella*-related illnesses in the U.S. More specifically, *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) are the most frequently associated with human disease (K. Lee et al., 2015; Löfström et al., 2016; Thung et al., 2016).

Similar to *E. coli*, no reported cases of salmonellosis have been associated with fermented vegetable consumption yet in the U.S. However, there have been incidents in which

Salmonella caused foodborne illnesses in fruit and fruit juices (CDC, 1999; 2012), as well as fresh vegetables (CDC, 2006; 2016).

1.6.2.1. Acid

The minimum pH at which *Salmonella* commonly grows at is a pH level of 4.5 (Ray & Bhunia, 2014). However, ATR was also found in *S.* Typhimurium, protecting the cells from severe acidic conditions (pH 3.0 - 4.0) (Foster & Hall, 1990). *S.* Typhimurium was able to induce cross-protection against heat, salt, and other environmental stresses, as ways to adapt to the presence of acids (Leyer & Johnson, 1993). As an example, *S.* Enteritidis acquired additional heat (50 °C) and salt (8% NaCl) tolerance after acid adaptation, by growing cells in acidified media (pH 4.5 - 6.0) (Ye et al., 2019). Acid-adapted cultures of *Salmonella* spp. were also found to have an increase in virulence and human cell invasion (Humphrey et al., 1996; Wilmes-Riesenberg et al., 1996; Gahan & Hill, 1999).

While acid adaptation induces cross-protection, not all acids have the same effect. One study showed that *S.* Typhimurium's ability to grow under acidic conditions was affected by the type of acid used to acidify the growth medium (Álvarez-Ordóñez et al., 2010). The researchers found that acetic acid was most effective in inhibiting or delaying the growth of *S.* Typhimurium, followed by lactic acid, citric acid, and hydrochloric acid. Similarly, acid-adapted *S.* Typhimurium was completely inactivated at a pH level of 3.5 with acetic acid within 60 - 120 minutes of treatment. Acetic acid was also able to induce ATR that increased resistance to osmotic stress in the form of either NaCl or KCl, in comparison to exposure to lactic acid (Greenacre & Brocklehurst, 2006).

In a study on fermented green table olives in brine, *S.* Enteritidis was able to survive in an environment of pH 4.2 and a high salt concentration (6.0%) for up to 21 days of storage

(Argyri et al., 2013). Similarly, *S.* Typhimurium was capable of surviving under low pH levels (pH 3.8 - 3.9) in spontaneously fermented cauliflower. This survival was thought to be triggered by the gradual decrease of pH, which could have activated the adaptive response. The high osmolarity of the product could have also triggered the development of cross-protection against low pH levels (Paramithiotis et al., 2012).

1.6.2.2. Salt

The addition of salt has been used to prevent the growth of *Salmonella* spp. Unlike *E. coli*, literature has indicated that a combined salt and acid treatment is fairly effective in eradicating this pathogen. Bae and Lee (2010) found that when *S.* Typhimurium was pre-exposed to NaCl (5%), the resistance to acetic acid (1%) was decreased. The physicochemical characteristics of naturally-fermented black olives at 6.0% NaCl (pH 3.95) were found to be inhospitable for *S.* Enteritidis, and *S.* Typhimurium, and these microorganisms were observed to be completely eliminated after 2 days of fermentation (Grounta et al., 2013).

1.6.3. *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive pathogen with the ability to adapt to a wide range of conditions such as refrigerated temperatures (2–4 °C), high acidity, and high salt (Doyle et al., 1997). The organism is ubiquitous in nature with the ability to form biofilms. For this reason, food producers are required to be diligent in maintaining facility and equipment cleanliness, as the FDA and USDA have implemented zero tolerance regulations in the food industry. *L. monocytogenes* causes listeriosis, which can lead to serious health implications such as meningitis, septicemia, spontaneous abortion, stillbirth or fetal death (Rocourt & Cossart, 1997). Infections in humans have been related to high mortality (15 - 20%), especially in young, elderly, and immunocompromised individuals. The total annual cost of foodborne illnesses in

2013 associated with *L. monocytogenes* contamination was estimated at $2.8 billion dollars in the U.S. (ERS USDA).

L. monocytogenes infection is often associated with high-sodium, refrigerated, ready-to-eat foods, such as deli meats and cheeses. While there are no reported outbreaks of *L. monocytogenes* associated with fermented vegetables, there have been prior foodborne illness reports related to fresh produce such as celery, tomatoes, lettuce, sprouts, and cabbage (Ho et al., 1986; CDC, 2015). *Listeria* spp. strains had been found to colonize plant roots, especially root-tuber vegetables, due to direct contact of plant tissue with *Listeria*-contaminated soil. Laboratory experiments, for example, confirmed successful colonization of carrot roots by *L. monocytogenes* (Kljujev et al., 2018). Therefore, the risk of *L. monocytogenes* contamination of other root vegetables, such as red beetroot, is possible.

1.6.3.1. Acid

The minimum pH allowing the growth of *L. monocytogenes* is at a pH level of 4.6 (Ray & Bhunia, 2014). *L. monocytogenes* is more sensitive to acidic environments compared to *E.coli*. In strawberry juice (pH 3.6) stored at 4°C over 3 days, *L. monocytogenes* population decreased, whereas *E.coli* O157:H7 population remained constant (Han & Linton, 2004). *L. monocytogenes* pH tolerance is highly dependent on the bacterial strain, the kind of acid used, and growth phase (Phan-Thanh et al., 2000). Despite the comparable pH and titratable acidity values in spontaneously fermented cauliflower, fermented cabbage and radish kimchi, survival of *L. monocytogenes* was documented in spontaneously fermented cauliflower, but not cabbage and radish kimchis (Paramithiotis et al., 2012; G. Cho et al., 2011). Differences in the production process and the raw materials used to make each product may have caused these contradicting results (Paramithiotis et al., 2012). Therefore, it is possible that the inactivation of *L.*

monocytogenes in fermented foods may not be just due to the decrease in pH levels, but a combination of other factors such as bacteriocins and/or antimicrobial activity from spices.

Similar to *E.coli* and *Salmonella*, *L. monocytogenes* was also found to exhibit acid tolerance response (ATR), which would prevent cell death at normally lethal acid dosages (pH 3.5) (Gahan et al., 1996). When *L. monocytogenes* was subjected to pH 5.5 media for 2 hours, researchers observed an increased resistance against heat shock (52°C), osmotic shock (25-30% NaCl), and alcohol stress (15%) (Phan-Thanh et al., 2000).

1.6.3.2. Salt

As previously mentioned, salt has been used in foods as a form of preservation by limiting available water and also disrupting the osmoregularity of cells, including *L. monocytogenes*. Although salt concentrations can limit bacterial growth, some bacteria have previously demonstrated osmoadaptation, when exposed to sublethal NaCl concentrations. Typically, NaCl concentration in foods are insufficient in limiting *L. monocytogenes* growth. However, this exposure may lead to greater osmo- and acid tolerance, although this conclusion is not universally consistent (Faleiro et al., 2003). Bae & Lee (2010) for example, found that *L. monocytogenes* pre-exposed to NaCl (5%), became less resistant to 1% acetic acid over time. Similarly, Lee and Kang (2016) observed a synergistic effect of acetic acid and NaCl against *L. monocytogenes*. The addition of NaCl caused a decrease in cytoplasmic pH of *L. monocytogenes*, which led to decreased survival. However, in fermented cabbage sauerkraut, no obvious association was found between salt concentration and the isolation of acid tolerant *L. monocytogenes* (Niksic et al., 2005). Therefore, it was suspected that the effects of acid and NaCl on the survival of *L. monocytogenes* may depend on the sequence of treatment. While the application of two treatments simultaneously may be more convenient for producers, it has the

lowest inactivation rates compared to treatments that were applied one after another (Shabala et al., 2008). Contrary to *E.coli* and *Salmonella*, *L. monocytogenes* has also been found to survive after selective enrichment in naturally-fermented black olives (pH 3.95; NaCl 6.05%) (Grounta et al., 2013). Therefore, the effects of osmoadaptation are highly dependent on the salt concentration, type, and treatment application.

1.7. Safety Concerns of Low Salt Brines in Fermented Vegetables

The preference for salt reduction in fermented vegetable products is emerging due to the health implications of high sodium intake. A recent assessment has found that 20% (59/295) of home fermenters in Maine felt that making low-sodium fermented foods was very or extremely important to them (Camire et al., 2019). Fermented vegetables have been regarded as a low-risk food product due to the naturally present competitive microorganisms (LAB), high acid/low pH environments, and sodium chloride (NaCl) as a processing ingredient. As previously mentioned, the reduction of salt in fermented vegetables may be a food safety concern because it could potentially affect pathogen survival (STEC, *Salmonella*, and *L. monocytogenes)*.

Paocai, a fermented Chinese cabbage product with a 3% salt level, was found to inhibit the survival and growth of *E. coli* O6, *Staphylococcus aureus*, *S. enterica*, and *L. monocytogenes* within 4.5 days. However, it is notable to mention the pH level of the product was well below pH 4.0 during the enumeration of these pathogens. Although this study showed promising results, there was a lack of information on the product pH level when tested for microbial safety, along with the information for low-salt paocai (1 - 2%), while claiming complete eradication of the pathogens (Zhang et al., 2016). Contrary, a study on low-salt fermented sauerkraut (1 - 2.5%) showed the survival of STEC and *S. aureus,* while *L. monocytogenes* was absent at pH levels of 3.70 (Khanna, 2019). Therefore, more studies need to be performed to further confirm the safety

of low-salt fermented vegetables. Currently, there are limited studies on the storage microbial safety, biogenic amine contents, along with the consumer acceptability of low-salt fermented vegetables.

Besides the risk of foodborne diseases caused by pathogens, the presence of LAB metabolites, such as biogenic amines, could contribute to possible chemical food safety concerns related to fermented vegetables. Although some studies have suggested that the levels of BA found in fermented vegetables were not high enough to cause acute poisoning, the accumulation during fermentation and storage time could potentially be a threat, especially to those who are on MAOI drugs and/or are sensitive or allergic to histamine.

Non-O157 STEC, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, and *Salmonella* were isolated from fresh raw red beetroot juice (Gómez-Aldapa et al., 2014). Due to the growth conditions of red beetroot, and the inclusion of skin during the fermentation of beet kvass, there is a risk of contamination with pathogens. Therefore, fermented beet kvass is a suitable food system to study the efficacy of fermentation at eradicating common foodborne pathogens.

1.8. Research Justification

In conclusion, the desire for low-salt fermented vegetable products creates the need for the investigation of the survival of bacterial pathogens (STEC, *Salmonella*, and *L. monocytogenes*) in naturally fermented vegetables, especially at lower salt concentrations. Although studies on low-salt fermented vegetables such as sauerkraut, table olives, and kimchi have been accomplished, the study of fermented beet kvass is not evident in literature. This is a fairly new product in the consumer marketplace that has not been standardized nor studied, making this research even more critical. The study of the microbial and chemical safety, along with consumer acceptability of lower salt

concentration fermented beet kvass is much needed. Better understanding of this product may generate additional opportunities to market value-added, naturally fermented foods at lower salt concentrations to an expanding market of health-conscious consumers.

1.9. Objectives

The overall goals of these research studies are to assess the safety, biochemical, and sensory perception of naturally-fermented vegetables at salt concentrations below 2.5%. The results from these studies will provide guidance to small, agricultural-based businesses, and home-fermenters alike, in producing safe yet appealing fermented foods with a lower sodium content. Therefore, this research may improve the economy of states with micro food businesses, including Maine.

Therefore, the objective of these research studies are as follows:

1) to determine the effects of three different salt (0.5%, 1.5%, and 2.5% NaCl (w/w)) and garlic (0%, 0.5%, and 1.0% (w/w)) concentrations on bacterial pathogen (STEC*, Salmonella,* and *L. monocytogenes*) survival during production and after storage of spontaneously fermented beet kvass,

2) to analyze the effects of salt (0.5%, 1.5%, and 2.5% NaCl (w/w)) and garlic (0%, 0.5%, and 1.0% (w/w)) concentrations on the biochemical properties such as fermentable sugars (glucose, fructose and sucrose), organic acids (lactic and acetic acids), and biogenic amines (agmatine, putrescine, tyramine, cadaverine, and histamine) during the production and after storage of spontaneously fermented beet kvass, and

3) to evaluate the effects of salt (1.5% and 2.5% NaCl (w/w)) and garlic (0% and 0.5% (w/w)) concentrations on the sensory perception of spontaneously fermented beet kvass and the possible impacts of health-related messaging on product consumer acceptability.

CHAPTER 2

EFFECTS OF SALT AND GARLIC CONCENTRATION ON THE MICROBIAL SAFETY OF SPONTANEOUSLY FERMENTED BEET KVASS

2.1. Abstract

Beet kvass is a value-added beverage gaining popularity among home fermenters and small-scale, commercial food processors. While salt is an essential ingredient for fermenting vegetables, high sodium intake is associated with increased blood pressure and kidney disease. However, despite the apparent nutritional benefits of reducing sodium content, the reduction of salt within a fermented vegetable formulation presents potential food safety concerns, as salt may increase pathogen survival during fermentation. In addition to salt, garlic also has known antimicrobial properties which could potentially mitigate the safety risks associated with a low-sodium, fermented vegetable product. Therefore, the objective of this research was to determine the effects of salt and garlic concentration on the microbial safety of beet kvass. Samples of beet kvass formulated with varying salt (0.5, 1.5, and 2.5% w/w) and garlic (0, 0.5, and 1% w/w) levels were inoculated with Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella*, and *Listeria monocytogenes* cocktails. The survival of these pathogens was then analyzed at the end of fermentation (pH ≤ 4.0). Samples were enriched in selective media and tested using qPCR. There were no significant differences in the survival rates of *Salmonella* or *L. monocytogenes* in beet kvass among the different treatments. However, a significantly higher STEC survival rate was observed compared to the other pathogens, and at the highest salt concentration treatment during storage. A decrease in survival rate of *Salmonella* and *L. monocytogenes* was observed after 30 days of storage at 4°C. No significant interactions between salt and garlic treatments were found. These data suggest that the pathogens tested can readily survive the fermentation of

beet kvass, and that a higher concentration of salt (within the tested range), and the addition of garlic would not increase the safety of the product against potential foodborne pathogen presence.

2.2. Introduction

Fermented vegetables have long been considered a fairly safe food commodity, due to acidification by primary fermentative microorganisms (lactic acid bacteria), which creates an adverse environment for competing microflora. However, despite this perceived safety, foodborne illness has recently been associated with some fermented vegetable-based products. For example, in South Korea, enterotoxigenic *E. coli* has been implicated in foodborne illnesses resulting from the consumption of improperly prepared kimchi, a cabbage-based fermented vegetable product (Cho et al., 2014; Shin et al., 2016). As a result of the changing inherent safety perceptions of fermented vegetable products, a reanalysis of critical preparation parameters is necessary. Practices of making fermented vegetables, such as salt concentration, cleanliness of raw material, and fermentation time are essential in assuring product safety.

Salt is an essential ingredient in fermented vegetables, as it provides a favorable flavor and environment for the growth of lactic acid bacteria, while inhibiting the growth of many other spoilage bacteria (Caplice & Fitzgerald, 1999; Doyle & Glass, 2010). However, salt has been associated with several adverse health effects, including kidney disease and increased blood pressure (Malta et al., 2018). The reduction of salt levels in a fermented vegetable formulation may affect the survival of foodborne pathogens. More specifically, the reduction of salt may increase competition from non-LAB microflora, delaying the acidification of fermented vegetables, and potentially producing a product that is unsafe. This theory is supported by previous studies which assessed the survival capabilities of pathogenic bacteria in fermented

vegetable products (Paramithiotis et al., 2012; Choi et al., 2018). Additionally, pathogen adaptability toward the stresses conferred in a fermented product, including low pH levels, has become an area of focus within the food safety community due to acquisition of acid resistance among microorganisms (Koutsoumanis & Sofos 2004; Bae et al., 2018).

Garlic is also a well-known ingredient used in many food products. Besides flavor, garlic is also known for possessing antimicrobial activity against both Gram-negative and Gram-positive bacteria (Ankri & Mirelman, 1999). Although there are no studies to date assessing the antimicrobial activity of crushed garlic during fermentation, there are proposed benefits of including this ingredient in formulations, such as decreasing levels of toxic biogenic amines, improving acidification, and increasing sensory quality of the finished product (Zhou et al., 2016; Mah et al., 2009; Bernbom et al., 2009). Therefore, the explicit functions of garlic in fermented vegetable formulations, including potential antimicrobial activity benefits during fermentation and storage, require further exploration.

Beet kvass, a fermented beverage made by infusing chopped red beetroot in salt and water, is gaining popularity among home fermenters due to its simple recipe, short preparation time, and proposed health benefits. These purported health benefits include lowering hypertension, enhancing exercise performance, and benefiting cardiovascular health (Kapil et. al., 2015; Jones, 2014; Kapil et al., 2014). Raw vegetables are a commodity with a high natural microbial load (Beuchat, 2002). Therefore, safety measures implemented by the Food and Safety Modernization Act (FSMA) are practiced when growing and producing these crops. Red beetroot is a root vegetable that is in direct contact with soil, a known fomite for foodborne pathogens (Mritunjay& Kumar, 2017). However, red beetroot does not fall under this regulation due to it being a “rarely consumed as raw” commodity (FDA, 2015). This may affect the safety of

fermented beetroot products, such as beet kvass, as it is cannot be thermally processed without compromising product quality, specifically the live active cultures in the product that consumers desire (Buck et al., 2003). Current peer reviewed literature is lacking in regards to the microbial safety this product. Therefore, given the increasing interest in fermented vegetables among small, agricultural-based businesses and home preservers, it is important to standardize the product formulation to better ensure its safety (Sloan, 2019).

Thus, the objectives of this study are to determine the effects of three different salt (0.5%, 1.5%, and 2.5% NaCl (w/w)) and garlic (0%, 0.5%, and 1.0% (w/w)) concentrations on bacterial pathogen (STEC*, Salmonella,* and *L. monocytogenes*) survival during both spontaneous fermentation and storage of beet kvass.

2.3. Materials and Methods

2.3.1. Experimental Design

Fermented beet kvass samples were produced at three different NaCl (0.5%, 1.5%, and 2.5% (w/w)) and garlic (0.0%, 0.5%, and 1.0% (w/w)) concentrations in a full factorial design. Figure 2.1. represents the various salt and garlic ratios used in each treatment formulation. Each treatment was independently replicated four times. The pH levels of the samples were tested every 24-48 hours until the targeted pH of ≤ 4.0 was reached. A set of uninoculated controls were also prepared to perform simultaneous analysis to quantify organic acids, sugars, alcohol, and biogenic amines.

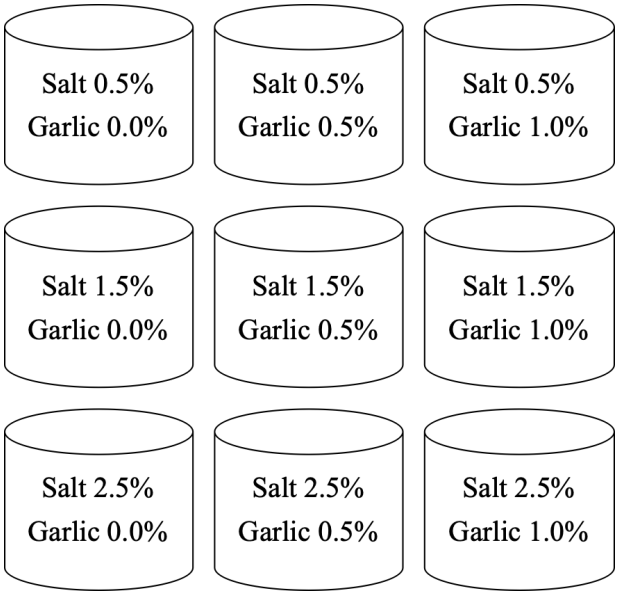

Figure 2.1. Salt and garlic treatments prepared with a final mass of 850 g (n = 4)

2.3.2. Bacterial Strains

The bacterial pathogen strains used in this study were obtained from American Type Culture Collection (Manassas, VA), including *E. coli* O111:H8 (CDC 2000-3025), *E. coli* O26:H11 (EHEC 1534), *Salmonella* Enteriditis (BAA-1045), *Salmonella* Typhimurium LT2 (BAA-2722), as well as *L. monocytogenes* Serotype 1/2a (19111) and 4b (19115). All strains were maintained and kept frozen at -80°C in 40% glycerol. They were sub-cultured twice in 10 mL tryptic soy broth (TSB) (Alpha Biosciences, Inc., Baltimore, MD) at 37°C (*E. coli* and *Salmonella*) or 32°C (*L.monocytogenes*), for 24 hours prior to use.

2.3.3. Preparation of Inocula

The population of the inocula was confirmed by plating serial dilutions of the cultured pathogens in 0.1% peptone (Difco, Sparks, MD). After sub-culturing, the three previously mentioned foodborne pathogen strains were plated and grown overnight on tryptic soy agar

(TSA) (Alpha Biosciences, Inc., Baltimore, MD) at 37℃, 37℃, and 32℃, respectively, for enumeration.

2.3.4. Sample Preparation

Fresh red beetroot and garlic were purchased from a local retailer, (Hannaford, Old Town, ME) prior to processing. Only medium-sized, undamaged, and mold-free beetroots were selected for this project. Prior to cubing, the beetroots were submerged in a tub of tap water and hand scrubbed to remove any visible soil. The tops and bottoms of the beetroots were removed manually before cutting into halves with a clean knife. The beetroots were cubed using a Robot-coupe (CL 50 series E, Robot-coupe USA. Inc., Ridgeland, MS) and placed in a clean stainless-steel container. Garlic cloves were peeled and blended with a Supreme Juicer model 6001 (Acme, Leymone, PA) immediately before addition to beet kvass samples to minimize loss of volatile compounds.

For each sample, 300 g of red beetroot were weighed into each of nine separate sterile quart-sized mason jars along with non-iodized pickling salt (Morton Salt Inc., Chicago, IL) and blended garlic according to the formulae in Figure 2.1. After placing all the ingredients into the jars, deionized water was added to create a total mass of 850 g. The beet kvass samples were mixed manually by stirring with a sterile spoon until the pickling salt was completely dissolved.

After stirring, the samples were inoculated (Section 2.3.5) with target of 10-100 CFU/g of each pathogen. The jars were then covered with a silicon airlock lid (Siliware, China), and secured with a metal lid ring. The jars were placed in an incubator shaker (Innova 4000, New Brunswick Scientific Co., NJ) for 10 minutes at a speed of 100 rpm, at 22°C to ensure uniform mixing of inocula. The jars were then incubated in a bacteriological incubator at an average temperature of 22°C to maintain a uniform fermentation temperature.

When samples achieve the targeted pH, a sterile wire gauze was used to separate the solids from the liquid kvass. The liquid kvass was stored in another sterile jar and placed in the refrigerator at 4°C for 30 days before subsequent testing.

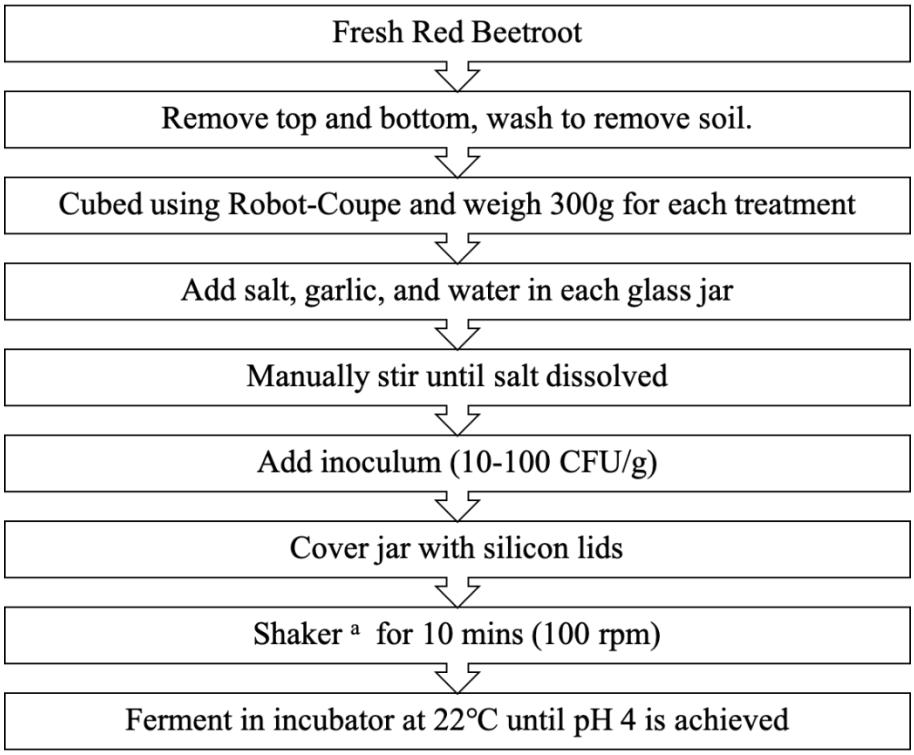

Figure 2.2. Preparation of beet kvass. Inoculation step was skipped for control samples (n=4)
[a] Incubator shaker (Innova 4000, New Brunswick Scientific Co., NJ)

2.3.5. Inoculation

After enumeration, a single colony of each bacterial pathogen was cultured in 10 mL of TSB in 15 mL conical centrifuge tubes (Fisher Scientific, Waltham, MA) and incubated at 37°C (*E. coli* and *Salmonella*) and 32°C (*L.monocytogenes*) for 24 hours. The tubes were centrifuged (Eppendorf 5810R, Hauppauge, NY) for 10 minutes at 15.550 x *g* and washed twice with 0.1% peptone. The similar, dual pathogen strains were then combined and diluted to 10^5 CFU in 1mL

of 0.1% peptone, and 0.1mL of the inocula was added into the jars of beet kvass to yield an inoculation of approximately 100 CFU/mL. The three bacterial genera were added separately.

2.3.6. Sample Collection and pH Analysis

Five mL of beet kvass were collected for pH analysis every 24 - 48 hours, as appropriate. First, jars of beet kvass were placed into the incubator shaker set at 100 rpm, for 5 mins before sampling to ensure homogeneity of microbiota. Sterile 10 mL Luer-Lok tip syringes (BD, Franklin Lakes, NJ) with 3-inch, 12-gauge hypodermic needles (Jorvet, Loveland, CO) were used to extract samples through the opening of the silicon top (Figure 2.3). Three milliliters were extracted and transferred into 15 mL conical centrifuge tubes for pH measurement. An additional 1.8 mL were extracted and transferred into 2 mL sterile centrifuge tubes for subsequent DNA extraction.

Figure 2.3. The opening on the silicon fermentation lid used for sample extraction to reduce the introduction of oxygen and contamination

An Edge Multiparameter pH meter with digital PEI body pH electrode (Hanna Instrument, Woonsocket, RI) was used for pH testing and was calibrated with pH 4 and 7 buffer solution prior to use on test days. The pH probe was cleaned with deionized water and 70% ethanol between samples.

2.3.7. Pathogen Detection

2.3.7.1. Enrichment

When the beet kvass samples achieved the target pH of ≤ 4.0, the presence of both healthy and injured bacterial cells were assessed for all inoculated pathogens. Kvass samples were strained to remove cubed beetroot. Twenty-five milliliters of the beet kvass were transferred into a stomacher bag along with 225 mL of multipathogen selective enrichment broth (SEL, a synthetic medium) for simultaneous detection of injured *Salmonella*, STEC, and *Listeria* cells (Suo & Wang, 2013). The composition of SEL was replicated from Kim and Bhunia (2008) as seen in Table 2.1. All antibiotics were diluted and filter sterilized with 0.25µm syringe filters (Midwest Scientific, MO). Antibiotics (Table 2.1) were added after 4 h of incubation at 37°C, and samples were left to incubate for another 16 h at 37°C. Separate enrichments were used after the first negative PCR test to validate the results. Enrichment with *Enterobactericeae* enrichment broth (EEB; Himedia Laboratories, Mumbai, India) at 37°C for the recovery of STEC and *Salmonella,* and buffered listeria enrichment broth (BLEB) were used at 32°C for the recovery of *L. monocytogenes*. The enrichment procedure is repeated with samples after 30 days of storage.

Table 2.1. Composition of SEL (*Salmonella, Escherichia, Listeria*) broth

Ingredients	Amount (g/L)	Comment
BLEB [a]	48.0	
Acriflavine [b]	0.01	Newly added; 0.5% (w/v) stock solutions in distilled water

Table 2.1. Continued

Cycloheximide [c]	0.05	Newly added; 1.0% (w/v) stock solution in 40% (v/v) solution of ethanol in water
Fosfomycin [d]	0.05	Newly added; 1.0% (w/v) stock solution in distilled water
Nalidixic Acid [e]	0.04	Newly added; 0.5% (w/v) stock solutions in distilled water

[a] Buffered listeria enrichment broth, BD Difco, Sparks, MD
[b] Acriflavine HCl, Sigma-Aldrich, St Louis, MO
[c] Cycloheximide, Millipore, Burlington, MA
[d] Fosfomycin disodium, Alfa Aesar, Haverhill, MA
[e] Nalidixic Acid (sodium salt), Sigma-Aldrich, St Louis, MO
Adapted from Kim and Bhunia (2008)

2.3.7.2. DNA Extraction

The extraction of genomic DNA was achieved using the Qiagen DNeasy PowerFood Microbial Kit (Hilden, Germany). After extraction, DNA samples were kept frozen (-20°C) prior to performing singleplex real-time PCR.

2.3.7.3. Real-time Polymerase Chain Reaction

The method of detection used in this experiment is based on a method developed by Bundidamorn et al. (2018) with the following modifications. Singleplex real-time PCR and melting temperature curve analysis were performed on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, USA). The reaction mixture for each real-time PCR tube is listed in Table 2.2.

Table 2.2. Reaction setup

Component	Volume (µL)
1X Precision Melt Supermix	5
Forward primer [a]	1
Reverse primer [a]	1

Table 2.2 Continued

Nuclease-Free water	2
Sample DNA	1
Final Volume	10

[a] Final concentration of 0.1µM, 0.2µM, 0.4µM for *inv*A, *stx*1,2, and *hly*A, respectively

Precision Melt Supermix with EvaGreen (BIO-RAD, Hercules, CA, USA), one set of specific forward and reverse primers, and nuclease-free water (Thermo Fisher Scientific, Waltham, MA) were combined and transferred into a thin walled PCR tube. Then 1 µL of DNA samples were added. The specific primers used for pathogen detection are listed in Table 2.3. Negative control samples were included in each run to minimize the likelihood of false-negative results. The following PCR cycle program was used: 95 °C for 2 min, followed by 30 cycles of 95 °C for 10 s, 57 °C for 30 s, and 72 °C for 30 s. Fluorescence melting temperature curve analysis was performed from 60 to 95 °C with gradual temperature increments (the slowest possible ramp rate) of 0.1 °C/s to determine peak fluorescence change over time.

Table 2.3: Target genes and primers used for the singleplex real-time PCR detection of *Salmonella* spp., STEC, and *L. monocytogenes*

Pathogen	Target gene	Primer [a]	Sequence (5'→3')
STEC	*stx*1, *stx*2 [b]	F	TTGARCGAAATAATTTATATGTG
		R	ACGAAATCCCCTCTGTATCTGCC
Salmonella	*inv*A	F	TTGARCGAAATAATTTATATGTG
		R	ACGAAATCCCCTCTGTATCTGCC
L. monocytogenes	*hly*A	F	GGGAAATCTGTCTCAGGTGATGT
		R	CGATGATTTGAACTTCATCTTTTGC

[a] F, forward; R, reverse
[b] Stx1, 2- F is degenerate primer, R = A or G
Adapted from Bundidamorn et al. (2018)

For the optimization and specificity of singleplex real-time PCR, each bacterial pathogen (100 CFU) was enriched in SEL for 24 hours then extracted for the testing of target pathogens. To determine the survival of bacterial pathogens, singleplex real-time PCR and melt curve analysis were performed for each replicate after fermentation and after storage.

2.3.8. Statistical Analyses

Data were analyzed using R studio (Version 1.1.456, Boston, MA). Logistic regression test for binomial data was used to assess the significant ($p < 0.05$) differences among treatments for the presence and absence of target pathogens. One-way ANOVA was used to determine significant differences ($p < 0.05$) among treatments for pH levels.

2.4. Results

2.4.1. pH

The average time for all beet kvass treatments to achieve the target pH of 4.0 or lower was 2.83 days (Table 2.4.). Salt concentration did not significantly contribute to the acidification of the beet kvass samples. However, the presence of garlic, regardless of the level, significantly ($p < 0.05$) decreased the fermentation time to an average of 2.13 days. The average starting pH was 5.54 ± 0.13. The average pH after 30 days of refrigerated storage was 3.74 ± 0.08. Samples with the addition of garlic, regardless of the level, had a significantly ($p < 0.05$) lower pH post-storage (pH 3.70 ± 0.08). No significant interactions were found between salt and garlic.

Table 2.4. The average pH levels of beet kvass samples (n=36)

Salt concentration %	Garlic concentration %	pH	
		End of Production	End of Storage
0.5	0	4.01 ± 0.02	3.83 ± 0.07
	0.5	3.95 ± 0.05	3.73 ± 0.08
	1.0	3.97 ± 0.05	3.76 ± 0.03
1.5	0	3.96 ± 0.06	3.82 ± 0.08
	0.5	3.91 ± 0.09	3.68 ± 0.01
	1.0	3.87 ± 0.07	3.70 ± 0.05
2.5	0	3.97 ± 0.07	3.82 ± 0.07
	0.5	3.84 ± 0.03	3.69 ± 0.04
	1.0	3.87 ± 0.05	3.66 ± 0.06

2.4.2. Pathogen Detection

2.4.2.1. Primer Specificity and Optimization

To validate the specificity of the primers and selective enrichment media chosen, low levels of inoculum (100 CFU) were enriched in SEL, then, singleplex and multiplex real-time PCR assays were performed. The genes targeted by the species-specific primers can be distinguished by their melting curves. The melting curve was obtained during PCR by monitoring the fluorescence of dsDNA-binding dyes as the temperature increased through the denaturation temperature. The melting curve temperature, T_m, is the peak of the negative derivative of fluorescence with respect to temperature ($-dF/dT$ vs T) as seen in Figure 2.4. Due to the close melting temperature of the target genes *inv*A and *stx*1,2, the melting curve analysis was unable to discriminate between those peaks in multiplex real-time PCR. Therefore singleplex real-time PCR was used for subsequent testings. The presence of the target pathogens were determined by T_m of target genes along with the exponential amplification before 25 annealing cycles.

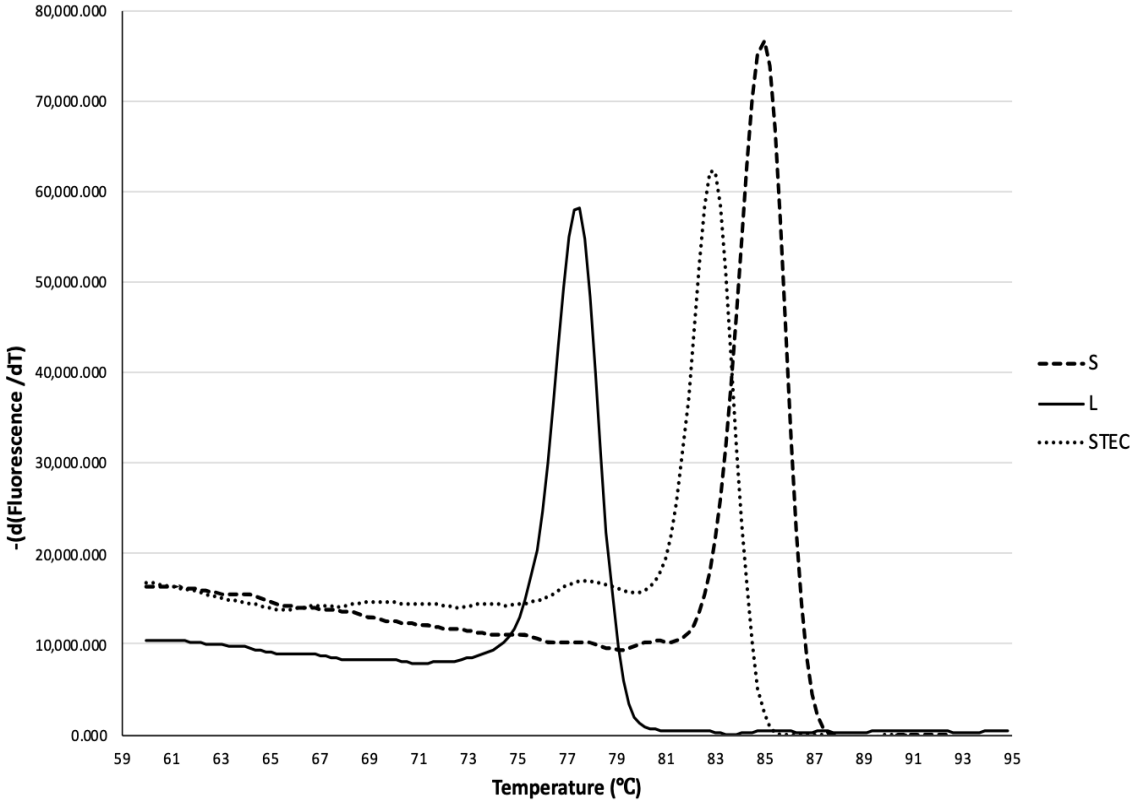

Figure 2.4. Melting temperature curve analysis for the detection of *E. coli* O26:H11 and *E. coli* O111:H8 (STEC), *Salmonella* Enteritidis and *Salmonella* Typhimurium (S), and *L. monocytogenes* 4b and *L. monocytogenes* 1/2a (L). Peaks obtained from target genes: STEC (*stx*1 and *stx*2; $T_m = 83.11 \pm 0.15$°C), *Salmonella* (*Inv*A; $T_m = 85.35 \pm 0.16$°C), and *L. monocytogenes* (*hly*A; $T_m = 77.74 \pm 0.21$°C)

2.4.2.2. Survival of Shiga Toxin-Producing *Escherichia coli* (STEC)

The number of samples with STEC recovery is recorded in Table 2.5. During beet kvass production, salt and garlic concentrations were observed to have no significant ($p \geq 0.05$) effect on the survival of STEC. However, salt concentration had a significant ($p < 0.05$) effect on the survival of STEC during storage, as a higher salt concentration was associated with a higher survival rate after 30 days of storage.

Overall, salt concentration had a significant effect during storage. There was a decrease in survival during production (81%) and after storage (64%), but the difference in survival between those two days was not statistically significantly different from each other. No significant interactions were found between salt and garlic.

Table 2.5. Beet kvass samples with STEC bacteria that were recovered by enrichment and qPCR (n = 36)

Salt Concentration %	Garlic concentration %	No. of isolates recovered[a]	
		Production	Storage
0.5	0	2/4	3/4
	0.5	4/4	1/4
	1.0	3/4	1/4
1.5	0	3/4	2/4
	0.5	4/4	2/4
	1.0	3/4	3/4
2.5	0	4/4	3/4
	0.5	4/4	4/4
	1.0	2/4	4/4

[a] Values are number of positive samples/number of replications
Detection limit 1 CFU/g

2.4.2.3. Survival of *Salmonella*

The number of samples with *Salmonella* recovery is shown in Table 2.6. Like STEC, salt and garlic treatments had no significant ($p \geq 0.05$) effect on the survival of *Salmonella* during beet kvass production and after storage. However, no *Salmonella* was recovered from the 1.5% salt treatment after storage, though this trend was not observed in other salt concentrations. A significant ($p < 0.05$) decrease of 77% in survival rate was observed after storage. There was a recovery rate of 72% (26 of 36 samples) post-fermentation and only 17% (6 of 36 samples) following storage. Overall, the higher the beet kvass pH level, regardless of treatment, resulted in a higher likelihood of survival. No significant interactions were found between salt and garlic.

Table 2.6. Beet kvass samples with *Salmonella* bacteria that were recovered by enrichment and qPCR (n = 36)

Salt Concentration %	Garlic concentration %	No. of isolates recovered[a]	
		Production	Storage
0.5	0	1/4	1/4
	0.5	3/4	1/4
	1.0	4/4	1/4
1.5	0	2/3	0/4
	0.5	3/4	0/4
	1.0	2/4	0/4
2.5	0	4/4	0/4
	0.5	3/4	2/4
	1.0	4/4	1/4

[a] Values are number of positive samples/number of replications
Detection limit 1 CFU/g

2.4.2.4. Survival of *Listeria monocytogenes*

The number of samples with *L. monocytogenes* recovery is shown in Table 2.7. Similar to STEC and *Salmonella*, salt and garlic treatments had no significant ($p \geq 0.05$) effect in the survival of *L. monocytogenes* during beet kvass production and after storage. *L. monocytogenes* had a survival rate of 78% (28 of 36 samples) after fermentation, and a decreased survival rate of 31% (11 of 36 samples) after storage. A significant ($p < 0.05$) decrease of 61% in survival rate was observed from production to storage. Like the other bacteria used in this analysis, overall, a higher product pH level was associated with higher survival rate. No significant interactions were found between salt and garlic.

Table 2.7. Beet kvass samples with *L. monocytogenes* bacteria that were recovered by enrichment and qPCR (n = 36)

Salt Concentration %	Garlic concentration %	No. of isolates recovered[a]	
		Production	Storage
0.5	0	3/4	1/4
	0.5	4/4	1/4
	1.0	3/4	1/4
1.5	0	3/4	1/4
	0.5	3/4	1/4
	1.0	3/4	2/4
2.5	0	3/4	0/4
	0.5	3/4	2/4
	1.0	3/4	2/4

[a] Values are number of positive samples/number of replications
Detection limit 1 CFU/g

2.5. Discussions

2.5.1. pH

Salt concentration did not affect the acidification of the beet kvass in this study. This trend was previously reported in a study which assessed the impact of salt reduction in fermented sauerkraut (Khanna, 2019). The addition of garlic slightly reduced the fermentation time, and resulted in lower pH products after storage. Although it was not examined in this study, garlic was observed to contribute to higher populations of lactic acid bacteria in fermented cabbage kimchi, and served as a carbohydrate source for LAB in a fermented fish product (Lim et al., 2015; Paludan-Müller et al., 1999). Hence, it is possible the addition of crushed garlic served the same role in this study, or that its antimicrobial activity contributed to the suppression of competing microflora.

2.5.2. Pathogen Detection

2.5.2.1. Shiga Toxin-Producing *Escherichia coli* (STEC)

The determination of the survival of bacterial pathogens is based on the presence or absence of both injured and healthy cells. Due to the potential recovery and possibility of becoming viable in a favorable environment, it is important to analyze both injured cells and healthy counterparts (Wu, 2008). Therefore, samples were first treated with an enrichment before DNA extraction and PCR. This challenge study was conducted with low levels of inoculum (10 - 100 CFU/g) due to the expected low level of pathogens on commercial vegetables, as well as the low infectious dose of bacterial pathogens STEC (< 10 CFU/g), *Salmonella* (10 - 100 CFU/g), and *L. monocytogenes* (unknown) (Kapperud et al., 1990; NACMCF, 1991; Hara-Kudo & Takatori, 2011). Therefore, the presence of the target bacterial pathogens, regardless of quantity, was considered sufficient to be considered a safety risk to consumers.

In this study, garlic did not have any effects on the survival of pathogens despite possessing antibacterial activity. This could be a result of the highly volatile nature of allicin, the antibacterial chemical found in garlic (Fujisawa et al., 2008). Besides its instability, allicin is often used in the form of a garlic extract, rather than crushed garlic. Therefore, the concentration of allicin present in crushed garlic may not be as effective in fermented beet kvass.

Acid stress during fermentation allows H^+ ions to cross bacterial cell membranes, creating a more acidic intracellular pH level (Booth, 1985). This low intracellular pH environment leads to an altered cell membrane structure with decreased activity of pH-sensitive enzymes (Davidson & Taylor, 2007). Likewise, undissociated organic acids can diffuse across the bacterial cell membranes and lower the internal pH upon dissociation in the cytoplasm. The pH equilibrium shift, which is common in fermented vegetables such as beet kvass, ultimately results in acid injury (Wesche et al., 2009).

A higher survival rate of STEC at higher salt concentration was observed, despite the acidic conditions. Studies have investigated the cross-protection that NaCl may provide to *E. coli* against acid stress. Chapman et al. (2006) reported that the coupling of Na^+ import to H^+ export permits STEC to maintain its internal pH, and allows for a longer survival period in acetic acid-based sauces. The researchers also found that water loss from the cytoplasm induced by salt resulted in a reduced cytoplasmic cell volume. This decrease in volume is believed to effectively concentrate the cytoplasmic constituents, and thereby raise the internal pH of the cell, allowing STEC to survive in an acidic environment, such as fermented beet kvass. This observation is further supported by Lee and Kang (2016) who observed an increase in the cytoplasmic pH of *E.coli* O157:H7 after the addition of salt in laboratory media, due to a perceived enhanced

balance of cytoplasm pH following exposure to organic acids. *E. coli* can use NaCl to counteract acidification of the cytoplasm by organic acids (Casey & Condon, 2002).

These studies offer potential explanations for why the survival rate of STEC at 2.5% NaCl beet kvass treatments did not significantly decrease after 30 days of storage, despite the low pH (3.72 ± 0.09) and low temperature (4°C). The ability of STEC to tolerate acidic environments has led to previous *E. coli* O157:H7 outbreaks, which include the consumption of contaminated apple cider (pH 3.7 - 3.9) and fermented dry salami (pH 5.0) (Besser et al., 1993; CDC, 1995). Therefore, the potential survival of STEC in fermented vegetable products, such as beet kvass, may present health risks to consumers if it present. Moreover, this pathogen is known to be transmitted by contaminated soil, having caused several high profile outbreaks associated with consumption of uncooked produce such as bagged salad, romaine lettuce, and spinach (CDC, 2006; Marder et al., 2014; Bottichio et al., 2019).

2.5.2.2. *Salmonella*

Our results show that *Salmonella* was the pathogen most adversely affected by the increase in acidity during storage compared to the other two pathogens we studied. This was also observed in a previous study that evaluated the effect of different pH conditions on the acid resistance of *L. monocytogenes*, *E.coli* O157:H7, and *S.* Typhimurium. When subjected to acidified laboratory media (pH 3.5), *S.* Typhimurium was significantly more sensitive to the acid treatment than the other two foodborne pathogens (Koutsoumanis & Sofos, 2004). Similarly, *S.* Enteritidis had the shortest survival (21 days) period in fermented green table olives brine (pH 4.2; 6% NaCl), compared to *E.coli* O157:H7 (27 days) and *L. monocytogenes* (31 days) (Argyri et al., 2013).

However, *Salmonella* was still recovered from several beet kvass samples despite the low acid and cold storage. This finding was also observed in other fermented vegetable products. For example, *S.* Enteritidis was able to survive in an environment of pH 4.2 and high salt concentration (6.0%) for up to 21 days of storage in fermented green table olives in brine (Argyri et al., 2013). *Salmonella* Typhimurium was also capable of surviving low pH levels (pH 3.8 - 3.9) in spontaneously fermented cauliflower (8% w/v NaCl). Although this study did not explore the effect of storage at low temperatures, the authors thought that survival was facilitated by the gradual decrease of pH, from pH 6.05 to pH 3.8 over 12 days, which could have activated an acid adaptive response (Paramithiotis et al., 2012).

Although *Salmonella* was proven to have higher sensitivity to acid, salt, and low temperature storage in this study, compared to the other two pathogens we studied, *Salmonella* still persisted and survived in our beet kvass samples. Hence, it is crucial that proper hygiene and sanitation practices are followed during processing. After 30 days of refrigerated storage, the survival of *Salmonella* was reduced significantly, with no detection in the 1.5% salt treatments, but viable cells were detected in other treatments. This could imply the higher salt concentration (2.5%) may have induced cross protective responses in *Salmonella*, while lower salt concentrations did not. However, *Salmonella* still survived in the 0.5% salt samples. The possible explanation for this observation is that 0.5% NaCl is too low to reduce the survival of *Salmonella* despite the low pH levels. Therefore, it is important to conduct further studies which are aimed to observe the protective response of *Salmonella*.

2.5.2.3. *Listeria monocytogenes*

Listeria monocytogenes presents many challenges to the food industry due to its wide distribution in the environment and resistance to diverse environmental conditions, including

being capable of growth at temperatures as low as -0.4°C (Walker et al., 1990; Ferreira et al., 2014). *Listeria monocytogenes* has been shown to survive at pH values as low as 3.8 (Sorrells et al., 1989). However, in a fermented food application, lactic acid produced by lactic acid bacteria (LAB) during fermentation may inhibit the growth or survival of *L. monocytogenes*. Conner et al. (1986) observed no viable cells of *L. monocytogenes* in salted cabbage juice (1.5 - 4.0% NaCl) after 15 days of incubation at 30 ℃, and cabbage juice supplemented with lactic acid (pH < 4.2) had a complete inactivation of *L. monocytogenes* after 8 days of incubation at 5℃. Although the study did not explore the combined effects of salt, pH, and temperature, it showed the potential for inactivation of *L. monocytogenes* under conditions such as low salt, and high acid with low temperature. Moreover, in a study of low salt sauerkraut (1.0 - 2.5% NaCl; pH < 3.70), *L. monocytogenes* population decreased over time and was absent after 6 days of fermentation and after 1 week of refrigerated storage (Khanna, 2019).

Nonetheless, this pathogen has demonstrated survival in naturally-fermented black olives (pH 3.95; NaCl 6.05%) (Grounta et al., 2013). In this study, survival in beet kvass samples occurred in the lowest salt treatment (0.5% NaCl), and at a pH level of ≤ 4.0, despite 30 days of refrigerated storage. This could be the result of a slightly higher pH level (pH 3.74), compared to other studies on fermented vegetables (pH 3.5 - 3.7), along with this organism's natural tolerance for low temperature (as a psychrotroph), which was also observed in the study by Conner et al. (1986). The survival of this pathogen is a safety risk specifically due to the unknown infectious dose of *L. monocytogenes* and the severity of the resulting illness. Listeriosis in animal models has occurred with doses as high as 10^9 and as low as 10 cells (NACMCF, 1991). Therefore, the complete inactivation of this pathogen is necessary, and it is required by to be absent in all ready-to-eat foods (USDA-FSIS, 2014).

Raw vegetables used for the production of fermented vegetable products could be contaminated at the farm and may serve as vehicles for transmission of pathogenic bacteria to consumers (Berger et al., 2010). Moreover, there is potential for foodborne pathogens, such as *L. monocytogenes* and STEC, to persist and proliferate on vegetable leaves and roots, such as red beetroot used to prepare beet kvass (Feng et al., 2014; Kljujev et al., 2018). Therefore, it is important to consider proper hygiene and sanitation practices for commercial processors and home fermenters, which include adequate cleaning of utensils and food contact surfaces, and proper washing of produce when producing fermented products. Good manufacturing and sanitation practices help to prevent or reduce the risk of pathogenic bacteria from contaminating fermented food products and reducing the risk of foodborne illness.

A possible approach to decreasing the safety risk of bacterial pathogens is by applying a sanitizer wash on raw red beetroots before production. The usage of sodium hypochlorite at 50 - 200 ppm concentration with a contact time of 1 - 2 mins have been used to sanitize produce surfaces (Parish et al., 2003). Although this method would decrease the load of native LAB on the surface of raw beetroot and increase fermentation time, spontaneous fermentation would occur due to the favorable environment provided by the formulation of beet kvass.

2.6. Conclusions

In this study, neither salt concentration (0.5%, 1.5%, and 2.5% NaCl) nor presence or concentration of garlic (0.0%, 0.5%, and 1.0 %) significantly affected the survival of STEC, *Salmonella,* or *L. monocytogenes* during the fermentation of spontaneously fermented beet kvass. Additionally, our study also analyzed the survival of these pathogens after 30 days of refrigerated (4°C) storage. Among the three pathogenic bacteria, STEC was the most acid resistant, with an increased resistance with the highest salt concentration during storage.

Refrigerated storage did not have a significant effect on the STEC, although there was a 21% decrease in survival from day 0 (81%) to storage day 30 (64%). These results indicate a potential risk of foodborne illness, irrespective of salt level, primarily because of the low infectious dose of STEC.

In contrast, *Salmonella* was the least acid resistant, with a 72% (26 of 36 samples) survival rate during production, and 17% (6 of 36 samples) following storage. Complete eradication of *Salmonella* after refrigerated storage was observed in beet kvass prepared with 1.5% NaCl. *L. monocytogenes* had a survival rate of 78% (28 of 36 samples) after fermentation, and a decreased survival rate of 31% (11 of 36 samples) following storage.

Overall, these results indicate that although fermented foods are typically considered safe, it is crucial to weigh the health benefits against the risks of foodborne illness as a result of their consumption. Based on this study, there is a potential risk for STEC, *Salmonella,* and *L. monocytogenes* survival during fermentation of beet kvass, regardless of low salt concentrations or garlic concentrations. These findings reinforce how important proper hygiene and sanitation practices must be followed to avoid contamination of beet kvass with pathogenic bacteria that may be present in raw materials or result from cross-contamination from the environment. Though survival of all pathogens decreased after 30 days of refrigerated storage, the survival rates were still considerably high, and it is not advised that fermentation be considered a sole method to ensure product safety. Therefore, to decrease the bacterial pathogen safety risk, it is recommended to apply a sanitization step before production.

CHAPTER 3

EFFECTS OF SALT AND GARLIC CONCENTRATION ON THE BIOCHEMICAL PROPERTIES OF SPONTANEOUSLY FERMENTED BEET KVASS DURING PRODUCTION AND STORAGE

3.1. Abstract

Fermentation is the process by which carbohydrates are converted into several byproducts, including acid, ethanol and/or gas by microorganisms. Often these byproducts result in the production of desirable food with unique flavor attributes. This is primarily achieved by lactic acid bacteria (LAB), which are organisms that are ubiquitous in the environment. Vegetables such as red beetroot are suitable commodities for fermentation, due to availability of sugar and nutrient sources to sustain LAB growth. However, this growth may also lead to the production of harmful biogenic amines, specifically histamine and tyramine. The present study was designed to determine the effects of salt and garlic levels on the production of organic acids, sugar, ethanol, and biogenic amines in fermented beet kvass during fermentation and storage. Our results indicate that both the salt and garlic concentration have significant effects on organic acids and fructose content. The addition of garlic in beet kvass produced samples with lower lactic acid, acetic acid, glucose, and fructose content, but higher ethanol levels. This biochemical profile suggests that garlic favors the growth of yeast in kvass. Although not explicitly analyzed in our study, these observations demonstrate the likely effects of formulation variability on microbial diversity among the treatments. Agmatine (< 0.015 mg/mL), putrescine (< 0.018 mg/mL), tyramine (< 0.018 mg/mL), cadaverine (< 0.011 mg/mL), and histamine (< 0.018 mg/mL), the targeted biogenic amines analyzed in beet kvass, were all below detection limits, regardless of formulation. Therefore, an accumulation of these compounds in a fermented beet

kvass beverage during production and after storage is not expected to pose obvious safety risks to consumers.

3.2. Introduction

Red beetroot is a root vegetable rich in carbohydrates, protein, micronutrients, and several functional constituents that have substantial health-promoting properties. Nutritionally, it is known as an important source of natural antioxidants and contains a considerable amount of both essential and non-essential amino acids (Nemzer et al., 2011). The vegetable has also been associated with a range of health benefits such as lowering hypertension, enhancing exercise performance, and benefiting cardiovascular health (Kapil et al., 2015; Jones, 2014; Kapil et al., 2014). As a result, beetroot-based products are currently under development and have been used as health supplements (Morgado et al., 2016; Panghal et al., 2017; Domínguez et al., 2018).

Lactic acid fermentation of vegetables has been found to improve sensory properties, extend shelf life, and increase the bioavailability of nutrients (Tamang et al., 2016; Septembre-Malaterre et al., 2018). Beet kvass is a fermented vegetable beverage made by infusing red beets in water and salt. Given the health implications associated with high salt intake, a recent assessment found that 20% (59/295) of surveyed Maine home fermentors feel that making low-sodium fermented food formulations is very or extremely important to them (Camire et al., 2019). Salt concentration, fermentation time, and manufacturing process are critical factors which affect the bacterial community composition in fermented kimchi (Lee et al., 2017). There are currently no previous works which assess the biochemical properties of fermented beet kvass. Concentrations of byproducts of fermentation such as organic acids, sugars, and alcohol may reveal the microbial dynamics in a fermented product. Therefore, it is important to determine the

effects of formulation on the biochemical properties of beet kvass, which may provide initial information on the microbial dynamics of this product.

Biogenic amines (BA) are the byproducts of the metabolism of proteins by LAB and other microorganisms that possess the appropriate decarboxylase enzymes. BA are often found in fish, fish products, fermented foods, and alcoholic beverages (Doeun et al., 2017). Histamine and tyramine specifically have been related to safety risks such as histamine poisoning and adverse interaction with monoamine oxidase inhibitors (MAOIs), drugs used for treatment of depression (ten Brink et al., 1990; McCabe-Sellers et al., 2006). LAB activity during fermentation may lead to accumulation of these compounds, which presents a safety concern for consumers of fermented vegetables. Fermented cabbage, for example, was found to have a higher tyramine concentration (4.9 mg/100 g) when compared to fresh cabbage (0.3 mg/100 g) (Moret et al., 2005). Therefore, it is important to assess the biogenic amines in fermented beet kvass.

Thus, the objective of this study was to analyze the effects of salt concentration and inclusion of garlic on the biochemical properties, including fermentable sugars (glucose, fructose and sucrose), organic acids (lactic acid and acetic acid), and biogenic amines (agmatine, putrescine, tyramine, cadaverine, and histamine), during the production and after storage of spontaneously fermented beet kvass.

3.3. Materials and Methods

3.3.1. Experimental Design

Fermented beet kvass was produced at three different NaCl concentrations (0.5%, 1.5%, and 2.5% (w/w)) along with three different garlic concentrations (0.0%, 0.5%, and 1.0% (w/w)),

in a full factorial design, to determine the effects of these formulation variables on the organic acids, sugars, alcohol, and biogenic amine presence in the finished product. The experiment was independently replicated in triplicate. The pH of the samples was tested every 24 - 48 h until samples reached the target pH of ≤ 4.0. Aliquots of beet kvass treatments were syringe-filtered (see below) and used for subsequent HPLC analyses as described in section 3.3.3.

3.3.2. Sample Preparation

Beet kvass samples were prepared as described previously (Section 2.3.4), but without inoculation. A summary of the preparation procedures is presented in Figure 3.1.

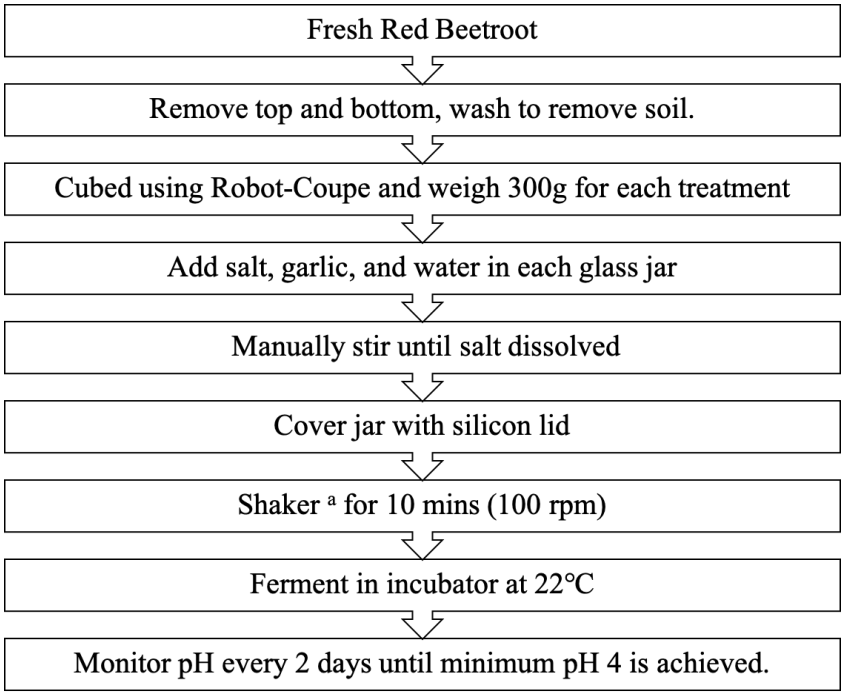

Figure 3.1. Beet kvass preparation steps (n= 3)
[a] Incubator shaker (Innova 4000, New Brunswick Scientific Co., NJ)

3.3.3. Sample Collection

A total of 7 mL of beet kvass was collected from each sample for pH and biochemical analyses. Three milliliters of the extracted sample was transferred into 15 mL sterile conical

centrifuge tubes for pH measurement. The remaining 4 mL were filtered with a 0.45µm nylon syringe filter (MDI Membrane, Harrisburg, PA), evenly divided into two separate 2 mL sterile centrifuge tubes and stored at -80℃ for subsequent biochemical analyses.

3.3.4. pH Analysis

pH analysis was repeated in accordance with section 2.3.7. Once the samples achieved the targeted pH of 4.0 or lower, samples were strained. The kvass was transferred into a clean 1-quart mason jar and stored under refrigeration (4℃) for 30 days. After 30 days of storage the testing was repeated.

3.3.5. High Performance Liquid Chromatography (HPLC) Analyses

3.3.5.1. Chemicals

The chemicals used for preparation of the standard stock solutions are listed as follows: D- Fructose (Specturm, Gardena, CA), glucose, sucrose, putrescine, agmatine, tyramine, cadaverine, DL-histamine (all purchased from Sigma, St Louis, MO), acetic acid and lactic acid (Fisher Scientific, PA), and ethyl alcohol 200-proof (Pharmco, Brookfield, CT).

3.3.5.2. Equipment

An Agilent Technologies (Santa Clara, CA) model 1100/1200 HPLC system with a Agilent Technologies degasser (1100 series), thermostatted column compartment (1200 series), refractive index detector (RID) (1200 series), and fluorescence detector (FLD) (1100 series) were used for this study. The HPLC system also included a 1100 series pump and autosampler. To obtain data readings, a Dell Optiflexx 755 formatted with Windows XP Professional, version 5.1.2600 was used with this system. The system was controlled byt and data collected using ChemStation Software for LC by 3D Systems, version 8.0401(481) (Agilent Technologies).

3.3.5.3. Standard Preparation

For HPLC calibration and performance evaluation, sugar standard solutions (fructose, glucose, and sucrose) and organic acids (acetic and lactic acid) were prepared by diluting the appropriate weight of each standard in 0.005N H_2SO_4 (mobile phase). Biogenic amine (agmatine, putrescine, tyramine, cadaverine, and histamine) standards were prepared by diluting as appropriate in HPLC grade water, then applying the Waters AccQ ultra derivatization kit (Waters, MA, USA). A 3-point standard curve was produced for each of the targeted analytes.

3.3.5.4. Sample Preparation

All kvass samples were thawed at 4°C overnight prior to analyses. Using a Pasteur pipette, ~ 1 mL of thawed sample was transferred into a small sample vial for HPLC analysis of sugars, organic acid, and alcohols. A separate set of samples were derivatized for biogenic amines analysis with amino acid derivatization kit. Each peak was integrated to determine peak area, and analyte concentrations were calculated by comparing sample/standard area ratios.

3.3.5.5. Chromatography Conditions

A refractive index detector (RID; Agilent Technologies 1200 Series) was used in this study for the analysis of organic acids, sugars, and alcohol. A flow rate of 0.6 mL/min with a run time of 25 minutes was used and 20 μL of sample or standards were injected into the system. The mobile phase was 0.005N H_2SO_4 dissolved in HPLC-grade water. The column used was a Hi-Plex, 300 x 6.5 mm with a 5 x 3 Hi-Plex H guard column (Agilent Technologies) operated at 35°C. Triplicate readings were averaged, and the concentration of targeted chemicals were reported in mg/mL.

A FLD (Fluorescence Detector, Agilent Technologies 1100/1200) was used to analyze the biogenic amine composition of the samples. A flow rate of 1.5 mL/min with a run time of 45

min was used and 10 µL of both sample and standards were injected into the system. The mobile phase was AccQ-Tag Eluent A (Waters, Milford, MA) diluted with HPLC-grade water according to manufacturer instructions. An AccQ-Tag Column (Waters, Milford, MA) operated at 37°C was also used. Triplicate readings were averaged, and the concentration of targeted chemicals were reported in mg/mL.

3.3.6. Statistical Analyses

Data were analyzed using R studio (Version 1.1.456, Boston, MA). The normality of the variables was assessed with Shapiro-Wilk normality test. When the variables were not found to be normal, Q-Q plot was used to determine the outliers. Two outliers were removed from variables (glucose, sucrose, fructose, ethanol) before any further statistical testing. One-way analysis of variance (ANOVA) was conducted to determine significant differences among salt and garlic interaction, at the start of fermentation (sucrose only), post-production, and after 30 days of storage, on observations and increases in metabolites. Tukey's Honest Significant Difference (HSD) test was selected for post-hoc analyses to determine significant differences among means. A significance level of $p < 0.05$ was chosen for all statistical analyses.

3.4. Results

3.4.1. pH

The average pH of all samples at the start of production was 5.61 ± 0.18. The pH of the samples decreased as they fermented yielding an average pH of 3.93 ± 0.07 at the end of fermentation. The post-storage samples had a significantly ($p < 0.001$) lower pH of 3.76 ± 0.05, compared to the samples post-fermentation. Salt concentration did not have a significant effect on the acidification of beet kvass.

Garlic concentration had a significant effect ($p < 0.05$) on the lactic acid to acetic acid ratio in the beet kvass samples. Samples with no garlic, in all salt levels, had a significantly lower lactic:acetic acid ratio compared to the samples with garlic, regardless of levels. Garlic concentration also had a significant effect ($p < 0.05$) on the days needed to complete the fermentation (defined as reaching pH ≤ 4.0). The addition of garlic, regardless of level, decreased the total time, from an average of 4.11 ± 0.78 days to 2.67 ± 0.97 days, as seen in Table 3.1. No significant interactions were found between salt and garlic concentrations.

Table 3.1. The average fermentation time and acid ratio for beet kvass samples at the end of fermentation

Garlic (%)	Salt (%)	Time (Days)	Acid ratio (Lactic:Acetic acid)
0	0.5	4.0 ± 1.0^{a}	5.4 ± 1.0^{a}
	1.5	4.0 ± 1.0^{a}	5.6 ± 1.3^{a}
	2.5	4.3 ± 0.6^{a}	5.2 ± 0.9^{a}
0.5	0.5	2.7 ± 1.2^{b}	6.8 ± 1.4^{b}
	1.5	2.7 ± 1.2^{b}	6.9 ± 1.0^{b}
	2.5	2.7 ± 1.2^{b}	7.7 ± 1.3^{b}
1.0	0.5	2.7 ± 1.2^{b}	7.1 ± 1.3^{b}
	1.5	2.7 ± 1.2^{b}	7.6 ± 0.1^{b}
	2.5	2.7 ± 1.2^{b}	8.7 ± 0.9^{b}

n =3, end of fermentation defined as achievement of pH ≤ 4.0.
Superscripts indicate significant differences across treatments within column

3.4.2. Biochemical Analysis

3.4.2.1. Standards

Three-point standard curves were prepared for each targeted compound. The retention time along with the line equation for each target compound are shown in Tables 3.2 and 3.3.

Table 3.2. Retention times and response curves of organic acid, sugar, and alcohol standards

Target compound	Retention time (mins)	Response curve
Lactic Acid	10.26	$y = 142151x + 977.27$
Acetic Acid	12.12	$y = 293362x - 1593.4$
Glucose	7.43	$y = 315086x - 22967$
Fructose	8.02	$y = 310488x - 21972$
Sucrose	6.49	$y = 312012x - 10930$
Ethanol	15.9	$y = 126986x + 2480.6$

Based on three point standard curve

Table 3.3. Retention times and response curves of selected biogenic amines

Target compound	Retention time (mins)	Response curve
Agmatine	22.90	$y = 30605x - 392.13$
Putrescine	31.87	$y = 67123x - 769.28$
Tyramine	30.41	$y = 23406x - 731.04$
Cadaverine	32.53	$y = 23470x - 1713.9$
Histamine	20.97	$y = 30849x - 246.81$

Based on three point standard curve

3.4.2.2. Organic Acids

The organic acids quantified in this study includes lactic and acetic acids. Time, salt, and garlic concentration all had significant ($p < 0.05$) effects on the lactic acid concentration of beet kvass. At the end of beet kvass fermentation, the lactic acid concentration for all samples ranged from 0.78 - 3.45 mg/mL, with the 0.5% NaCl (no garlic added) treatment having the highest average lactic acid concentration of 2.88 ± 0.28 mg/mL. By the end of refrigerated storage, the lactic acid concentration for all samples were higher and ranged from 1.42 - 4.19 mg/mL, with

the 0.5% NaCl (1.0% garlic) treatment having the highest average lactic acid level of 3.94 ± 0.28 mg/mL. Overall, lactic acid concentration was significantly ($p < 0.05$) lower in the highest salt concentration treatment (2.5%), compared to samples with the lowest salt concentration sample (0.5%). This trend had a significantly greater effect ($p < 0.001$) when controlling for garlic (0.5% and 1%), indicating a possible interaction between salt and garlic (Figure 3.2).

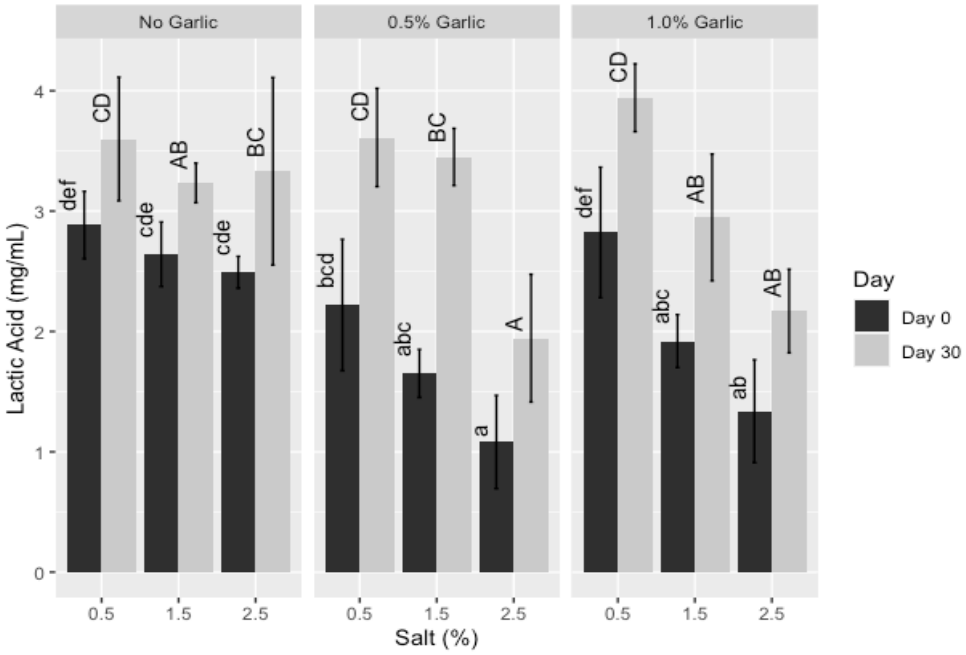

Figure 3.2. Mean lactic acid concentration (mg/mL) in beet kvass samples at the end of fermentation (Day 0; 22°C) and post-refrigerated storage (Day 30; 4°C).
Error bars denote standard deviation, n = 9, letters above bars indicate significant differences across treatments within time

The lactic acid concentration in samples post-storage was significantly higher ($p < 0.001$) than samples post-fermentation, regardless of treatment. When comparing the increase in the lactic acid concentration between treatments over time (Table 3.4), garlic concentration, but not salt concentration, had a significant effect. The increase in lactic acid between post-fermentation and post-storage samplings was significantly higher ($p < 0.05$) in samples formulated with 1.5% salt and 0.5% garlic, when compared to 1.5% salt and 1% garlic (Table 3.4).

Table 3.4. Average increase in organic acids in beet kvass samples after 30 days of refrigerated storage

Salt (%)	Garlic (%)	Lactic Acid (mg/mL ± SD)	Acetic Acid (mg/mL ± SD)
0.5	0	0.72 ± 0.26 ^{bc}	0.14 ± 0.06 ^{de}
	0.5	1.39 ± 0.38 ^{ab}	0.33 ± 0.09 ^{ab}
	1.0	1.12 ± 0.40 ^{ab}	0.29 ± 0.11 ^{abc}
1.5	0	0.59 ± 0.19 ^{bc}	0.11 ± 0.04 ^{e}
	0.5	1.80 ± 0.24 ^{a}	0.35 ± 0.10 ^{a}
	1.0	1.03 ± 0.48 ^{b}	0.24 ± 0.15 ^{abcd}
2.5	0	0.84 ± 0.72 ^{bc}	0.22 ± 0.07 ^{e}
	0.5	0.86 ± 0.35 ^{bc}	0.18 ± 0.09 ^{bcde}
	1.0	0.83 ± 0.21 ^{bc}	0.18 ± 0.08 ^{bcde}

n = 3, superscripts indicate the significant differences between treatments within acid type

Similar to the observed lactic acid trends, time (post-fermentation versus post-storage), salt and garlic concentration also had significant effects on the acetic acid concentration in samples. At the end of fermentation, the acetic acid concentration in beet kvass ranged from 0.10 - 0.59 mg/mL, with 0.5% NaCl (no added garlic) beet kvass again having the highest average acetic acid level of 0.54 ± 0.05 mg/mL. By the end of refrigerated storage, the acetic acid concentration for all samples ranged from 0.18 - 0.81 mg/mL, with the 0.5% NaCl (1.0% garlic) treatment having the highest average acetic acid level of 0.70 ± 0.16 mg/mL. Although the interaction between salt and garlic was not significant for this beet kvass model, acetic acid concentration after storage was significantly ($p < 0.05$) lower in samples with the higher salt concentrations (1.5 and 2.5%), compared to the 0.5% NaCl when treated with 1% garlic, as seen

in Figure 3.3.

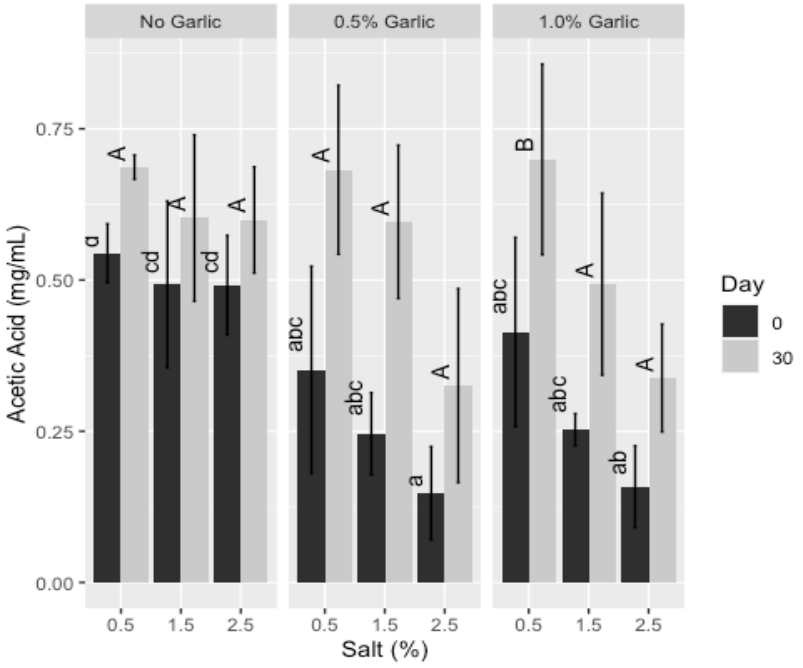

Figure 3.3. Mean acetic acid concentration (mg/mL) in beet kvass samples at the end of fermentation (Day 0; 22°C) and post-refrigerated storage (Day 30; 4°C).
Error bars denote standard deviation, n = 9, letters above bars indicate significant differences across treatments within time.

All beet kvass samples, post-storage, had a significantly ($p < 0.05$) higher acetic acid concentration compared to post-fermentation. This increase in acetic acid concentration over time was significantly greater ($p < 0.05$) in samples treated with garlic (0.5% and 1.0%) than samples with no garlic, when controlled for salt concentration (0.5% and 1.5%) (Table 3.4).

3.4.2.3. Sugars

The sugars quantified in this study includes sucrose, glucose, and fructose. Sucrose was present in all beet kvass samples after production with an average concentration of 2.99 ± 0.59 mg/mL. By the end of beet kvass fermentation, the sucrose concentration for all samples ranged from 0.90 - 18.98 mg/mL, with the 2.5% NaCl (no garlic) treatment having the highest average sucrose concentration of 10.08 ± 7.94 mg/mL. By the end of refrigerated storage, the sucrose

concentration for all samples ranged between 0.95 - 16.03 mg/mL, with the 1.5% NaCl (no garlic) treatment having the highest average sucrose level of 8.62 ± 6.56 mg/mL. However, two outliers were removed from further statistical analyses. The sucrose concentration in all samples post-fermentation and post-storage were higher than samples after production. However, no significant trends or differences in regards to time (post-fermentation versus post-storage), salt, or garlic concentration were observed.

In contrast, glucose was not detectable (detection limit of 0.07 mg/mL) on production day. By the end of beet kvass fermentation, the glucose concentration for all samples ranged from 0.07 - 1.66 mg/mL, with the 0.5% NaCl (no garlic) treatment having the highest average glucose concentration of 0.92 ± 0.71 mg/mL. By the end of refrigerated storage, the glucose concentration for all samples ranged from 0.07 - 1.44 mg/mL, with the 1.5% NaCl (no garlic) treatment having the highest average glucose concentration of 0.93 ± 0.30 mg/mL. Glucose concentration increased from production day to post-fermentation in the majority of samples, however, time did not have a significant effect on glucose levels in beet kvass samples. Differences in glucose concentration between day 0 (post-fermentation) and 30 (post-storage) were not significantly different within any individual treatment. Garlic concentration did have a significant effect ($p < 0.001$) on the glucose concentration. When samples were controlled for salt, those formulated with garlic (0.5% and 1.0%) had significantly lower glucose concentrations (Figure 3.4). No significant interaction was found between salt and garlic concentration. The change in glucose concentration over time did not differ significantly across treatments. The different salt and garlic concentrations did not have an effect on the change of

glucose concentration in beet kvass samples.

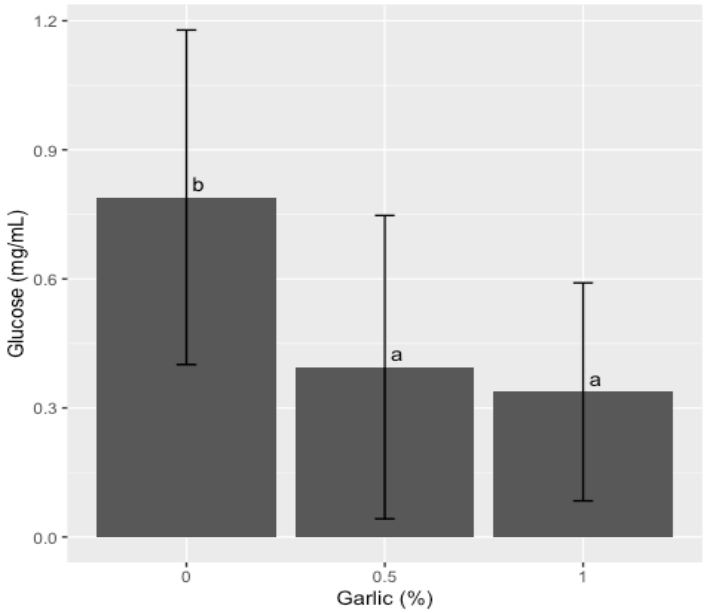

Figure 3.4. Mean glucose concentration (mg/mL) in beet kvass samples. Error bars denote standard deviation, n=18, letters above bars indicate significant differences across garlic treatments

Contrary to the sucrose results, time (post-fermentation versus post-storage), salt and garlic concentration did have a significant effect on sample fructose levels. Fructose was not detectable on production day, but by the end of beet kvass fermentation, the fructose concentration for all samples ranged from 1.17 - 10.89 mg/mL, with the 0.5% NaCl (no garlic) treatment, having the highest average fructose concentration of 7.55 ± 2.90 mg/mL (Figure 3.5). By the end of refrigerated storage, the fructose concentration for all samples ranged from 2.05 - 10.42 mg/mL, with the 0.5% NaCl (1% garlic) treatment, having the highest average fructose level of 8.15 ± 2.05 mg/mL. The samples treated with 2.5% salt (0.5% and 1.0% garlic added) had a significantly lower fructose concentration, compared to the samples treated with 0.5% salt

(no garlic). There was no significant interaction found between salt and garlic.

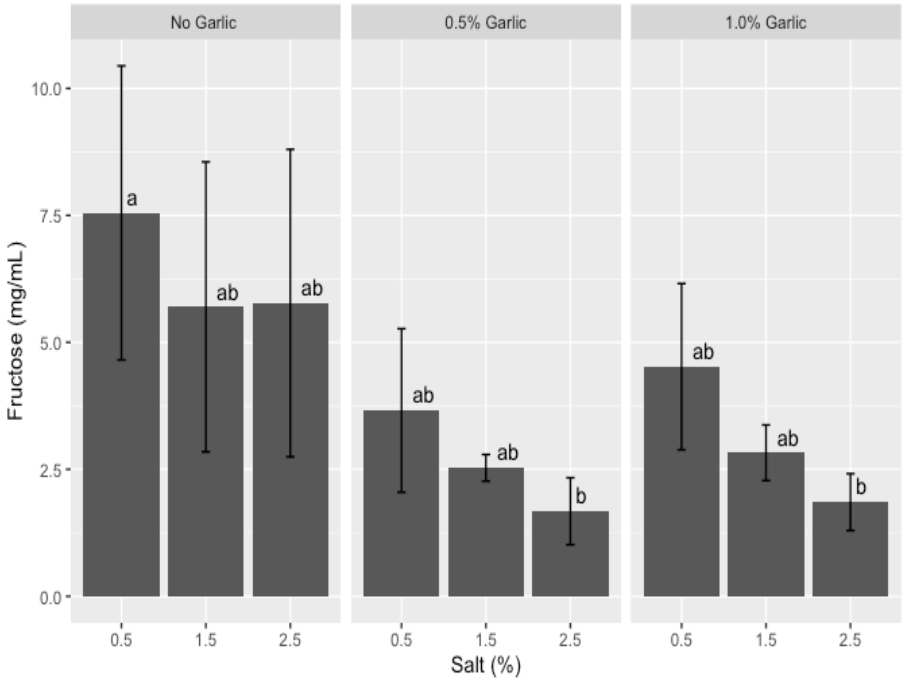

Figure 3.5. Mean fructose concentration (mg/mL) in beet kvass samples at the end of fermentation (Day 0; 22°C). Error bars denote standard deviation, n = 9, letters above bars indicate significant differences across treatment

As the samples fermented, fructose concentration increased in most samples. When comparing this increase in fructose levels over time, garlic had a significant effect (Table 3.5). Beet kvass samples formulated with garlic (regardless of level), at the lowest salt concentration (0.5%), had a significantly higher increase in fructose from post-fermentation to post-storage, than samples with no garlic added.

Table 3.5. Average fructose increase in beet kvass samples after 30 days of refrigerated storage

Salt (%)	Garlic (%)	Fructose (mg/mL ± SD)
0.5	0	1.53 ± 0.60* ^{de}
	0.5	3.71 ± 1.66 ^{ab}
	1.0	3.63 ± 1.95 ^{abc}
1.5	0	1.11 ± 0.52* ^{e}
	0.5	3.82 ± 1.10 ^{a}
	1.0	2.91 ± 1.47 ^{abcde}
2.5	0	0.99 ± 0.16 ^{e}
	0.5	1.81 ± 0.80 ^{bcde}
	1.0	1.74± 0.49 ^{bcde}

*n= 2, an outlier was omitted.
n = 3, superscripts indicate significant differences between treatments

3.4.2.4. Alcohol

Time (post-fermentation versus post-storage) and garlic concentration both had significant ($p < 0.001$) effects on beet kvass ethanol levels, while salt concentration did not have a significant effect. By the end of beet kvass fermentation, the ethanol concentration for all samples ranged between 0.10 - 0.48%, with the 0.5% NaCl (1% garlic) treatment having the highest average ethanol concentration of 0.42 ± 0.04%. By the end of refrigerated storage, the ethanol concentration for all samples ranged between 0.16 - 0.66%, with the 0.5% NaCl (1% garlic) treatment having the highest average ethanol concentration of 0.63 ± 0.02%. At the end of fermentation, when controlling for salt, samples treated with higher garlic concentration (1.0%) had significantly higher ethanol concentration, compared to lower (0.5%) and no garlic treatments. Over time, the ethanol appeared to accumulate in beet kvass samples, however, the

increase in ethanol over time was not significantly different among treatments.

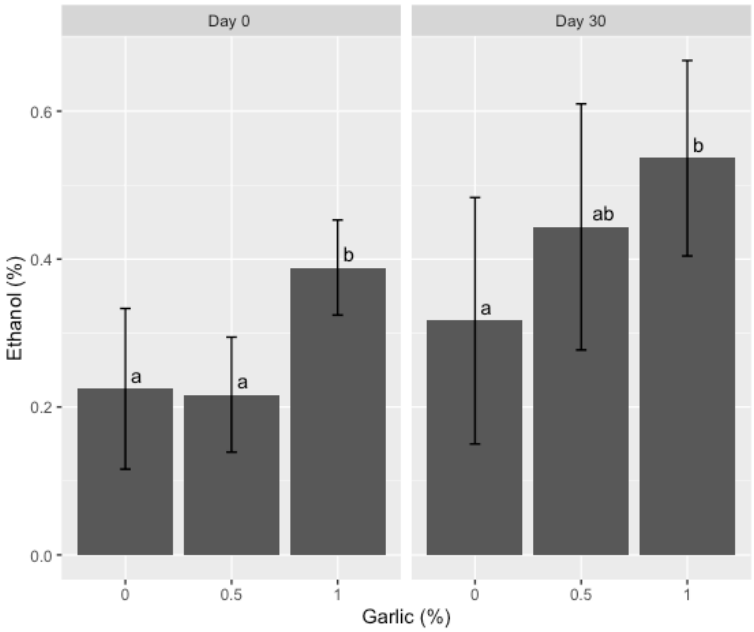

Figure 3.6. Mean ethanol concentration (% wt) in beet kvass samples at the end of fermentation (Day 0) and post-refrigerated storage (Day 30); n = 9

3.4.2.5. Biogenic Amines

The biogenic amines quantified in this study included agmatine, putrescine, tyramine, cadaverine, and histamine. All biogenic amines analyzed in the beet kvass samples were below the detection limit (Table 3.6) across all treatments. However, a suspected peak of α-aminobutyric acid (GABA) was found. Due to the absence of a mixed standard, the concentration of GABA was unable to be determined.

Table 3.6. The detection limit of biogenic amines with HPLC

Biogenic Amine	Concentration (mg/mL)
Agmatine	1.48E-02
Putrescine	1.79E-02
Tyramine	1.75E-02
Cadaverine	1.11E-02
Histamine	1.77E-02

3.5. Discussions

3.5.1. pH

Previously published literature has suggested that garlic may either contribute additional lactic acid bacteria to the fermented product, and/or act as an additional carbon source for their growth (Paludan-Müller et al., 1999; Lim et al., 2015). Garlic decreased the time needed to conclude the fermentation period (defined as a pH level ≤ 4.0) of beet kvass. However, due to the absence of lactic acid bacteria quantification in the present study, the explicit role of garlic on LAB levels is unknown. The acidity of fermented vegetables is mainly the result of lactic (and to a lesser extent acetic) acid accumulation, which is produced by the major LAB fermenters (Pardali et al., 2017). The average lactic:acetic acid ratio of beet kvass for all treatment after the end of fermentation was 7:1, higher than the ratio found in sauerkraut. Several authors have reported a target lactic:acetic acid ratio of 4:1 for sauerkraut, indicating the end of fermentation of high-quality sauerkraut (Pederson & Albury, 1969; Khanna, 2019). Due to the lack of information on fermented beet kvass, more research needs to be completed to determine the ideal lactic:acetic acid ratio for a high quality product.

Despite low storage temperatures, the accumulation of lactic and acetic acid during refrigeration (4°C) is indicative of this LAB activity, although it is lower than activity at room

temperature. As sugars in beet kvass are being fermented, these LAB continue producing metabolites, such as lactic and acetic acids. Therefore, it is unsurprising that our samples after storage had a significantly lower pH than the samples prior to refrigerated storage.

3.5.2. Biochemical Analysis

3.5.2.1. Organic Acids

The soluble sugars in raw red beetroot are mostly comprised of sucrose (91.6%) (Dolores Rodríguez-Sevilla et al., 1999). These beetroot sugar results are similar to what Wruss et al. (2015) found, with the average total sugar content being 7.75 g/100 g and the vast majority being sucrose (94.8%). These sugars act as carbon sources for autochthonous LAB to utilize during fermentation. As mentioned from the pH analysis of our samples, the accumulation of lactic and acetic acid in fermented vegetables is indicative of the LAB activity in the beet kvass samples. When garlic concentration is controlled, the increasing concentration of salt was found to be correlated with a decreasing amount of lactic and acetic acid. This observation is similar to work conducted by Xiong et al. (2016), which found that higher salinity (2%, 5%, 8%) had a negative effect on the lactic acid and acetic acid content of Chinese sauerkraut. They determined that salt concentration significantly affected the early stages of fermentation, with 2% salt yielding more LAB and its metabolites (lactic and acetic acid) in samples. This negative effect could be a result of osmotic stress on lactic acid bacteria at higher salt concentrations. In another study, Guan and team (2020) observed this trend when investigating the physicochemical differences between two different fermented vegetables, fermented bamboo shoot (Suansun) and Chinese sauerkraut (Suancai). The researchers observed the lactic acid and acetic acid content was significantly higher in fermented vegetables with lower salinity (0.47% NaCl) compared to fermented vegetables with higher salinity (2.12% NaCl). It should be noted that the preparation of these

vegetables is a stark contrast from beet kvass preparation due to the ingredient and formulation differences. Therefore, salt concentration may be only one of many factors contributing to the increased organic acid production.

Besides salt concentration being a factor affecting organic acid production, garlic played an important role in the acidification of beet kvass. Samples treated with garlic, at all salt levels, fermented in a shorter time period, in comparison to samples without garlic. However, these garlic-treated samples lower amounts of lactic and acetic acid concentrations at similar pH levels. This observation suggests there may other organic acids produced that lowered the pH levels of the samples such as succinic, propionic, malic acids, that have been found in fermented kimchi and soybean products (Shim et al., 2012; Shukla et al., 2010). This further reaffirmed the hypothesis that garlic may be a possible factor in modulating the diverse community of fermenters.

3.5.2.2. Sugars

The sucrose content increased as the beverage fermented, illustrating the release of these compounds as the red beetroot was immersed in water over time. The diffusion of sucrose through the pores of red beetroot into water is affected by different factors such as time, particle size, and temperature. Fick's second law of diffusion: $\frac{\delta C}{\delta t} = D\frac{\delta^2 C}{\delta x^2}$, where C is concentration, t is time, D is diffusion coefficient, and x is the particle diameter, states the driving force for diffusion is the concentration gradient between the particles and solvent. The rate of diffusion increases with a larger concentration gradient. This rate can be altered by increasing diffusion coefficient D, that is dependent on temperature and material, or reducing the particle diameter, x. In a prior study, sugar beets were found to have a diffusion coefficient at room temperature and 1

mm particle size of $1.6\mathrm{m}^2/\sec \times 10^{10}$. This value increases as temperature increases, and so would be expected to slow during refrigeration (Gertenbach, 2002).

When cubed red beetroot is submerged into brine, the major sugar, sucrose, diffuses into the solvent. Assuming all the other factors in the system are controlled, the sucrose concentration in the red beetroot and brine should achieve equilibrium and eventually be the same. However, the data suggest this is not the case. Assuming the total sucrose content in red beetroot is 6.69 g/100 g (value determined by Dolores Rodríguez-Sevilla et al., 1999), each jar of beet kvass should have 20.07 g of sucrose, producing a theoretical value of 23.6 mg/mL of sucrose in each sample. However, the sucrose concentration (2.99 ± 0.59 mg/mL) measured in this study is significantly lower than the theoretical value. The particle size and surface area of the red beetroot are much larger than the 1 mm sized particle Gertenbach (2002) discussed in his book chapter. In order to achieve equilibrium, the cubed red beetroot would have to be submerged in water for a longer period of time or be cut into smaller sizes. However, due to the removal of cubed red beetroot after completing fermentation, sucrose concentration did not achieve equilibrium. In addition, sucrose concentration post-fermentation and post-storage were highly variable indicating the inconsistent nature of spontaneous fermentation due to the presence of lactic acid bacteria, salt, and garlic.

Salt concentration had a significant effect on glucose and fructose levels. Higher salt concentration was associated with higher glucose and fructose levels in samples. However, these effects were not as significant as those exerted by garlic. Samples with the addition of garlic had lower fructose and glucose levels. This is based on an increase in the breakdown of sucrose into glucose and fructose, along with the rapid utilization of the simple sugars, which in turn rapidly increases ethanol levels. Although not tested in this study, this observation may indicate the

presence of *Saccharomyces* or other yeasts. One study, which assessed the metabolite changes in kimchi, observed a rapid decrease in glucose and fructose concentrations after 30 days of fermentation. However, there was no increase in lactate, acetate, or mannitol levels. Glycerol and ethanol levels on the other hand were increased throughout fermentation, while the growth of *Saccharomyces* was observed (Jeong et al., 2013), as these metabolite changes are typically caused by the growth and metabolism of *Saccharomyces* (Barnett, 1976; Jeong et al., 2013). Although glycerol was not monitored in this experiment, the increase in garlic was associated with an increase in ethanol levels, while glucose and fructose levels was simultaneously decreased. Similar to the study by Jeong et al. (2013), the results in this experiment indicated the possible presence and growth of *Saccharomyces.*

3.5.2.3. Alcohol

The increase in ethanol accumulation in all samples indicated the possible activity of both heterofermenters and yeast. Some samples exceeded the regulatory threshold for non-alcoholic beverages which is set at less than 0.5% alcohol by volume (FDA, 2005a). Hence, it is important for small businesses to monitor the fermentation of beet kvass in order to be compliant with the regulations. It was initially hypothesized that the addition of garlic would decrease microbial activity, specifically bacteria and fungi, due to the presence of antimicrobial compounds, 3-vinyl-1,2-dithiacyclohex-5-ene and 3-vinyl-1,2-dithiacyclohex-4-ene (Chen et al., 2018). However, this was not observed in our study. Instead, samples with the addition of garlic had a higher ethanol content, lower lactic and acetic acid content, and lower glucose and fructose content, when compared to samples prepared without garlic. These observations indicate a potentially higher yeast population in garlic treated samples, which may have resulted in the

decrease in LAB activity due to competition, as seen in the lower lactic and acetic acid levels of those samples.

3.5.2.4. Biogenic Amines

The accumulation of biogenic amines is directly proportional to microbial activity due to the decarboxylation of amino acids (Halász et al., 1994). Salt, garlic, and storage time were thought to have an effect on the LAB activity, which would directly impact the biogenic amine formation and accumulation. However, based on this study, we could not conclude any of the effects due to the low amounts of biogenic amines.

Low levels of biogenic amines have been previously found in fermented vegetables, such as sauerkraut and table olives (Tofalo et al., 2012; Majcherczyk & Surówka, 2019). The safety limit of histamine (500 mg/kg) was established by the FDA for fish and fishery products based on its toxicity (FDA, 2005b). However, there are currently no regulations on biogenic amines in fermented vegetables. In this study, biogenic amines were below the detection limit in any of the fermented beet kvass samples. Therefore, biogenic amines in fermented beet kvass are not expected to pose a safety risk to consumer health.

The results of this study have demonstrated the potential for the presence of γ-aminobutyric acid (GABA) in the samples. GABA is a non-protein amino acid that is produced by the decarboxylation of glutamic acid. Contrary to the other biogenic amines, GABA is considered safe and has the possibility of providing health benefits when ingested, such as blood pressure regulation and neurotransmitter inhibition (Diana et al., 2014). In fermented kimchi, GABA was produced by naturally present LAB (Jeong et al., 2013). Although not explicitly determined in our analysis, the amount of glutamic acid (0.428 g/100 g of edible portion), which is the precursor of GABA found in red beetroot, suggests fermented beet kvass may contain

levels of GABA and confirmation and quantification of GABA should be the focus in future studies (Nemzer et al., 2011).

3.6. Limitations and Pitfalls

The values represented in this study may not be exact due to a few things, including the improper storage of samples and the 3-point standard curve that did not encompass all the values of compounds examined. It is possible due to the extended storage in the refrigerator of certain samples, some of the targeted chemicals deteriorated while other continued to accumulate over time, creating a system that may not be representative. Specifically, lactic acid fermentation could have occurred despite filtration and low temperature storage. Therefore, all samples should be filter sterilized with 0.25 μm syringe filters, before frozen storage to improve accuracy.

However, the results still reflect the biochemical system in each samples due to the relative peaks and area identified through HPLC. Therefore, this study should be repeated with a 5-point standard curve for HPLC analysis using expected concentrations of each compounds to obtain more accurate values.

3.7. Conclusions

The results from this study indicate that spontaneously fermented beet kvass can be produced successfully at low sodium levels (0.5% - 2.5%). During the fermentation process, a sharp decline in pH and an increase in organic acids occurred in all samples, regardless of salt and garlic treatments, suggesting adequate growth of lactic acid bacteria. Changes in metabolites occurred at different salt and garlic concentrations, as well as at different time points. Specifically, the addition of garlic in beet kvass formulations produced samples that reached a target pH level more quickly, and were suspected to have a higher yeast population, based on these metabolite changes. It is necessary to analyze the microbial succession overtime, to better

understand the fermentation kinetics of a fermented beet kvass, and if faster fermentation can influence kvass quality characteristics. Lastly, we determined that biogenic amines are not a safety risk in fermented beet kvass, based on the formulation used in this study and chemical analyses have suggested the possible presence of GABA, a biogenic amine with potential health benefits.

Sensory acceptability is an important component in the formulation of new food products. Therefore, the sensory acceptability of beet kvass was assessed in the next chapter to determine the ideal salt and garlic concentrations that will produce a product that has the highest sensory quality to provide a more complete investigation on beet kvass since the literature is lacking on this fermented food product.

CHAPTER 4

EFFECTS OF SALT AND GARLIC CONCENTRATION ON THE SENSORY PERCEPTION OF BEET KVASS

4.1. Abstract

The consumer acceptability of a food product is dependent on both customer needs and satisfaction (Heldman, 2004). Salt is an important ingredient to successfully produce a fermented vegetable product, because it provides flavor and contributes to the creation of an ideal environment for fermentation. However, due to the health implications of high salt consumption, such as high blood pressure and kidney disease, consumers are urged to decrease dietary salt intake (Malta et al., 2018; Nerbass et al., 2015). A recent assessment found that 20% (59/295) of Maine home fermentors, value the concept of a low sodium diet, and reported feeling that production of low-sodium fermented foods were very or extremely important (Camire et al., 2019). Beet kvass, a value-added beverage made by infusing red beetroot in salt and water is a fermented vegetable product gaining popularity among home fermenters and commercial processors (Sarnacki, 2018). Therefore, the present work compared the consumer sensory perception, overall liking, and health related claim acceptance of beet kvass formulations prepared with varying concentrations of salt and garlic. Specifically, panelists assessed samples for color and flavor, intensity of tartness, acidity (vinegar), garlic, salt, and overall product acceptability. The results indicated that beet kvass prepared with 1.5% salt and 0.5% garlic had the highest overall liking score of 6.12 on a 9-point hedonic scale. Salt concentration was a significant deciding factor for the overall acceptance of the beet kvass product. The study showed sensory preference for lower salt beet kvass formulations. Inclusion of information on the potential health benefits of the product increased panelists' interest in consuming the product,

indicating that participants may be willing to compromise sensory characteristics for health benefits. Therefore, additional work assessing the health mediated effects of this modified beet kvass formulation is necessary to maximize product marketability.

4.2. Introduction

Probiotic foods, a sector of the functional food products market, have increased in both consumer demand and scientific research interest due to potential health benefits including gastroenterology effects associated with their consumption (Cremon et al., 2018; Ljungh & Wadström, 2006; Yeung et al., 2008). Beet kvass is a fermented beverage made by infusing chopped red beetroot in salt water. Red beetroot consumption has been associated with several health benefits such as lowering hypertension, enhancing exercise performance, and improving cardiovascular health (Jones, 2014; Kapil et al., 2014; Kapil et al., 2015). Consumer interest in beet-containing products as a result of these possible health mediated effects is reflected in the growing research initiatives surrounding beetroot-based functional foods and supplements (Morgado et al., 2016; Panghal et al., 2017; Wootton-Beard & Ryan, 2011). However, there is relatively limited research on the sensory acceptability of beet kvass.

Salt is a crucial ingredient in the formulation of fermented vegetables. However, high sodium intake has been associated with increased blood pressure and kidney disease risk (Malta et al., 2018). In 2010, the U.S. Institute of Medicine (IOM) recommended the reduction of sodium in processed foods (McGuire, 2010). The 2015-2020 “Dietary Guidelines for Americans” suggested 2300 mg as the maximum sodium intake per day (USHHS, 2015). Beyond food industry applications, the desire for sodium reduction in the form of salt has also been expressed by home cooks as well. A recent assessment found that 20% (59/295) of Maine based, home fermenters feel that preparation of low-sodium fermented food products is are very

or extremely important (Camire et al., 2019). Therefore, with growing consumer interest in beet kvass, it is necessary to evaluate the sensory acceptance of different formulations due to the current lack of information and standardization of safe formulations.

The objectives of this study were to evaluate the sensory characteristics of beet kvass prepared with varying salt and garlic concentrations in terms of (i) overall acceptance and (ii) the impact of health-related messaging on product acceptability. There is currently no information or any previous research to date assessing the consumer acceptability of beet kvass.

4.3. Materials and Methods

4.3.1. Experimental Design

This study was completed in two parts. First, a survey was conducted to determine the demographics of individuals who were interested in fermented products, specifically vegetables, in order to gain insights for future educational programming. These results were also leveraged in designing the sensory test. Then, a sensory test was carried out to evaluate the acceptability of the product prepared with varying salt and garlic concentrations.

4.3.2. Survey

A survey (Appendix A) was conducted to assess consumer habits with regard to the making or purchasing fermented products, familiarity with beet kvass, and whether information regarding health benefits of fermented foods would affect the perception of the product concept. Approval for testing was provided by the University of Maine Institutional Review Board for the Protection of Human Subjects (2019-09-15). The survey, which was administered through Qualtrics Survey Software (Provo, UT), was active for two weeks to provide sufficient time for responses. An informed consent form was displayed on the front page of the survey (Appendix B). Survey participants (n = 258) were recruited through postings on social media, mass-emails

through University of Maine School of Food and Agriculture, and physical posters displayed on the University of Maine campus (Appendices C and D). Individuals with interest in fermented foods or who identified as physically active were encouraged to complete the survey to gain information on their interests in a sports nutrition fermented product. Participants were incentivized to complete the survey by eligibility to enter in a raffle for two gift cards.

4.3.3. Sample Preparation

Beet kvass samples were prepared as indicated in section 2.3.4., with the following modifications. Four beet kvass formulas (Table 4.1) were prepared and fermented in 1-gallon glass jars with air lock jar lids (Figure 4.1) in the University of Maine Commercial Kitchen at ambient temperature (between 20°C and 22°C).

Table 4.1. Beet kvass formulation used for the sensory evaluation

Treatment	Salt (%)	Garlic (%)
1	1.5	0
2	1.5	0.5
3	2.5	0
4	2.5	0.5

Figure 4.1. Samples of fermenting beet kvass in 1-gallon glass jars with airlock lids

All utensils were washed in soapy water and rinsed prior to preparation. Samples were prepared 9 days before the testing date to ensure complete fermentation. The conclusion of fermentation was defined by achievement of a pH level of 4.0 or less. Kvass pH levels were monitored throughout the fermentation period. Following the fermentation period, samples were strained into clean 1-gallon jars and held under refrigerated (4°C) storage until testing day. Prior to the sensory test, all samples were tested for the presence of foodborne-pathogens (STEC, *Listeria monocytogenes*, and *Salmonella*) using the procedure previously described in section 2.3.7. No targeted foodborne pathogens were detected in any of the samples.

4.3.4. Sensory Evaluation

Sensory evaluation was conducted to determine consumer acceptability of beet kvass formulations prepared with varying salt and garlic concentrations (Appendix E). SIMS 2000 Sensory Software (Version 6, Berkeley Heights, NJ) was used to design the questionnaire, establish testing, design, and execution. To provide a balanced testing design and avoid potential panelist biases, samples were coded with randomized three digit codes which were generated using the SIMS Software. Approval for testing was provided by the University of Maine Institutional Review Board for the Protection of Human Subjects (2019-11-15). Panelists received no training prior to participating in the study.

Sensory panelists (n = 66) were recruited from both the University of Maine Sensory Evaluation Center mailing list and from a pool of willing participants who had previously completed the survey (Appendices F & G). Individuals recruited from the mailing list were required complete a pre-screening questionnaire to assure eligibility for the sensory test (Appendix E&H). The targeted panelists were any individual aged 18 or above, with interest in fermented foods, and not allergic to product ingredients. Panelists who completed the sensory test were monetarily compensated for their participation in the study.

Each panelist evaluated samples in private booth (Figure 4.2) at The University of Maine Sensory Evaluation Center with positive air flow to prevent any aroma bias from the kitchen. Samples were served at 4.0 ± 0.4°C. To confirm panelist consent, participants were asked to read an informed consent statement before entering the testing center (Appendix I). The panelists answered sensory ballot questions on Hewlett Packard Windows-based Elite Pro tablets

(California, US) installed with SIMS 2000 sensory software.

Figure 4.2. Panelists in designated booths at the sensory evaluation center

Panelists were first required to answer a series of demographic based questions, familiarity with fermented beet kvass, and habits concerning the consumption of fermented foods, and were then provided with four approximately 1-oz samples served in 2-oz clear plastic cups. Samples were assigned randomly-generated 3-digit codes and presented in a randomized order determined by the SIMS software. The samples were delivered simultaneously to panelists on beige colored trays with a napkin and plastic cup of spring water (Poland Springs, Poland Spring, ME) (Figure 4.3).

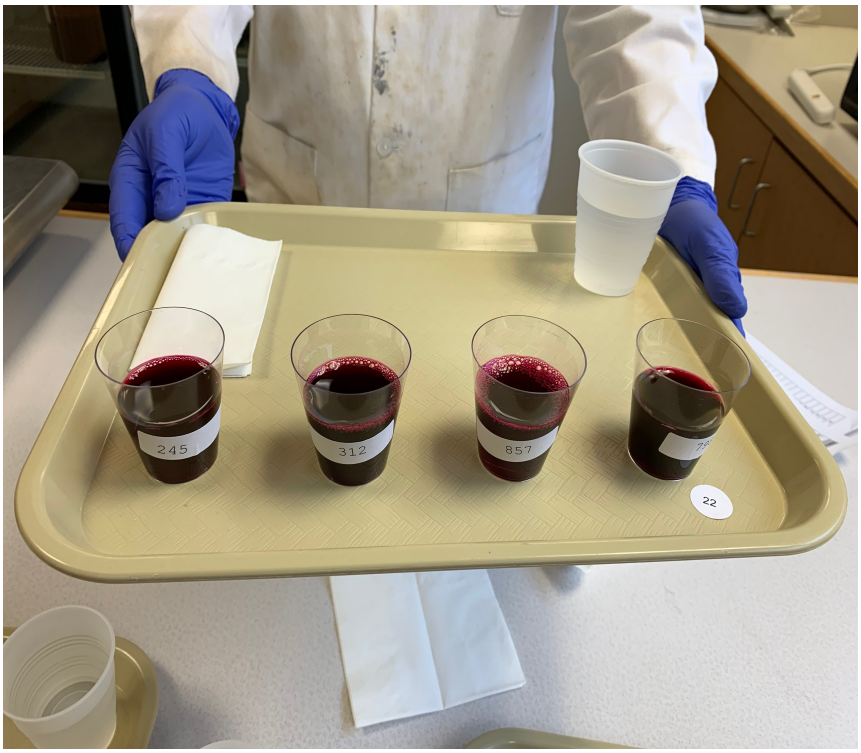

Figure 4.3. Samples were presented in a randomized order and clearly labeled with three digit codes for identification. Water was provided to panelists to cleanse their palates between samples

Each of the four samples was evaluated for garlic flavor, saltiness, tartness and vinegar flavor on a five point just-about-right (JAR) scale, while color and overall liking attributes were evaluated based on the 9-point hedonic scale. The 5-point JAR scale used was (1) “Much Too” and (5) “Not” and (3) “Just about right” in the middle (Li et al., 2014). This scale is a method to determine if the attribute’s intensity is at an optimal level based on consumer perception. The 9-point hedonic scale, developed by Peryam and Giradot (1952), used was as follows (1) “dislike extremely”, (2) “dislike very much” (3) “dislike moderately” (4) “dislike slightly” (5) “neither like or dislike” (6) “like slightly” (7) “like moderately” (8) “like very much” and (9) “like extremely”. Panelists were then given an opportunity to type open ended comments about the sample using a comment box. After answering the first set of questions, panelists were instructed to take a sip of water between samples to prevent flavor carryover.

After completing the series of questions associated with sensory evaluation of the samples, panelists were asked if they would purchase the products after tasting them. Then, information regarding potential health benefits related to the consumption of beet kvass was displayed, and panelists were asked if knowing the potential health benefits and the presence of live cultures would increase their intent of purchasing or producing the product.

4.3.5. Statistical Analyses

Analysis of the survey data was completed using R studio. Pearson's product moment correlation was used to determine the correlations between factors. Sensory evaluation data were analyzed on SIMS software with SPSS (Chicago, IL). One-way analysis of variance (ANOVA) was conducted to determine significant differences among garlic flavor, saltiness, tartness and vinegar flavor, liking of color, and overall liking of beet kvass and the garlic and salt concentration treatments. Tukey's Honest Significant Difference (HSD) test was selected for post-hoc analyses to determine significant differences among means. A significance level of $p < 0.05$ was chosen for all statistical analyses.

4.4. Results

4.4.1. Survey

The majority of survey participants were between the ages of 18 and 25 (34%), and mostly female (83%). Out of the 258 participants, 81% of participants (209) indicated that they currently purchase and/or consume fermented foods (Table 4.2.). There were no significant correlations however, between the demographics of the participants and their choice of consumption of fermented foods ($p \geq 0.05$).

Table 4.2. Survey panelist demographics and fermented food production/consumption habits

Questions	Response	Panelists (n = 209)
Gender	Male	41 (16%)
	Female	213 (83%)
	Other	3 (1%)
	Prefer not to answer	1 (> 1%)
Age	18 -25	87 (34%)
	26-35	49 (19%)
	36-45	40 (16%)
	46-55	26 (10%)
	> 56	47 (18%)
	Prefer not to answer	9 (3%)
Do you currently purchase and/ or consumer fermented foods?	Yes	209 (81%)
	No	49 (19%)
Do you ferment foods at home?	Yes	97 (38%)
	No	161 (62%)

When prompted to select which fermented foods were currently purchased or consumed, yogurt and cheese were most frequently reported (Figure 4.4.). Garlic (3), miso (2), olives (1), and mushrooms (1), were some of the other fermented vegetables provided by the panelists as open ended responses.

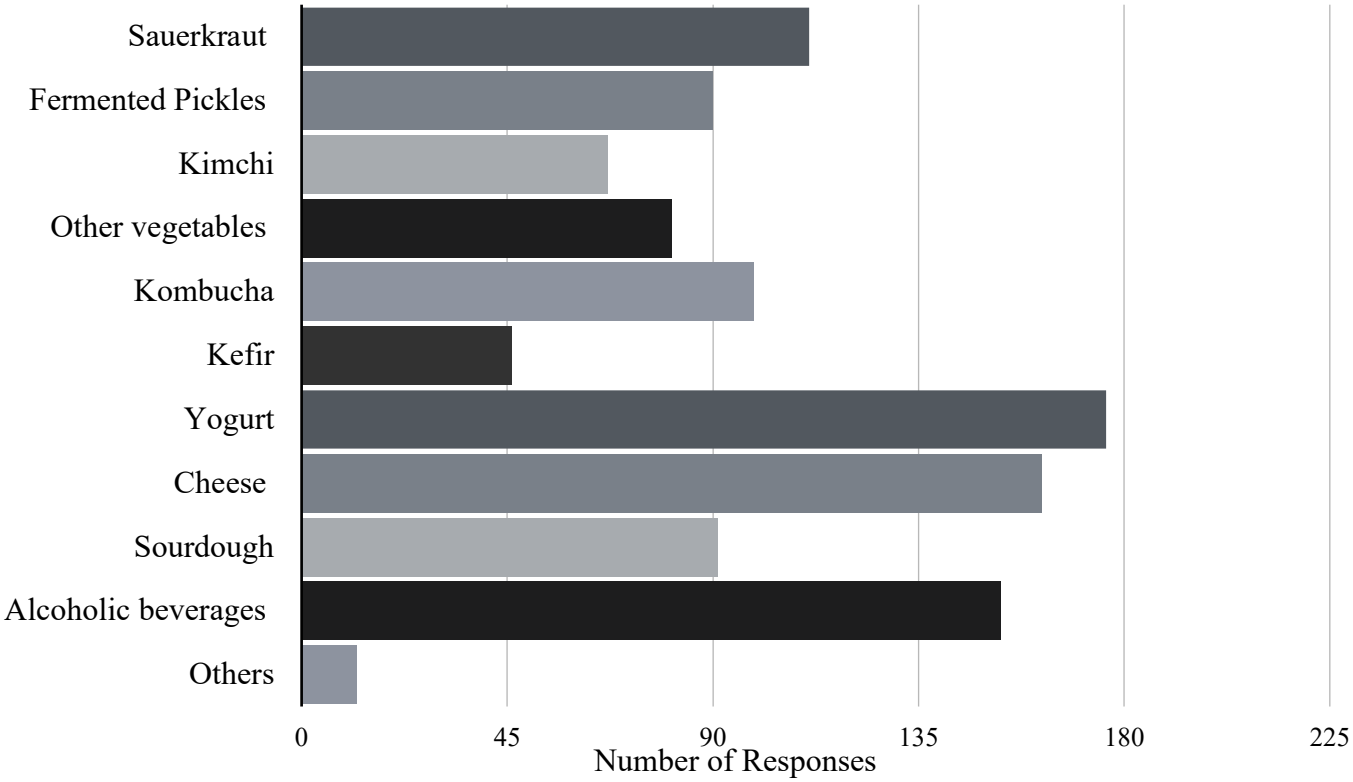

Figure 4.4. Fermented foods currently purchased or consumed by participants (n = 209)

Among participants who currently purchase or consume fermented foods, 192 participants (91%) indicated that taste was the most important reason for the consumption of fermented foods. Following sensory appeal gut health benefits (66%) was the second most important factor. Of the panelists who consume, but do not prepare fermented foods (45% of consumers), inconvenience and lack of knowledge were the most common reasons for

avoiding fermentation at home (Table 4.3). Additionally, participants also indicated their preference for store-bought products and unsuccessful attempts at home fermentation. Among participants who reported fermenting at home, the most popular fermented products were vegetable-based including beans, beets, carrots, and sauerkraut.

Table 4.3. Responses to why survey respondents do not ferment foods at home

Why do you not ferment foods at home? (n=161)	Number of responses (% total responses)
Inconvenient (e.g. time and supplies)	62 (39)
Do not know how to ferment foods	55 (34)
Do not like fermented foods	18 (11)
Unsure about the safety of fermented foods	14 (9)
Others	12 (7)

Less than half of all participants (45%, 117/258) were aware of the potential health benefits associated with red beetroot consumption (Table 4.4). Such knowledge would likely increase consumer interest, with one hundred and fifty-four participants (60%), responding that they would be more likely to purchase and/or consume beet kvass if they understood the positive health effects. However, it is important to note that among those who currently do not consume or purchase fermented foods, 64% (32/50) were either unsure or unlikely to purchase and/or consume beet kvass despite understanding the health benefits.

Table 4.4. Awareness of health benefits of red beetroot on consumption/purchase intent

Questions	Responses	Number of responses
Are you aware of the benefits of consuming red beets?	Yes	117 (45%)
	No	107 (42%)
	Unsure	34 (13%)
Would understanding the health benefits of red beets make you more likely to consume and/or make beet kvass?	Yes	154 (60%)
	No	56 (22%)
	Unsure	48 (18%)

Because some commonly cited benefits of beet consumption are related to enhancement of exercise performance, participants were asked if they consider themselves to be physically active (Jones, 2014; Wruss et al., 2015). Two hundred and thirteen (83%) participants answered that they were moderately to very active. Among those participants, 23% (48) indicated that they were currently purchasing and/or consuming sports nutrition supplements and/or beverages. Self-reporting of "moderately active" or "very active" lifestyle was correlated ($p < 0.05$) higher likelihood to purchase and/or consume sports nutrition supplements and/or beverages.

4.4.2. Sensory Evaluation

The majority of sensory panelists were between the ages of 26 and 35 (39%), approximately 58% were female and 42% were male. Out of the 66 panelists, only 37 (56%) were familiar with beet kvass (Table 4.5).

Table 4.5. Sensory panelist demographics and product concept familiarity

Questions	Response	Panelist (n = 66)
Gender	Male	28 (42%)
	Female	38 (58%)
	Other	0
	Prefer not to answer	0
Age	18 -25	17 (26%)
	26-35	26 (39%)
	36-45	6 (9%)
	46-55	6 (9%)
	> 56	11 (17%)
	Prefer not to answer	0
How familiar are you with beet kvass?	Very familiar	1 (1%)
	Moderately familiar	36 (55%)
	Have never heard of it	29 (44%)

Panelists were instructed to take a sip of each sample, and rate the intensity of garlic flavor, saltiness, tartness and vinegar flavor on a five point just-about-right (JAR) scale. Panelists rated the color and overall liking of the samples using a 9-point hedonic scale (Table 4.6).

Table 4.6. JAR scores of flavor perception of beet kvass samples (mean ± sd) treated with different salt and garlic concentrations (n = 66)

Sample	Garlic	Salt	Tart	Vinegar
1[w]	2.18 ± 1.04[a]	3.14 ± 1.01[a]	2.36 ± 1.08	2.48 ± 1.01
2[x]	3.29 ± 0.97[b]	3.15 ± 0.86[a]	2.42 ± 0.99	2.65 ± 0.90
3[y]	2.26 ± 1.00[a]	3.80 ± 1.14[b]	2.42 ± 1.15	2.59 ± 1.19
4[z]	3.58 ± 1.01[b]	3.83 ± 1.14[b]	2.29 ± 1.15	2.47 ± 1.10

Subscripts following the means represent the significant difference, within the same column, between treatments
[w]1.5% Salt and 0% Garlic
[x] 1.5% Salt and 0.5% Garlic
[y] 2.5% Salt and 0% Garlic
[z] 2.5% Salt and 0.5% Garlic

There were significant differences in panelists' liking of the saltiness and garlic flavor between treatments, but no differences in liking the tartness and vinegar flavor. Results indicated that participants clearly identified the different salt and garlic concentrations. Specifically, JAR score distribution results revealed that the higher salt samples were associated with "too much salt", and samples prepared without garlic were designated as containing "not enough garlic" (Figure 4.5 and 4.6).

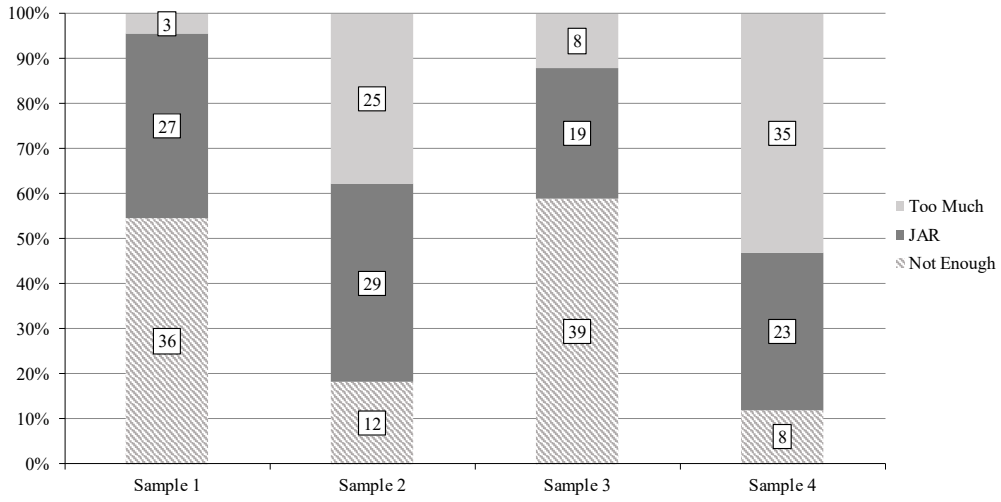

Figure 4.5. JAR score distribution of garlic flavor in beet kvass samples (n = 66). Sample formulation: 1) 1.5% salt and 0% garlic, 2) 1.5% salt and 0.5% garlic 3) 2.5% salt and 0% garlic 4) 2.5% salt and 0.5% garlic

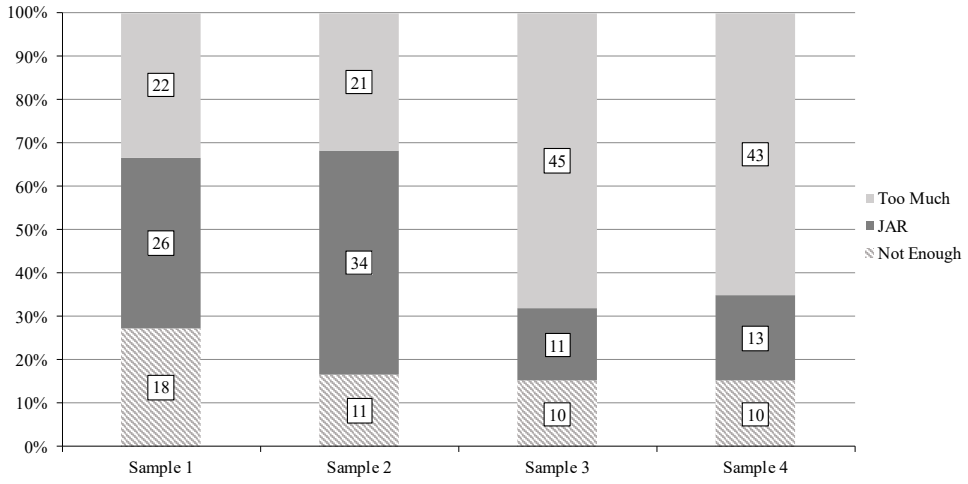

Figure 4.6. JAR score distribution of saltiness in beet kvass samples (n = 66) Sample formulation: 1) 1.5% salt and 0% garlic, 2) 1.5% salt and 0.5% garlic 3) 2.5% salt and 0% garlic 4) 2.5% salt and 0.5% garlic

The color of the product consistently received a score of 7 (like slightly) or above (Table 4.7). Although there were significant differences in the liking of the color of beet kvass between the different treatments, these differences were not related to the salt or garlic concentrations. Overall, lowest salt (1.5%) beet kvass received significantly higher overall liking scores than the

highest salt (2.5%) beet kvass, as seen in Table 4.7. Within the lower salt formulations, the overall liking of the samples with and without the addition of garlic were not significantly different. Therefore, beet kvass prepared with 1.5% salt was considered more acceptable compared to the 2.5% salt treatment.

Table 4.7. The hedonic scores for color and overall liking of beet kvass samples (mean ± sd) prepared with different salt and garlic concentrations (n = 66)

Sample	Color	Overall
1[w]	7.52 ± 1.34[ab]	6.06 ± 1.88[b]
2[x]	7.70 ± 1.38[b]	6.12 ± 1.85[b]
3[y]	7.21 ± 1.52[a]	5.00 ± 1.78[a]
4[z]	7.23 ± 1.58[a]	4.98 ± 2.07[a]

[w] 1.5% Salt and 0% Garlic
[x] 1.5% Salt and 0.5% Garlic
[y] 2.5% Salt and 0% Garlic
[z] 2.5% Salt and 0.5% Garlic

The most common descriptors in the panelist comments for each sample are listed in Table 4.8. Similar to the overall liking scores, Sample 4 (2.5% salt and 0.5% garlic) was most frequently described as “too salty” and “garlicky”, while Sample 2 (1.5% salt and 0.5% garlic) was most frequently described as the preferred sample with balanced flavors. Samples prepared with 1.5% salt, with or without garlic, were found to have a significantly higher number of intended consumer purchases (39%), compared to the samples prepared with 2.5% salt, with or without garlic (0.5%).

Table 4.8. Main descriptors mentioned in the comments written by sensory participants (n = 66)

Sample	Descriptors
1[w]	Salty (4); Beet (3)
2[x]	Preferred (5); Balanced (2)
3[y]	Salty (11); Dirt flavor (2)
4[z]	Salty (9); Too much garlic (7)

Values in brackets are the numbers of mentions by the participants in each samples.
[w] 1.5% Salt and 0% Garlic
[x] 1.5% Salt and 0.5% Garlic
[y] 2.5% Salt and 0% Garlic
[z] 2.5% Salt and 0.5% Garlic

There were no significant correlations between the demographics of the participants and their overall liking of samples. However, as expected, there was a significant correlation between increased overall liking score and increased purchase intent. Upon completion of the taste test, participants were asked to answer a few questions regarding their intention of producing and purchasing the product (Table 4.9). Fifty-two participants (79%) were either moderately likely or very likely to produce or purchase beet kvass after learning of the health benefits associated with product consumption. Whereas only 36 participants (55%) were either moderately likely or very likely to produce and purchase beet kvass after learning of the presence of "live, active bacterial cultures" in the product.

Table 4.9. Sensory panelist purchase of intent after taste test and reading the potential health benefits associated with beet kvass (n= 66)

Questions	Responses	Panelist (n = 66)
Would knowing the health benefits of this sample make you more likely to make or purchase this product? [a]	Very likely	17 (26%)
	Moderately likely	35 (53%)
	Neither likely nor unlikely	6 (9%)
	Moderately unlikely	4 (6%)
	Very unlikely	4 (6%)
Would knowing this (beet kvass contains live active bacterial cultures) make you more likely to make or purchase this product? [b]	Very likely	12 (18%)
	Moderately likely	24 (36%)
	Neither likely nor unlikely	18 (27%)
	Moderately unlikely	7 (11%)
	Very unlikely	5 (8%)

Statements displayed before the questions above were asked:

[a] "Red beets have been associated with health benefits such as lowering hypertension, enhancing exercise performances, and benefitting cardiovascular health."

[b] "Beet kvass is a fermented beverage that contains live, active bacterial cultures."

4.5. Discussions

4.5.1. Survey

The majority of survey participants were college-aged females. This may indicate that the survey did not fully represent the targeted population of home fermenters or physically active individuals. Most of the participants (91%) reported that they consume fermented foods due primarily to product taste. This finding offers a potential explanation of why participants who do not currently purchase fermented foods would not eat them despite the potential health benefits. Therefore, recruiting sensory panelists who were interested in fermented food

products was necessary for this study in order to eliminate potentially biased product opinions.

Slightly more than half of survey participants (55%) who reported consuming fermented foods indicated that they purchased the products rather than made them at home. This finding highlights the market potential for product formula standardization among small businesses as well as home fermenters and hobbyists, because vegetable-based fermented products were reported as popular products among this sector of participants (n=96).

The low number of people who reported currently purchasing and/or consuming sports nutrition supplements and/or beverages suggests that the commercial potential of a beet kvass product marketed as a sports enhancement product is limited based on these panelists surveyed. Despite previous research which has associated sports enhancement with red beetroot consumption, future studies on beet kvass should focus on general health and probiotic benefits (Ferguson et al., 2013; Pinna et al., 2014). This is because fermented vegetable is more commonly associated with gut health and probiotic benefits, instead of a nutrition supplement.

4.5.2. Sensory Evaluation

The overall acceptance of a food product is dependent on both its sensory and non-sensory based attributes. While previous studies have indicated that consumers consider sensory characteristics including flavor, taste, color, and texture to be the most important factor in selecting a food product, non-sensory attributes are becoming increasingly important. In fact, non-sensory attributes such as “feeling good and safety” and “health and nutrient content”, have been associated with consumer’s choice of consumption of functional foods in Uruguay (Ares et al., 2007). Other factors, including price, production methods, nutritional information, and

branding, also influence consumers' food product expectations (Iop et al., 2006; Jaeger, 2006). Therefore, combining both sensory and non-sensory factors is essential to fully understanding the consumer response in our study.

Results have shown that different salt (1.5% or 2.5%) and garlic (0% or 0.5%) treatments were easily identifiable by panelists, specifically by perceived salt and garlic intensity ratings. However, appropriateness of tart and vinegar taste were statistically undistinguishable among treatments. This finding suggests that neither salt nor garlic levels affected the perception of tartness or vinegar taste in the product, or that consumers have a wider range of acceptability for these attributes. Based on our previous product biochemical analyses in Chapter 3, the amounts of lactic and acetic acid were variable among treatments. Specifically, samples with garlic had lower lactic and acetic acid contents compared to samples without garlic. Hartwig and McDaniel (1995) reported that final product pH level, and more specifically the dissociated form of present acids, significantly affected the flavor profile of organic acids. This suggests that the different levels of organic acid within beet kvass samples may contribute to its unique flavor profile.

Salt is an important ingredient in food due to both its sensory and safety implications. It offers a favorable environment for the rapid growth of lactic acid bacteria (LAB) (Caplice & Fitzgerald, 1999). Besides, the reduction of salt in many foods may lead to the change in flavor and decreased acceptability (Lucas et al., 2011; Nguyen & Wismer, 2019). However, this study showed that panelists preferred both beet kvass samples with less salt. Although not significantly different compared to Sample 1 (1.5% salt without garlic), Sample 2 (1.5% salt with 0.5% garlic) had the highest overall acceptance score, compared to the other samples we tested. This suggests that garlic may have a slight positive impact on consumer acceptability of beet kvass, but that salt is the primary driver of acceptability. Additionally the perceived samples saltiness was

determined to be a deciding factor for the purchase intent among consumers. Samples prepared with 1.5% salt, with or without garlic, were found to have a significantly higher number (39%) of intended consumer purchases, compared to the samples prepared with 2.5% salt, with or without garlic (25%). Overall low purchase intent may be attributed to a lack of product familiarity among consumers. In addition, this product is unique compared to other fermented vegetable products due to the fact that it is consumed as a beverage. Popular fermented vegetables, such as sauerkraut and kimchi for example, are often consumed whole, while the beetroot is strained and removed from the product to produce kvass. Therefore, saltiness is more prominent in a beverage-based product compared to the other previously mentioned fermented vegetable based options. Likewise, the majority of beverages in the consumer market have sweet or tart flavor profiles. Savory beverages are relatively limited in terms of market share and consumption.

Beetroot has a unique sensory profile with distinct earthy aroma and flavor attributes. This earthiness, often associated with soil and dirt, is caused by geosmin (*trans*-1,10-dimethyl-*trans*-(9)-decalol), a volatile bicyclic alcohol. This compound also provides an earthy off-odor in some water, fish, dry beans, and wine products (Acree et al., 1976; Lu et al., 2003). In a previous study which evaluated the sensory perception of vegetable and berry juice products, beetroot juice was commonly associated with the taste of soil (Waehrens et al., 2018). Studies on beetroot processing to decrease geosmin and overall product bitterness suggest consumers prefer less these earthy flavors in beetroot-based products (Tyler et al, 1979; Bach et al., 2014). While a negative effect on sensory acceptance is associated with samples that taste too earthy, the absence of this taste also had a negative product perception. Absence of this flavor in cooked beetroot juice had previously been described as lacking in both character and flavor (Tyler et al.,

1979). Similar to the sensory evaluation of beetroot juice with different geosmin contents, some panelists in the present beet kvass study commented on the earthy beetroot flavor as a desirable product attribute, while other panelists prefer it to be masked. For example, in the comments section for Sample 3, a panelist stated that "This literally tasted like the ground. There was no balance of flavor." while another panelist said "This sample did also have a bit more of a beet flavor than (another sample), which I liked." The presence of garlic in this study was employed to enhance product flavor, while also masking the often undesirable earthy flavor compounds of beets. While the taste of garlic was prominent, it was not a deciding factor on the overall acceptance of the beet kvass samples. Therefore, the garlic flavor in beet kvass did not significantly affect the overall sensory acceptance of this product.

The overall appearance of this product was somewhat variable when being served. It is unclear if it affected the scoring of the product because this variability was not monitored, and was brought to attention after reading the comments by several participants (3). Specifically, this inconsistency is attributed to the appearance of bubbles within some of the samples. Two participants mentioned that bubble presence was a positive product attribute and generally rated these samples with a higher score. However this finding was not universal as another participant indicated in the comments that the bubble presence had a negative effect on the color and overall acceptability of the product. To have an unbiased evaluation, all samples should have been tapped after dispensing to eliminate the presence of bubbles and to ensure all samples looked the same.

In addition to these sensory attributes, non-sensory factors among beet kvass samples were also assessed. Specifically following sample tasting, panelists were instructed to consider potential health benefits associated with the product. The majority of respondents (79%)

expressed interest in beet kvass if it possessed health benefits. The intent of purchase increased from an average of 32% after tasting to 79% after knowing that beet kvass may possess health benefits. This finding is similar to previous research which compared consumer acceptability of juice-based products. Vidigal et al. (2011) determined a higher consumer acceptance score for exotic Brazilian fruit juices when information on health benefits was provided, compared to the fruit juices without this information. However, this finding was not true for all products, as an increase in acceptance was not observed for camu-camu juice. Camu-camu juice, reported to have a very intense bitter and acidic taste, was scored unacceptable in both cases. Therefore, the inclusion of health benefit claims is only effective when the product has overall acceptable sensory attributes. Similarly, Carrillo et al. (2012) also determined that panelists were unwilling to compromise sensory characteristics in enriched digestive biscuits for the perceived health benefits, specifically high fiber biscuit with no added sugar. Therefore, the increase in intent of purchase may indicate the sensory acceptance of beet kvass.

Additionally, the type of health claim is also an important consideration to consumer purchase intent. In our study, while 79% of panelists expressed interest in beet kvass if it possessed health benefits, only 55% of participants were interested in the product specifically for the presence of live cultures. The taste of beetroot has been associated with health promotion by sensory panelists (Waehrens et al., 2018). Hence, the differences in the number of respondents in those two health claims could be attributed to the more well-known health benefits of beetroots, the main ingredient, than the presence of probiotics that fermented products often claim.

4.6. Conclusions

This study was designed to standardize beet kvass formulations based on sensory acceptance with varying salt and garlic concentrations and to determine what other factors may

contribute to consumer purchase intent of a commercial beet kvass. Based on the sensory perception results, beet kvass formulated with lower salt concentration (1.5%), with or without garlic, was preferred. The lack of peer-reviewed information on this product indicates the apparent need for future work to focus on the health benefits and processing recommendations for small-scale agricultural businesses and home fermenters to successfully product beet kvass.

Overall, there is a potential for beet kvass to be a marketable value-added product. Despite the general lack of consumer familiarity, survey participants who reported to be currently consuming fermented foods were more likely to have a positive attitude toward beet kvass. Health claims increased sensory panelists' interest in consuming the product, despite the low overall acceptance score of 4.98 - 6.12 (a rating of 7.0 on the 9-point scale is considered the benchmark for acceptable products). Although our survey respondents were generally willing to compromise on sensory components in order to achieve these potential health-mediated effects, sensory considerations are still necessary in future research initiatives to increase sensory acceptance among consumers.

CHAPTER 5

OVERALL CONCLUSIONS AND RECOMMENDATIONS

The results from the first two studies indicate the successful spontaneous fermentation of beet kvass at low salt concentrations (0.5%, 1.5%, and 2.5% NaCl) along with the addition of garlic (0%, 0.5%, and 1.0%). The achievement of pH level 4.0 or lower for all treatments within 5 days of fermentation indicates the success of fermentation. However, low salt concentrations used in this study along with the addition of garlic appeared to have no significant effect on the survival of foodborne pathogens STEC, *Salmonella*, and *L. monocytogenes* during fermentation. The survival of these pathogens persisted despite 30 days storage at refrigeration temperature (4°C). These pathogens showed potential adaptability to low-pH environments in the presence of salt and the addition of garlic, and low temperature storage. This suggests a potential risk of foodborne illness to consumers even at low levels (~100 CFU/g). The United States has a zero-tolerance policy for *L. monocytogenes* in processed foods. Hence, home fermenters and industry members should adapt alternative sanitation practices such as soaking and washing red beetroot in a sanitation solution to reduce the safety risk.

The biochemical analyses showed no safety risk from biogenic amines, often accumulated in foods with high microbial activity. However, alcohol accumulation of more than 0.5% ABV was detected in some samples after 30 day storage. Hence, industry members should closely monitor the fermentation and storage of these products to be in compliance with the alcohol threshold (< 0.5% ABV) of non-alcoholic beverages.

Lastly, the consumer perception tests have revealed a potential market opportunity of these products, specifically when accompanied with health-related messages. Based on the

results, the ideal formulation of beet kvass should contain lower salt (< 1.5% NaCl), with or without garlic. Unlike other popular fermented vegetable such as sauerkraut and kimchi that are often consumed as a condiment, beet kvass is intended to be consumed as a beverage rather than a condiment. Hence, the saltiness of this product is more prominent. To further increase the acceptability and marketability of this product, more studies on the health benefits, probiotic potential, and healthful biogenic amines in this product should be conducted.

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APPENDIX A: FERMENTED FOODS ONLINE SURVEY QUESTIONNAIRE

1. Please indicate your gender. (Choose one answer)
    - Male
    - Female
    - Other
    - Prefer to not answer
2. Please indicate your age bracket based on your last birthday. (Choose one answer)
    - Under 18 [Directs user to the end of the survey]
    - 18-25
    - 26-35
    - 36-45
    - 46-55
    - 56 years or older
    - Prefer not to answer
3. Do you currently purchase or consume fermented foods?
    - Yes
    - No

      (If no, skip to Question 6)
4. If yes, which type of fermented product do you currently purchase and/or consume?
    - Sauerkraut
    - Kimchi
    - Fermented pickles

- Sourdough
- Kombucha
- Alcoholic beverages (e.g., beer, wine, cider, mead) o Yogurt
- Kefir
- Cheese
- Other vegetables (e.g, beans, beets, carrots)

5. Why do you consume fermented products? (Mark all that apply)
    - Gut health (probiotics)
    - Like the taste
    - As a food supplement
    - Others : (Specify)
6. Do you ferment foods at home?
    - Yes
    - No

      (If no, skip to Question 8)
7. If yes, which type of foods do you ferment at home? (Mark all that apply)
    - Sauerkraut
    - Kimchi
    - Fermented pickles
    - Sourdough
    - Kombucha
    - Alcoholic beverages (e.g., beer, wine, cider, mead) o Yogurt
    - Kefir

- Cheese
- Other vegetables (e.g, beans, beets, carrots) (continue to Question 9)

8. If no, why do you not ferment foods at home?
    - Do not like fermented foods
    - Do not know how to ferment foods
    - Unsure about the safety of fermented foods
    - Inconvenient (e.g time and supplies) o Other: (specify)
9. How physically active do you consider yourself?
    - Very Active (More than 30 minutes of moderate-intense activity, 5 days a week)
    - Active (At least 30 minutes of moderate-intense activity, 5 days a week)
    - Moderately Active (At least 30 minutes of moderate-intense activity, 3 days a week)
    - Sedentary (Less than 30 minutes of moderate-intense activity, 3 days a week)
    - Unsure/prefer not to answer
10. Do you currently purchase and/or consume sports nutrition supplements and/or beverages?
    - Yes
    - No
    - Unsure
11. Are you aware of the benefits consuming of red beets?
    - Yes
    - No
    - Unsure

Read the statements below:

1: “Beet kvass is a fermented beverage made by infusing red beets in water along with salt and spices like garlic.”

2: “Red beets have been associated with health benefits such as lowering hypertension, enhancing exercise performances, and benefiting cardiovascular health.”

12. Would understanding the health benefits of red beets make you more likely to consume and/or make beet kvass?
    - Yes
    - No
    - Unsure

    (If no or unsure, skip to Question 14)

13. If yes, do you think you would be more likely to make or purchase?
    - Make
    - Purchase
    - Both
    - Unsure

(Next page)

As part of this research, we may ask consumers to participate in a taste test at the University of Maine Sensory Evaluation Center. Would you be interested in an invitation to participate in this type of study? If yes, please provide an email address for notifications. Your personal information is confidential and is linked to your responses to confirm eligibility for future sensory evaluation, but will not be used in publication.

o Yes : (enter email)

o No

(Next Block)

If you would like to be entered in a raffle to win one of two $25 Amazon gift cards, please enter your email below. Your responses are confidential. Gift cards will be awarded via email.

Email:

Thank you for your time and answers.

APPENDIX B: ONLINE SURVEY INFORMED CONSENT FORM

You are invited to take part in online survey for a research project. The goal is to learn how people perceive of fermented foods. This project will be done by graduate student Abigail Hing and faculty sponsor Dr. Jennifer Perry from the School of Food and Agriculture. You must be at least 18 years old to participate.

What Will You Be Asked to Do?

If you choose to take part in this study, you will be asked to answer a survey about yourself, your perception and interest towards the safety and health benefits of fermented foods. You may not skip any questions. All responses are confidential and will take approximately 15 mins to complete.

Risks

Time and inconvenience are the risks to you from participating in this study.

Benefits

While this survey will have no direct benefits to you, the results from this study will allow researchers to help Maine home fermenters and processors develop safer fermented products.

Compensation

All participants who complete the survey and provide their contact information for the raffle will be eligible to enter a raffle for one of two $25 Amazon gift cards. No compensation will be pro-

vided if you decide not to complete the survey, or if you do not provide contact information for the raffle. All responses are confidential.

Confidentiality

This study is confidential. You will be given the option to provide contact information so that you can participate in future studies and to enter a raffle. Personal information will be linked to your responses to confirm eligibility for future sensory evaluation, but will not be used in publication and stored separately from publicized data. All data will be kept on a password-protected computer indefinitely.

Voluntary

Taking part in this study is voluntary. If you choose to take part in this study, you may stop at any time, but you must complete the survey to enter the raffle. You may not skip any questions. Submission of the survey implies consent to participate.

Contact Information

If you have any questions about this study, please contact:

• Abigail Hing, email: abigail.hing@maine.edu

• Dr. Jennifer Perry (Faculty sponsor), email: jennifer.perry@maine.edu

If you have any questions about your rights as a research participant, please contact the Office of Research Compliance, University of Maine, 207-581- 2657 (or e-mail umric@maine.edu).

APPENDIX C: ONLINE SURVEY RECRUITMENT NOTICE

Participation needed for a brief online survey on perceptions and interests towards fermented foods.

Hello-

You are receiving this email because you are in the emailing list of (School of Food and Agriculture, Cooperative Extension's Constant Contact, or the School of Kinesiology, Physical Education and Athletic Training).

You are invited to participate in a research project being conducted by graduate student Abigail Hing and faculty sponsor Dr. Jennifer Perry of the University of Maine School of Food and Agriculture. If you are least 18 years old, and have access to the internet, please help researchers learn about the perception of consumers towards fermented foods and the health benefits they may provide.

This study should take no more than 15 minutes to complete and participants who complete this survey may enter in a raffle to win one of two $25 Amazon gift cards. You may not skip any questions. All responses are confidential. If participants agree to participate in future sensory evaluation, personal information will be linked to your responses to confirm eligibility, but will not be used in publication .The data files will be archived in Digital Commons and kept indefinitely so that other researchers may access the anonymous, federally- funded data.

If you have any questions, please contact Abigail Hing at abigail.hing@maine.edu or Dr. Jennifer Perry (Faculty sponsor) at jennifer.perry@maine.edu.

APPENDIX D: ONLINE SURVEY RECRUITMENT POSTER

THE UNIVERSITY OF MAINE

SCHOOL OF FOOD AND AGRICULTURE

Participation needed for a brief online survey on perceptions and interests towards fermented foods.

Hello-

You are receiving this email because you are in the emailing list of Cooperative Extension's Constant Contact.

You are invited to participate in a research project being conducted by graduate student Abigail Hing and faculty sponsor Dr. Jennifer Perry of the University of Maine School of Food and Agriculture. If you are least 18 years old, and have access to the internet, please help researchers learn about the perception of consumers towards fermented foods and the health benefits they may provide. **This survey is available until October 30, 2019.**

Link to the survey : https://umaine.qualtrics.com/jfe/form/SV_emND1p9VuVWjye1

This study should take no more than 10 minutes to complete and participants who complete this survey may enter in a raffle to win **one of two $25 Amazon gift cards**. You may not skip any questions. All responses are confidential. If participants agree to participate in future sensory evaluation, personal information will be linked to your responses to confirm eligibility, but will not be used in publication. The data files will be archived in Digital Commons and kept indefinitely so that other researchers may access the anonymous, federally- funded data.

If you have any questions, please contact Abigail Hing at abigail.hing@maine.edu or Dr. Jennifer Perry (Faculty sponsor) at jennifer.perry@maine.edu.

APPENDIX E: SENSORY EVALUATION BALLOT

Welcome to the Sensory Evaluation Center at the University of Maine! Thank you for taking the time to participate in our research. Please evaluate the samples in the order they are displayed to you on the computer screen. Please make sure the 3-digit code on your sample matches the code on your computer screen. For each samples, please take at least two sips. Please take a sip of water before tasting and evaluating each sample.

1. Please indicate your gender. (Choose one answer)

- Male
- Female
- Other
- Prefer to not answer

2. Please indicate your age bracket based on your last birthday. (Choose one answer)

Under 18 [Directs user to the end of the survey]

- 18-25
- 26-35
- 36-45
- 46-55
- 56 years or older
- Prefer not to answer

3. How familiar are you with beet kvass?

- Very familiar
- Moderately familiar

- Have never heard of it

[The following questions will be displayed for all samples]

Please evaluate this sample and rate the intensity of each flavor listed:

Garlic

- Much too garlicky
- Little too garlicky
- Just about right
- A little garlicky
- Not garlicky

Salt

- Much too salty
- Little too salty
- Just about right
- A little salty
- Not salty

Tartness

- Much too tart
- Little too tart

- Just about right
- A little tart
- Not tart

Vinegary

- Much too vinegary
- Little too vinegary
- Just about right
- A little vinegary
- Not vinegary

How much do you like the color of this sample?

- Dislike Extremely
- Dislike very much
- Dislike moderately
- Dislike Slightly
- Neither like nor dislike
- Like slightly
- Like moderately
- Like very much
- Like extremely

How much do you like this product overall?

- Dislike Extremely
- Dislike very much
- Dislike moderately
- Dislike Slightly
- Neither like nor dislike
- Like slightly
- Like moderately
- Like very much
- Like extremely

How likely would you be to purchase/make this product?

- Very likely
- Moderately likely
- Neither likely nor unlikely
- Moderately unlikely
- Very unlikely

Is there anything else that you would like to say about this sample? Please type the sample's three-digit code in your comments.

[Appears after the evaluation of samples]

“Red beets have been associated with health benefits such as lowering hypertension, enhancing exercise performances, and benefitting cardiovascular health.”

Would knowing the health benefits of this sample make you more likely to make or purchase this product?

- Very likely
- Moderately likely
- Neither likely nor unlikely
- Moderately unlikely
- Very unlikely

“Beet kvass is a fermented beverage that contains live, active bacterial cultures.”

Would knowing this make you more likely to make or purchase this product?

- Very likely
- Moderately likely
- Neither likely nor unlikely
- Moderately unlikely
- Very unlikely

Thank you for your time and opinions. Please raise the window slightly to let the kitchen staff know that you are done.

APPENDIX F: SENSORY EVALUATION INVITATION NOTICE

Participation needed for sensory evaluation of beet kvass.

Hello-

You are receiving this invitation because you completed the survey on beet kvass that was held from October 16, 2019 to October 31, 2019, and have agreed to participate in future testing.

You are invited to participate in a research project being conducted by graduate student Abigail Hing and faculty members Dr. Jennifer Perry and Dr. Mary Ellen Camire of the University of Maine School of Food and Agriculture. If you are least 18 years old, please help researchers learn about the consumer acceptability of beet kvass. If you are allergic to red beetroots, garlic, or salt, please refrain from participating.

This study should take no more than 15 minutes to complete and participants who complete this sensory evaluation may receive a compensation of $5. All responses will be anonymous.

When? **(Enter times)**

Where? The Sensory Evaluation Center located in Rooms 158A and 158B in Hitchner Hall at the University of Maine, Orono, Maine.

If you have any questions, please contact Abigail Hing at abigail.hing@maine.edu, Dr. Jennifer Perry (Faculty sponsor) at jennifer.perry@maine.edu or Dr. Mary Ellen Camire at camire@maine.edu

APPENDIX G: SENSORY EVALUATION RECRUITMENT NOTICE

Participation needed for a sensory evaluation on beet kvass. You are receiving this email because you are in the sensory evaluation center email list.

Hello-

You invited to participate in a research project being conducted by graduate student Abigail Hing and faculty members Dr. Jennifer Perry and Dr. Mary Ellen Camire of the University of Maine School of Food and Agriculture. If you are least 18 years old, please help researchers learn about the consumers acceptance towards beet kvass.

Please complete a pre-survey questionnaire that would determine your eligibility for this sensory evaluation. (Enter link and QR code)

This questionnaire should take no more than 5 minutes, while the sensory evaluation study should not take no more than 15 minutes to complete. Participants who have successfully completed the sensory evaluation would be compensated with $5.

If you have any questions, please contact Abigail Hing at abigail.hing@maine.edu, Dr. Jennifer Perry (Faculty sponsor) at jennifer.perry@maine.edu or Dr. Mary Ellen Camire at camire@maine.edu

APPENDIX H: SENSORY EVALUATION PRE-SCREENING QUESTIONNAIRE

Thank you for showing interest in participating in our sensory evaluation. Please complete this pre-screening questionnaire to determine your eligibility to participate in our sensory evaluation.

1. Please indicate your age bracket based on your last birthday (Choose one answer)

- Under 18
- 18-25
- 26-35
- 36-45
- 46-55
- 56-65
- 66 years or older

Skip To: End of Survey If Q1 = Under 18

2. Do you currently purchase and/or consume fermented foods?

- Yes
- No

Skip To: End of Survey If Q2 = No

3. Are you allergic to red beetroot, garlic, and/or salt?

- Yes
- No

Skip To: End of Survey If Q3 = Yes

4. Please click on the link below for the sensory evaluation time and location.

(link)

End of survey: Thank you for your interest in this research. You are currently not eligible to participate in this sensory evaluation

APPENDIX I: SENSORY EVALUATION INFORMED CONSENT FORM

You are invited to take part in a sensory evaluation for a research project. The goal is to learn the acceptance and taste preference of consumers towards beet kvass. This project will be done by graduate student Abigail Hing and faculty members Dr. Jennifer Perry and Dr. Mary Ellen Camire from the School of Food and Agriculture. You must be at least 18 years old to participate. If you are allergic to red beetroots, garlic, or salt, please refrain from participating.

What Will You Be Asked to Do?

If you choose to take part in this study, you will be asked to try four different beet kvass. Please take at least two sips of each sample. For each sample, you answer a few questions about the taste of the sample. Please click on the boxes you identify the flavor of the samples with. You may not skip any questions. It may take up to 15 minutes of your time.

Risks

Time and inconvenience are the risks to you from participating in this study.

Benefits

While this sensory evaluation will have no direct benefits to you, the results from this study will allow researchers to better understand the acceptance and preference of beet kvass.

Compensation

All participants who complete the evaluation will be compensated with $5. No compensation will be provided if you decide not to complete the evaluation.

Confidentiality

All data will be collected anonymously and store indefinitely on a secure password protected computer. All data will be archived to Digital Commons for other researchers to access.

Voluntary

Taking part in this study is voluntary. If you choose to take part in this study, you may stop at any time, but you must complete the evaluation to be compensated. You may not skip any questions.

Contact Information

If you have any questions about this study, please contact:

- Abigail Hing, email: abigail.hing@maine.edu
- Dr. Jennifer Perry (Faculty sponsor), email: jennifer.perry@maine.edu

- Dr. Mary Ellen Camire: camire@maine.edu

If you have any questions about your rights as a research participant, please contact the Office of Research Compliance, University of Maine, 207-581- 2657 (or e-mail umric@maine.edu).

BIOGRAPHY OF THE AUTHOR

Abigail Wei Jing Hing was born in Kuala Lumpur, Malaysia on November 26, 1996. She was raised in Kuala Lumpur, Malaysia and moved to Pennsylvania to pursue her undergraduate degree. She graduated from Messiah University, PA, with a Bachelor's degree in Biochemistry in 2018. After graduating, she moved to Maine and attended the University of Maine. Abigail plans to work in the food industry and move to the Pacific Northwest when opportunity arises. She is a candidate for the Master of Science degree in Food Science and Human Nutrition from the University of Maine in August 2020.