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Isolation and Identification of Foodborne Pathogens and Spoilage Bacteria in Environmental and Food Samples

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ISOLATION AND IDENTIFICATION OF FOODBORNE PATHOGENS AND SPOILAGE
BACTERIA IN ENVIRONMENTAL AND FOOD SAMPLES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
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Doctor of Philosophy

in

School of Nutrition and Food Sciences

by
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To Francisco & Emma

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Escherichia coli O157:H7 has become an important problem for human health in the United States. Scientific studies indicate cattle herds as primary reservoirs of *E. coli* O157:H7. To diagnose this pathogen, proper isolation and identification methods are crucial. From five different culture media, CT-SMAC and CHROMagar™ O157 results analyzed simultaneously were the best option for an effective detection of environmental *E. coli* O157. Despite extensive research at feedlots and dairy farms, there is limited information available on the prevalence of *E. coli* O157:H7 at cow/calf operations. From 28 small-scale cow/calf operations in the state of Louisiana, we observed an 8% prevalence of this pathogen, with no significant difference between fecal matter, water, and swabs from surfaces as a source of contamination. In a different context but also important for the state of Louisiana, hot sauce has become a large industry with more than 35 brands available in the market. Production of hot pepper sauce may require fermentation of red hot pepper mash in barrels from 2 weeks to 3 years. Physicochemical and microbiological changes during mash natural fermentation were studied over a period of 18 months. A significant reduction of pH was observed, which had a reverse correlation with production of lactic acid. There were minor changes in color of mash. Aroma was analyzed based on six volatile compounds which had a significant increase during the first 60 days of fermentation. We observed four stages during the fermentation of the mash, where LAB and yeast were the main microorganisms isolated, presenting a symbiotic association that stopped at 18 months of fermentation. As part of the fermentation process, 7% of red hot chili pepper mash will develop undesired changes in color, texture, and aroma. *Bacillus firmus, Bacillus pumilus, Brevibacillus laterosporus, Enterococcus avium*, and *Aerococcus viridans* were isolated from spoiled mash. When fresh pepper mash was inoculated with this isolates, spoilage of mash was produced after 60 days of storage at 35°C.

These microorganisms were also isolated from naturally fermented not spoiled pepper mash. In conclusion, to prevent loses due to spoilage of the mash, fermentation should be stopped after 18 months.

CHAPTER 1.   LITERATURE REVIEW

Microorganisms found in food can have either a negative or positive effect; some cause spoilage of foods, others can cause foodborne illness, and some can transform food in a beneficial way (food fermentation). Microorganisms have specific growing requirements that include nutrients, environmental temperature, presence/absence of oxygen, pH, moisture, etc. Therefore, changes in these factors will determine which microorganisms will survive and colonize a food product. The methods we choose to isolate and identify these microorganisms will depend on the type of microorganism and type of sample we are analyzing.

1.1. *Escherichia coli* O157:H7

E. coli bacteria are environmental microorganisms that have been isolated from human and animal intestines, and are considered an important part of a healthy human intestinal tract. These bacteria were discovered in the colon of humans in 1885 by Dr. Theodor Escherich, who named it *Bacterium coli commune*. It was not until 1954 when *Escherichia coli* name was recognized. *E. coli* is a large group of bacteria, of which most are harmless and have been broadly used in microbiology laboratories in research and food safety purposes. Pathogenic *E. coli* can cause a broad range of human diseases that span from gastrointestinal tract to urinary tract, bloodstream, and central nervous system (CDC, 2015; Croxen et al., 2013). Certain serotypes of *E. coli* produce a cytotoxin known as Shiga toxin (Stx).Organisms from this group are named Shiga toxin producing *E. coli* (STEC). Some strains of STEC are associated with hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans; this last group is called enterohemorrhagic *E. coli* (EHEC). *E. coli* O157:H7 is the main EHEC serotype associated with foodborne outbreaks in United States (Bettelheim, 1998; CDC, 2014; Feng et al., 2011; LeJeune et al., 2004; Stanford et al., 2005).

E. coli O157:H7 was reported within the top five pathogens contributing to domestically acquired foodborne illnesses in hospitalization (CDC, 2011). Several studies have indicated cattle as the main reservoir of *E. coli* O157:H7 worldwide, finding that the main sources are feces, feed bunks, water troughs and incoming water supplies (Dargatz et al., 1997; Hancock et al., 1997; Sargeant et al., 2003; Van Donkersgoed et al., 2005).

1.1.1. Isolation and Identification of *E. coli O157:H7*

In the Bacteriological Analytical Manual (BAM), the FDA recommends the isolation and identification of *E. coli* O157:H7 before testing for specific virulence traits (Feng et al., 2011). To reduce nontarget organisms during culture, selective enrichment has been used for the recovery of *E. coli* O157:H7 from food, animal and environmental samples (Barkocy-Gallagher et al., 2002; Chapman, 2000). Modified tryptone broth (mTSB) has been used as a selective enrichment for *E. coli* O157:H7 in foods and environmental samples. (Doyle and Schoeni, 1987; Sallam et al., 2013).

When *E. coli* O157:H7 contamination is suspected in samples that contain high levels of competing microbial flora, it has been also recommended to use immunomagnetic separation (IMS). Specific *E. coli* O157 IMS kits available in the market allow rapid selective separation of *E. coli* O157 from food and environmental samples, which improves the quality of sample preparation and sample testing process (Chapman, 2000; Chapman et al., 1994; Keen et al., 2006).

Several media have been used for the isolation of *E. coli* O157:H7.Sorbitol MacConkey Agar (SMAC) has been recommended for the differentiation of slow or non-sorbitol fermenters like *E. coli* O157:H7 (Farmer and Davis, 1985; Fedio et al., 2011; March and Ratnam, 1986; Rappaport and Henig, 1952). More recently CHROMagar®O157 was introduced in the market for the detection of *E. coli* O157 which has been used for testing food and environmental samples (Bettelheim, 1998; Fratamico and DebRoy, 2010; Keen et al., 2006; Tanaro et al., 2014). Rainbow agar has been also used for the isolation of *E. coli* O157:H7 in carcass, beef, and environmental samples (Barkocy-Gallagher et al., 2002; Van

Donkersgoed et al., 2001; Vipham, 2011). Both, CHROMagar and Rainbow agar principle of differentiation is β-glucuronidase activity. Modifications to media have been recommended to improve selectivity of media; novobiocin, cefsulodin, cefixime, vancomycin, and potassium tellurite have been used in SMAC, CHROMagar and Rainbow agar for the isolation of *E. coli* O157:H7 in food, carcass, feces and environmental water samples (Barkocy-Gallagher et al., 2002; Chapman et al., 1994; Fedio et al., 2011; Keen et al., 2006; LeJeune et al., 2004; Sallam et al., 2013; Sargeant et al., 2003; Tanaro et al., 2012; Wallace et al., 1997).

Real-time multiplex PCR (RT-PCR) assays have been developed to detect *E. coli* O157:H7 in food and environmental samples targeting virulence genes *stx1*, *stx2*, O157, and H7, which offers several advantages over traditional end-point PCR methods (Fedio et al., 2011; Fratamico and DebRoy, 2010; Jinneman et al., 2003; Qin et al., 2010). Multiplex RT-PCR offers the amplification of two or more DNA regions at the same time, and there is no need for post PCR processing steps to analyzed PCR products.

1.2. Microorganisms associated with food fermentation

1.2.1. Lactic Acid Bacteria

Lactic acid bacteria (LAB) play an important role in the food industry, which have been isolated from fermented vegetable foods, beverages, dairy products, and meats; however, they can also be responsible for spoilage. LAB have been used as starter culture in food fermentation, contributing to food preservation, taste and texture. *Streptococcus*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, and *Leuconostoc* are all LAB. They are fastidious organisms requiring many vitamins, amino acids, purines, and pyrimidenes for their growth; they normally depend on sugar fermentation for energy. LAB are generally recognized as facultative anaerobes. They grow under slightly acidic conditions, when pH is between 4.5 and 6.4. (Deguchi and Morishita, 1992; Garver and Muriana, 1993; Prescott et al., 2002; Teusink et al., 2005).

Several media are recommended for the isolation, cultivation and identification of LAB; Lactobacillus MRS, Reinforced Clostridial Medium, M17, Ragosa, APT within others (Dave and Shah,

1996; Dicks et al., 2004; Fleming et al., 1983; Holzapfel, 1997; Zimbro et al., 2009). MRS has been largely used for the isolation and cultivation of LAB from red hot chili pepper mash, olives, sauerkraut, carrots, wine, dairy products, etc. (Halász et al., 1999; Kakiomenou et al., 1996; Kun et al., 2008; Lonvaud-Funel, 1999; Tassou et al., 2002; Tofalo et al., 2012; Wang et al., 2002). MRS agar contains peptone, dextrose, polysorbate 80, acetate, magnesium and manganese, which provide growth factors for culturing LAB, and it can be used for direct count or as broth enrichment (Zimbro et al., 2009).

Within the microorganisms involved during food product fermentation, *Lactobacillus plantarum* has been isolated and used during fermentation of several vegetable foods, such as black olives, sauerkraut, pepper mash, cucumbers, and soybean (Halász et al., 1999; Panagou et al., 2008; Plengvidhya et al., 2007; Sarkar et al., 1994; Tamang et al., 2005; Yoon et al., 2006). Moreover, *Lactococcus lactis* has been also isolated from dairy products, olives, meat products (Ayad et al., 1999; Caplice and Fitzgerald, 1999; Garver and Muriana, 1993; Holzapfel, 1997; Leroy and De Vuyst, 2004) . *L. lactis* is also related to production of aromatic compounds such as short chain fatty acid esters, responsible for fruity flavors in dairy products (Hiu et al., 2010; Holland et al., 2005; Liu et al., 2004).

Lactobacillus curvatus has been identified and used in the production of fermented meats, and more recently it has also been isolated from sauerkraut (Caplice and Fitzgerald, 1999; Garver and Muriana, 1993; Leroy and De Vuyst, 2004). *Lactococcus brevis* was also isolated during sauerkraut fermentation, black olives, and from traditionally fermented vegetable products in the Himalayas (Halász et al., 1999; Leroy and De Vuyst, 2004; Plengvidhya et al., 2007; Tamang et al., 2005; Tassou et al., 2002). *Pediococcus acidilactici* has been isolated from several vegetable products and fermented meats as well (Garver and Muriana, 1993; Holzapfel, 1997; Leroy and De Vuyst, 2004; Tamang et al., 2005).

1.2.2. Yeast

Yeasts have been associated with fermented foods, such as bread, alcoholic beverages, rice, soy sauce, olives, red hot chili pepper, and cheese (Aidoo et al., 2006; Arocha, 1984; Gotcheva et al., 2000;

Paul Ross et al., 2002; Tamang et al., 2005; Tofalo et al., 2012; Tsuyoshi et al., 2005). Yeast alone, or in combination with LAB and/or molds have a significant effect on food quality parameters such as taste, texture, and aroma (Aidoo et al., 2006). For the isolation and cultivation of yeast, Potato Dextrose Agar/Broth, Brain Heart Infusion, Dextrose Agar, Sabouraud Media, YM media, etc. have been recommended (Zimbro et al., 2009). In addition to traditional methods, 3M Petrifilm™ Yeast and Mold Count Plate (3M, Saint Paul MN) method has been probed as an equivalent to conventional methods for the enumeration of yeast in food products (Beuchat et al., 1991, 1990; Knight et al., 1997).

Candida has been identified as the primary yeast involved in the natural fermentation of red hot chili pepper mash (Arocha, 1984). Within this genus, *C. glabrata* was isolated from fermented cereal based beverages and food products (Aidoo et al., 2006; Gotcheva et al., 2000; Hammes et al., 2005; Tsuyoshi et al., 2005). *Candida krusei* was previously isolated from cucumbers (Costilow et al., 1955) and is an important starter culture in sourdough (Salim-ur-Rehman et al., 2006). *C. norvegensis* has not been isolated from vegetable products but is important as a starter culture in sourdough bread (Salim-ur-Rehman et al., 2006). Additionally, *Rhodotorula spp.* has been isolated from red chili pepper mash, bread products, alcoholic beverages, and cucumbers (Aidoo et al., 2006; Arocha, 1984; Costilow et al., 1955). *Saccharomyces cerevisiae* has been isolated from several vegetable products such as pickles, olives, cereal based products, alcoholic beverages (Aidoo et al., 2006; Caplice and Fitzgerald, 1999; Hammes et al., 2005; Holzapfel, 1997; Salim-ur-Rehman et al., 2006; Tofalo et al., 2012; Tsuyoshi et al., 2005), and is also an important producer of esters (Liu et al., 2004).

1.3. Red hot chili pepper mash

Hot pepper sauce is made from red hot chili peppers that can be fermented for weeks to years depending on the processor. Food fermentation goes through several modifications that include physical, chemical and microbiological changes that will affect the quality and safety of product. In the majority of fermentation processes of food products, the prevailing microbial groups are lactic acid bacteria (LAB) and/or yeast, the relative population of which defines the characteristics of the final product. The

5

fermentation of red hot chili mash results in approximately 7% of the barrels having spoiled pepper mash that have changes in color, texture, and aroma. These changes include black or white pigmentation on the top or alongside the walls of the barrels, slime formation and an unpleasant aroma. The spoilage causes an economical loss to the hot sauce industry of 2 million dollars per year in the state of Louisiana.

1.3.1. Spoilage microorganisms in red hot chili peppers

Bacillus firmus, *Bacillus pumilus*, *Enterococcus avium*, *Brevibacillus laterosporus*, and *Aerococcus viridans* are environmental microorganisms isolated from pepper mash, and when conditions in the mash are optimum for their growth, they will lead to spoilage of the mash. *Bacillus firmus* is a Gram-positive, spore forming rod naturally found in soil. It has been used as natural pesticide and nematicidal, used in residential outdoor and greenhouse applications to fruit, vegetables, and field crops, including non-food crops such as turf and ornamentals. It is intended to protect roots from nematode infections, when applied directly to the soil, foliar treatment to turf, and as seed treatments (Bacchus, 2008; Giannakou et al., 2004; Terefe et al., 2009). Use of *B. firmus* meets the standards for pesticides and food use under the Federal Insecticide, Fungicide, and Rodenticides Act (FIFRA), as amended by the Food Quality Protection Act (FQPA) of 1996 (Bacchus, 2008). It produces a protease used in the brewing, detergent, food and leather industries (Moon and Parulekar, 1991; Puri et al., 2002; Rao and Narasu, 2007) Proteolysis in fermented products increases the pH due to production of basic non-protein nitrogen (Bover-Cid et al., 1999).

Bacillus pumilus is a Gram-positive bacterium that is aerobic, spore-forming, rod-shaped and commonly isolated in soil, plants and environmental surfaces. It naturally occurrs in soil and plant tissue (Gutiérrez-Mañero et al., 2001). When applied to soybean seeds, the bacterium protects the roots of the soybean plant against certain fungi (Nielsen and Sorensen, 1997). No harm to humans or the environment is expected from use as a pesticide active ingredient (Bottone, 2003). *B. pumilus* has been found to produce proteases (Huang et al., 2003; Kumar, 2002).

6

Enterococcus avium is a Gram-positive, cocci-shape bacterium. Enterococci are facultative anaerobic organisms that can survive and grow in many environments. Enterococci are part of the normal intestinal flora of humans and animals, *E. avium* was isolated from the bee (*Apis mellifera*) intestinal tract and bee bread (Audisio et al., 2005).

Brevibacillus laterosporus is a Gram-positive, rod shape bacterium; formerly classified as *Bacillus laterosporus* (Shida et al., 1996), it was also isolated from honey, dead honeybee larvae, and in healthy worker-bee intestine (Alippi and Reynaldi, 2006). It is used as a biological control against beetles, mosquitos, flies, and nematodes (Alippi and Reynaldi, 2006; Oliveira et al., 2004; Ruiu et al., 2007).

Aerococcus viridans is a cocci-shape Gram-positive bacterium. It has been isolated in air and dust (Cetin et al., 2007). It was also isolated from spoiled meat curing brines showing ability to produce a greenish discoloration in spoiled cured meat products (Deibel and Niven C. F., 1959; Peirson et al., 2003).

1.3.2. Volatile Compounds in red hot chili pepper mash

Consumers often base their choice of food on flavor, aroma, as well as appearance. Volatile compounds are used to evaluate the quality and determine if the fermentation process has been successful in producing the desired final product. Currently, aroma has been used as an important parameter for the quality of fresh fruits and vegetables. The aroma is measured by the extraction, isolation and identification of volatile compounds. Volatile compound content of the red hot chili pepper is very low, ranging from 0.1 to 2.6 % in the fruit, contributing to the overall aroma of the pepper. There have been more than 125 volatile compounds identified in fresh and processed red hot chili pepper (Pino et al., 2006). Simultaneous distillation extraction (SDE) and solid phase microfiber extraction (SPME) have been used for the extraction of volatile compounds from fresh habanero chili peppers during different ripening stages and in different colors of peppers in Latin America. One approach for the isolation and identification of volatile compounds is the gas chromatography and mass spectrometry (GC-MS) analysis.

SPME is a fast and simple method of extraction that can be done without the use of solvents. SPME is an equilibrium extraction technique that can be used as an alternative to conventional sample extraction techniques. In SPME, a polymer-coated fused fiber is introduced into the headspace above the sample until equilibrium is reached among the sample matrix, the headspace, and the fiber. Components of SPME are shown in Figure 1. After extraction, SPME fiber is retracted and transferred to the injection port of a separating instrument, such as a gas chromatograph (GC), where desorption of the analyte takes place and analysis is carried out. The procedure on how to collect sample and the desorption procedure are shown in Figures 2 and 3.When properly stored, samples can be analyzed days later in the laboratory without significant loss of volatiles. Volatile compounds concentrated on the fiber are rapidly delivered to the column, minimum detection limits are improved and resolution is maintained. Headspace, volume, and airflow rate are important variables when using SPME as extraction method. Desorption from the fiber depends on temperature of the GC-MS injector, the time, the gas flow, and the depth at which the fiber is inserted in the port (King et al., 2003).

Volatile compounds isolated from chili peppers include but are not limited to *n*-hexanol, 4-methyl-1-pentyl-isobutyrate, (*Z*)-3-hexenyl isopentanoate, heptyl isobutanoate, heptyl pentanoate, 3,3-dimethylcyclohexanol. *N*-hexanol or hexyl alcohol, is an organic alcohol with green and herbaceous, woody, or sweet organoleptic properties (Burdock, 2010), and was isolated in fresh Tabasco and Habanero peppers (Pino et al., 2006; Wendell-Haymon and Aurand, 1971). 4-methyl-1-pentyl-isobutyrate or 4-methylpentyl 2-methylpropanoate is an ester predominant in fruit aromas and contributes to the overall aroma of Tabasco peppers (Wendell-Haymon and Aurand, 1971). (*Z*)-3-hexenyl isopentanoate or 3-hexenyl isovalerate is an ester, colorless liquid insoluble in water. Its organoleptic characteristics include a powerful, sweet, green odor of apple and a buttery, apple-like taste. It occurs naturally in mint and Tabasco peppers, and was previously identified as a constituent of Habanero and Tabasco peppers (Burdock, 2010; Pino et al., 2006; Wendell-Haymon and Aurand, 1971).
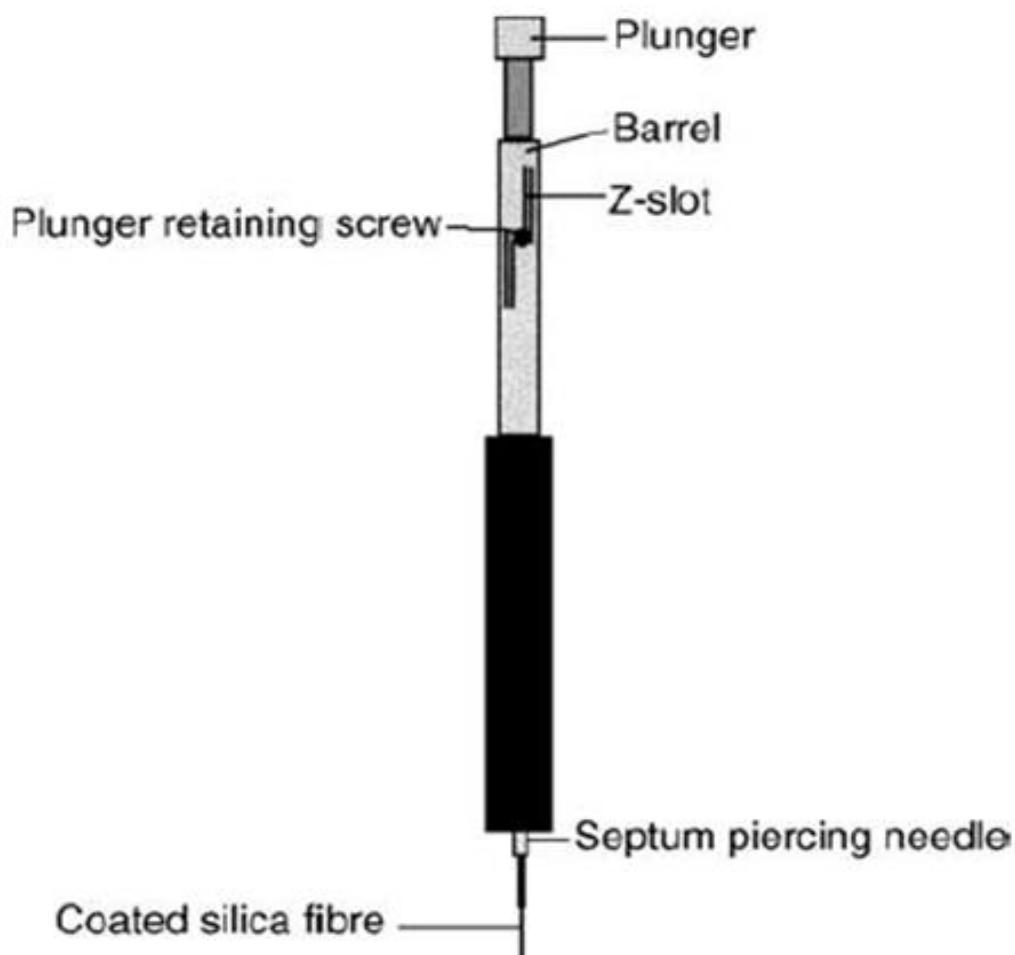
Figure 1. Components of SPME (King et al., 2003)

Heptyl isobutanoate or heptyl isobutyrate is an ester, colorless liquid insoluble in water, and has a characteristic woody odor with distinctly herbaceous, sweet aroma, and has also been characterized as having a sweet, herbaceous, fruity, and slightly warm floral taste. It occurs naturally in the essential oil of hops (Burdock, 2010). Heptyl pentanoate, or heptyl valerate is also an ester, colorless to pale yellow liquid with a fruity aroma (Burdock, 2010). 3,3-dimethylcyclohexanol has been described as having a "fried onion" aroma (Ferreira and Lopez, 2013). The chemical structure of these compounds is shown in Figure 4. Aroma descriptions of esters found in nature are illustrated in Figure 5.
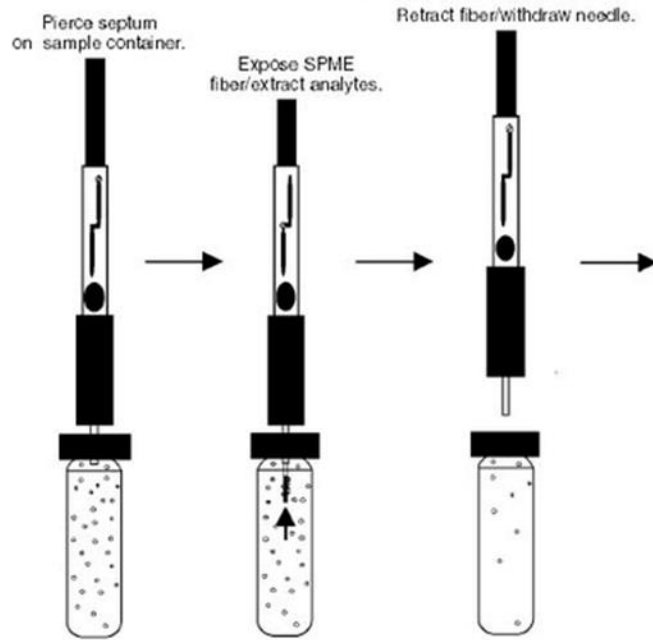
Figure 2. Collecting sample procedure using SPME (King et al., 2003).
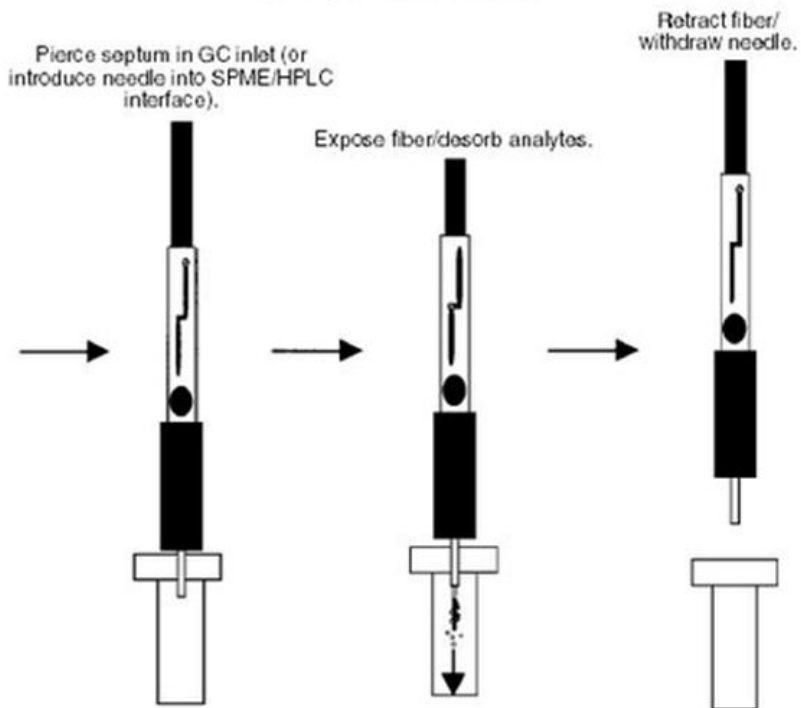
Figure 3. SPME Desortion Procedure (King et al., 2003).
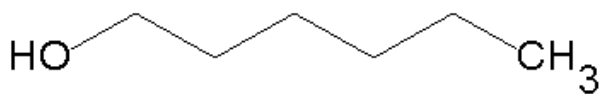
n-hexanol
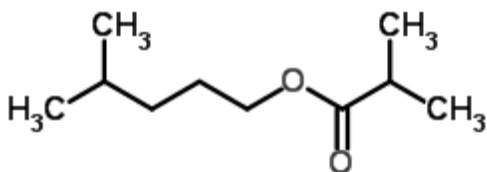
4-methyl-1-pentyl-isobutyrate
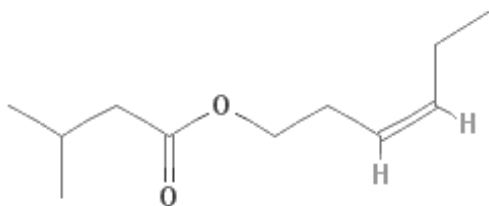
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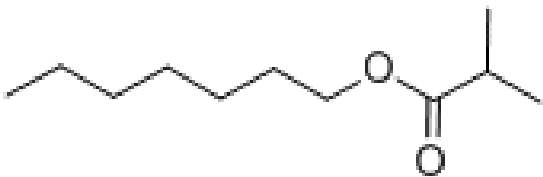
Heptyl isobutanoate
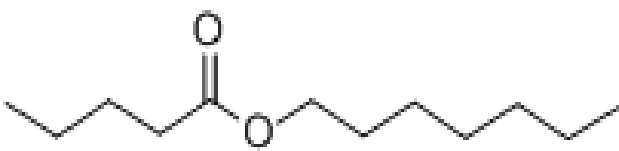
Heptyl pentanoate
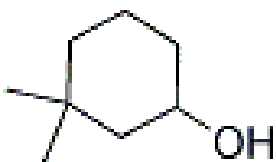
3,3-dimethyl cyclohexanol

Figure 4. Chemical structure of volatile compounds in red hot chili pepper mash.

from the alcohol (first word)

	methyl 1 carbon	ethyl 2 carbons	propyl 3 carbons	2-methyl propyl-	butyl 4 carbons	pentyl 5 carbons	hexyl 6 carbons	benzyl benzene ring	heptyl 7 carbons	octyl 8 carbons	nonyl 9 carbons
methanoate 1 carbon	ETHEREAL	BACARDI		ETHEREAL			"GREEN"				?
ethanoate 2 carbons								JASMINE			?
propanoate 3 carbons											?
2-methyl propanoate 4 carbons, branched		ETHEREAL	BACARDI								?
butanoate 4 carbons											?
pentanoate 5 carbons				ETHEREAL						?	?
hexanoate 6 carbons											
benzoate benzene ring	YLANG-YLANG		NUTS	BALSAMIC						?	
heptanoate 7 carbons					?						?
salicylate from salicylic acid			MINT	WINTERGREEN	STRONG			DIFFERENT PEOPLE PERCEIVE DIFFERENT AROMAS!	?		?
octanoate 8 carbons											
phenylacetate benzene ring + 2 carbons	STRONG							JASMINE	none!		?
nonanoate 9 carbons										?	
cinnamate benzene ring + propenol											
decanoate 10 carbons			OIL		JACK DANIEL'S Old No. 7			?	?	?	?

from the carboxylic acid (second word)

Figure 5. Esters commonly found in nature and their smells (Kennedy, 2013).

1.3.3. Color in red hot chili peppers

Carotenoids are responsible for the red color in chili pepper. The carotenoid synthesis takes place during the ripening stage. Capsanthin (red) is the major pigment of the red pepper. Other major pigments include β-carotene (orange), violaxanthin (yellow), and antheraxantin (Ahmed et al., 2002; Arocha, 1984; Hornero-Méndez and Mínguez-Mosquera, 2000; Howard et al., 2000; Tian et al., 2014). Hunter values (L*, a*, and b*) have previously been used as a valuable tool to describe visual color in food products (Skerede, 2006). Additionally, hue is an element of the color wheel, where change in hue from red to yellow goes

from 0° to 90°, yellow to green from 90° to 180°, green to blue from 180° to 270° and blue to red 270° to 360°. Hunter values a* and b* are coordinates that reflect hue ($h=\tan^{-1}b*/a*$) (Figure 6) (McGuire, 1992).
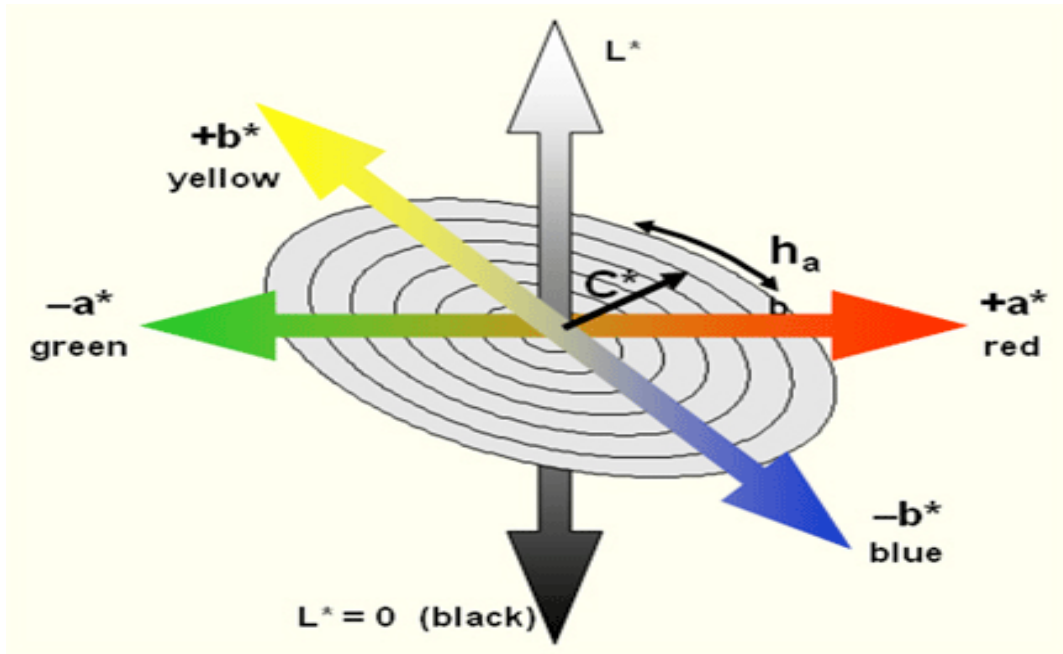
Figure 6. Hunter values (L*, a*, and b*) and hue diagram (Engineering360, 2016)

1.4. Justification

CDC reported that *E. coli* O157:H7 was within the top five pathogens contributing to domestically acquired foodborne illnesses resulting in hospitalization (CDC, 2011). Considerable evidence associates *E. coli* O157:H7 outbreaks with foods of bovine origin such as, ground beef and raw milk hard cheese; cattle have been identified as primary reservoir (Bach et al., 2005). Most studies on *E. coli* O157:H7 have been performed in feedlots, cattle and dairy farms, where researchers use strains adapted to laboratory conditions showing good resistance to media supplements. Additionally, there is limited information on the prevalence of *E. coli* O157:H7 in cow/calf operations. During preliminary studies isolating *E. coli* O157 naturally occurring in environmental samples from cattle farms, we found that some selective media inhibited its growth. First, it was important to identify which media was/were the best option for the isolation and presumptive identification of environmental *E. coli* O157 from cow/calf farms, and to evaluate if the

different environmental samples (fecal matter, water, and water trough surface swabs) had a significant effect on the probability of media in correctly detecting *E. coli* O157. And secondly, to establish the prevalence of *E. coli* O157:H7 in small-scale cow/calf operations in the state of Louisiana and to determine the primary source of *E. coli* O157:H7 in these types of farms.

In a different context, but also important for the state of Louisiana, hot pepper sauce is made from red hot chili peppers and is fermented for weeks to years depending on the processor. Food fermentation goes through several modifications that include physical, chemical and microbiological changes that will affect the quality and safety of product. In the majority of fermentation processes of food products, the prevailing microbial groups are lactic acid bacteria (LAB) and yeast, the relative population of which defines the characteristics of the final product. Limited information is available regarding the bacteria and yeasts involved in the fermentation, and the impact that they have on physicochemical changes occurring during natural fermentation of red hot chili pepper mash. Moreover, the fermentation of red hot chili pepper mash results in approximately 7% of the barrels having spoiled pepper mash that has changes in color, texture, and aroma. With this, the second part of my research was focused on isolating and identifying spoilage microorganisms from normally fermented and spoiled fermented red hot chili pepper mash, in addition to studying the impact of microorganisms in physicochemical changes in the mash during fermentation.

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CHAPTER 2.   ASSESSMENT ON THE SENSITIVITY AND SPECIFICITY OF FIVE CULTURE MEDIA IN THE DETECTION OF *ESCHERICHIA COLI* O157

2.1. Introduction

According to the CDC (CDC, 2015), *Escherichia coli* is a large and diverse group of bacteria, most of which are harmless, but some can be highly pathogenic. Enterohemorrhagic *Escherichia coli* (EHEC) are identified by the production of Shiga toxins (Stxs), but only the ones that have been clinically associated with hemorrhagic colitis (HC) are designated as EHEC. Hemolytic uremic syndrome (HUS) is caused by Stxs produced in the intestine which acts systemically on kidney cells and other organs. Shiga-toxin producing *Escherichia coli* (STEC) is an important group of zoonotic pathogens, and its prototype and most frequent serotype associated with HC and HUS is *E. coli* O157:H7 (CDC, 2015; Fratamico and DebRoy, 2010; Hancock et al., 1994).

To diagnose this pathogen, proper isolation and identification methods are crucial. Prior to confirming the presence of *E. coli* O157:H7 in a sample, the isolation and identification of *E. coli* O157 in culture media are necessary. Currently, there are several options of selective and differential media for the isolation and presumptive identification of *E. coli* O157, including Sorbitol MacConkey Agar (SMAC), Cefixime-Tellurite Sorbitol MacConkey Agar (CT-SMAC),   CHROMagar™ O157 (CHROMagar), Tellurite CHROMagar™ O157 (T-CHROMagar), Vancomycin Cefixime Cefsoludin CHROMagar™ O157 (VCC-CHROMagar). Although all media have been claimed by various authors to be effective in isolating and identifying *E. coli* O157, most of those studies were performed in food, inoculated samples, or in environmental samples from feedlots (Franz et al., 2007; Fratamico and DebRoy, 2010; Hancock et al., 1994).

SMAC was first reported for the identification of *E. coli* O111 and O55 that fermented sorbitol slowly or not at all (Rappaport and Henig, 1952). Later it was suggested that it could also be effective for the detection of *E. coli* O157 in stool samples from adults and children submitted for enteric culturing (Harris et al., 1985; March and Ratnam, 1986). Since then it has been used in research, and multiple studies

suggested that it be supplemented with cefixime and potassium tellurite to make it more selective for the isolation and identification of *E. coli* O157 from environmental sources such as feedlots and dairy cattle (Sargeant et al., 2003; Van Donkersgoed et al., 2001). It has also been used in challenge studies in feces from corn- and barley-bed steers (Bach et al., 2005).

More recently, chromogenic chemical substrates for identification of microbial colonies were patented (Rambach, 1979) which triggered an introduction of a wide range of media for detection of pathogens. Within this type of media, CHROMagar™ O157 and Rainbow agar O157 are widely used, which selectively promote the growth of *E. coli* O157 and differentiate *E. coli* O157 from others due to a chromogenic β-glucuronide substrate (Rambach, 2000). CHROMagar™ O157 has been used for the detection of *E. coli* O157 in food as well as environmental samples, and can be more selective with the addition of potassium tellurite, or vancomycin, cefixime and cefsoludin (Barkocy-Gallagher et al., 2002; Fratamico and DebRoy, 2010; Keen et al., 2006; Wallace et al., 1997).

The success of a selective and differential medium to detect a pathogen depends on its sensitivity and specificity. Sensitivity is also known as true positive rate, which measures the proportion of actual positive results; it is the probability of a positive result that indicates growth of a suspected bacteria. On the other hand, specificity is known as true negative rate, which measures the proportion of actual negative results; it is the probability of a negative result that indicates no growth of bacteria. A highly sensitive medium will give a high number of false positives results, while a highly specific medium will give a high number of false negative results (Lalkhen and McCluskey, 2008; Saah and Hoover, 1997).

During preliminary studies isolating *E. coli* O157 naturally occurring in environmental samples from cattle farms, we found that some selective media was inhibiting the growth. This behavior differed from the one observed in challenge and recovery studies performed in feedlots, cattle and dairy farms, where researchers use strains adapted to laboratory conditions showing good resistance to media supplements. The purposes of this study were to establish sensitivity and specificity of five commercial media for the detection of E. *coli* O157 in the cattle farm environment, to identify which of the five media was/were the

best option for the isolation and presumptive identification of environmental *E. coli* O157 from cattle farms, and to evaluate if the different environmental samples (fecal matter, water and water trough surface swabs) had a significant effect on the probability of media in detecting *E. coli* O157.

2.2. Methodology

For this study, 138 samples were collected from six small-scale cow/calf operations in the State of Louisiana during the months of June and July in 2011. At each operation, a set of 15 samples of fresh fecal matter were collected from the ground, 6 samples of water were collected from water troughs and ponds, and 2 swabs from water trough surface.

2.2.1. Collection of Environmental Samples

To collect the fecal matter from the ground, gardener sterile spoons were used, and then the samples were placed in 13 oz, Nasco Whirl-Pak® write-on bags. Water samples were taken from water troughs and ponds using a sterile plastic ladle, and the samples were placed in VWR® 4 oz Polypropylene Specimen containers with Polyethylene lid. For the swabs, 18 oz Nasco Whirl-Pak® Speci-Sponge® hydrated with Phosphate Buffer Saline (PBS) were used to swab water troughs, hay and salt bunks. All the samples were transported on ice chests to the Food Safety Laboratory located in Baton Rouge, LA, on the Louisiana State University Campus. Samples were placed into enrichment broth the same day of collection.

2.2.2. Isolation of *E. coli* O157

Enrichment in Tryptic Soy Broth (TSB) was used for the isolation of *E. coli* O157 from all the environmental samples collected. Pre-enrichment was as follows: 5 g of fecal matter samples was added to 50 ml of TSB, 30 ml of environmental water samples was added to 30 ml of double strength TSB, and environmental sponge samples were added to 90 ml of TSB. All the samples were incubated overnight at $35\pm2°C$. After incubation, Immunomagnetic Separation (IMS) was performed using Dynadeads® MAX *E.coli* O157 Kit, following a manual protocol suggested by the manufacturer (Invitrogen Co., Carlsbad,

CA). The product from a single IMS was plated on SMAC (Difco™ MacConkey Sorbitol Agar), CT-SMAC (Cefuxune Tellurite Supplement, 77981 Fluka Analytical), CHROMagar (CHROMagar™ O157, CHROMagar), T-CHROMagar (Potassium Tellurite, 02100427 MP Biomedicals, and VCC-CHROMagar (Vancomycin Cefixime Fefsulodin Selective Supplement, 80704 Fluka Analytical). The approximate formula for SMAC, CHROMagar, and concentration of supplements per liter of medium are described in Tables 1, 2, and 3, respectively. Suspected colonies from selective/differential media were cultured and tested for the presence of stx_1, stx_2, and wzy_{O157} genes by Real-Time Polymerase Chain Reaction (RT-PCR). Since CHROMagar™ O157 and Rainbow agar O157 both use the same enzymatic reaction for detecting *E. coli* O157, we chose CHROMagar™ O157 to represent the chromogenic β-glucuronide substrate reaction.

Table 1. SMAC approximate formula per liter (Zimbro et al., 2009)

Ingredient	Amount (g)
Peptone	15.5
Proteose Peptone	3.0
D-Sorbitol	10.0
Bile Salts	1.5
Sodium Chloride	5.0
Agar	15.0
Neutral Red	0.03
Crystal Violet	1.0

Table 2. CHROMagar™ O157 approximate formula per liter Source (CHROMagar, 2013)

Ingredient	Amount (g)
Agar	15.0
Peptone and Yeast extract	13.0
Chromogenic mix	1.2

27

Table 3. Supplement concentration per liter of selective/differential media

	Cefixime	Potassium Tellurite	Cefsoludin	Vancomycin
CT SMAC	0.050 mg	2.5 mg	n/a	n/a
T CHROMagar O157	n/a	2.5 mg	n/a	n/a
VCC CHROMagar ™ O157	0.025 mg	n/a	5.0 mg	4.0 mg

n/a: not applicable

2.2.3. Identification of the strain

All cultures from suspected colonies were analyzed by RT-PCR. FDA Bacteriological Analytical Manual (BAM) protocol was used for the DNA extraction (Feng et al., 2011). SmartCycler II platform was used, targeting stx_1, stx_2, and wzy_{O157} genes with product sizes of 200, 140, and 112 bp, respectively (Table 4). The PCR was performed using 6.2 µl of OmniMix HS for Cepheid SmartCycler® Systems (TaKaRa Bio Inc.), which was prepared as recommended by the manufacturer. The concentration of primers and probes used for the assay were 0.25 µM and 0.125 µM, respectively, and the volume of the sample was 2.5µl. The protocol used for the PCR was 2 min at 94°C, followed by 40 cycles at 94°C for 20 s, 60°C for 30 s, and 72°C for 50 s (Fratamico and DebRoy, 2010). The threshold between positive and negative was set at 30 fluorescence units.

2.2.4. Statistical analysis

Contingency tables were used to analyze the sensitivity and specificity of each medium individually. To analyze the results of two media, Logistic Regression was applied, modelling the presence/absence of *E. coli* O157 as a dependent variable, and the culture media (SMAC, CT-SMAC, CHROMagar, T-CHROMagar, and VCC-CHROMagar) as regressor variables of the model. Moreover, the effects of different environmental samples on the detection of *E. coli* O157 were evaluated using logistic regression models. Statistical Analysis Software® (SAS 2012) at alpha=0.05 was used for all data analysis.

Table 4. Oligonucleotide primers and probes used in the Real-Time Multiplex PCR assay for detection of E. coli O157 (Fratamico and DebRoy, 2010)

	Primer sequences 5' to 3'	PCR product size	GenBank accession no.
Primer sets			
stx1-418-F	CTCGACTGCAAAGACGTATG	200	U47614
stx1-617-R	TTCGTTCAACAATAAGCCGTAGATT		
stx2-314-F	ACGATAGACTTTTCGACCCAACAA	140	M16626
stx2-453-R	AAATAACTGCCCGGTGGGGT		
wzyO157-528-F	CCTGTCAAAGGATAACCGTAATCC	112	X07865
wzyO157-639-R	TTGTTCTCCGTCTTGTCCTAAACT		
Probes			
stx1-P	5'-Texas Red-CGCTGAATGTCATTCGCTCTGCA-BHQ2		
stx2-P	5'-Texas Red-AACAGACACCGATGTGGTCCCCTGAGAT-BHQ2		
wzyO157-P	5'-FAM-AAAACAACGAGCATACAACCCCTACCAAT-BHQ1		

2.3. Results

The sensitivity of each medium to detect *E. coli* O157 in environmental samples from small-scale cow/calf operations in Louisiana is displayed in Figure 7, where SMAC showed the highest sensitivity having 109 suspected positive results from 138 samples, followed by CT-SMAC with 53 suspected positive samples, and CHROMagar with 14 suspected positive samples. Furthermore, T-CHROMagar and VCC-CHROMagar were unable to detect *E. coli* O157 in all the environmental samples tested in the present study (Figure 7).

	SMAC	CT-SMAC	CHRomagar	T-CHRomagar	VCC-CHRomagar	RT-PCR (validation)
■ positive	109	53	14	0	0	27
■ negative	29	85	124	138	138	111

Figure 7. Detection of *E. coli* O157 in environmental samples from small-scale cow/calf operations in the State of Louisiana; comparing results from SMAC, CT-SMAC, CHROMagar, T- CHROMagar, VCC-CHROMagar with RT-PCR. SMAC had higher sensitivity (109/138) followed by CT-SMAC (53/138), CHROMagar (14/138), and T- CHROMagar and VCC- CHROMagar (0/138).

The interaction between each culture medium and RT-PCR was analyzed to establish the specificity of each medium to detect *E. coli* O157 in environmental samples from cattle farms in Louisiana. To achieve this, contingency tables were used, where the positive and negative results from each medium and the positive and negative results from RT-PCR were crossed to obtain positive, negative, false positive and false negative as marginal totals. For RT-PCR results, positive detection of stx_1, stx_2 and wzy_{O157} was considered as positive *E. coli* O157. The total of *E. coli* O157 positive results detected using RT-PCR as a validation method was 27 from 138 environmental samples from cattle farms (Figure 7). When the RT-PCR results were compared with each medium individually, it was found that T-CHROMagar and VCC-CHROMagar had the highest number of *E. coli* O157 false negative results with 27 for both of them, followed by CHROMagar with 23, CT-SMAC with 13, and SMAC with 7 false negative results (Table 5). The logistic regression model found that media had a significant effect on the predicted probability of correct detection of *E. coli* O157 in environmental samples from cattle farms in the present study (media $P < 0.0001$). Additionally, the probability of detecting *E. coli* O157 in environmental samples using CHROMagar or CT-SMAC was significantly higher than using SMAC, and CHROMagar (odds ratio over SMAC = 7.296) more effective than CT-SMAC (odds ratio over SMAC = 3.789) (Table 6).

Table 5. Detection of *E. coli* O157 in environmental samples from small-scale cow/calf operations in the State of Louisiana; comparing results from SMAC, CT-SMAC, CHROMagar, T- CHROMagar, VCC-CHROMagar with RT-PCR. Negative, positive, false negative and false positive results based on contingency tables comparing results from each media with RT-PCR results.

	SMAC	CT-SMAC	CHROMagar	T CHROMagar	VCC CHROMagar	RT-PCR
Negative	29	85	124	138	138	111
Positive	109	53	14	0	0	27
False negative	7	13	23	27	27	n/a
False positive	89	39	10	0	0	n/a
Concur with RT-PCR	42	86	105	111	111	n/a

Table 6. Logistic Regression analysis on selected media for detection of *E. coli* O157 in environmental samples

Media	$P>\chi^2$	Odds ratio	C.I.L (95%)	C.L.U (95%)
CHROMagar vs SMAC	<0.0001	7.296	4.277	12.444
CT-SMAC vs SMAC	<0.0001	3.789	2.297	6.250

C.I.L. = Lower confidence interval
C.I.U. = Upper confidence interval

After evaluating each medium individually, it was decided to assess results of a combination of two media simultaneously to see if the detection level of *E. coli* O157 could be improved and the false negative results could be reduced. Logistic regression was used to analyze the probability of two media in detecting *E. coli* O157 in environmental samples in this study simultaneously. Where the presence and absence of *E. coli* O157 was used as a dependent variable, and each medium as regressors, the odds ratio obtained from the logistic regression were converted into probability (%) to detect *E. coli* O157 in the samples. Due to the high selectivity of T-CHROMagar and VCC-CHROMagar, and no detection of any *E. coli* O157 in the samples analyzed in the present study, these two media were excluded from the logistic regression analysis. From all the samples that were positive based on RT-PCR results, SMAC, CT-SMAC and CHROMagar were analyzed individually, then results from different combinations of two media were analyzed simultaneously. The calculated probability of correctly detecting *E. coli* O157 was 18% with SMAC, 26% with CT-SMAC and 29% with CHROMagar. All these compared with samples tested positive by RT-PCR. This percentage increased when a combination of two media was assessed simultaneously: 52% for SMAC and CHROMagar, followed by 57% for SMAC and CT-SMAC, and 79% for CT-SMAC and CHROMagar (Table 7).

Another important aspect in this study was the effect of different environmental samples on the detection level of *E. coli* O157 using each different medium. This was also analyzed using logistic regression, where the presence/absence of *E. coli* O157 was used as a dependent variable, and fecal matter,

Table 7. Probability* of detecting correctly *E. coli* O157 in environmental samples, either analyzing results from each medium individually or two media simultaneously.

Media	Probability of detection
SMAC	18%
CT-SMAC	26%
CHRomagar	29%
SMAC & CHRomagar	52%
SMAC & CT-SMAC	57%
CT-SMAC & CHRomagar	79%

*based on logistic regression analysis

water, swabs, SMAC, CT-SMAC, and CHROMagar as regressors. The logistic regression model found that different environmental samples did not have significant effect on the predicted probability of correct detection of *E. coli* O157 in environmental samples from cattle farms in the present study (type of sample $P = 0.7420$).

2.4. Discussion

The present study found that while SMAC, CT-SMAC, and CHROMagar allowed the growth of *E. coli* O157 in environmental samples from small-scale cow/calf operations in the State of Louisiana (Figure 7), results from CT-SMAC and CHROMagar analyzed simultaneously appeared to be the best option for the detection of *E. coli* O157 (Table 7). Furthermore, T-CHROMagar and VCC-CHROMagar were unable to detect *E. coli* O157 in the envi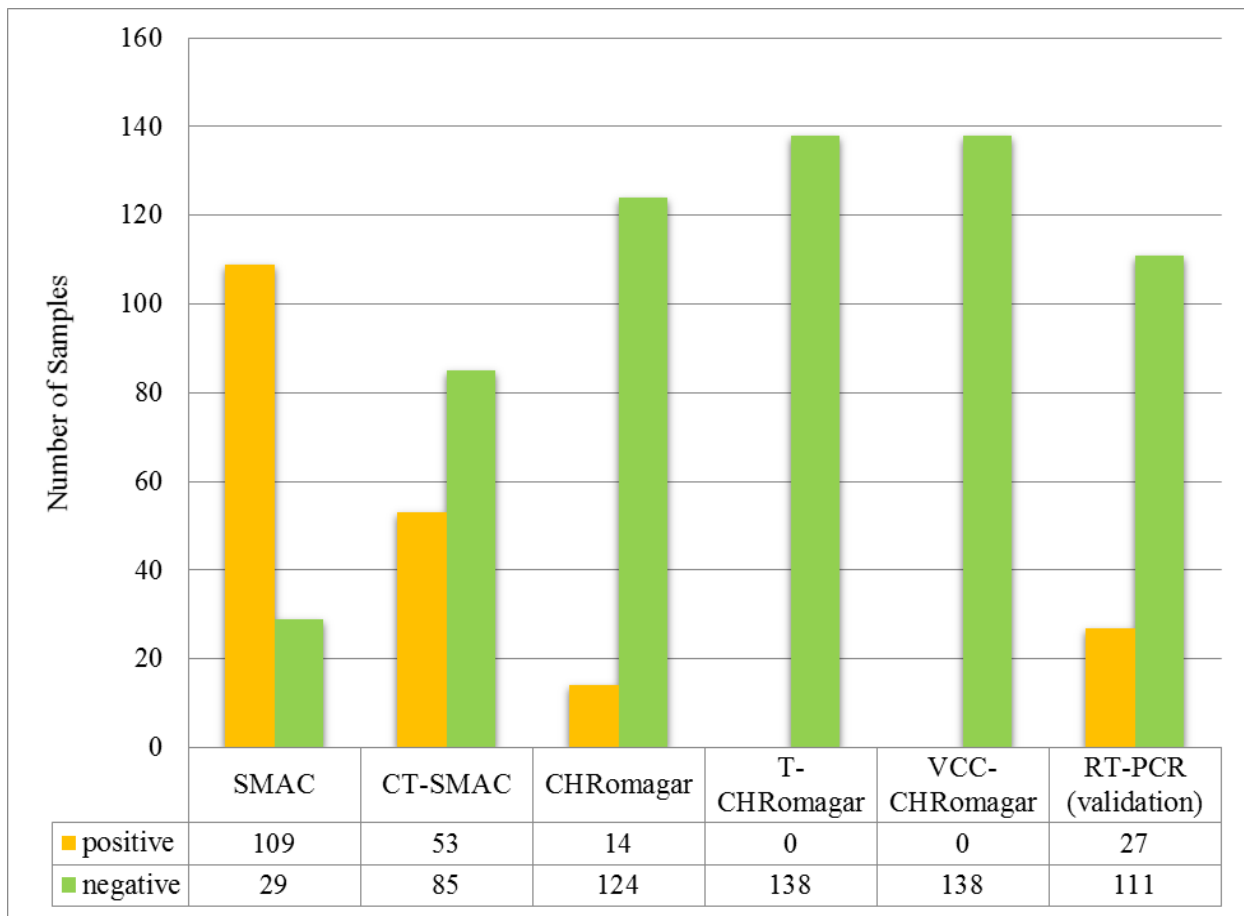ronmental samples that were tested in this study (Figure 7). Although SMAC appeared to be the best medium in terms of sensitivity, it showed a high number of false positive results for environmental samples. However, when supplemented with Cefixime and Potassium Tellurite as recommended for the analysis of environmental samples, it became more selective, finding less false positives results (Chapman et al., 1994; Fedio et al., 2011; Hancock et al., 1994; Van Donkersgoed et al., 2001; Zimbro et al., 2009).

33

CHROMagar had a good sensitivity and specificity in the detection of *E. coli* O157 in the environmental samples analyzed in this study. CHROMagar has been used as a selective medium for the isolation, differentiation and presumptive identification of *E. coli* O157 from stool specimens, foods, veterinary and environmental samples (Barkocy-Gallagher et al., 2002; Bettelheim, 1998; Fratamico and DebRoy, 2010; Keen et al., 2006). Due to the chromogenic substances in the medium, colonies of *E. coli* O157 will produce mauve color, thus allowing presumptive identification. The addition of potassium tellurite (2.5mg/l), or vancomycin, cefixime and cefsoludin (4.0, 0.025, and 5.0 mg/L, respectively) as recommended by several authors (Fratamico and DebRoy, 2010; Invitrogen, 2008; Zimbro et al., 2009) resulted in growth inhibition of *E. coli* O157 naturally found in environmental samples from small-scale cow/calf operations in Louisiana. Even though potassium tellurite has been used as supplement in SMAC agar for the isolation of *E. coli* O157 (Chapman et al., 1994; Fedio et al., 2011; Hancock et al., 1994), when it was used with CHROMagar, it inhibited the growth of *E. coli* O157 in environmental samples from small-scale cow/calf operations in Louisiana (Table 5).

While the matrix of each environmental sample analyzed in the present study was different, it was found that there was no significant effect of the type of samples (fecal matter, water and swabs) on the probability of each medium to correctly detect the presence of *E. coli* O157 in environmental samples.

In conclusion, culture medium analyzed individually is less likely to correctly detect environmental *E.coli* O157, compared to when results of two different media were analyzed simultaneously. The best combination of two media used in this study for detection of *E.coli* O157 was CT-SMAC and CHROMagar. Furthermore, the findings suggested that T-CHROMagar and VCC-CHROMagar may not be a good option because they did not allow growth of *E.coli* O157 in environmental samples from small-scale cow/calf operations in Louisiana. Collectively, it is recommended to consider results of CHROMagar and CT-SMAC simultaneously as a tool for the detection of STEC O157 naturally found in environmental samples from cattle farms.

2.5. Acknowledgments

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CHAPTER 3.   PREVALENCE OF *ESCHERICHIA COLI* O157:H7 IN SMALL-SCALE COW/CALF OPERATIONS IN LOUISIANA

3.1.   Introduction

According to CDC *Escherichia coli* is a large and diverse group of bacteria, most of them are harmless, but some strains can be highly pathogenic (CDC, 2015). Enterohemorrhagic *Escherichia coli* (EHEC) are considered the main cause of hemorrhagic colitis (HC). EHEC are identified by the production of Shiga toxins (*Stxs*), but only the ones that have been clinically associated with HC are designated as EHEC. Hemolytic uremic syndrome (HUS), results from *Stxs* produced in the intestine and act systemically on kidney cells and other organs. Shiga-toxin producing *Escherichia coli* (STEC) is an important group of zoonotic pathogens, with the prototype and most frequent serotype associated with HC and HUS, *E. coli* O157:H7 (Bach et al., 2002; Bilge et al., 1996; Franz et al., 2007; Fu et al., 2005; Laegreid et al., 1999).

Foodborne illnesses have been associated with *E. coli* O157:H7 since this organism was first reported in 1982 (Fedio et al., 2011; Hancock et al., 1997). CDC reported that *E. coli* O157:H7 was within the top five pathogens contributing to domestically acquired foodborne illnesses requiring hospitalization (CDC, 2011). Considerable evidence associates *E. coli* O157:H7 outbreaks with foods of bovine origin such as, ground beef and raw milk hard cheese; cattle have been identified as primary reservoir (Bach et al., 2005).

Epidemiological studies on the prevalence and survival of *E. coli* O157:H7 has mainly been conducted at feedlots (Bach et al., 2005; Dargatz et al., 1997; Hancock et al., 1998; Sargeant et al., 2003; Van Donkersgoed et al., 2005). A study conducted at 73 feedlots located in four states (Kansas, Nebraska, Texas, and Oklahoma) found 10.2% of fecal samples were positive for *E. coli* O157:H7, water or water tank-sediment had 13.1% of positives (Sargeant et al., 2003). However, in an earlier study conducted in the Northwestern USA, there was a lower prevalence of *E. coli* O157:H7 (1.6%) in fecal samples obtained from the feedlots (Dargatz et al., 1997).

The reservoirs responsible for the colonization of cattle with *E. coli* O157:H7 are still poorly understood, and the reported prevalence of *E. coli* O157:H7 within the environment of the feedlots and farms can vary greatly (Jinneman et al., 2003; Wells et al., 2014). Reservoirs of *E. coli* O157:H7 in feedlot areas include feces (0.8%), feed bunks (1.7%), water troughs (12%), and incoming water supplies (4.5%) (Sargeant et al., 2003; Van Donkersgoed et al., 2001). Additionally, research indicates diet can influence shedding of *E. coli* O157:H7 (Dargatz et al., 1997; Wells et al., 2014).

In Louisiana, calves are raised with cows mainly on grass forage until shipped to finishing sites in other states. Knowledge of the prevalence of *E. coli* O157:H7 associated with small-scale cow/calf farms can help assessing risks and developing management strategies needed to control the colonization of this pathogen within the farm environment (Bach et al., 2005; Franz et al., 2007).

The purpose of this study was to establish the prevalence of *E. coli* O157:H7 in small-scale cow/calf operations in the state of Louisiana and to determine the primary source of *E. coli* O157:H7 in these operations.

3.2. Methodology

A total of 644 samples were collected from 28 small-scale cow/calf operations in the State of Louisiana between the months of June and December in 2011. At each farm a set of 15 samples of fresh fecal matter were collected from the ground, 6 samples of water were collected from water troughs and ponds, and 2 swabs from the surface of water troughs, hay and salt bunks.

3.2.1. Collection of Environmental Samples

To collect the fecal matter from the ground, gardener sterile spoons were used, and then the samples were placed in 13 oz, Nasco Whirl-Pak® write-on bags. Water samples were taken from water troughs and ponds using a sterile plastic ladle, and the samples were placed in VWR® 4 oz Polypropylene Specimen containers with Polyethylene lids. For the swabs, 18 oz Nasco Whirl-Pak® Speci-Sponge® hydrated with Phosphate Buffer Saline (PBS) were used to swab water troughs, hay and salt bunks. All the samples were

transported in ice chests to the Food Safety Laboratory located in Baton Rouge, LA on the Louisiana State University Campus. Samples were placed into enrichment broth the same day of collection.

3.2.2. Isolation of *E. coli* O157:H7

Enrichment in Tryptic Soy Broth (TSB) was used for the isolation of *E. coli* O157:H7 from all the environmental samples collected. Enrichment was as follows: 5 g of fecal matter samples was added to 50 ml of TSB, 30 ml of environmental water samples was added to 30 ml of double strength TSB, and environmental sponge samples were added to 90 ml of TSB. All the samples were incubated overnight at $35\pm2°C$. After incubation, Immunomagnetic Separation (IMS) was performed using Dynadeads® MAX *E.coli* O157 Kit, following a manual protocol suggested by the manufacturer (Invitrogen Co., Carlsbad, CA). The product from IMS was plated on CT-SMAC (Cefuxune Tellurite Supplement, 77981 Fluka Analytical) and CHROMagar (CHROMagar™ O157, CHROMagar). Suspected colonies from selective/differential media were cultured and tested for the presence of stx_1, stx_2, Wzy_{O157} and $flic_{H7}$ genes by Real-Time Polymerase Chain Reaction (RT-PCR).

3.2.3. Identification of the strain

All cultures from suspected colonies were analyzed by RT-PCR. FDA Bacteriological Analytical Manual (BAM) protocol was used for the DNA extraction (Feng et al., 2011). SmartCycler II platform was used, targeting stx_1, stx_2, Wzy_{O157}, and $fliC_{h7}$ genes with product sizes of 200, 140, 112, and 247 bp, respectively (Table 8). The PCR was performed using 6.2 μl of OmniMix HS for Cepheid SmartCycler® Systems (TaKaRa Bio Inc.), which was prepared as recommended by the manufacturer. The concentration of primers and probes used for the assay were 0.25 μM and 0.125 μM, respectively, and the volume of sample was 2.5μl. The protocol used for the PCR was 2 min at 94°C, followed by 40 cycles at 94°C for 20 s, 60°C for 30 s, and 72°C for 50 s (Fratamico and DebRoy, 2010). The threshold between positive and negative was set at 30 fluorescence units.

Table 8. Oligonucleotide primers and probes used in the Real-Time Multiplex PCR assay for detection of *E. coli* O157:H7 (Fratamico and DebRoy, 2010)

	Primer sequences 5' to 3'	PCR product size	GenBank accession no.
Primer sets			
FLICH71038-F	TACCATCGCAAAAGCAACTCC	247	X60439
FLICH71314-R	GTCGGCAACGTTAGTGATACC		
Stx1-418-F	CTCGACTGCAAAGACGTATG	200	U47614
Stx1-617-R	TTCGTTCAACAATAAGCCGTAGATT		
Stx2-314-F	ACGATAGACTTTTCGACCCAACAA	140	M16626
Stx2-453-R	AAATAACTGCCCGGTGGGGT		
WzyO157-528-F	CCTGTCAAAGGATAACCGTAATCC	112	X07865
WzyO157-639-R	TTGTTCTCCGTCTTGTCCTAAACT		
Probes			
FLICH7-P	5′-TET-CGGCTGCCGCGACATCTTCAAT-BHQ1		
Stx1-P	5'-Texas Red-CGCTGAATGTCATTCGCTCTGCA-BHQ2		
Stx2-P	5′-Texas Red-AACAGACACCGATGTGGTCCCCTGAGAT-BHQ2		
WzyO157-P	5′-FAM-AAAACAACGAGCATACAACCCCTACCAAT-BHQ1		

3.2.4. Statistical analysis

Descriptive statistics were used to calculate the prevalence of *E. coli* O157:H7. To determine the primary source (fecal matter, water or surfaces) of *E. coli* O157:H7 Logistic Regression was applied, using as a model presence and absence of *E. coli* O157:H7 and as regressors fecal matter, water and swabs. Statistical Analysis Software® (SAS 2012) at alpha=0.05 was used for the data analysis.

3.3. Results

To calculate the prevalence of *E. coli* O157:H7 the total of positive samples was divided by the total of samples analyzed. From the 644 samples analyzed from 28 small-scale cow/calf operations in Louisiana, 420 samples were fecal matter, 168 water, and 56 swabs. *E. coli* O157:H7 tested positive in 38 (9%) of fecal matter samples, 12 (7%) of water samples and 1 (2%) of swabbed surfaces, for a total of 51 (8% of the 644) positive samples (Figure 8). From those 51 *E. coli* O157:H7 positive samples, 38 (74%) percent were fecal matter, 12 (24%) were water and 1 (2%) was from swabbed surfaces (Figure 8). From the 28 farms sampled, 13 (46%) tested at least one positive sample to *E. coli* O157:H7.

An important aspect in the epidemiology of *E. coli* O157:H7 is to identify the primary source of contamination within the farm environment. Logistic regression was used to analyze which of the environmental samples collected was the primary source, or if all of them have a significant input to the prevalence of the pathogen in small-scale cow/calf operations. Presence and absence of *E. coli* O157:H7 was used as dependent variable, and fecal matter, water and swabs as regressors. The logistic regression model found that there is no significant difference between fecal matter and water as a primary source of *E. coli* O157:H7 in environmental samples from small-scale cow/calf operations in Louisiana (source of sample *P* > chi squared = 0.0837).

To establish if there was a relationship between positive results and the location of the farms, the operations were grouped into Northwest, Central and Southwest, and the source of samples were plotted individually. It was observed that the prevalence of the pathogen was higher in the farms located in the Central region with a 13% of fecal matter, 11% of water, and 4% of swabs tested positive (Table 9).

	Fecal Matter	Water	Swabs
■ Positives	38	12	1
■ Negatives	382	156	55
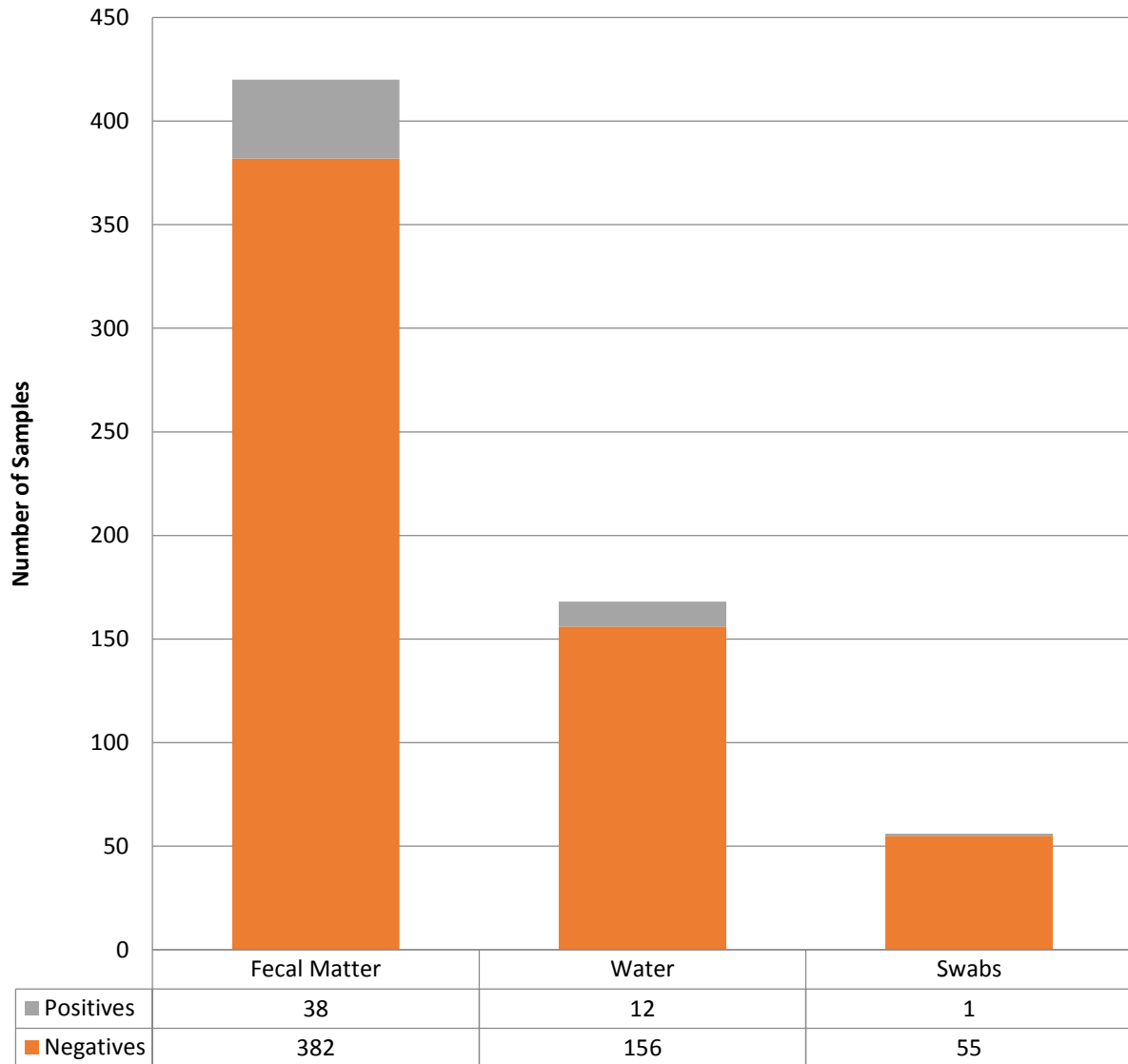
*Samples collected from 28 small/scale cow-calf farms.

Figure 8. Different environmental sources tested for presence of *E. coli* O157:H7 in small-scale cow/calf operations in the state of Louisiana*.

Additionally, we also grouped the prevalence within different months of sample to evaluate if there was an effect between season and prevalence. The monthly prevalence of *E. coli* O157:H7 on small-scale cow/calf operations in the State of Louisiana was 14% in June, 2% in August, 14% in October, 10% in November and 7% in December.

Table 9. Percentage of positive *E. coli* O157:H7 in environmental samples in small-scale cow/calf operations in different regions of Louisiana, from June to December 2011*.

Region	Fecal Matter	Water	Swabs
Northwest	1%	2 %	0 %
Central	13%	11%	4 %
Southwest	11%	6%	0%

* 644 samples were collected from 28 small/scale cow-calf farms in Louisiana.

During each visit to the small-scale cow/calf operations in the State of Louisiana a survey was filled out to collect information regarding different animal husbandry practices at each farm. From this survey we found that the source of water available for the cattle in different farms was from wells/windmill, runoff capture and municipal water. In addition, cattle had access to water in free-flowing creeks, cement/metal stock tanks, and small capacity/continuous flow stock tank. Furthermore, cattle were fed with grass and minerals in block or loose in all the farms. Information regarding the population and acreage was collected as well, the population of animals in the surveyed small-scale cow/calf operations ranged from 6 to 60 animals plus calves with an animal density from 0.4 to 5.5 heads/acre. All surveyed small-scale cow/calf operations had cross bread only with black and red as primary hide color. Regarding cleaning practices in the surveyed small-scale cow/calf operations trailers for hauling animals were not cleaned; feed bins were not cleaned or cleaned sometimes; water troughs were never cleaned, rarely or sometimes; and chutes and heavy equipment were actually cleaned more often than the rest of facilities. During the time samples were taken, the air temperatures were in a range from 9.5 to 35.3°C, and the water from 6.0 to 31.7°C (Figure 9).

3.4. Discussion

The main objective of this study was to estimate the prevalence of *E. coli* O157:H7 in small-scale cow/calf operations in Louisiana. Twenty eight small-scale cow/calf operations were sampled to provide an estimate of the prevalence in these farms; twenty three samples were taken at each farm. It was found that the prevalence of *E. coli* O157:H7 in small-scale cow/calf operations in Louisiana was 8%.
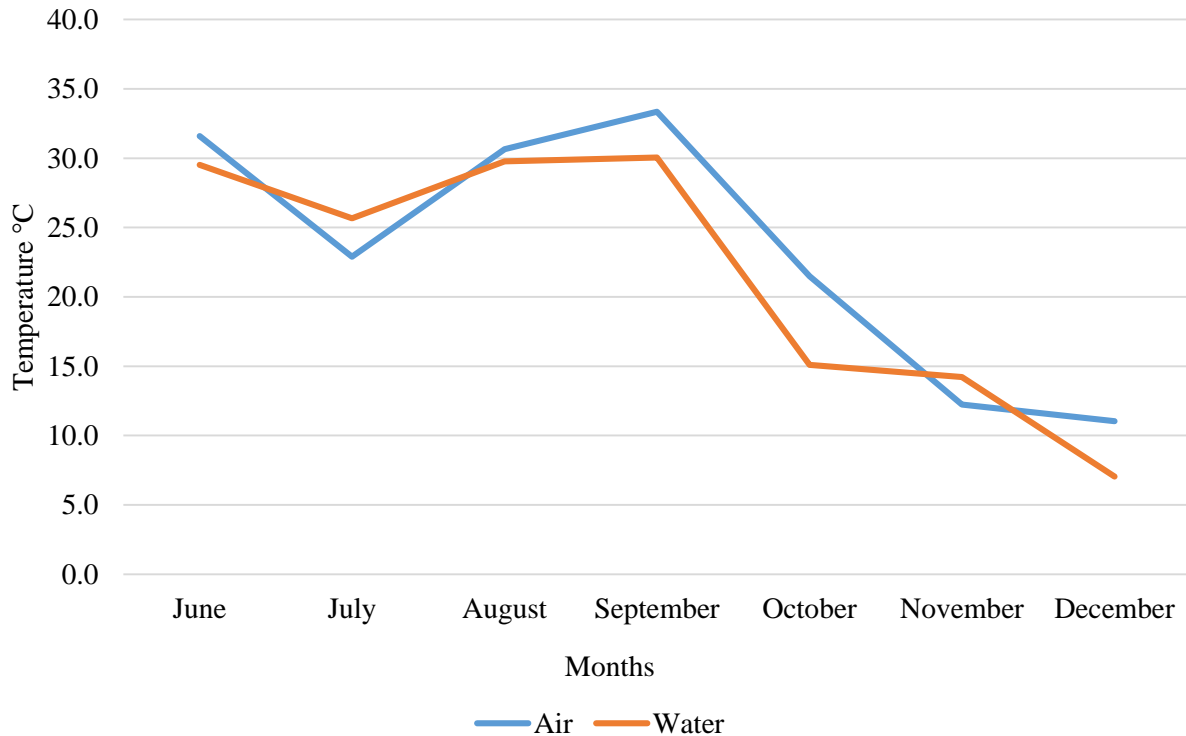
Figure 9. Average temperature (°C) of air and water at point of sampling in small-scale cow/calf operations in the state of Louisiana.

Previous studies at feedlots found the prevalence of *E. coli* O157:H7 was 10.2 % to 13% for fecal matter samples, and 13.1% to 21.5% for water samples (LeJeune et al., 2004; Sargeant et al., 2003). The prevalence of *E. coli* O157:H7 at small-scale cow-calf operations in Louisiana was 9% for fecal matter and 7% for water which was lower then what was observed for feedlots. Another study in feedlots found 63% of feedlot operations tested had at least one positive sample for *E. coli* O157:H7 (Dargatz et al., 1997) compared to a 46% operations that tested positive in the present study. These results suggest that the prevalence of *E. coli* O157:H7 in cattle feedlots is higher, compared with small-scale cow/calf operations. The higher prevalence could be due to animal density; 500-250 ft2/head in feedlots (Mader and Colgan, 2007) compared to 0.4-5.5 heads/acre in small-scale cow/calf operations surveyed in our study.

A study on cattle water troughs and surface water indicated that water can be an important reservoir of *E. coli* O157, and that the pathogen can survive at least 245 days in sediments and stay infectious for young calves (Johnson et al., 2003; LeJeune et al., 2001; Tanaro et al., 2014, 2012), which makes cleaning

45

water troughs and limiting access to ponds and free running creeks an important strategy for the reduction and dissemination of the *E. coli* O157:H7 in cattle farms.

Small-scale cow/calf operations should implement effective on-farm management strategies within the farm environment to reduce *E. coli* O157:H7 in cattle. Some strategies that can be used for the control of *E. coli* O157:H7 in cattle farms are: provide water to animals in cement/metal stock tanks, reduce cattle access to water ponds and free-flowing creeks, implement cattle pasture rotation, carry out cleaning of water tanks, feed bins, trailers, and chutes practices.

3.5. Acknowledgments

3.6. References

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CHAPTER 4.   PHYSICAL/CHEMICAL CHANGES OF NATURALLY FERMENTED RED HOT CHILI PEPPER MASH

4.1. Introduction

Hot pepper sauce is made from red hot chili peppers that go through a fermentation process that varies from weeks to years. Fermentation of food goes through several changes that include physical, chemical and microbiological that will affect the quality and safety of the product. In most fermentation processes of food products, the prevailing microbial groups are lactic acid bacteria (LAB) and/or yeast, the relative population of which defines the characteristics of the final product. When LAB outgrow yeasts, lactic acid fermentation is favored, rendering a more acidic product with a lower pH, which is greatly desirable. The opposite happens when yeast or other spoilage microorganisms become the prevailing group (Panagou et al., 2008; Tofalo et al., 2012). During the fermentation of red hot chili pepper mash, it is believed that LAB come from the hot red chili peppers naturally and also from the fermenting barrel. However, these cultures exhibit diverse metabolic activities, which vary even among strains, including differences in growth rate, adaptation to a particular substrate, antimicrobial properties, flavor, aroma and quality attributes and competitive growth behavior in mixed cultures (Holzapfel, 1997). There is limited information available regarding physical/chemical changes of naturally fermented red hot chili pepper mash.

Tabasco like hot sauce is mostly made utilizing the small (20-30 mm) bullet-shaped pepper which matures from yellow-green to bright red (*Capsicum frutescens*). The pepper comes from a tall, vigorous perennial plant that grows in tropical areas (Crosby, 2008). The composition of the fresh fruits varies within species, cultivar, environmental conditions of growth, and stage of maturity. It contains fatty acids, volatile compounds, pigments, and pungent elements among other components. Carotenoids are responsible for the red color of the pepper. The carotenoid synthesis takes place during the ripening stage. Capsanthin (red) is the major pigment of the red pepper. Other major pigments include β-carotene (orange), violaxanthin (yellow), and antheraxantin (Ahmed et al., 2002; Arocha, 1984; Hornero-Méndez and

Mínguez-Mosquera, 2000; Howard et al., 2000; Tian et al., 2014). Hunter values (L*, a*, and b*) have previously been used as a valuable tool to describe visual color in food products (Skerede, 2006). Additionally, hue is an element of the color wheel, where change in hue from red to yellow goes from 0° to 90°, yellow to green from 90° to 180°, green to blue from 180° to 270° and blue to red 270° to 360°. Hunter values a* and b* are coordinates that reflect hue (h=tan^{-1}b*/a*) (McGuire, 1992).

Consumers often base their choice of food on flavor, aroma, as well as appearance. Volatile compounds are used to evaluate the quality and determine if the fermentation process has been successful in producing the desired final product. Currently, aroma has been used as an important parameter for the quality of fresh fruits and vegetables. The aroma is measured by the extraction, isolation and identification of volatile compounds. Volatile compound content of the red hot chili pepper is very low, ranging from 0.1 to 2.6 % in the fruit, contributing to the overall aroma of the pepper. There have been more than 125 volatile compounds identified in fresh and processed red hot chili pepper (Pino et al., 2006). Simultaneous distillation extraction (SDE) and solid phase microfiber extraction (SPME) have been used for the extraction of volatile compounds from fresh habanero chili peppers during different ripening stages and in different colors of peppers in Latin America. One approach for the isolation and identification of the volatile compounds is gas chromatography and mass spectrometry (GC-MS) analysis.

Researchers have been working to identify volatiles on *C. annuum, C. chinense,* and *C. frutescens.* Studies identifying volatile compounds of habanero pepper in Yucatan found a range of 136-300 compounds (Pino et al., 2006; Rodríguez-Burruezo et al., 2010). The volatile compounds found in the majority of fresh red hot chili peppers are (*E*)-2-Hexenal, Hexyl isobutanoate, Hexyl 2-methylbutanoate, Hexyl isopentanoate, Heptyl buanoate, (*Z*)-3-hexenyl isopentanoate, Heptyl isobutanoate, Heptyl pentanoate, 3,3-dimethylcyclohexanol, ϒ-Himachalene, Germacrene D, (*E*)-β-Ionane, Benzyl benzoate and Hexadecanal (Table 10) (Pino et al., 2007).

Table 10. Volatile compounds in Habanero chili pepper cultivars (mgKg^{-1} dry fruit) (Pino et al., 2007).

Compound	KI	Red 1	Red 2	Red 3	Red 4	Orange 1	Orange 2	Orange 3	Orange 4	Orange 5	Brown
(E)-2-Hexenal	854	-	0.01	0.01	t	0.02	0.02	0.03	0.01	0.02	0.03
Hexyl isobutanoate	1149	0.02	0.04	-	0.01	0.05	0.07	0.06	0.02	0.02	0.09
Hexyl 2-methylbutanoate	1236	0.08	0.25	0.09	0.07	0.24	0.37	t	0.18	0.40	0.39
Hexyl isopentanoate	1244	0.80	1.95	1.62	0.67	2.05	2.50	2.50	2.42	3.04	1.69
Heptyl butanoate	1291	0.10	0.11	0.10	0.11	0.12	0.10	0.10	0.11	0.11	0.13
(Z)-3-hexenyl isopentanoate	1295	0.31	0.77	0.24	0.16	0.18	0.63	0.72	0.54	1.34	1.40
Heptyl isobutanoate	1300	0.05	0.06	0.08	0.07	0.06	0.08	0.06	0.05	0.09	0.07
Heptyl pentanoate	1376	0.02	0.16	0.11	t	0.42	0.49	0.48	0.15	0.69	0.45
3,3-dimethylcyclohexanol	1392	0.07	0.60	1.98	0.07	2.05	1.84	1.59	1.36	1.52	1.90
Ƴ-Himachalene	1483	0.03	0.13	0.09	0.03	0.11	0.38	0.22	0.28	0.27	-
Germacrene D	1485	0.01	0.02	t	-	0.04	0.14	0.09	0.02	0.09	t
(E)-β-Ionane	1489	0.01	0.02	0.01	t	0.05	0.11	0.06	0.05	0.18	0.05
Benzyl benzoate	1762	0.01	0.01	0.02	0.01	0.02	0.01	0.01	0.02	t	0.01
Hexadecanal	1811	0.01	0.02	0.02	0.02	0.02	0.01	t	0.02	0.01	0.01

KI: Kovac's index

-Not detected; t means lower than 0.01 mg kg^{-1} dry fruit.

51

Due to limited information on microbiological impact on physicochemical changes occurring during natural fermentation of red hot chili pepper mash, the purpose of the present study was to evaluate microbial impact on physicochemical characteristics of red hot chili pepper mash during the fermentation process. Changes in pH, acid content, color, and aroma of red hot chili pepper mash were analyzed during its natural fermentation process for a period of 18 months.

4.2. Methodology

4.2.1. Collection of Samples

The red hot chili pepper mash was obtained from McIlhenny Co., Avery Island, LA. Mash was kept in 5 gallon bucket at temperatures ranging from 16.5°C to 28°C. There was no climate controlled conditions during the storage of the mash. Mash was stored in a warehouse (Baton Rouge, LA) similarly as it is stored at Avery Island LA, under natural environment conditions typical of south of Louisiana; where warm temperatures and high humidity are predominant factors during summer, and cool and also humid during winter. Samples were collected at the beginning of the fermentation process, once a week for one month, once a month during the first year, followed by a last sampling at 550 days from the naturally fermented mash. To obtain the samples, a one ½ inch PVC pipe was used, pushing down into the mash, pipe was pulled out and sample was collected from the bottom end of the pipe. Two samples were collected at each time. For each mash sample pH, titratable acidity, color, and volatile compounds were determined.

4.2.2. pH and titratable acidity

Before each pH and titratable acidity measurement, Orion 2 Star pH Benchtop (Thermo Scientific, Waltham, MA) was calibrated using buffer solutions pH 10, 7, and 4. For the pH, 10 g of pepper mash was weighed in a 100 ml flask, pH was measured in triplicate. After measuring pH, titratable acidity (TA) was determined by adding 10 ml of distilled water to 10 g of mash, stirring constantly. A solution of 0.1

N of NaOH was used for the titration until solution reached a pH of 8.2. Volume of NaOH was measured using a 50 ml burette. The TA was expressed as percentage of lactic acid (%TA) (Wilson, 2012).

$$\%TA = \frac{(\text{ml base titrant})(\text{N of base in mol/L})(\text{Acid Equiv. Weight})}{(10)(\text{grams of sample})}$$

4.2.3. Color

Objective color measurement was made using a Baking meter BC-10 colorimeter (Konica Minolta) based on three color coordinates, namely L*, a*, and b*. The instrument was calibrated with white reference tile (Konica Minolta 12695, L*=95.3; a*=-0.3; b*=4.7). Each sample was placed in 1 oz. container and L*, a*, and b* values were recorded. Five color measurements were taken and average values in triplicate were used for calculation. Hue angles were calculated using h=tan^{-1}b*/a* (Mclellan et al., 1994).

4.2.4. Volatile compounds Extraction

The extraction of volatile compounds was performed by SPME adding pepper mash in a 250 ml round bottom flask. A 1 ml aliquot of internal standard (IS) (2,4,6-collidine) 100 ppm solution was spread on 2 g of pepper mash and mixed well by shaking. Flask was placed in a 60°C water bath and capped using a cork wrapped in aluminum foil; cork had a perforation of 2 mm diameter in the center. A SPME needle with polydimethylsiloxane (PDMS) fiber was inserted through perforation in the cork to reach the neck of the flask. Then the fiber was exposed to the sample headspace to perform the volatile extraction for 30 min. After the extraction, the fiber was retracted for GC-MS analysis. Fiber was conditioned for 5 min at 250°C before next use.

4.2.5. Volatile compounds Analysis

GC/MS system used in this study consisted of Varian CP3800 GC and Saturn 2000 MS (Varian, Inc., Walnut Creek, CA) with an SPB-5-fused silica capillary column (60 m ¥ 0.25 mm ¥ 0.25 mm film

hickness) (Supelco, Inc.). Helium was used as a carrier gas at a column linear flow rate of 1.5 ml/min. The injection port temperature was at 250°C and in a splitless mode. The initial temperature of the GC oven was held at 40°C for 5 min. Then, the temperature was increased by 2°C/min to a final temperature of 200°C and held for 20 min. The MS detector was operated at an ionization voltage of 70 eV and ion source temperature of 200°C. The volatile compounds were identified by comparison of the mass spectra and retention time of IS, and National Institute of Standards and Technology database library. Ratio between each subject volatile compound vs. IS peak height (PH) was calculated to obtain relative concentration of each compound. Analysis was run in triplicate for each sample.

4.2.6. Statistical analysis

Mean values were calculated from the data obtained on trials. A longitudinal study was perform to evaluate the changes and trends of pH, %TA, color, and volatile compounds during 550 days of natural fermentation of red hot chili pepper mash. To determine significant difference, data were analyzed by Tukey's test at α= <0.05 using statistical software SAS 9.4 (SAS Institute Inc. Cary, NC, USA). Additionally, correlation matrix was used to analyze data and determine if there was a correlation between variables using Data Analysis function of Microsoft Excel 2013®.

4.3. Results

4.3.1. pH and titratable acidity

The initial pH of the pepper mash was 4.651, which during the first month of fermentation had a reduction to 4.468. After the first month of fermentation the longitudinal effect of natural fermentation of red hot chili pepper mash had a slight reduction to 4.456. On the other hand the titratable acidity expressed on percentage of Lactic Acid (%TA) increased through all the fermentation process from 0.54% up to 1.22%. In general, %TA augmented and the pH declined as the fermentation time progressed. Longitudinal effect of natural fermentation of red hot chili pepper mash on pH and %TA through time can be observed

in Table 11 and Figure 10. From correlation matrix was found that pH and %TA have a reverse correlation (R^2=-0.58, Appendix).

Table 11. Changes in pH and Titratable acidity expressed on percentage of Lactic Acid (%TA) during 550 days of natural fermentation of red hot chili pepper mash ± Standard Deviation (SD).

Day	7	14	21	30	60	90	120	150
pH	4.65±0.01	4.57±0.01	4.52±0.01	4.47±0.02	4.52±0.03	4.52±0.01	4.47±0.01	4.49±0.01
% TA*	0.54±0.00	0.65±0.03	0.65±0.01	0.65±0.00	0.79±0.03	0.93±0.14	0.95±0.02	0.93±0.00

Day	180	210	240	270	300	330	550
pH	4.55-±0.04	4.49±0.01	4.51±0.02	4.50±0.02	4.51±0.01	4.47±0.03	4.46±0.01
% TA*	1.03±0.11	0.82±0.02	1.04±0.19	1.18±0.02	1.14±0.04	1.16±0.03	1.22±0.02

*Percentage of Lactic Acid
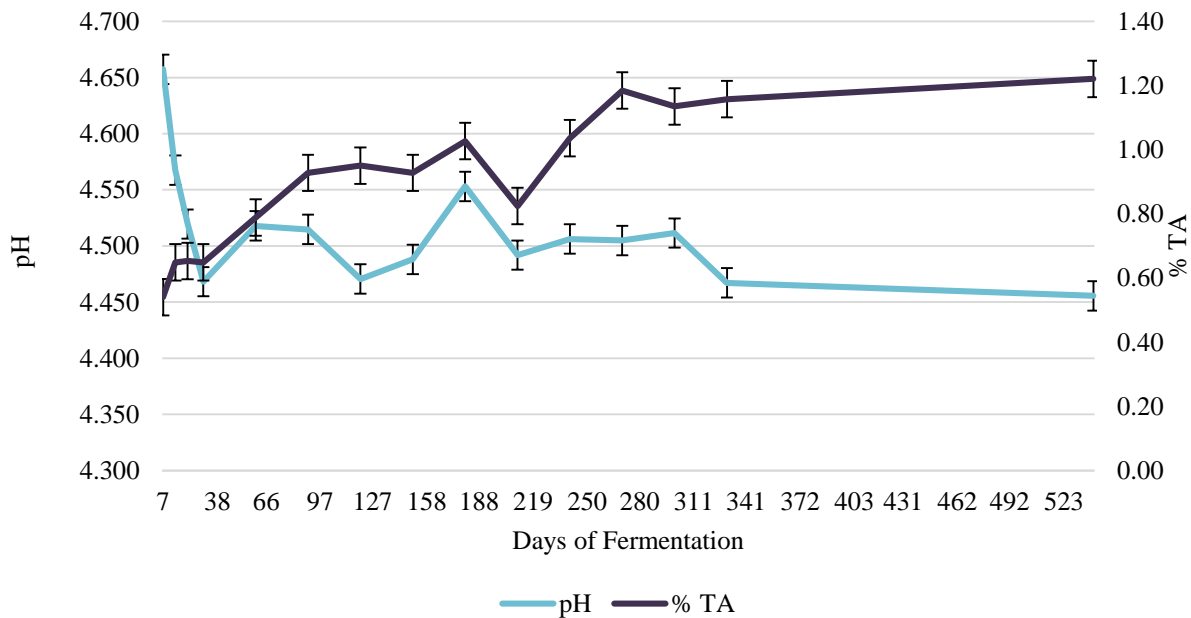
*Data from 2 samples analyzed in triplicate.

Figure 10. Longitudinal effect of pH and percentage of Lactic Acid (%TA) in natural fermentation of red hot chili pepper mash.

4.3.2. Color

In general red hot chili pepper mash had a red color throughout the 550 days of natural fermentation, which changed from a bright red to a dull red where a* went from 24.8 to 20.8. The yellow intensity (b*) was also reduced from 16.5 to 12.4. At the end of the process it was observed a slight darkening of the mash, that was confirmed with the change on L* from 53.5 to 52.1. Hue angle started at 33.64 degrees increasing to 35.57 after two weeks of fermentation, then had another peak after 180 days (Figure 11 and Table 12).   From correlation matrix was found that pH and hue angle have a direct correlation ($R^2=0.59$, Appendix), it was found that a* and b* had also a direct correlation ($R^2=0.93$, Appendix).
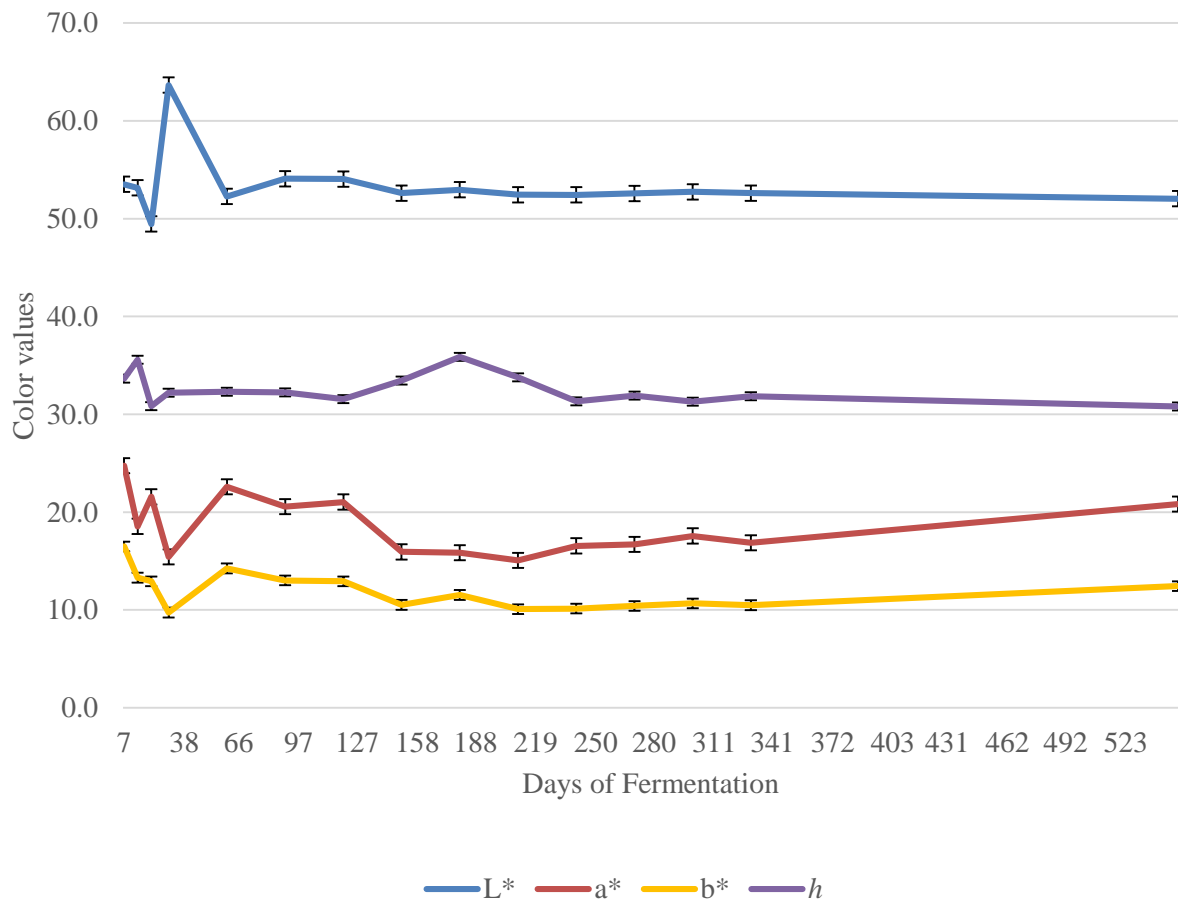
Figure 11. Longitudinal effect of Hunger L*, a*, b* values and hue (*h)* in natural fermentation of red hot chili pepper mash.

Table 12. Changes in color based on Hunter values (L*, a*, & b*) and hue (*h*) during 550 days of natural fermentation of red hot chili pepper mash ± Standard Deviation (SD).

	7	14	21	30	60	90	120	150
L*	53.5±2.6	53.2±1.2	49.5±1.1	63.7±1.2	52.3±2.3	54.1±0.4	54.1±0.6	52.6±0.5
a*	24.8±1.3	18.6±2.3	21.6±1.0	15.4±1.6	22.6±3.5	20.6±0.6	21.0±0.9	15.9±0.6
b*	16.5±1.5	13.3±3.1	12.9±1.6	9.7±1.0	14.3±2.9	13.0±0.7	12.9±1.0	10.5±0.7
h^1	33.64	35.57	30.85	32.21	32.32	32.25	31.56	33.44

	180	210	240	270	300	330	550
L*	53.0±1.0	52.5±0.5	52.4±0.5	52.6±0.9	52.8±0.6	52.6±0.5	52.1±0.9
a*	15.9±2.1	15.1±0.2	16.6±1.4	16.7±0.5	17.6±1.3	16.9±1.1	20.8±1.6
b*	11.5±2.0	10.1±0.8	10.1±0.8	10.4±0.9	10.7±1.3	10.5±0.7	12.4±1.6
h^1	35.88	33.78	31.32	31.91	31.30	31.85	30.80

[1] Calculated based on the averaged values of a* and b*.

4.3.3. Volatile compounds

After evaluating chromatograms and comparing main peaks with compounds previously detected in fresh peppers, the volatile compound profile to analyze in this study was based on *n*-hexanol, 4-methyl-1-pentyl-isobutyrate, (*Z*)-3-hexenyl isopentanoate, heptyl isobutanoate, heptyl pentanoate, 3,3-dimethylcyclohexanol. The most prevalent compound was 4-methyl-pentyl-isobutyrate with a ratio of 1.60 at the beginning of the study, but after 30 days of fermentation had a reduction. (*Z*)-3-hexenyl isopentanoate was the less concentrated compound at the beginning of the study with a ratio of 0.01, the concentration of this compound had two peaks during the fermentation process, first at 60 days and second at 330 days. Heptyl isobutaonate has a ratio of 1.23 at the beginning of the study, the concentration of this compound had two peaks as well, first at 60 days and second at 180 days. Concentration of *n*-hexanol during study was low, ratio at the beginning was 0.86 with the highest peak of 1.61 at 30 days of fermentation, with a final ration of 0.13. Heptyl pentanoate started with a ratio of 0.04, having two peaks, first at 30 days and second at 330 days. 3,3-dimethyl cyclohexanol had a ratio of 0.03 at the beginning of the study, presenting three peaks through the study, first after 90 days, second after 180 days, and last at 330 days of fermentation. All the compounds showed an increment during first 30 days of the study,

especially 4-methyl-1-pentyl-isobutirate. After this time, all the compounds had high peaks at different times, but all had a reduction in concentration toward the end of the study. At the end of the study the most concentrated compound was (*Z*)-3-hexenyl isopentanoate and the less prevalent compound was *n*-hexanol. The largest increase in concentration in the compounds was observed between the 21st and 30th day of fermentation (Figure 12 and Table 13). From correlation matrix was found that heptyl pentanoate and (*Z*)-3-hexenyl isopentanoate had a direct correlation (R^2=0.881, Appendix), and 3,3-dimethyl cyclohexanol and heptyl isobutanoate had a direct correlation as well (R^2=0.793, Appendix).

Chromatogram from fresh mash compared to 4 week chromatogram presented an increase in some compounds and the creation of new ones, especially after 30 min of isolation, (Figure 13, and 14). After 300 days of fermentation there was a reduction in number and concentration of volatile compounds (Figure 15). Compounds that were subject to this study had a retention time between nineteen and thirty min.
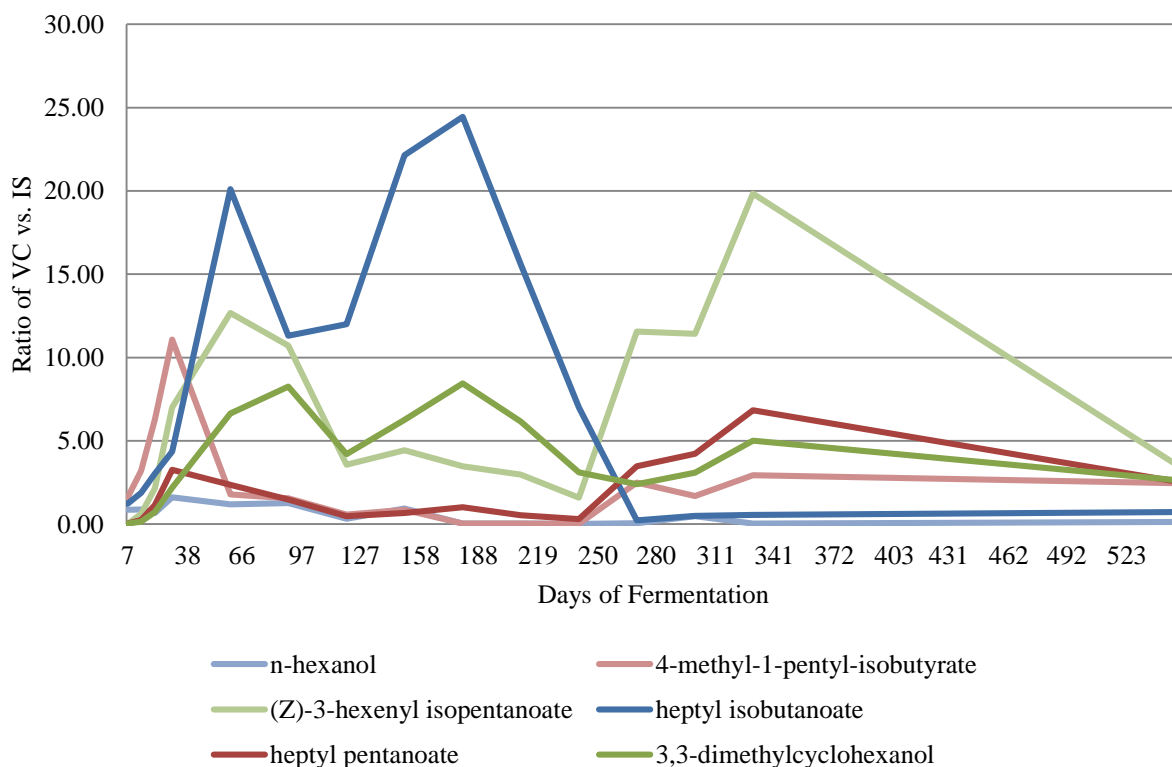
Figure 12. Longitudinal effect of Volatile Compounds during 550 days of natural fermentation of red hot chili pepper mash (ration of volatile compound vs internal standard).9

Table 13. Changes in Volatile compounds during 550 days of natural fermentation of red hot chili pepper mash (ration of volatile compound vs internal standard)*.

Compound	RT	Days of Fermentation														
		7	14	21	30	60	90	120	150	180	210	240	270	300	330	550
n-hexanol	19.13	0.86	0.88	0.66	1.61	1.19	1.27	0.33	0.93	0.03	0.03	0.02	0.05	0.48	0.04	0.13
4-methyl-1-pentyl-isobutyrate	26.88	1.60	3.20	6.28	11.08	1.78	1.54	0.57	0.86	0.03	0.04	0.02	2.49	1.69	2.94	2.45
(*Z*)-3-hexenyl isopentanoate	28.87	0.01	0.61	2.14	7.00	12.66	10.72	3.57	4.44	3.48	2.97	1.59	11.56	11.43	19.84	3.47
heptyl isobutanoate	29.19	1.23	1.88	3.02	4.36	20.11	11.30	12.00	22.14	24.45	15.62	7.00	0.23	0.49	0.54	0.73
heptyl pentanoate	29.67	0.04	0.25	1.15	3.26	2.36	1.47	0.48	0.66	1.02	0.53	0.29	3.47	4.22	6.83	2.53
3,3-dimethylcyclohexanol	30.09	0.03	0.15	0.73	2.18	6.63	8.24	4.20	6.26	8.44	6.17	3.11	2.40	3.09	5.00	2.62

RT: Retention time on SPB-5-fused silica capillary column (60 m ¥ 0.25 mm ¥ 0.25 mm film hickness) (Supelco, Inc.)

*Data from two samples analyzed in duplicate.
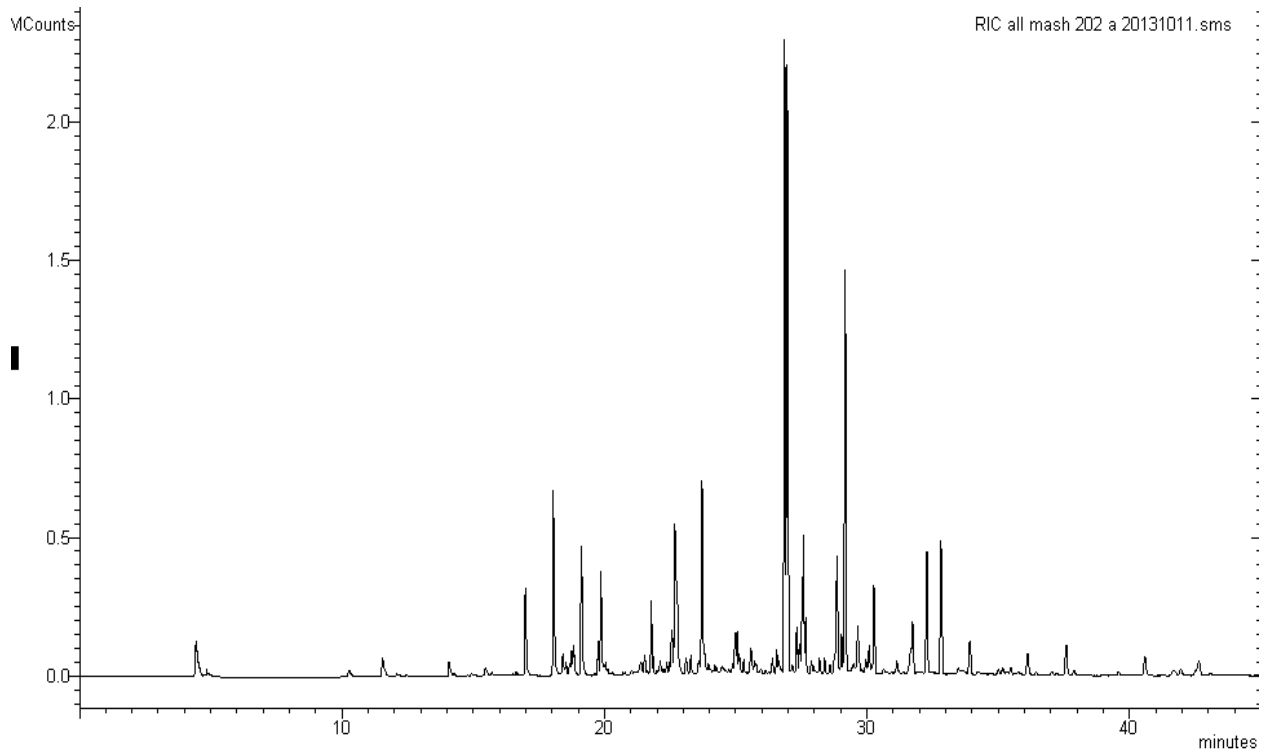
Figure 13. Chromatography from initial red hot chili pepper mash
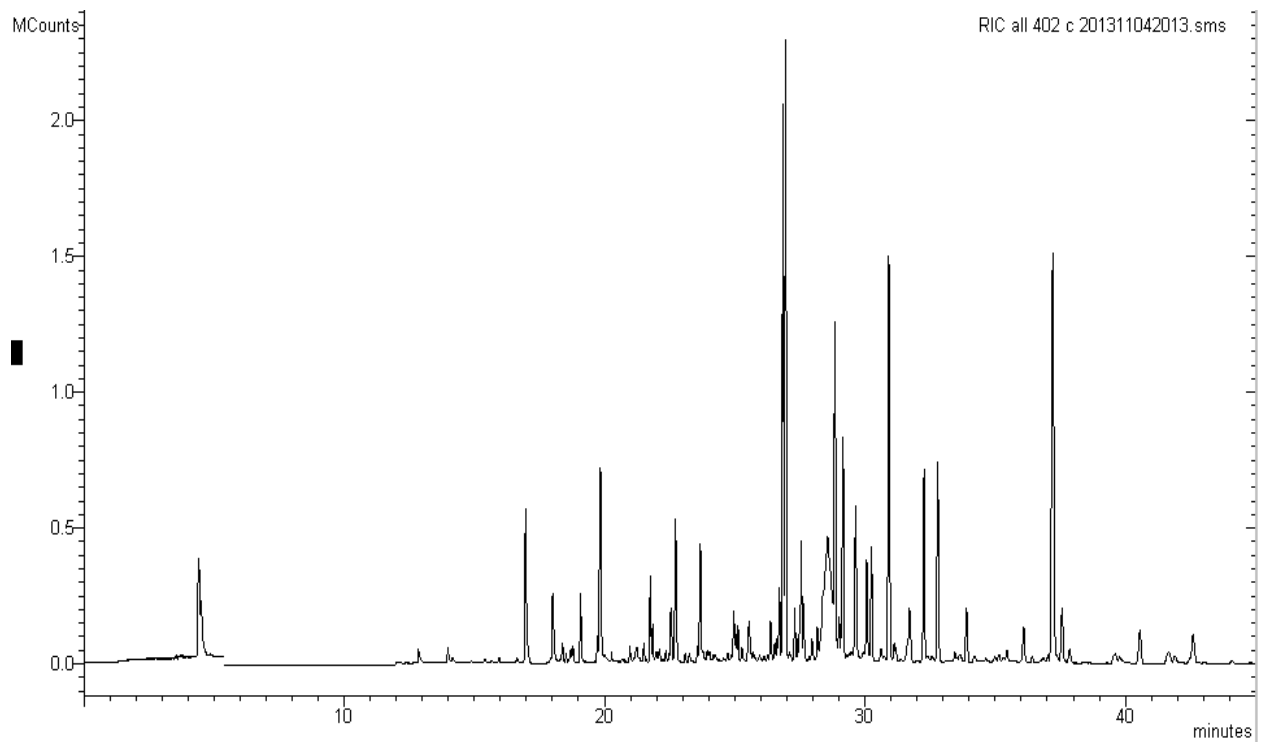
Figure 14. Chromatography after 4 weeks of fermentation of the red hot chili pepper mash.
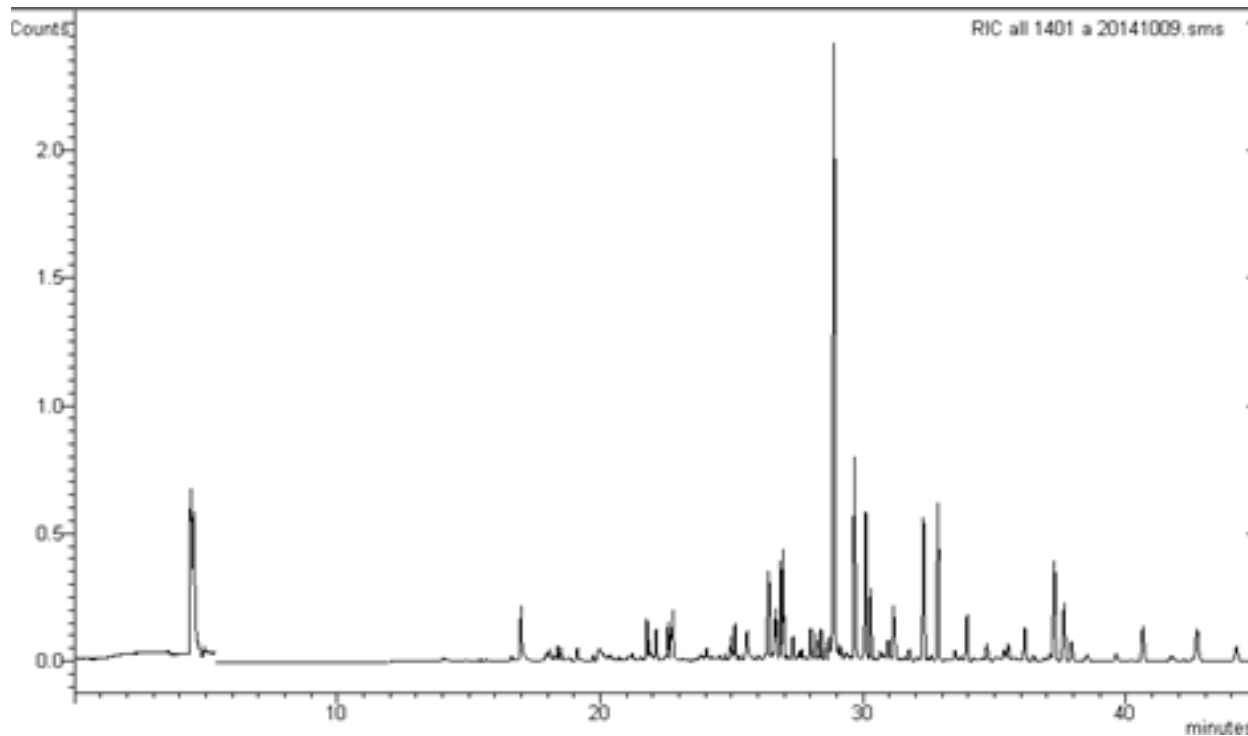
Figure 15. Chromatography after 300 days of fermentation of the red hot chili pepper mash.

4.4. Discussion

During the 550 days we studied the natural fermentation of red hot chili pepper mash we found that pH was consistently below 4.6, which classifies the mash as an acid and acidified product as established by FDA and Codex Alimentarius Commision FAO/OMS specifications (Alimentareus, 2007; FDA, 2000). Reduction in pH from 4.6 to 4.4 was likely due to the production of lactic acid as result of LAB growing in mash. We found an increase in the percentage of lactic acid during natural fermentation of red hot chili pepper mash from 0.54% to 1.22%, which agrees with previous studies completed in red bell pepper (1.53%), jalapeno peppers (1.5%), and red hot chili pepper mash (1.04% and 2.10%) (Fleming et al., 1983; Koh, 2005). A reversed correlation between percentage of lactic acid and pH during fermentation of red hot chili pepper mash was expected, due to production of acid promotes reduction in pH (Anthon and Barrett, 2012; Xu et al., 2012).

Carotenoids contribute to red, orange, and yellow color in fruits and vegetables, which depend on conjugated double bonds and functional groups contained in the molecule. The isomerization and esterification of carotenoids with fatty acids could affect color intensity (Bartley and Scolnik, 1995; Hornero-Méndez and Mínguez-Mosquera, 2000; Khoo et al., 2011; Lancaster et al., 1997; Minguez-Mosquera and Hornero-Mendez, 1994; Moraru and Lee, 2005). Correlation between pH and hue can be explained by changes in pH during the natural fermentation of red hot chili pepper which can lead to changes from *trans*-molecules to *cis*-isomers of carotenoids, which affects the way a molecule reflects light (Khoo et al., 2011). Carotenoids are also susceptible to oxidation due to exposure to changes in temperature during fermentation and pH (Ahmed et al., 2002; Bartley and Scolnik, 1995; Howard et al., 2000; Khoo et al., 2011; Lee and Coates, 1999; Moraru and Lee, 2005; Vega-Gálvez et al., 2009).

We identified six main volatile compounds during the fermentation of the mash, the first was *n*-hexanol which is an organic alcohol with green and herbaceous, woody, or sweet organoleptic properties (Burdock, 2010). This finding agrees with previous studies that isolated *n*-hexanol in fresh Tabasco and Habanero peppers (Pino et al., 2006; Wendell-Haymon and Aurand, 1971). The second compound we isolated was 4-methyl-1-pentyl-isobutyrate, which is an ester predominant in fruit aromas and contributes to the overall aroma of Tabasco peppers (Wendell-Haymon and Aurand, 1971). The third compound in our list was (*Z*)-3-hexenyl isopentanoate which is an ester, colorless liquid insoluble in water. Its organoleptic characteristics include a powerful, sweet, green odor of apple and a buttery, apple-like taste. It occurs naturally in mint and Tabasco peppers (Burdock, 2010), it was previously identified as a constituent of Habanero and Tabasco peppers (Pino et al., 2006; Wendell-Haymon and Aurand, 1971).

We also isolated heptyl isobutanoate which is an ester, colorless liquid insoluble in water, has a characteristic woody odor with distinctly herbaceous, sweet aroma, and has also been characterized as having a sweet, herbaceous, fruity, and slightly warm floral taste; it occurs naturally in the essential oil of hops (Burdock, 2010). Heptyl pentanoate was another compound we isolated from the mash, this is also an ester, colorless to pale yellow liquid with a fruity aroma (Burdock, 2010). Farther more we isolated 3,3-

dimethylcyclohexanol which has been described as having a "fried onion" aroma (Ferreira and Lopez, 2013).

Production of esters during the fermentation of red hot chili pepper mash is due to the presences of yeast, *L. lactis*, and other LAB, which have been associated with development of esters in dairy products (Hiu et al., 2010; Holland et al., 2005; Kennedy, 2013; Liu et al., 2004). Presence of LAB and yeast also explains direct correlation between heptyl pentanoate and (*Z*)-3-hexenyl isopentanoate. Esters that were isolated during natural fermentation of red hot chili pepper mash have powerful fruity aromas (Hiu et al., 2010; Kennedy, 2013).

Volatile compounds that were isolated from mash during natural fermentation correspond with compounds found in Habanero chili peppers (Pino et al., 2007). The composition of volatile compounds of the habanero chili peppers differed clearly in different cultivars, but major volatile compounds in all cultivars tested were hexyl isopentanoate, (*Z*)-3-hexenyl isopentanoate, hexyl pentanoate, and 3,3-dimethylcyclohexanol (Pino et al., 2007). Additionally, these compounds were also found in a study of Habanero chili peppers at two ripening stages, where 102 compounds were identified (Pino et al., 2006).

In this longitudinal study on natural fermentation of red hot chili pepper mash, we observed reduction in pH, increase in percentage of lactic acid concentration, slight changes in Hunter values (L*, a*, & b*) and hue in the mash. Additionally different changes of concentration of 6 volatile compounds could offer quality characteristics for the production of hot sauce.

4.5. Acknowledgments

4.6. References

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CHAPTER 5. MICROBIOLOGICAL CHANGES DURING NATURAL FERMENTATION OF RED HOT CHILI PEPPER MASH

5.1. Introduction

Hot pepper sauce is made from hot red peppers and used for seasoning on many foods due to its pungent flavor. Tabasco pepper (*Capsicum. frutescens*) is used to make hot pepper products worldwide. The hot sauce industry is one of the largest food industries in in America (Juffingtonpost, 2012; Yglesias, 2012). For the past decades, the hot sauce industry has been concentrated in Louisiana, mainly in the New Iberia, Mantinville, and Lafayette area. Avery Island remains as the most important production center. Hot sauces, due to their wide popularity, have also been produced in other regions in US. Hot sauce is manufactured by several plants under different brand names utilizing slightly different processes. The most popular pepper species used for the hot sauce production are *Capsium fructescens* and *Capsium annum.* The Tabasco brand of hot pepper sauce is made utilizing the small (20-30 mm) bullet-shaped pepper which matures from yellow-green to bright red. *Capsium frutescens* is cultivated commercially on a large scale. The pepper comes from a tall, vigorous perennial plant that grows in tropical areas (Crosby, 2008).

The exact procedure to produce hot sauce may vary between companies, and is considered to be a guarded trade secret. The main differences between products are: the amount of salt added, the fermentation time, the degree of grinding, and the percentage of vinegar added. In general, the production of hot pepper sauce requires the fermentation of red hot pepper mash in wooden and/or plastic barrels for a period between two to three years. Peppers used for the sauce are grown in Guatemala, Mexico, Honduras, Nicaragua, Panama, Columbia, Ecuador, Peru, South Africa, Zambia, Zimbabwe, and Mozambique. In these countries the peppers are handpicked, ground and 8 to 20 percent of salt is added. The mash ferments for a month before they are shipped to Louisiana. Once at the processors in Louisiana, the "pepper mash" is aged in ex-bourbon and plastic barrels that are fermented from several weeks to three years, depending on the industry. After fermentation/aging, pepper mash is mixed with vinegar and aged for an additional 28 days before bottling.

67

In most fermentation of food products the prevailing microbial groups are lactic acid bacteria (LAB) and yeast, the relative population of which defines the characteristics of the final product. When lactic acid bacteria outgrow yeasts, lactic acid fermentation is favored, rendering a more acidic product with a lower pH, which is greatly desirable. During the fermentation of the hot pepper sauce, it is believed that the lactic acid bacteria comes from the hot red chili peppers naturally and also from the fermenting barrel. However, these cultures exhibit diverse metabolic activities, which vary even among strains, including differences in growth rate, adaptation to a particular substrate, antimicrobial properties, flavor, aroma and quality attributes and competitive growth behavior in mixed cultures (Holzapfel, 1997).

Fermented foods are the result of the activity of a few species of microorganisms. Organisms isolated from some popular fermented vegetable products, include *Leuconostoc mesenteroides Lactobacillus brevis, Pediococcus cervisiae,* and *Lactobacillus plantarum* (Chen et al., 2005; Holzapfel, 1997; Leroy and De Vuyst, 2004; Tamang et al., 2005; Tassou et al., 2002). *Candida etchellsii* and *Pediococcus pentosaceus* were reported as responsible for fermentation of red hot pepper during the early stages, and *Lactobacillus* spp and *Candida versatilis* are probably the organisms responsible to maintain low pH and high titratable acidity during the aging process (Arocha, 1984).

Limited information is available regarding the bacteria, yeasts and molds involved in the fermentation of the red hot chili pepper mash. For improving the fermentation process and production of a high quality product and consistent final product, you need to identify the microorganisms involved in fermentation of the food product. The main objective of this study was to determine the fermentation microbiological profile, quantifying and identifying bacteria and yeast involved in natural fermentation process of red hot chili pepper mash.

5.2. Methodology

5.2.1. Collection of Samples

The red hot chili pepper mash was obtained from McIlhenny Co., Avery Island, LA. Mash was kept in 5 gallon bucket at temperatures ranging from 16.5°C to 28°C. There was no climate controlled conditions during the storage of the mash. Mash was stored in a warehouse (Baton Rouge, LA) similarly as it is stored at Avery Island LA, under natural environment conditions typical of south of Louisiana; where warm temperatures and high humidity are predominant factors during summer, and cool and also humid during winter. Samples were collected at the beginning of the fermentation process, once a week for one month, once a month during the first year, followed by a last sampling at 550 days from the naturally fermented mash. To obtain the samples, a one ½ inch PVC pipe was used, pushing down into the mash, pipe was pulled out and sample was collected from the bottom end of the pipe. Two samples were collected at each time.For each mash sample it was determined aerobic plate count (APC), Lactic Acid Bacteria (LAB) counts, and yeast and mold counts. pH and titratable acidity expressed in percentage of lactic acid (%TA) were monitored in parallel to microbiological analyses.

5.2.2. Quantification of bacteria and yeast

For Aerobic Plate Count direct spreading method was used, where 25 g of the sample was mixed with 25 ml of Phosphate Buffer Saline (PBS), then 1 ml of mixed solution was transferred in to 9 ml blank tubes containing PBS then serial diluted from 10^{-1} to 10^{-8}. Each dilution was spread directly onto the surface of Standard Method Agar (SMA) (Acumedia®, Neogen Lansing MI) in duplicate. Samples were incubated at 35 ± 2 °C for 48 h (AOAC®990.12).

Yeast and Mold Petrifilm™ (3M®, Saint Paul, MN) was used for Yeast and Mold quantification. Where 25 g of the sample was mixed with 25 ml of PBS, then 1 ml of mixed solution was transferred in to PBS 9 ml blank tubes and serial dilutions were made from 10^{-1} to 10^{-8}. Each dilution was spread directly onto the Petrifilm surface in duplicate following manufacturers recommended procedure. Samples were

incubated at 20-25°C for 3 to 5 days (AOAC®997.02). If negative results were obtained, Petrifilm were kept up to 15 days to look for slow growing microorganisms.

Pour plate method was used to quantify LAB, adding 25 g of the sample to 25 ml of PBS, then 1 ml of mixed solution was transferred in to PBS 9 ml blank tubes to prepare serial dilutions from 10^{-1} to 10^{-8}. Each dilution was spread directly onto the surface of Lactobacilli MRS agar™ (MRS) (Acumedia®, Neogen, Lansing, MI) in duplicate. Samples were incubated under anaerobic conditions using BBL™ GasPack™, H_2+CO_2 (VWR, Radnor, PA) at 30 ± 2 °C 48 h.

5.2.3. Isolation of lactic acid bacteria

Two methods were used for the isolation of LAB in the red hot chili pepper mash: the pour plate method and the enrichment method. For the pour plate method, an approach as mentioned earlier on the LAB quantification was used. For enrichment method, 5 g of each sample were added to 25 ml of MRS broth, and then incubated under anaerobic conditions using BBL™ GasPack™, H_2+CO_2 at 30 ± 2 °C for 48 h. After incubation 1 ml from each enrichment was transferred in to PBS 9 ml blank tubes and serial dilution were made from 10^{-1} to 10^{-8}. Each dilution was spread directly onto the surface of MRS agar in duplicate. Samples were incubated under anaerobic conditions using BBL™ GasPack™, H_2+CO_2 at 30 ± 2 °C for 48 hours (up to 7 days when growth was negative).

5.2.4. Isolation and storage of isolates

Morphologically distinct colonies were picked from SMA and MRS plates and isolates were purified by successive streaking onto Brain Heart Infusion Agar (BHI) (Acumedia®, Neogen Lansing, MI) and MRS agar, respectively. Isolates from BHI were tested with Gram stain and Catalase, and classified as Gram positives rods and cocci. Isolates from MRS were tested with Gram stain and Catalase, and only Gram positive and Catalase negative bacteria were kept. Pure cultures were stored at -80° C in BHI and MRS broth with 30% glycerol for later identification.

5.2.5. Identification of isolates

Physiological and biochemical tests were performed to identify the isolates. The identification of isolates from APC was performed by examining the cell shape and fermentation of carbohydrates using API® 50 CHB/E (Biomérieux, Inc., Durham, NC) for bacillus and API® 20 Strep (Biomérieux, Inc., Durham, NC) for cocci. The identification of LAB was be performed by examining the cell shape, fermentation of carbohydrates, using API® 50 CHL (Biomérieux, Inc., Durham, NC) for rod shaped and API® 20 Strep (Biomérieux, Inc., Durham, NC) for cocci. Isolates from Yeast and Mold Petrifilm™ were identified using API® 20 C AUX (Biomérieux, Inc., Durham, NC). Final identification of the microorganisms were obtained by calling APIweb™ (Biomérieux, Inc., Durham, NC) database service provided by manufacturer.

5.2.6. Statistical analysis

The mean values and the standard deviations were calculated from the data obtained. A longitudinal study was perform to evaluate the changes and trends of APC, LAB, and yeast counts during 550 day of natural fermentation of red hot chili pepper mash. To determine significant differences, data were analyzed by Tukey's test at $\alpha= <0.05$ using statistical software SAS 9.4 (SAS Institute Inc. Cary, NC, USA). Additionally, correlation matrix was used to analyze data and determined if there was a correlation between APC, LAB, and yeast count using Data Analysis function of Microsoft Excel 2013®. And correlation matrix was also used to analyze correlation between microbiology, pH, and %TA.

5.3. Results

It is important to mention that the red hot chili pepper mash obtained during this study was already over 7 days into the fermentation process so we were unable to perform analysis at the beginning of fermentation process. We found that APC bacterial counts were 2.09 Log CFU/g at 7 days of fermentation, increased to 2.66 Log CFU/g at 180 days of fermentation, and declined to 2.20 Log CFU/g after 550 days

during natural fermentation of the mash, there was no significant difference between counts at day 7 and counts at day 550 of fermentation (Figure 16 and Table 14).

Counts for LAB at the beginning of the study were 1.59 Log CFU/g, having three significant growth peaks during the fermentation process. The first growth peak in LAB counts was at day 60 with 4.0 Log CFU/g, second at day 240 with 4.4 Log CFU/g, and the third at 550 days with 4.8 Log CFU/g. LAB had a significant reduction in bacterial counts at 90 days of fermentation (0.8 Log CFU/g) (Figure 16 and Table 14).

Yeast was the only microorganism isolated from the Yeast and Mold Petrifilm^{TM} (3M®), which had initial counts of 1.57 Log CFU/g, increasing to 5.93 Log CFU/g after 330 days of fermentation. Data from yeast quantification showed a marked reduction in yeast counts at day 550, with a count of 0.7 Log CFU/g (Table 14). % TA showed a closer correlation to LAB and yeast counts (R^2=0.62 and 0.56, respectively, Appendix) compared to pH (R^2=-0.22 and -0.17, respectively, Appendix). Changes in % TA are presented in Figure 16.

Bacillus spp. and *Brevibacillus spp.* microorganisms were isolated throughout the fermentation of red hot chili pepper mash (Table 15). For the LAB, initially the fermentation involved *Lactobacillus plantarum* and *Lactococcus lactis,* which had a reduction in bacterial counts at day 90 and then *Lactobacillus curvatus, Pediococcus acidilactici, and Lactococcus* brevis started growing (Table 14). The most prevalent microorganism isolated from the mash during its natural fermentation was yeast and Candida was the most common genus isolated which included the following species; *Candida spp.* in the first phase, and *C. glabrata, C. krusei and C. norvegensis* in the second phase of fermentation. Additionally, *Rhodotorula mucilanginosa* and *Saccharomyces cerevisiae* were also isolated during the second phase of fermentation of red hot chili pepper mash as well (Table 15).

72
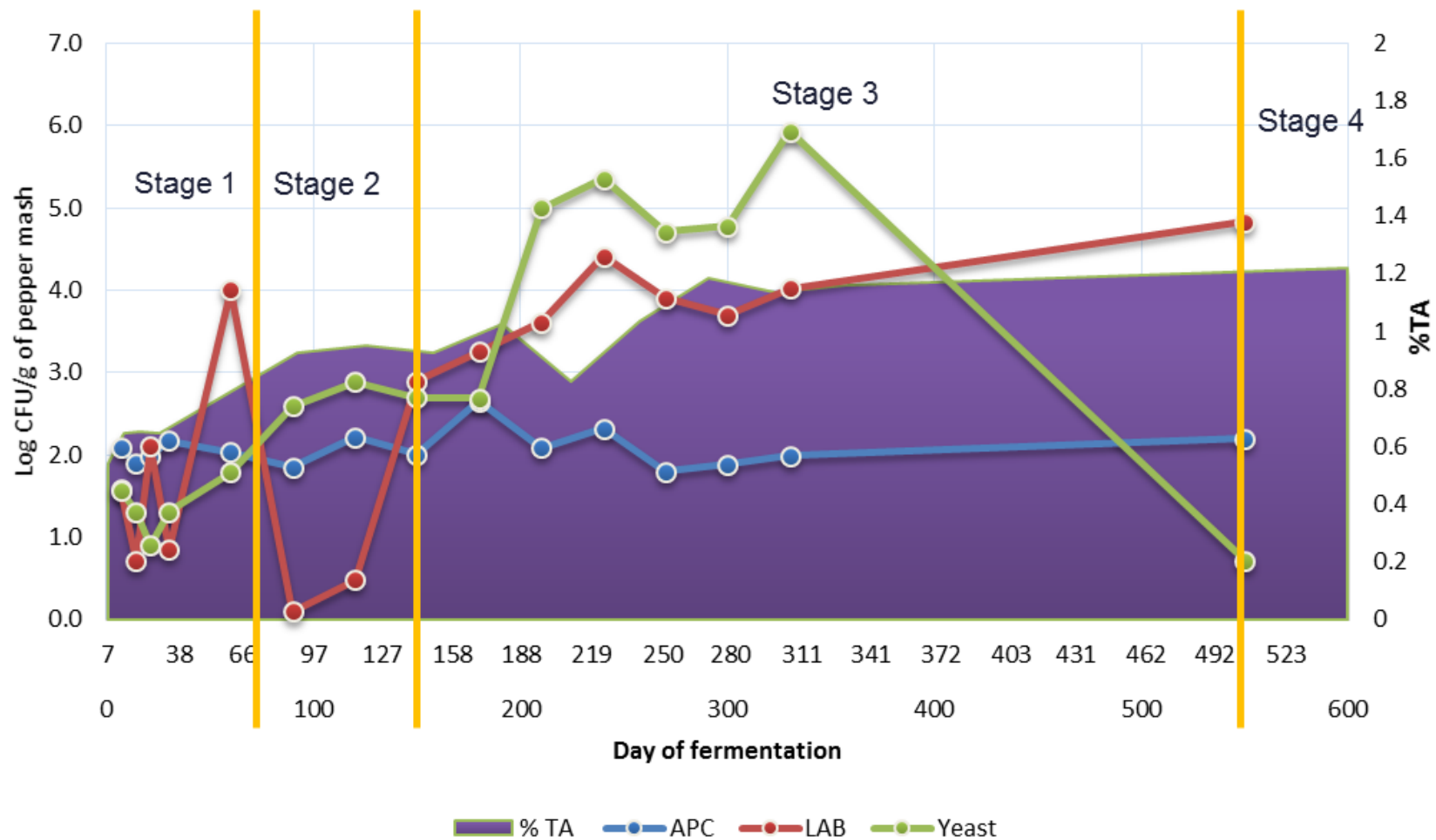
*Yellow lines separate different stages during pepper mash fermentation.

Figure 16. Aerobic plate count (APC), lactic acid bacteria counts (LAB), yeast counts, polynomial function for APC, %TA, and stages during 550 days of red hot chili pepper fermentation.

Table 14. Microbial growth in red hot chili pepper mash during 550 days (d) of natural fermentation. Results are mean values ± Standard Deviation (S.D.) of duplicate determinations from two separate samples.

	Log CFU/g													
	7 d			14 d			21 d			30 d			60 d		
APC*	2.09	±	1.34	1.90	±	1.11	1.99	±	0.77	2.17	±	1.30	2.03	±	1.15
LAB**	1.59	±	1.36	0.72	±	0.40	2.10	±	1.71	0.85	±	0.30	4.00	±	3.31
Yeast	1.57	±	0.94	1.31	±	0.66	0.90	±	0.62	1.31	±	0.74	1.79	±	0.49

	Log CFU/g													
	90 d			120 d			150 d			180 d			210 d		
APC*	1.85	±	1.09	2.22	±	1.73	2.00	±	1.01	2.66	±	2.18	2.09	±	0.65
LAB**	0.10	±	0.30	0.48	±	0.41	2.89	±	2.75	3.25	±	2.18	3.61	±	3.23
Yeast	2.59	±	1.74	2.89	±	2.37	2.69	±	2.30	2.69	±	2.02	5.00	±	4.45

	Log CFU/g													
	240 d			270 d			300 d			330 d			550 d		
APC*	2.32	±	1.39	1.80	±	0.88	1.88	±	1.05	1.98	±	1.01	2.20	±	1.23
LAB**	4.41	±	3.36	3.90	±	3.66	3.70	±	2.91	4.02	±	3.75	4.83	±	4.45
Yeast	5.35	±	5.01	4.71	±	4.72	4.78	±	4.22	5.93	±	5.52	0.72	±	1.05

*Aerobic Plate Counte
**Lactic Acid Bacteria

5.4. Discussion

The main objective of our study was to establish the microbial changes in red hot chili pepper mash during fermentation, and establish the main microorganisms involved during this process. During the 550 days of natural fermentation of chili pepper mash we observed four different stages. First stage from day 7 to day 60 where LAB was the predominant microorganisms in the mash, subsequently a second stage from day 60 to day 240, where yeast was the microorganisms with higher counts. There was a third stage from day 240 to day 330, where both LAB and yeast were involved. Followed by a final stage at day 550 were LAB and yeast counts started to decline.

Table 15. Isolates identified at each stage during 550 days of natural fermentation of red hot chili pepper mash.

Stage	Aerobic	LAB	Yeast
1st stage		*Lactobacillus plantarum*	*Candida spp.*
		Lactococcus lactis	
2nd stage		*Lactobacillus curvatus*	*Rhodotorula mucilanginosa*
	Bacillus pumilus		*Candida glabrata*
3rd stage	*Bacillus firmus*	*Pediococcus acidilactici*	*Sacharomyces cerevisiae*
	Bacillus spp.	*Lactococcus brevis*	*Candida krusei*
	Brevibacillus spp.		*Candida norvegensis*
4th stage		*Pediococcus acidilactici*	*Candida spp.*
		Lactococcus curvatus	*Candida krusei*
		Lactococcus lactis	

During the first stage we observed LAB counts were significantly higher compared to yeast counts, and % TA increased in the mash. LAB are known for production of organic acids during food fermentation which increases %TA and prepares mash for the growth of yeast (Panagou et al., 2008). Then, during the second stage of fermentation due to the concentration of lactic acid in the mash LAB counts decreased, and yeast counts started to increase. Yeast can grow in presence of acid and consume lactic acid in the mash (Deák, 2008; Roller, 1999). This is when we observed a third stage in the mash fermentation, with both LAB and yeast started growing at about same rate creating a symbiosis, where LAB produced lactic acid, yeast consumed the lactic acid and kept it low enough to allowed LAB multiplication and more lactic acid production (Panagou et al., 2008; Tassou et al., 2002) After certain time this equilibrium breaks down when LAB and yeast reached death phase, which marked a fourth phase in the fermented pepper mash. Once

mash reaches this stage, fermentation stops and spoilage microorganisms would be able to grow in the mash.

Within the microorganisms involved during the natural fermentation of red hot chili pepper mash, we identified *L. plantarum* during initial fermentation, *L. plantarum* has been isolated and used during fermentation of several vegetable foods (Fleming et al., 1983; Garver and Muriana, 1993; Holzapfel, 1997; Panagou et al., 2008; Tamang et al., 2005; Yoon et al., 2006). Moreover, we isolated *L. lactis* from the mash at the beginning of the fermentation process, which has been also isolated from fermented products (Ayad et al., 1999; Caplice and Fitzgerald, 1999; Garver and Muriana, 1993; Holzapfel, 1997; Leroy and De Vuyst, 2004) . *L. lactis* is also related to production of aroma compounds such as short chain fatty acid esters, responsible for fruity flavors in dairy products (Hiu et al., 2010; Holland et al., 2005; Liu et al., 2004).

During third stage of red hot chili pepper mash fermentation we identified, *L. cuvatus, P. acidilactici* and *L. brevis*. *L. curvatus* has been identified and used in the production of fermented meats, more recently it has also been isolated from sauerkraut (Caplice and Fitzgerald, 1999; Garver and Muriana, 1993; Leroy and De Vuyst, 2004). *L. brevis* was also isolated during sauerkraut fermentation, black olives, also from traditionally fermented vegetable products in the Himalayas (Halász et al., 1999; Leroy and De Vuyst, 2004; Plengvidhya et al., 2007; Tamang et al., 2005; Tassou et al., 2002). *P. acidilactici* has been isolated from several vegetable products and fermented meats as well (Garver and Muriana, 1993; Holzapfel, 1997; Leroy and De Vuyst, 2004; Tamang et al., 2005).

Our findings of *Candida* as the primary yeast involved in the natural fermentation of red hot chili pepper mash, which was also isolated from fermented pepper mash in a previous study (Arocha, 1984). Within this genus we isolated *C. glabrata*, *C. krusei* and *C. norvegensis*. *C. glabrata* whish have been also isolated from other fermented vegetable based products (Aidoo et al., 2006; Costilow et al., 1955; Gotcheva et al., 2000; Hammes et al., 2005; Tsuyoshi et al., 2005). Additionally, we isolated *Rhodotorula mucilanginosa* during the second stage that was also isolated previously from pepper mash (Arocha, 1984).

76

And finally we isolated *Saccharomyces cerevisiae* during second and third stage of fermentation, which is an important producer of esters *(Liu et al., 2004).*

We also isolated the Genus *Bacillus* and *Brevibacillus* throughout the pepper mash fermentation; bacteria from these genus are aerobic spore forming rods commonly isolated in environment and used in many crops as biological control against infestation of fungi, insects, and nematodes (Alippi and Reynaldi, 2006; Bottone, 2003; Giannakou et al., 2004; Gutiérrez-Mañero et al., 2001; Huang et al., 2005; Moon and Parulekar, 1991; Oliveira et al., 2004; Puri et al., 2002).

Our study found that red hot chili pepper mash fermentation occurs in four stages, where a symbiosis between LAB and yeast makes possible the fermentation of the mash, intensifying desirable volatile compounds important for the quality of aroma and flavor of hot sauce. At the end of the fermentation process, we observed a reduction of LAB and yeast population and presence of aerobic microorganisms as potential spoilage agents. At this stage, we recommend to stop fermentation of the mash and start processing the hot sauce.

5.5. Acknowledgments

5.6. References

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CHAPTER 6. SPOILAGE OF FERMENTED RED HOT CHILI PEPPER MASH

6.1. Introduction

Hot pepper sauce is made from red hot chili peppers that fermented for weeks to years depending on the processor. Food fermentation goes through several modifications that include physical, chemical and microbiological changes that will affect the quality and safety of product. In the majority of fermentation processes of food products the prevailing microbial groups are lactic acid bacteria (LAB) and/or yeast, the relative population of which defines the characteristics of the final product. When LAB grow during fermentation of food products, lactic acid fermentation is favored, rendering a more acidic product with a lower pH, which is greatly desirable. The opposite happens when yeast or other spoilage microorganisms become the dominant group (Garver and Muriana, 1993; Kakiomenou et al., 1996; Panagou and Tassou, 2006; Panagou et al., 2008; Tofalo et al., 2012).

During the fermentation of the hot pepper sauce it is believed that LAB comes from the hot red chili peppers naturally and also from the fermenting barrel. However, these cultures exhibit diverse metabolic activities, which vary even among strains, including differences in growth rates, adaptation to a particular substrate, antimicrobial properties, flavor, aroma and quality attributes and competitive growth behavior in mixed cultures (Hiu et al., 2010; Holzapfel, 1997; Panagou et al., 2008; Tamang et al., 2005).

The initial microorganisms in hot sauce fermentation are probably the same as in other agricultural products harvested under similar conditions of soil and weather. Besides the microorganisms found in the plant and soil where the peppers are grown, the peppers could be contaminated from dust, fecal contamination from birds, rodents, insects, and from other sources during transportation, and processing of the red hot pepper mash (Arocha, 1984). Researchers found that *Bacillus* is the most common isolate in spoiled hot red pepper sauce (Draughon et al., 1981). Other microorganism included *Streptococcus*, *Leuconostoc*, and *Micrococcus* species among bacteria, and *Saccharomyces*, *Candida*, and *Rhodotorula* species among yeast. The most common fungi reported being present on red peppers were *Aspergillus*, *Penicillium*, and *Rhizopus* (Christensen et al., 1967; Draughon et al., 1981)

The fermentation of red hot chili mash results in approximately 7% of the barrels having spoiled pepper mash that have changes in color, texture, and aroma. These changes include black or white pigmentation on the top or alongside the walls of the barrels, slime formation and an unpleasant aroma. The spoilage causes an economical loss to the hot sauce industry of 2 million dollars per year in the state of Louisiana. Our research will help reduce this loss to the industry by identifying the bacteria, yeasts and molds causing the spoilage, the source of the spoilage microorganism, and establishing the cause of spoilage. Information regarding normal and spoilage microorganisms of the red hot chili peppers is limited.

6.2. Methodology

6.2.1. Isolation and identification of spoilage bacteria in fermented red hot chili pepper mash

A hot sauce manufacturer from south of Louisiana approached the Food Safety Laboratory asking for help establishing causes of spoilage in red hot chili pepper mash during fermentation. When the manufacturer opened barrels after two years of fermentation, they observed spoilage of mash on the top layer and walls of barrels. Spoiled mash had rotten aroma, black color, and slime layer. We collected six samples of spoiled pepper mash from wooden barrels and transported samples to the Food Safety Laboratory in an ice chest to analyze samples within 24 h. Streak plate method was used to isolate colonies on Brain Hearth Infusion Agar (BHI) (Acumedia®, Neogen Lansing MI), MacConkey Agar (MAC) (Acumedia®, Neogen Lansing MI), and Lactobacilli MRS Agar (MRS) (Acumedia®, Neogen Lansing MI). This process was repeated until isolated colonies grew in each plate. Once isolated, microorganisms were classified using gram stain and doing direct observation under the microscope. API® 20 STREP (Biomérieux, Inc., Durham, NC) biochemical test identification system was used according to the manufacturer's instructions. Final identification of the microorganisms were obtained by calling APIweb™ (Biomérieux, Inc., Durham, NC) database service provided by manufacturer.

6.2.2. Inoculation of fresh red hot chili pepper mash with spoilage isolates

Fresh pepper mash was inoculated under controlled conditions with isolated bacteria from spoiled mash, and observed for microbiological changes and spoilage during 120 days. This was achieved by inoculating each strain into normal fresh mash. 240 pounds of fresh pepper mash were separated into 7 different subgroups. Subgroups 1 through 5 were inoculated with 7 Log CFU/g of the isolated bacteria individually, subgroup 6 was inoculated with 7 Log CFU/g of a mixed culture of all isolated bacteria, and subgroup 7 was used as control. Mash was put in 4.5 oz. specimen container (Kendall Precission™, VWR Radnor PA), inoculated and stirred. Containers were stored at 35°C ± 2°C, for 120 days. On day 0 Aerobic Plate Count (APC) were performed for each group, and every 15 days during 120 days.

6.3. Results

6.3.1. Isolation and identification of spoilage bacteria in fermented red hot chili pepper mash

From the microbiological analysis of spoiled red hot chili pepper mash *Bacillus firmus*, *Bacillus pumilus*, *Enterococcus avium*, *Brevibacillus laterosporus*, and *Aerococcus viridans* were isolated and identified. *Bacillus firmus* is a Gram-positive, rod-shaped bacterium. Colonies of *B. firumus* in BHI agar had a white translucent pigmentation, circular in shape, with undulated margins, and pulvinate elevation. When manipulated with a needle, the colonies were viscous (Figure 17).

Bacillus pumilus is a Gram-positive, aerobic, spore-forming, rod-shaped bacterium commonly isolated in soil, plants and environmental surfaces. Colonies of *B. pumilus* in BHI agar had a white pigmentation, surface of colonies was smooth and opaque, were puntiform with entire margins, and had a convex elevation. When manipulated with a needle, the colonies were butyrous (Figure 18).

Enterococcus avium is a Gram-positive, cocci-shape bacterium. Enterococci are facultative anaerobic organisms that can survive and grow in many environments. Colonies of *E. avium* in BHI agar has a white pigmentation, surface was smooth and opaque, circular in shape with entire margin, and convex elevation. When manipulated with a needle, the colonies were butyrous (Figure 19).

Brevibacillus laterosporus is a Gram-positive, rod shape bacterium. Colonies of *B. laterosporum* in BHI agar had a dull white translucent pigmentation, with a smooth surface, circular in shape with undulated margin and raised elevation. When manipulated with a needle, the colonies were butyrous (Figure 20).

Aerococcus viridans is a Gram-positive cocci-shape bacterium. Colonies of *A. viridans* in BHI agar had an white opaque pigmentation, with smooth surface, circular in shape with entire margin, and convex elevation. When manipulated with a needle, the colonies were butyrous (Figure 21).

Figure 17. *Bacillus firmus* isolated form spoiled red hot chili pepper mash

Figure 18. *Bacillus pumilus* isolated form spoiled red hot chili pepper mash

Figure 19. *Enterococcus avium* isolated form spoiled red hot chili pepper mash

85

Figure 20. *Brevibacillus laterosporus* isolated form spoiled red hot chili pepper mash

Figure 21. *Aerococcus viridans* isolated form spoiled red hot chili pepper mash

6.3.2. Inoculation of spoilage microorganisms in fresh red hot chili pepper mash with spoilage isolates

APC of inoculated mash increased during first 15 days from 6.79 log CFU/g to 9.29 log CFU/g, and then started declining down to 7.92 log CFU/g on day 120. Non-inoculated mash counts declined from 6.79 log CFU/g to 3.98 log CFU/g in first 15 days, then increased to 5.25 log CFU/g at 75 days, and finally a reduction to 4.77 log CFU/g after 120 days (Table 16 and Figure 22).

Table 16. Aerobic Plate Counts (APC) in inoculated and non-inoculated red hot pepper mash (Log CFU/g)*.

Inoculate	Day of Analysis							
	0	*15*	*3*	*45*	*60*	*75*	*90*	*120*
	APC Log CFU/g							
Bacillus firmus	6.91	7.73	7.86	7.74	7.30	7.72	7.75	7.03
Bacillus pumilus	6.91	8.09	7.66	7.24	7.34	7.91	6.59	7.24
Aerococcus viridans	6.91	9.46	6.86	8.00	7.36	7.02	2.30	7.30
Enterococcus avium	6.91	9.37	7.27	7.60	7.15	7.07	7.25	8.23
Brevibacillus laterosporus	6.91	6.34	6.98	6.58	6.38	7.66	7.36	7.46
Mix	6.91	9.54	6.99	6.89	6.92	6.45	5.30	5.60
Average Inoculated mash	6.79	9.29	7.92	7.38	7.37	7.63	7.45	7.92
Control mash	6.79	3.98	4.01	4.81	5.18	5.25	4.61	4.77

*based on duplicate experiment.

At day 30 after inoculation the *Bacillus* species produced a slimy white layer on top of the mash. On day 60 the odor and color of the inoculated mash samples were different from the control, especially for *Enterococcus avium and Brevibacillus laterosporus*. All inoculated mash presented darker color and undesirable odor after 120 of inoculated (Figure 23).
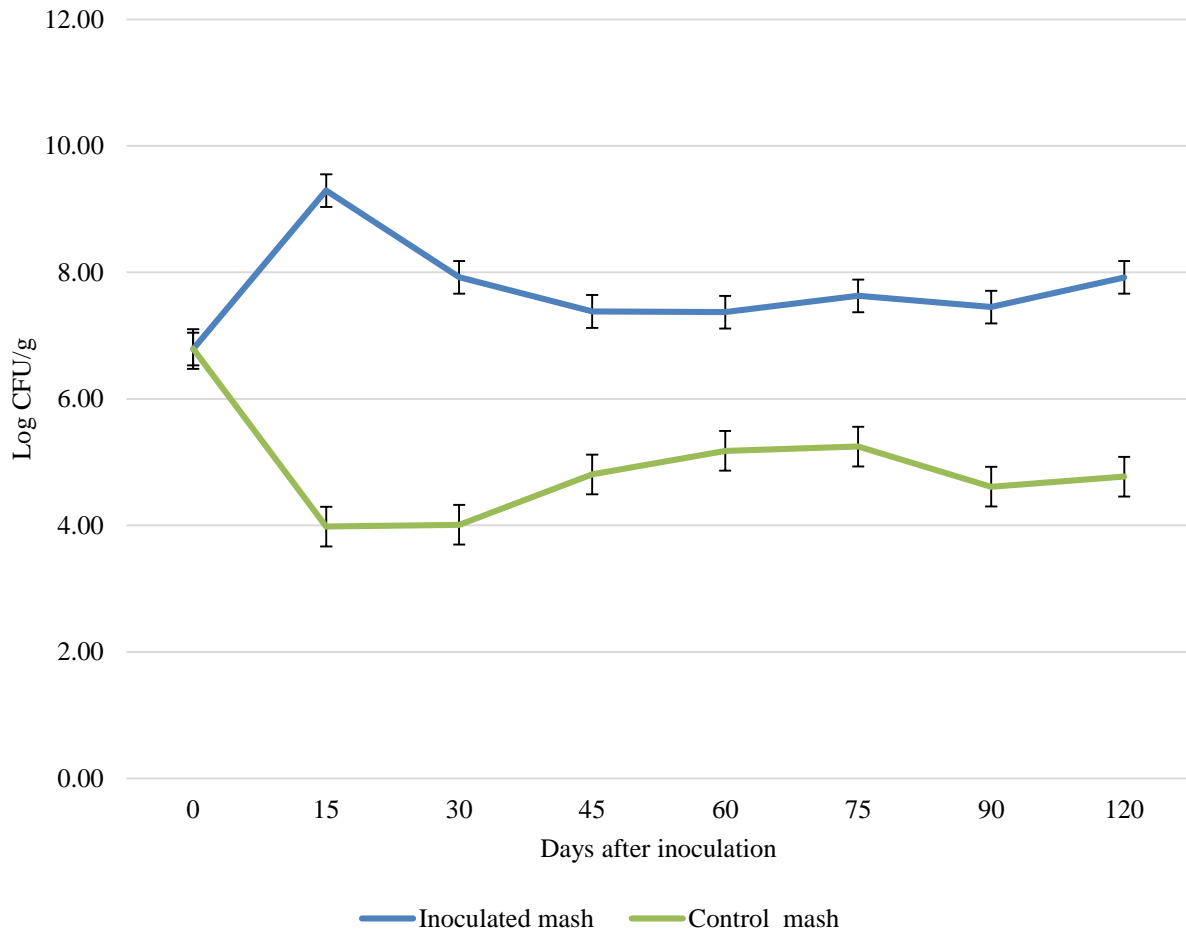
Figure 22. Comparison of aerobic plate count (APC) between inoculated mash and non-inoculated mash.

6.4. Discussion

The objective of our study was to stablish causes of spoilage in fermented red hot chili pepper mash. The first step was to isolated and identified microorganisms present in spoiled mash, followed by inoculation of isolates into fresh pepper mash and subsequent observation for spoilage changes in inoculated mash. From the isolation step we successfully identified *Bacillus firmus*, *Bacillus pumilus*, *Enterococcus avium*, *Brevibacillus laterosporus*, and *Aerococcus viridans.* This microorganisms have been isolated as normal flora in environment and have been used as biological controls against fungi, insects, and nematodes in several crops.

88

*Mash stored at 35°C ± 2°C to accelerate spoilage process.

Figure 23. Color changes after 120 days of inoculation, Top layer of mash (top pictures), and bottom layer (bottom pictures).

Production of proteases from *B. firmus* and *B. pumilus* during fermentation increase pH due to basic non-protein nitrogen compounds formed as a result of enzymatic proteolysis. Increase of pH during the fermentation of pepper mash will lead to spoilage (Bover-Cid et al., 1999; Chantawannakul et al., 2002; Moon and Parulekar, 1991; Puri et al., 2002; Rao and Narasu, 2007). *Aerococcus viridans* has been reported to produce greenish discoloration in spoiled meat products (Deibel and Niven C. F., 1959; Peirson et al., 2003), which could be also associated with the spoilage of fermented pepper mash.

The natural presence of these microorganisms in the chili pepper environment eases their way to pepper mash. Additionally, undesirable process related activities such as, excessive dust in air and open currents of air in processing area during mashing and filling of barrels can lead to the introduction of this microorganisms in the mash. Barrels are not sanitized between fermentation because it is believed that part to the fermentation microorganisms come from the barrels. Which can also facilitate the introduction of environmental microorganisms into the mash.

The same microorganisms isolated from spoiled pepper mash were isolated in a parallel study from normal fermented pepper mash as well. In parallel study we observed that after 730 days (2 years) of fermentation; LAB counts were reduced to a non-detectable level and yeast counts were increasing, which favored increase of pH and proliferation of environmental microorganisms which could lead to spoilage of pepper mash (Figure 24). All these support the hypothesis that additionally to the presence of these microorganisms in the mash, there are also environmental conditions during the fermentation process that could lead to spoilage of the mash.
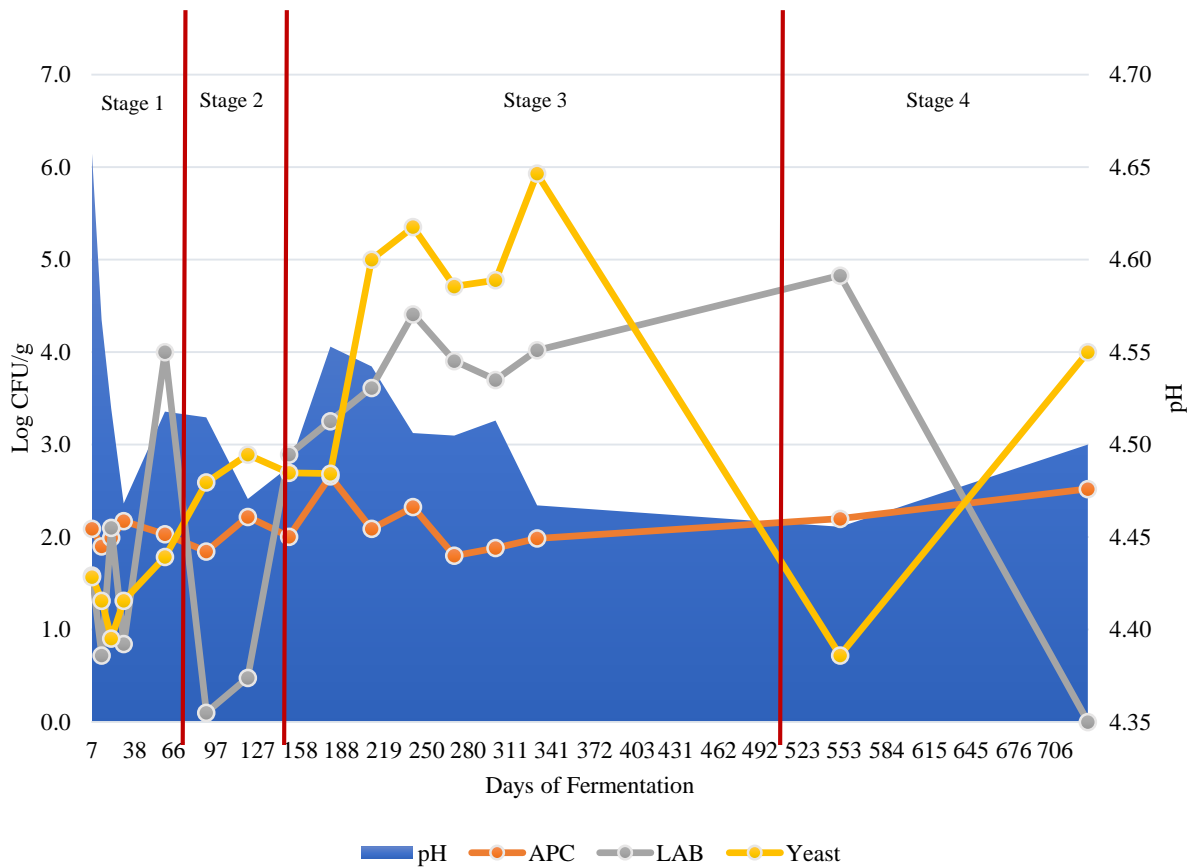
*Red lines separate different stages during pepper mash fermentation.

Figure 24. Aerobic plate count (APC), lactic acid bacteria counts (LAB), yeast counts, pH, and stages during 730 days (2 years) of red hot chili pepper fermentation.

In conclusion, microorganisms isolated from spoiled red hot chili pepper mash are commonly found in nature, therefore very likely to find them in pepper mash. These microorganisms might come originally from peppers or might be introduced during processing and storage in barrels. When fresh pepper mash was inoculated with these microorganisms and stored at 35°C ± 2°C, mash developed spoilage characteristics similar to those observed after two years of natural fermentation.

6.5. Acknowledgments

6.6. References

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CHAPTER 7. CONCLUSIONS

E. coli O157:H7 is responsible for a large proportion of domestically acquired foodborne illnesses which ends in hospitalization in the United States. Several studies indicate cattle as the main reservoir of *E. coli* O157:H7 worldwide, finding that the main sources are feces, feed bunks, water troughs and incoming water supplies. Most studies on *E. coli* O157:H7 have been in performed in feedlots, cattle and dairy farms, where researchers use strains adapted to laboratory conditions showing good resistance to media supplements. This study provides important information regarding the isolation and identification of environmental *E. coli* O157 from cow/calf farms and prevalence of this pathogen in small-scale cow/calf operations in Louisiana. It was found that results from two different culture media should be considered simultaneously for detecting environmental E*. coli* O157. It was also determined that the type of environmental samples does not affect probability of detecting this pathogen.

Cow/calf farms are the beginning of the beef chain, and even though extensive research has been completed at feedlot stage, we are missing information of *E. coli* O157:H7 prevalence in cow/calf operations. If we start working on the reduction and control of *E. coli* O157:H7 from the beginning of the food chain at cow/calf farms, it could make a positive impact in the reduction of the pathogen on the rest of the chain. An initial step is to establish *E. coli* O157:H7 prevalence in cow/calf farms and additionally to determine the main source of contamination at this stage. We found that *E. coli* O157:H7 prevalence in small-scale cow/calf operations in Louisiana is 8%, and that there is no significant difference between fecal matter, water, and swabbed surfaces as sources of contamination.

Hot sauce is a fast growing industry in the U.S., where Louisiana is one of the bigger producers with more than 35 different brands available in the market. Information regarding fermentation of pepper mash is required to improve the fermentation process, production of a high quality final product, and reduction of loses due to spoilage of pepper mash. With this research we were able to identify the microorganisms involved in the fermentation process of red hot chili pepper mash and their impact in physical/chemical properties such as, pH, %TA, color, and volatile compounds concentration in the mash.

Red hot chili pepper mash can be divided in four stages, where LAB and yeast act in a symbiotic environment, where we observed an intensification of aromas in the mash. Additionally, microorganisms isolated from spoiled mash were also isolated in naturally fermented mash with no signs of spoilage. Therefore, we conclude that optimal growing conditions are needed to trigger the spoilage of the mash, conditions observed after 18 months fermentation, when there is no LAB to stop yeast from increasing pH in the mash. With this, we recommend to stop mash fermentation at this point and start processing hot sauce. Findings from red hot chili pepper mash natural fermentation can be used in future research developing a controlled fermentation targeting more desirable flavor and aroma, additionally to a faster fermentation.

	APC	LAB	Yeast	pH	Lactic Acid	L	a	b	Hue	n-hexanol	4-methyl-1-pentyl-isobutyrate	(Z)-3-hexenyl isopentanoate	heptyl isobutanoate	heptyl pentanoate	3,3-dimethylcyclohexanol
APC	1														
LAB	0.16	1													
Yeast	-0.09	0.45	1												
pH	0.02	-0.22	-0.17	1											
Lactic Acid	0.05	0.62	0.56	-0.58	1										
L	0.13	-0.44	-0.18	-0.19	-0.27	1									
a	-0.17	-0.26	-0.55	0.37	-0.36	-0.28	1								
b	-0.07	-0.37	-0.60	0.62	-0.51	-0.25	0.93	1							
Hue	0.30	-0.25	-0.13	0.59	-0.35	0.06	-0.23	0.15	1						
n-hexanol	-0.31	-0.62	-0.59	0.10	-0.65	0.55	0.29	0.32	0.05	1					
4-methyl-1-pentyl-isobutyrate	-0.17	-0.33	-0.43	-0.25	-0.40	0.65	-0.05	-0.13	-0.23	0.55	1				
(Z)-3-hexenyl isopentanoate	-0.41	0.26	0.47	-0.44	0.48	0.06	-0.15	-0.28	-0.34	0.01	0.09	1			
heptyl isobutanoate	0.46	0.01	-0.04	0.01	-0.05	-0.06	-0.19	-0.05	0.44	0.08	-0.41	-0.11	1		
heptyl pentanoate	-0.31	0.37	0.40	-0.50	0.51	0.15	-0.24	-0.39	-0.42	-0.11	0.31	0.88	-0.40	1	
3,3-dimethylcyclohexanol	0.27	0.13	0.28	-0.24	0.35	-0.06	-0.29	-0.24	0.20	-0.06	-0.45	0.36	0.79	0.05	1

Where coefficient of correlation close to 1 means positive correlation, close to -1 negative correlation, and close to 0 no correlation.
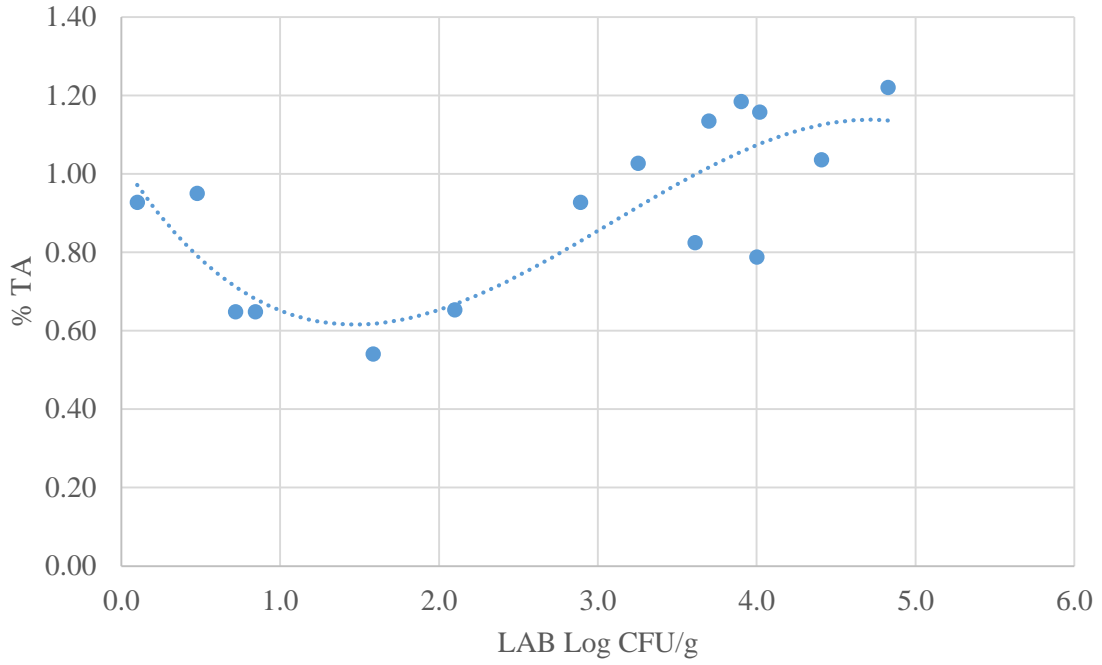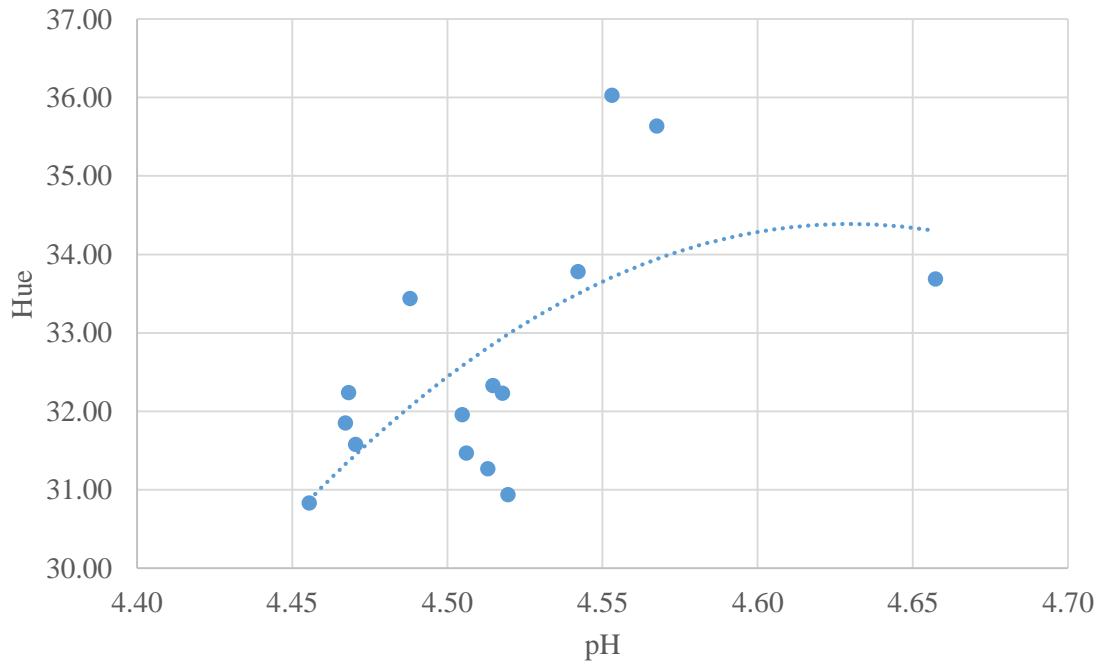
Correlation between a* and b*
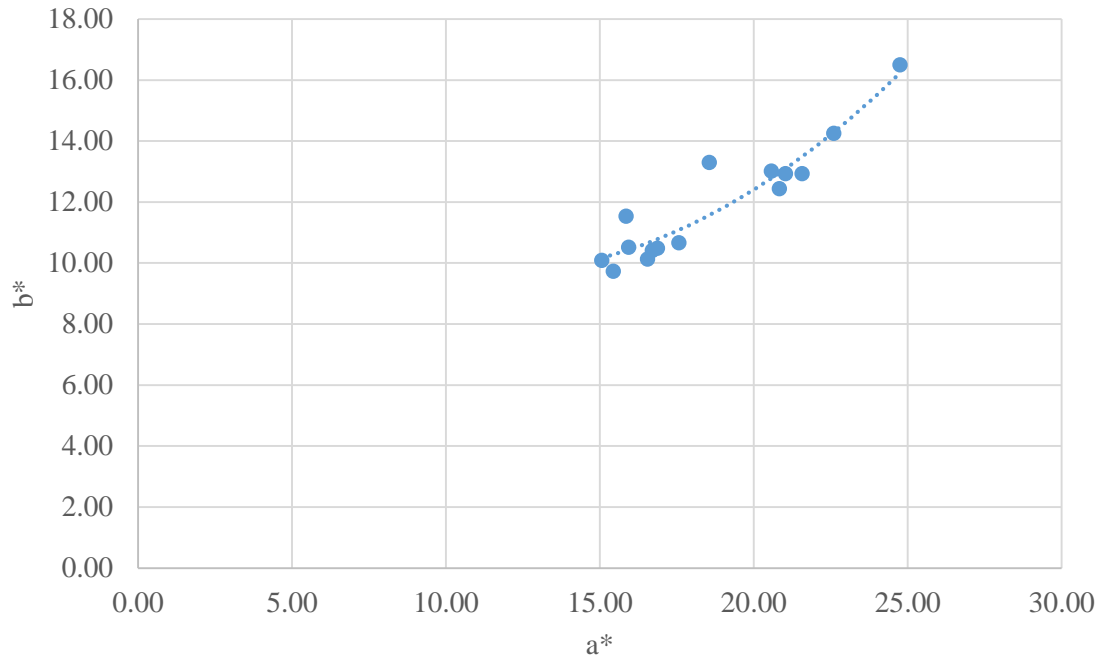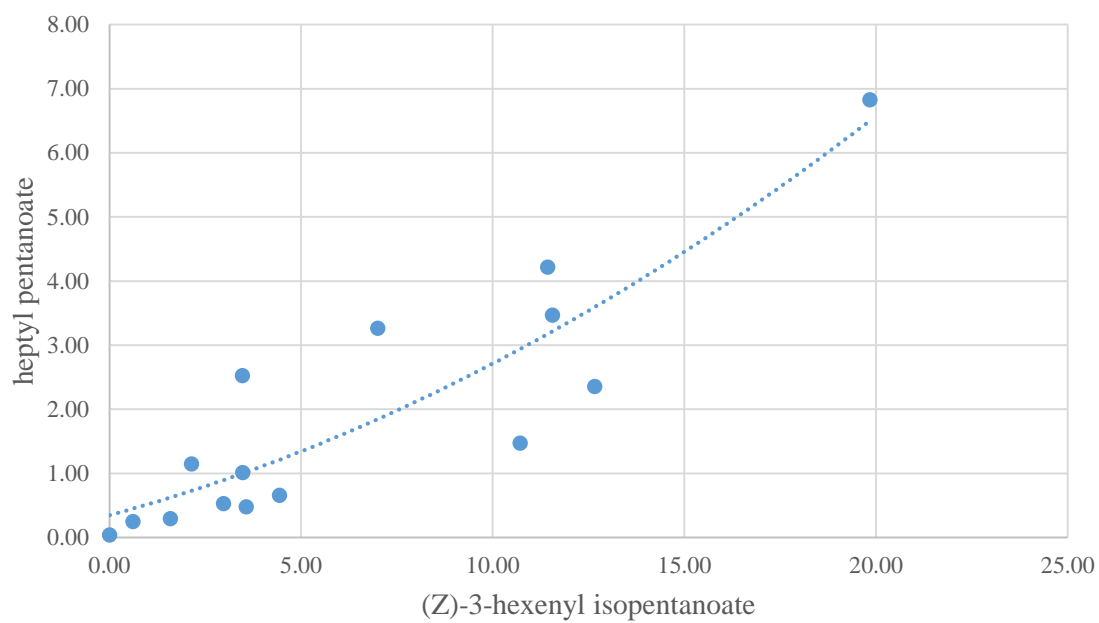

Correlation between TA and pH
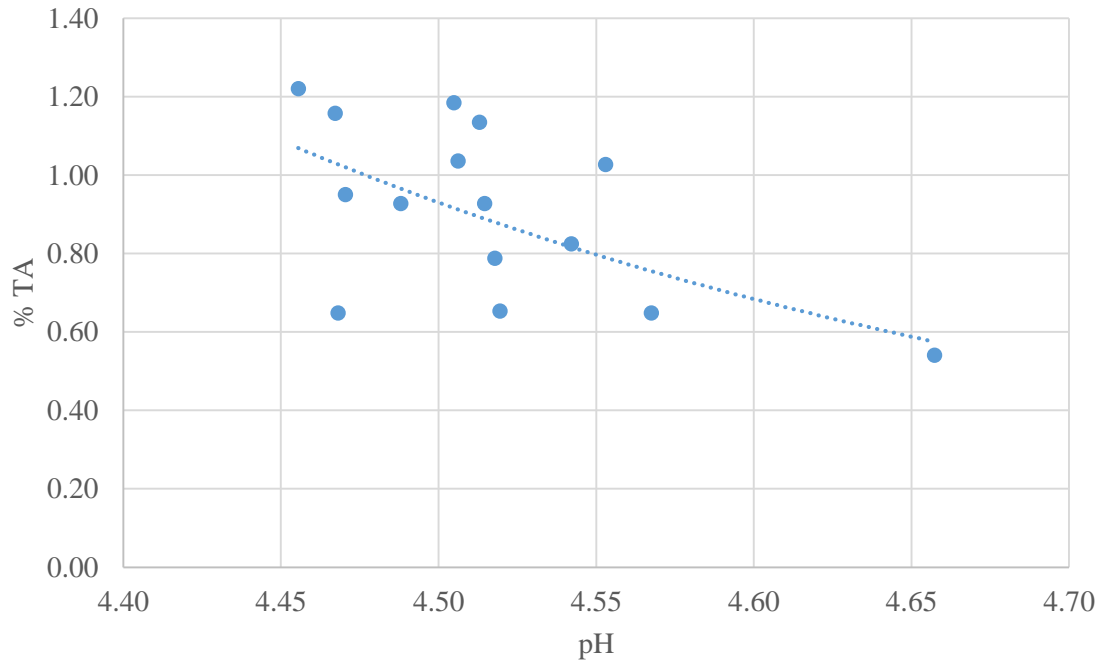
Correlation between TA and LAB

Correlation betwen pH and Hue

Correlation between heptyl pentanoate and (Z)-3-hexenyl isopentanoate

VITA

Myriam Evelyn Gutierrez Barberena was born in Guatemala City, Guatemala in 1977. She earned degree on Veterinary Medicine in 2001 from University of San Carlos in Guatemala, Guatemala. She worked for a few years in the food industry as QC supervisor, where she found her passion for food safety. Then she earn her master's degree in Food Safety also at the University of San Carlos in Guatemala. In 2011 she moved to U.S. and started working on her PhD degree in Food Science at Department of Food Science, Louisiana State University AgCenter, and conducted her research isolating and identifying microorganisms from environment and food samples. Currently, she is a candidate to receive her doctorate of philosophy in May 2016.

During her graduate career, she has been actively involved in multiple research projects and active member in the International Association for Food Protection and Institute of Food Technology. She has given two poster presentations and one technical presentation at the International Association of Food Protection Annual Meetings, and one poster presentation at the Institute of Food Technology Annual meeting, and plans to continue her professional career as a food safety specialist.