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Characterization of Volatile and Metabolite Compounds Produced by Lactococcus lactis in Low-Fat and Full-Fat Cheddar Cheese Extract

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CHARACTERIZATION OF VOLATILE AND METABOLITE COMPOUNDS PRODUCED BY *LACTOCOCCUS LACTIS* IN LOW-FAT AND FULL-FAT CHEDDAR CHEESE EXTRACT

by

Michael J. Young

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ABSTRACT

Characterization of Volatile and Metabolite Compounds Produced by *Lactococcus lactis* in Low-Fat and Full-Fat Cheddar Cheese Extract

by

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This study was conducted to compare and contrast potential aroma compounds in the headspace and small molecule metabolites produced as a result of starter culture metabolism in a full-fat and low-fat cheddar cheese model system. Past studies have indicated differences in the headspace flavor compound profiles between full-fat and low-fat Cheddar cheeses with no indication as to what compounds were produced as a result of starter culture metabolism.

Starter cultures were incubated in a Cheddar cheese extract environment that was made up of the water-soluble portion of Cheddar cheese with environmental conditions mimicking full-fat and low-fat Cheddar cheese by altering the levels of salt and milk fat globular membrane in the system. Incubation times were up to 14 days at 30°C and samples were taken at days 0, 1, 7, and 14. Headspace analysis was accomplished using solid phase micro-extraction coupled with GC-MS and small metabolites were monitored using metabolomic methods coupled with GC-MS.

Results indicate that the starter culture was responsible for an increase in the concentration of propan-2-one, heptan-2-one, 3-methylbutanal, heptanal, benzaldehyde, 2-ethylhexanal, and dimethyl trisulfide in both the full-fat and low-fat medias when compared to their respective controls. While heptanal was present at a higher concentration in the full-fat treatments compared to the low-fat treatments and 2-ethylhexan-1-ol and isothiocyanato cyclohexane were present at higher concentrations in the low-fat treatments compared to the full-fat treatments.

Principal component analysis for the headspace compounds showed a clear separation of the treatments with heptanal, p-cymene, nonan-2-one, and undecan-2-one contributing the most to the variation between the full-fat and low-fat samples, while 3-methylbutanal, heptan-2-one, benzaldehyde, 2-ethylhexan-1-ol, 2,6-dimethylheptan-4-ol, and 3-methylbutanol contributed the most to the variation between the controls and treatments.

The metabolomics data for both the bacteria and Cheddar cheese extract did not provide a clear separation between the full-fat and low-fat samples.

(91 pages)

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I give special thanks to my friends and family, especially my wife, Heather, and son Noah. You gave me the strength to see this project to the end and I couldn't have done it without you both.

Michael J. Young

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ABBREVIATIONS AND DEFINITIONS

CCE	Cheddar Cheese Extract
SPME	Solid Phase Micro-Extraction
GC-MS	Gas Chromatography-Mass Spectrometry
HS	Headspace
NSLAB	Non Starter Lactic Acid Bacteria
S/M	Salt in Moisture
FF	Full-Fat
LF	Low-Fat
FFC	Full-Fat Control
LFC	Low-Fat Control
FFT	Full-Fat Treatment
LFT	Low-Fat Treatment
PCA	Principal Component Analysis

INTRODUCTION

The CDC reported in 2008 that Colorado was the only state with a prevalence of obesity less than 20%. There were 32 states with prevalence rates equal to or greater than 25%. This has been a cause of concern due to the health risks associated with obesity. Dieticians have suggested a reduction in total calories in the diet as a way to reduce obesity. Recent dietary guidelines indicate that fat should account for less than 30% of the total energy intake in the American diet (McDonald 2000). As a result of these guidelines, demand for low-fat (LF) and reduced-fat food alternatives has risen.

According to the Code of Federal Regulations, Cheddar cheese is a high fat food. Most traditional Cheddar cheeses contain 9 grams of total fat per one ounce serving, which translates to 14% of the daily intake of total fat in a 2000 calorie diet. As the consumer searches for food options, with this increase in awareness of the need to reduce total caloric intake due to fat, labels that contain a high amount of fat may deter purchase.

The increased demand for lower fat alternatives to full-fat (FF) Cheddar cheese has resulted in the development of reduced-fat Cheddar cheese products. The primary difference between reduced-fat Cheddar cheese and FF Cheddar cheese is the reduction of fat content. The fat has to be replaced to maintain body and in most cases this is accomplished by increasing the moisture content.

The reduction of fat produces a product that has a lower consumer acceptance due to its rubbery texture and its bland and bitter flavor (Banks 2004). The flavor, aroma, and texture are important components of the overall sensory experience of Cheddar cheese. In 1998 the sales of LF and reduced-fat cheese accounted for 20% of the total cheese sold in United States supermarkets (Mistry 2001). It is has been suggested that the growth of

LF and reduced-fat cheese may have stalled due to consumer demands for a higher quality product and the consumer's memory of poor quality in the initial LF and reduced-fat cheese products (Guinee and others 1998).

The development of Cheddar cheese flavor is a complicated process that occurs primarily during ripening. The main constituents of milk interact with native enzymes in the milk as well as added components such as rennet and starter culture to form volatile compounds. Some of these products contribute to the overall flavor of the cheese, which is determined by not only whether or not certain compounds are found in the product but also by the proportion at which it is present compared to other compounds (Fox and Wallace 1997).

The complex nature of cheese makes the monitoring of aroma compounds produced by specific bacterial strains very difficult, especially when alterations in important intrinsic parameters like pH, redox, salt, and lactate levels are considered. If conducting research with natural cheese, blocks of each treatment would be required and the aroma compounds contributed by any specific bacteria would be hard to distinguish from other sources. To overcome this problem, a Cheddar cheese model system was developed that allows for the alteration of parameters important to both flavor production and bacterial growth (Diaz-Muniz and others 2006). Cheddar cheese extract (CCE) is essentially the aqueous phase of cheese and it is hypothesized that the microorganisms contributing to the flavor of cheese utilize components in this phase. The CCE can be inoculated with the bacterium of interest and grown under very specific conditions while monitoring the effect of that bacterium on aroma production.

The hypothesis of this study is:

Lactococcus lactis incubated in CCE, adjusted to mimic LF and FF conditions, will produce different volatile headspace (HS) and metabolite profiles.

The research aims addressed in this thesis are:

1. Characterization of volatile compounds in the HS of both LF and FF CCE inoculated with *Lactococcus lactis* using solid phase micro-extraction (SPME) gas chromatography mass spectrometry (GC-MS).
2. Characterization of small molecule metabolites in the bacteria and the media using metabolomics techniques coupled with GC-MS.

LITERATURE REVIEW

Cheddar Cheese Flavor

Flavor production in Cheddar cheese occurs primarily during the ripening stage of the cheese making process. During the ripening stage, key components of the cheese are broken down to produce different flavor compounds. This process occurs as a result of both added components, such as starter cultures and rennet, and components not intentionally added such as non-starter lactic acid bacteria (NSLAB) and native enzymes of the milk system. Key flavor precursors include proteins, fats, citrate, and residual lactose. Flavor development in cheese results from a combination of microbial and biochemical activities that lead to the formation of a heterogeneous mixture of volatile and nonvolatile flavor compounds (Fox and Wallace 1997). This process takes time and is dependent on intrinsic and extrinsic factors including temperature, redox potential, pH, and salt/moisture (S/M) (Fox and others 2000).

The characteristic Cheddar cheese flavor has not been attributed to a single compound; rather it is a result of a balance between a variety of flavor compounds in the cheese. This has been termed the "component balance theory" which states that the Cheddar cheese flavor comes from the correct proportion or balance of all the flavor compounds in the Cheddar cheese (Mulder 1952). To date, there has not been a successful synthetic recreation of the Cheddar cheese flavor, which indicates its complexity.

Although the overall desirable flavor of Cheddar cheese has not been defined in exact chemical makeup, the characterizations of off flavors have been identified with

moderate success. The off flavors arise due to a disproportional amount of certain compounds in the cheese. For example, bitterness mainly arises due to an increase in hydrophobic peptides, rancidity arises due to fatty acids, and fruitiness is due to ester formation (Fox and others 2000).

Low-Fat Cheddar Cheese Flavor

Low-fat cheese has a characteristic lack of desirable flavor intensity when compared to its FF counterpart. Instead, the flavor profile of LF cheese is dominated by off flavors, characterized as bitter, meaty, brothy, unclean, and barnyard like (Banks et al 1992). These off flavors are likely due to an imbalance of flavor compounds (Mistry 2001). Three proposed theories explain the loss of characteristic FF Cheddar flavor in the LF product.

The first theory proposes starter culture physiology and the resulting metabolic end product profiles are the same in all cheeses, but sensory perception of those metabolites is altered by differences in the physico-chemical environment (e.g. fat, moisture, or S/M values).

Research has shown that fat in food plays an important role in the delivery of flavor (Li and others 1997; Prindiville and others 2000; Roberts and others 2003; Carunchia Whetstine and others 2006). Most flavor compounds are classified as nonpolar and hydrophobic. Being classified as such, flavor compounds tend to associate with the fat portion in food systems (Relkin and others 2004). Van der Walls and hydrophobic interactions characterize the interactions between fat and hydrophobic flavor compounds (Plug and Haring 1993).

Recent research conducted by Carunchia Whetstine and others (2006) indicates that the aroma compounds in Cheddar cheese are present more in the aqueous phase of the cheese rather than the fat phase. In this study fat was removed from Cheddar cheese, using a novel process, and the aroma compounds of both the remaining cheese and removed fat were tested. The results showed that aroma compounds were present more in the cheese than the fat indicating that the fat does not play as critical a role in binding aroma compounds as previously thought.

Each flavor compound has different physical and chemical properties and only those compounds that are present in a high enough concentration to stimulate olfactory receptors are above sensory threshold and therefore sensed by the consumer (Carunchia Whetstine and others 2006). During the mastication process, the release of flavor and thus the sensory perception of aroma compounds are dependent on the rate of release from the cheese matrix, which is influenced by the fat content (Delahunty and others 1996). An increase in fat, holding the flavor compound concentration constant, would increase the sensory threshold due to the added interactions between flavor and fat. These interactions decrease the available amount of flavor in the HS of the sample and therefore a lower concentration of flavor compounds is needed to elicit a similar sensory threshold in a LF product due to the decreased availability of fat flavor interactions (Carunchia Whetsine and others 2006).

The second theory proposes microbial physiology itself, and thus overall metabolism, is altered by differences in the physico-chemical environment in ways that affect the production of flavor and aroma active metabolites.

The change in the physico-chemical environment of LF cheese could alter the

overall metabolism of the starter culture, which would in turn alter the proportion of flavor compounds of the final product. Thus, any difference in the proportion of flavor compounds found in the FF and LF cheeses would contribute to the differing flavor profile between the two products. Any differences in metabolic activity of the starter culture in FF versus LF cheese would likely be due to the increase in the moisture and the reduction of the S/M in the LF cheese when compared to the FF cheese. Table 1 demonstrates the differences between FF and LF Cheddar cheese physico-chemical environment conditions.

Research conducted by Milo and Reineccius (1997) showed a difference in the proportions of flavor compounds between FF and LF Cheddar cheese. They found that the meaty/brothy off-flavor of the LF Cheddar cheese was caused in part due to the increased amounts of methional, furaneol, and especially homofuraneol. They proposed that this change came about due to the increased bacterial growth as a consequence of increased moisture.

Table 1 Differences of full-fat and low-fat Cheddar cheese

	Full-fat	Low-fat
Moisture (%)	37.0	50.3
Fat (%)	33.8	7.00
Salt (%)	1.76	1.85
Salt in moisture (%)	4.75	3.68
pH	5.26	5.21

*Values were taken from the analysis of full-fat and low-fat Cheddar cheese used in Cheddar cheese extract preparation

The third theory proposes the physico-chemical environment of LF cheese may produce major changes in the gross microbiology of cheese.

Broadbent (2009) showed that there was a difference in the growth of both starter culture and NSLAB in FF and reduced-fat and LF Cheddar cheeses. The growth of the starter in the reduced-fat and LF cheeses showed signs of stability up to 3 months before showing signs of decline, while the starter cultures in the FF had declined by at least two orders at 3 months. The growth of the NSLAB in the LF cheese reached levels of 10^6 by six weeks while in FF cheese these levels were not attained by the 3 to 6 month time period. The difference in the growth of both the starter culture and the NSLAB in the FF and LF Cheddar cheese are most likely due to the differences in the environment shown in Table 1. The difference in the growth of both the starter culture and the NSLAB in the FF versus the LF cheese would likely result in a difference in the metabolism of the microorganisms and consequently a shift in the flavor compounds.

The most likely scenario is that all three of these effects play a role in the development of differences in FF and LF Cheddar cheese. The microbial growth of both the starter cultures and the NSLAB are affected by the alteration of the environment found in LF cheese leading to an increase in growth and possible changes in metabolic activity. The changes in growth lead to an alteration in the proportion of the flavor compounds found in the LF cheese when compared to the FF cheese due to alteration of metabolic activity. To further complicate the problem, fat, which may act to mask off flavors in FF cheese, is replaced with water in LF cheese. The reduction of fat decreases the flavor threshold of flavor compounds, making potentially undesirable compounds

perceived by the consumer. This increased perception of undesirable compounds leads to an unsatisfactory sensory experience for the consumer.

Lactococcus lactis Characteristics

Lactococcus lactis is a common starter culture that is added to milk in the production of Cheddar cheese. Some of the benefits of *L. lactis* strains include rapid acid production, salt sensitivity, and ripening activity (Fox and others 2000). Steady acid production throughout the cheese making process ensures the suppression of undesirable bacterial growth (Beresford and Williams 2004). *L. lactis* growth in an hour reaches levels greater than 10^8 cfu /g. As cheese ripens, the *L. lactis* levels decrease to levels of 10^7 to 10^4 cfu/g (Beresford and Williams 2004).

Hassan and Frank (2001) describe the characteristics of *L. lactis*. *L. lactis* is cocci and usually occurs in chains. They are homofermentive when grown in milk with 95% of their end products being lactic acid. Growth occurs at 10°C but not at 45°C. They are weakly proteolytic and can use milk proteins and lactose for growth. They hydrolyze milk casein by the extracellular proteinase PrtP. In addition, they can produce acid from lactose, galactose, maltose, and ribose and can grow in the presence of 4% salt.

Flavor Production of Lactococcal Strains

Starter cultures in Cheddar cheese have the primary role of reducing the pH by producing lactic acid from lactose. The production of flavor compounds by starter cultures occurs as a result of the major biochemical events during the cheese ripening process. These processes are categorized into four groups: (1) glycolysis of residual lactose and further catabolism of lactic acid, (2) catabolism of citrate, (3) lipolysis and the

subsequent catabolism of free fatty acids, and (4) proteolysis and the catabolism of amino acids (Fox and others 2000). The importance of starter cultures role in the development of flavor was shown by Reiter and others (1966) where the absence of Cheddar flavor was shown in glucono delta-lactone acidified cheese and typical Cheddar cheese flavor was shown in starter culture only cheese.

Lactose and citrate metabolism

The fermentation of lactose by *L. lactis* primarily produces lactate, which is accomplished via glycolysis. Lactate contributes to acidic flavor, especially in young cheeses (McSweeney and Sousa 1999). The branch point of potential flavor compounds in the production of lactate is pyruvate, a metabolic intermediate. Potential flavor compounds produced from pyruvate include acetoin, formate, acetaldehyde, ethanol, and acetate. Research conducted by Melchiorsen and others (2000) showed that the production of formate, acetate, and ethanol by *L. lactis* was dictated by the concentration of lactose present. Initially the lactose concentration is high enough to promote homo-fermentation of lactose to L-lactic acid, but as the concentration of lactose decreases during the ripening, the production of formate, acetate, and ethanol along with L-lactic acid occurs.

Lactococcal strains that are citrate positive have the ability to metabolize citrate in the presence of a fermentable sugar to acetate, diacetyl, acetoin and 2, 3-butanediol (Fox and others 2000). According to Curioni and Basset (2002) these compounds have been identified as contributing flavor compounds to Cheddar cheese. Citrate is a minor constituent of milk with a majority of it lost in the whey. Cheddar cheese contains 0.2%

Table 2. Some potential flavor byproducts of lactose and citrate metabolism

Compound	Flavor[a]
Acetaldehyde	Sweet, pungent
3-Hydroxybutan-2-one	Sour milk
Butane-2,3-dione (diacetyl)	Cheesy, caramel
Acetic acid	Vinegar
Ethanol	Dry dust

[a](Curioni and Basset 2002)

to 0.5% (wt/wt) citrate (McSweeney and Fox 2004). Table 2 contains some potential aroma compounds resulting from fermentation of lactose and citrate.

Fatty acid metabolism

Fatty acids in milk come primarily in the form of triacylglycerols. In order for the fatty acid to contribute to the flavor of the cheese it first be acted upon by a lipase or esterase to release the fatty acid from the glycerol backbone. Lipolytic agents come from 6 sources: milk, rennet, starter bacteria, secondary starter microorganisms, NSLAB, and exogenous lipase preparations (Collins et al 2004). Milk fat has a high proportion of fatty acids that are either short or medium length that when liberated contribute directly to the flavor of the cheese (McSweeney and Sousa 1999). Fatty acids can be substrates for further catabolic reactions, which produce methyl ketones, secondary alcohols, lactones, ethyl esters, aldehydes, acids, and alcohols. Table 3 lists some potential flavor compounds and their respective flavor and Figure 1 shows the potential pathways followed to make these flavor compounds.

Table 3. Some potential flavor byproducts of fatty acid metabolism

Class	Compound	Flavor[a]
Free fatty acid	Butanoic acid	Rancid, cheesy
	Hexanoic acid	Pungent
	Octanoic acid	Wax, goat, musty
Methyl ketone	Heptan-2-one	Fruity, foral, musty
	Propan-2-one	Fruity, foral, musty
	Nonan-2-one	Fruity, foral, musty
Secondary alcohol	Propan-2-ol	
	Butan-2-ol	
	Octan-2-ol	
Lactone	δ-Decalactone	Peachy, coconut
	γ-Decalactone	Coconut
Ethyl ester	Ethyl butyrate	Bubble gum, fruity
	Ethyl hexanoate	Young cheese
	Ethyl octanoate	Fruity
Aldehyde	Pentanal	Chemical
	Heptanal	Soapy
	Nonanal	Green, fatty
Alcohol	Propan-1-ol	Sweet (candy)
	Pentan-1-ol	Fruity

[a]Curioni and Basset 2002

Proteolysis and the catabolism of amino acids

Proteolysis in ripening cheese comes primarily from the following sources: coagulant, indigenous milk proteases, starter culture proteases, and NSLAB proteases. Initially in the cheese making process, the primary hydrolysis of milk proteins is accomplished by the action of the coagulant and to a lesser extent by the native milk enzyme, plasmin. The products of the initial hydrolysis are large peptides which are further broken down by action of the coagulant protease and enzymes contributed by the starter and NSLAB. Proteinases and peptidases of the starter culture and NSLAB further hydrolyze the peptides into shorter peptides and amino acids (McSweeney and Sousa 1999).

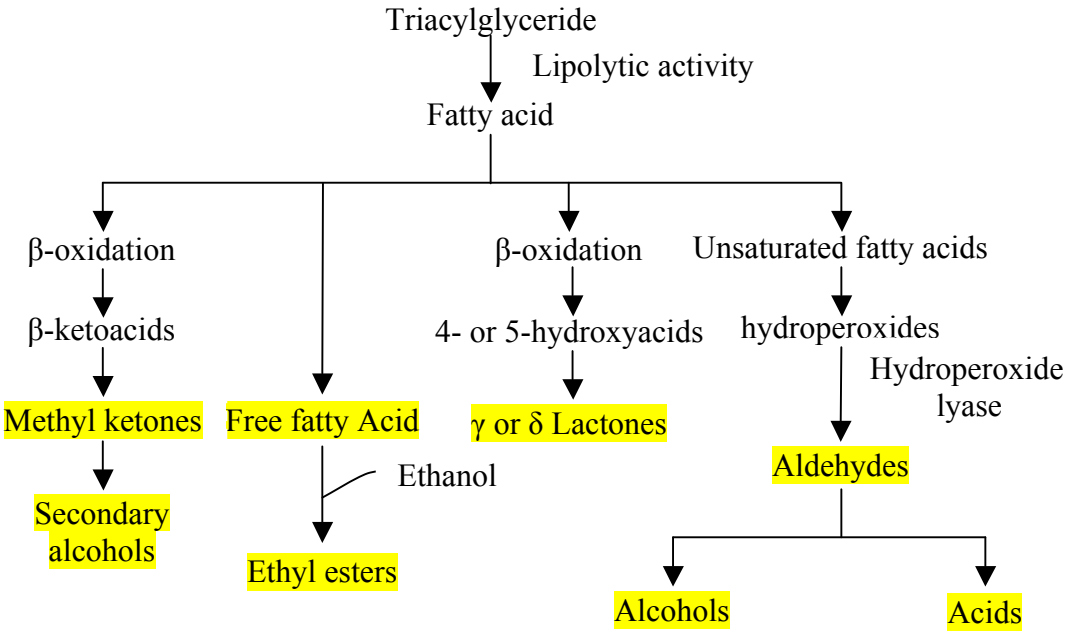


Figure 1. Potential pathways of fatty acid flavor development
Highlighted compounds contribute to cheese flavor.

Amino acids are further metabolized to form additional compounds that contribute to the flavor. According to a review by Yvon and Rijnen (2001), aromatic amino acids (phenylalanine, tyrosine, tryptophan), branched-chain amino acids (leucine, isoleucine, and valine), and methionine are major precursors to important cheese flavor compounds. There are 2 mechanisms by which amino acids are degraded by lactic acid bacteria, the first mechanism involves the cleaving off of amino acid side chains catalyzed by amino acid lyases, and the second method involves amino acid aminotransferases (Yvon and Rijnen 2001).

The use of amino acid lyases in *L. lactis* is primarily used in the degradation of methionine, although this is not believed to be the primary method of the degradation methionine in this organism (Yvon and Rijnen 2001). The two enzymes exhibiting lyase

activity in *L. lactis* are cystathionine-β-lyase and cystathionine-γ-lyase (Curtin and McSweeney 2004). cystathionine-β-lyase catalyzes the conversion of cystathionine to homocysteine, pyruvate, and ammonia, while cystathionine-γ-lyase catalyzes the conversion of cystathionine to cysteine, α-keto butyrate, and ammonia (Curtin and McSweeney 2004).

Aminotransferases catalyze the transfer of the amino group from an α-amino acid to an α-keto acid (Curtin and McSweeney 2004). Often the α-keto acid acceptor is α-ketoglutarate, which is transformed to glutamate upon reaction with an amino acid (Yvon and Rijnen 2001). Aminotransferases are pryridoxal-5'-phosphate dependent enzymes that have broad substrate specificity and can catalyze reverse reactions (Weimer and others 1999).

There appears to be three types of aminotransferases found in *L. lactis* bacteria, branched chain amino acid aminotransferase and aromatic amino acid aminotransferase (Yvon and Rijnen 2001). branched chain amino acid aminotransferase uses the following substrates Ile, Leu, Val, and Met, while aromatic amino acid aminotransferase uses Leu, Tyr, Phe, Trp, and Met. The resulting α-keto acid products are further degraded to form potential flavor compounds. Table 4 contains some potential flavor compounds that could originate from amino acids.

Environmental Conditions

The intent of this study is to monitor the aroma compound production of *L. lactis* in CCE under FF and LF conditions. The variables that will differ between the FF and

Table 4. Some potential flavor compounds from amino acid metabolism

Class	Compound	Flavor[a]
Aldehyde	3-Methyl butanal	Slightly caramel, nutty
	2-Methyl propanal	Floral
Alcohol	3-Methyl-1-butanol	Alcoholic, green
	3-Methyl-2-buten-ol	Cheese, fruity, green
	Phenylethanol	Rosey
Acid	1-Methyl propanoic acid	
	3-Methyl butanoic acid	Swiss cheese
Other	Indole	stable
	Skatole	Fecal
	2-Isopropyl-3-methoxypyrazine	Earthy
	Methional	Baked potato
	Methanethiol	Sulfury
	Dimethylsulphide	Cabbage like
	Dimethyltrisulfide	Putrid
	Dimethyltetrasulfide	Putrid
	p-Cresol	Cowy, barny

[a]Curioni 2002

the LF CCE will be fat and S/M. The following paragraphs describe the effects on flavor caused by altering the ripening temperature, S/M, and fat level.

Ripening temperature

Temperature is a major factor in the rate of biochemical reactions including enzymatic reactions. The growth of the starter cultures is also dependent on ripening temperature. The temperature used in the ripening of the cheese is a tradeoff between the growth of starter culture (good) and the growth of spoilage bacteria (bad). The elevation of temperature can be used as a method to accelerate ripening, but risks skewing microbial population dynamics, as indicated above. McSweeney and others (1996) showed that for Cheddar cheeses ripened at 8, 12, and 16°C, the 16°C ripened cheese exhibited the highest flavor score.

Salt level

The use of salt as a means of food preservation dates to pre-historic times. The S/M in cheese plays several critical roles in the development of the cheese. The added salt helps to determine the final pH of cheese by reducing starter culture metabolism. The salt also affects the overall flavor and texture by influencing proteolysis (Guinee and Fox 2004).

Lawrence and others (1984) have suggested a S/M value of 4% to 6% in the grading of premium New Zealand Cheddar for long term ripening. The increase of water associated with the decrease in fat in LF cheeses causes a reduction in the S/M to be as low as 3.5%. Mistry and Kasperson (1998) studied the effect of varying salt concentrations of reduced-fat cheeses on bacterial growth, pH, and proteolysis during the ripening process. They made reduced-fat Cheddar cheese with 2.7%, 3.7%, and 4.5% S/M and allowed them to ripen for 24 weeks testing the pH, microbial count, and proteolysis at 1, 4, 12, and 24 weeks. Their results showed a decrease in the microbial count of all varying level of S/M during the 24 week ripening period with a greater decrease in the microbial count in the 4.5 compared to the 2.7 and 3.7% S/M. The pH of the cheese was also affected by the salt concentration. The pH was lower in the 2.7% S/M and increased with an increase in salt, indicating an increase in the microbial activity in the low salt conditions.

In LF cheese the S/M contained in the final product is decreased due to the increase in water to the product. Salt is used as a method of keeping the bacterial growth in the cheese to a desirable level. In LF cheese the growth and metabolic activity of the

starter culture as well as NSLAB maybe accelerated. Off flavors can be observed potentially as a result of the increased bacterial growth and activity.

Volatile and Metabolite Molecule Identification

The following paragraphs detail the methods that were used in the identification of both the aroma volatiles and the small molecules. In both methods the detection method is the same but the extraction and derivatization processes differ.

Headspace solid phase micro-extraction

In a review of applications of SPME Kataoka and others (2000) describe SPME as a solvent less extraction method in which a silica fused fiber is placed above the HS of the sample to trap volatiles. The volatile compounds then partition to the fiber until equilibrium is achieved amongst the three phases in the system, fiber, HS, and sample or a predetermined amount of time has elapsed. The fiber is then removed and inserted into the analytical instrument, such as liquid chromatograph or GC. The use of SPME can greatly reduce the sample preparation time as well as eliminate potentially toxic solvents used to isolate compounds of interest as compared to other sample preparation techniques such as liquid-liquid or solid phase extraction. A limiting factor in the use of SPME in volatile analysis is the selectivity of the fiber chosen for analysis, as the analytes obtained from the sample may not be a complete representative of all the volatile compounds in the HS. The selectivity of different fibers is specific for certain types of compounds based primarily on the polarity and size of the compounds of interest. Polar compounds are absorbed by polar fibers and likewise the same is true for nonpolar compounds, which

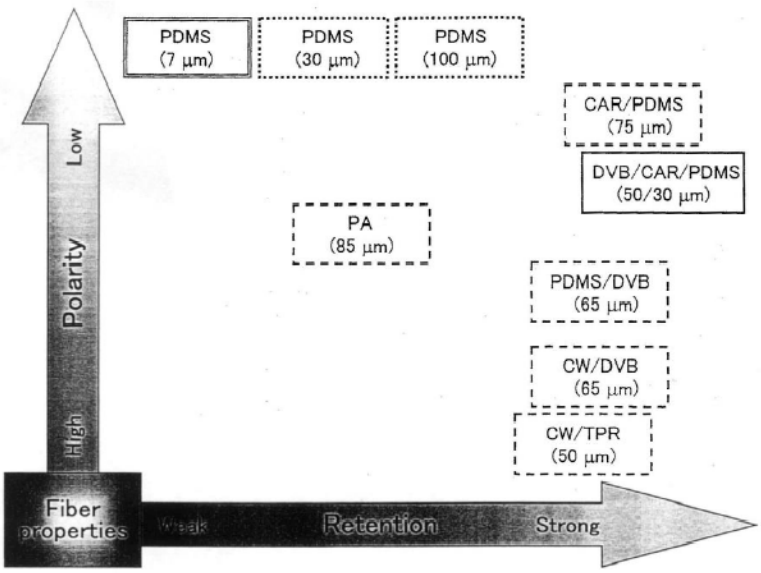


Figure 2. Comparison of polarity and retention of solid phase micro-extraction fibers: polydimethylsiloxane (PDMS), carboxen/polydimethylsiloxane (CAR/PDMS), divinylbenzene/carboxen/polydimethylsiloxane (CVB/CAR/PDMS), polyacrylate (PA), polydimethylsiloxane/divinylbenzene (PDMS/DVB), carbowax/divinylbenzene (CW/DVB), carbowax/templated resin (CW/TPR)

are absorbed by nonpolar fibers. In Figure 2, the different fiber types are shown with their relative polarity (Kataoka and others 2000).

The use of HS SPME has been used in the analysis of dairy products. Chin and Rosenberg (1997) used HS SPME to compare the HS of FF and reduced-fat Cheddar cheeses. Lee and others (2007) used HS SPME to monitor sulfur containing compounds in Cheddar cheese products.

Metabolomics

Metabolomics is an emerging field of study in which the small organic molecules of the system in question can be monitored. The metabolomic method that was used in this study involves a quenching of the cells and media to bring about a halt in cellular

processing; this is typically done with methanol (Pieterse and others 2005). The intracellular components of the cell are then extracted from the cells using a chloroform solution (Tian and others 2009). The solution is then centrifuged and the supernatant consisting of the methanol is then taken and dried. In order to volatilize the small molecules the dried sample is first subjected to an oximation reagent followed by a silylation step (Koek and others 2006). The sample is then run through a GC-MS to separate and identify the metabolites. Past research has shown that the use of oximation and syliation is a powerful tool to derivatize alcohol, aldehyde, acid, and amino groups of metabolites (van der Werf and others 2005).

Gas chromatography mass spectrometry

GC-MS is a method to first separate the analytes of interest by use of the GC, followed by the identification of the analytes using MS. The compounds are separated in a capillary column in which the inside has been coated with a liquid stationary phase. The compounds are introduced into the column and a flow of gas pushes the compounds through the column. The separation occurs as the analytes that have an increased affinity for the stationary phase will move slower than the compounds with a decreased affinity for the stationary phase. The MS detects the compound by fragmenting the compound and measuring the produced fragments. The peaks detected are then identified by comparison to authentic standards and libraries.

Automated mass spectral deconvolution and identification system

Automated Mass Spectral Deconvolution and Identification System (AMDIS) is a freely available computer program that extracts spectra of individual components in a

GC/MS data file and identifies target compounds by matching the spectra to that of the spectra in a target library. AMDIS works by conducting these four steps: noise analysis, component perception, deconvolution, and compound identification (Stein 1999).

SpectConnect

SpectConnect is a freely available service found at the website http://spectconnect.mit.edu. An example of the use of SpectConnect is given by Styczynski and others (2007). This service tracks potential known and unknown metabolites or flavor compounds across replicates and different sample conditions without the use of reference spectra. SpectConnect compares every spectrum in each sample to each spectra in every other sample. Compounds are conserved across the replicates and different treatments by having similar mass spectrum and retention times. Stycznski and others (2007) hypothesized that the important compounds will be conserved across most or all replicates, while the noise will not be conserved. Figure 3 shows the process in which SpectConnect finds potential biomarker candidates that would otherwise not be analyzed due to a deficient library.

SpectConnect requires the use of AMDIS to deconvolute the GC-MS chromatogram and a .ELU file is downloaded to the site. SpectConnect also requires that replicates for each condition be performed. The output obtained from the site includes retention time, relative abundance, integrated signal, and base peak for each compound analyzed.

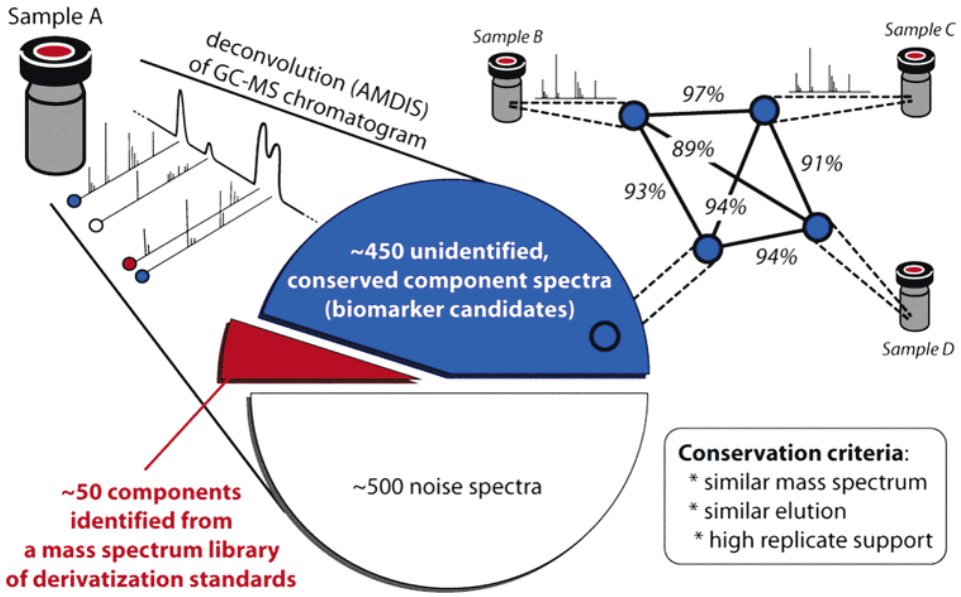


Figure 3. A representation of the SpectConnect process (Styczynski and others 2007).

MATERIALS AND METHODS

Cheddar Cheese Extract Make Procedure

Cheddar cheese extract was produced by extracting the water soluble portion of the cheese matrix. The cheese used as the starter material for the CCE in this project was a LF Cheddar cheese variety produced in the Gary Haight Richardson Dairy Products Laboratory at Utah State University. The composition of the cheese can be seen in Table 1. The cheese was ripened to a point at which the lactose was below detectable limits, which took 6 weeks, to produce a medium that would imitate ripened cheese.

The first step to make the CCE was to shred the ripened cheese and add it along with distilled water to a steam jacketed vat in a 1:2 ratio of cheese to water. The mixture was then heated slowly to 50°C with constant stirring to prevent burning and held or 20 minutes. The cheese came together and formed a dough like structure and was then removed from the mixture and discarded.

The liquid portion then went through a series of filtration steps. The first step was a diafiltration step using an ultrafilter, where the permeate was kept and the retentate was returned back to the filtration system, where with added distilled water it continued to be filtered until the permeate appeared to be mostly water. The pore size of the ultrafilter excluded any large molecules such as proteins from entering the permeate while allowing small molecules such as lactose to pass. The permeate from the ultrafiltration step was then concentrated using reverse osmosis, where the water was removed as the permeate and the retentate cycled through the filter to further concentrate the extract. This process continued until the reverse osmosis membranes reached maximum capacity due to the

decrease in water. The retentate was then collected and frozen. The reverse osmosis process concentrated the CCE to 1.6 X concentration. Figure 4 outlines the CCE make procedure.

Milk Fat Globular Membrane Make Procedure

Milk fat globular membrane in the original cheese was lost as a result of the filtration process during the CCE production. MFGM was isolated from buttermilk produced at West Point Dairy Products, LLC (Hyrum, Utah). The isolation of MFGM was accomplished using a method similar to Sachdeva and Buchheim (1997). The buttermilk was heated to 36.5°C then a 20% (wt/wt) solution of calcium chloride in water was added to the buttermilk in the amount of 0.1% (wt/wt) of the total buttermilk. The mixture was then incubated for 30 minutes at 36.5°C and rennet was added at

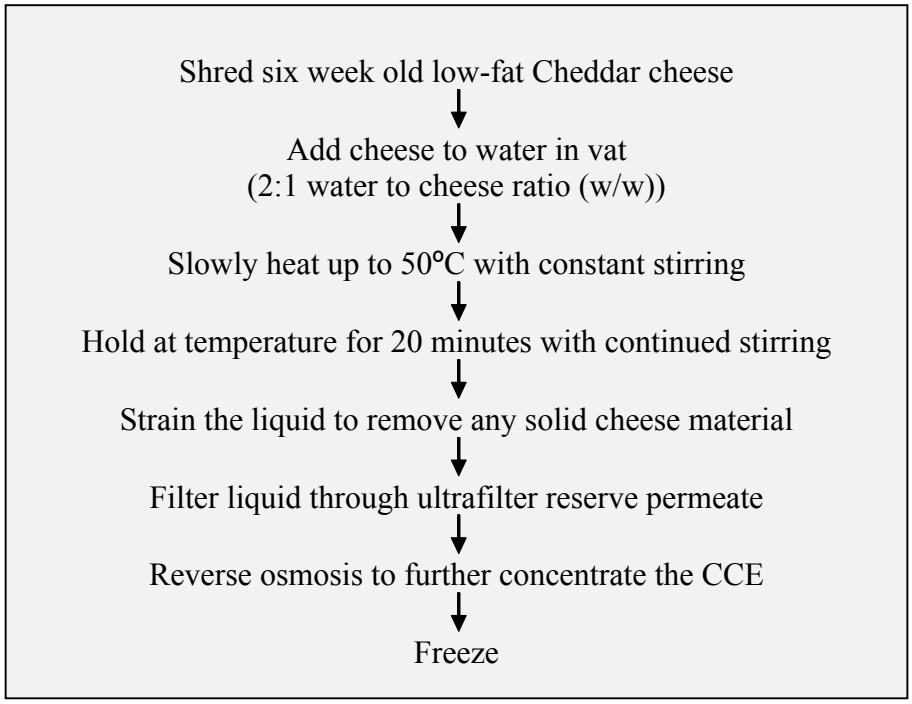


Figure 4. Cheddar cheese extract make procedure.

concentration of 0.03% (wt/wt) to the mixture. The curd was allowed to settle overnight at refrigeration temperature. The top layer was siphoned off and reserved. Distilled water was added to the curd and the top layer was again siphoned off after the curd had sufficiently settled. The siphoned top layers were then filtered through a 100K filtration unit produced by Millipore (Billerica, MA). The retentate was concentrated and subjected to a 2X difiltration process to remove excess lactose. The retentate was then freeze dried and stored in a -80°C freezer. Figure 5 shows the make procedure of the MFGM. The amount of MFGM to add to both the FF and LF samples were determined from the amount of fat in the original cheese and the calculations can be found in Table 5.

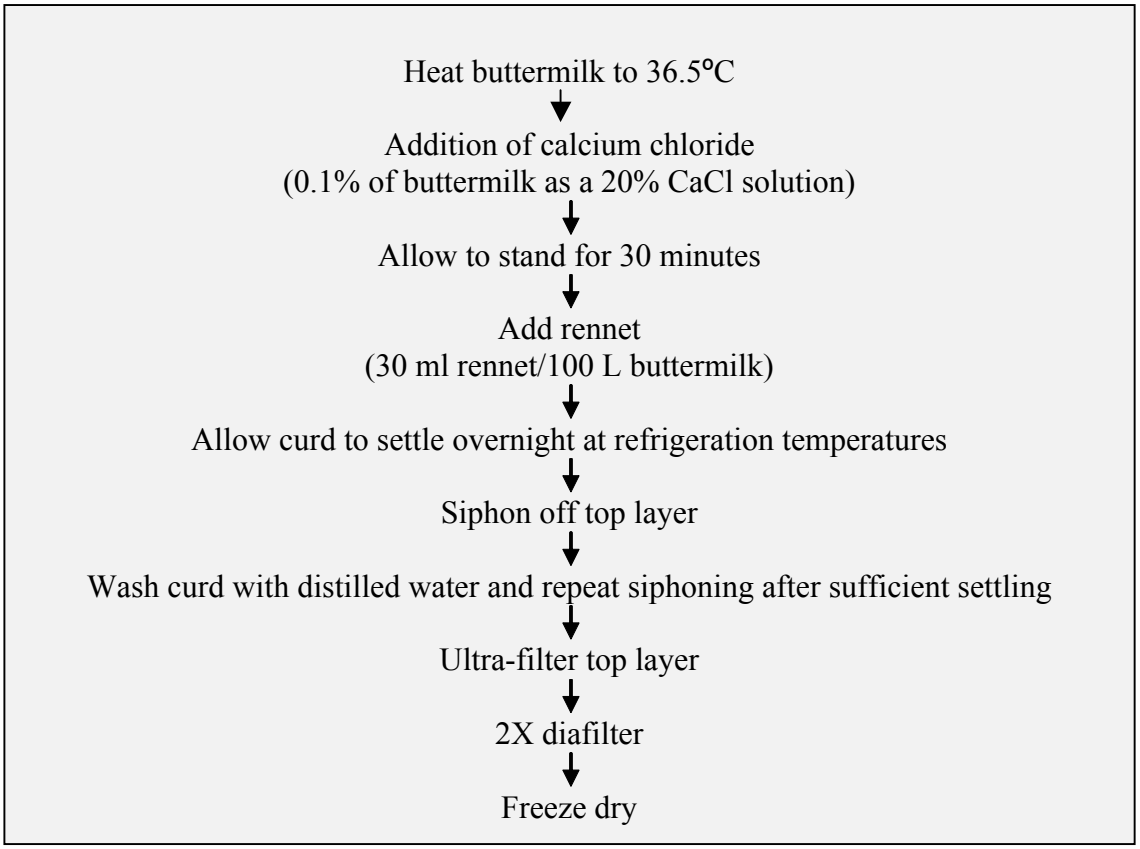


Figure 5. Milk fat globular membrane make procedure.

Table 5. Milk fat globular membrane calculations in full-fat and low-fat samples

	Low-fat	Full-fat
%fat in cheese	6%	33%
Fat amount in 1000 g cheese	60 g	330 g
Total phospholid (0.8% of fat is phospholipid*) in 1000 g cheese	0.48 g	2.64 g
PL associated with the MFGM (65% of PL associated with MFGM*) in 1000 g cheese	0.312 g	1.716 g
Total MFGM (Composition of MFGM 25% PL and 70% protein*) in 1000 g cheese	1.1856 g	6.5208 g
MFGM % in cheese*	0.11856%	0.65208%
Amount of cheese equivalent to 1 liter of CCE	2000 g	2700 g
MFGM % in CCE	0.237%	1.76%

*Percentages were taken from Keenan and Mather (2006).

Cheddar Cheese Extract Preparation for Runs

The CCE media is primarily made up of the 1.6 X CCE and distilled water. Each run had 500 ml of 1.6 X CCE and 300 ml of distilled water. The dried MFGM was added to half of the distilled water (150 ml) and autoclaved at 237°F for 10 min. The salt, lactic acid, and lactose was added to the remaining distilled water (150 ml) and mixed. The 500 ml of 1.6 X CCE was then added to the salt, lactic acid, lactose, and water mixture and sterilized by passage through a 0.45 micron filter (Thermo Fisher Scientific, Waltham, MA). The MFGM mixture was then added to the filtered mixture to make the final CCE media. The CCE media was then added to the fermenters and the pH was then adjusted to 5.1 and maintained throughout the fermentation.

Starter Culture Preparation

Lactococcus lactis M70 used in this study was obtained from Danisco and was

chosen for use in this study due to its use in a 2006 LF platform project (Drake and others 2010). A stock culture was maintained frozen at -80°C and working cultures were prepared by adding 0.1% of the frozen culture to M17 lactose broth and incubating at 30°C for 24 hours. 0.1% of the M17 lactose broth was then transferred to UHT milk and incubated for 24 hours at 30°C, then used to inoculate (2%, vol/vol) 50 ml of 1 X CCE with 2% lactose and incubated for 24 hours at 30°C. The inoculated CCE was then centrifuged at 3000 X G and the CCE was drained off. The remaining bacterial pellet was then re-suspended in 0.1% peptone buffer prior to inoculation of the CCE in a Biostat B Plus fermenter (Sartorius Stedim Biotech, Aubagne, France).

Experiment Design

Two cheese environments were created, LF treatment (LFT) and FF treatment (FFT). The S/M and MFGM level were adjusted in the media to reflect the values in the LF and FF cheese environments. The lactate and pH were also adjusted to that of the cheese environment. Table 6 shows the environmental conditions of the LF and FF model.

Table 6. Cheese environmental conditions

Environmental condition	Low-fat model	Full-fat model
S/M (%)[a]	3.7%	4.75
MFGM	0.12%	0.88%
Lactose	0.21%	0.21%
Lactate[a]	5500 ppm L-lactate 600 ppm D-lactate	5500 ppm L-lactate 600 ppm D-lactate
Temperature	30ºC	30ºC
pH[b]	5.1	5.1

[a]Reflects typical S/M (at press) and lactate contents (at 3 months) of washed curd low-fat and full-fat cheese made and analyzed under the 2006 DMI collaborative low-fat platform project (D.J. McMahon and J.L. Steele, pers. comm.)

[b]Initial pH; adjusted after lactate addition

Each fermentation took place at 30ºC with a pH of 5.1 maintained over a period of 2 weeks with sampling at days 0, 1, 7, and 14. With each LF and FF replicate, a low-fat control (LFC) and FF control (FFC) at each time point were also kept at 30°C during the duration of the fermentation with HS sampling taking place simultaneously with the treatment samples. Each condition was replicated in triplicate making a total of six batch runs.

Cell Recovery and Enumeration

Recoverable colony forming units were determined from each treatment at 0, 1, 7, and 14 days. Samples were serially diluted with sterile phosphate buffered saline and plated (0.1 ml), in duplicate, on M17 agar. The plates were incubated anaerobically at 30ºC for 48 hours. Colonies were counted from plates with counts between 30 and 300 colonies to calculate the total colony forming units in the CCE for each treatment at the specified time point.

Headspace Solid Phase Micro-Extraction Gas Chromatography Mass Spectrometry Methods

The CCE HS was analyzed using the following procedure: 5 ml of CCE liquid that had been centrifuged was added to a 20 ml HS vial along with an internal standard, 2-methyl-3-heptanone; the vial was then capped and equilibrated for 25 minutes at 45ºC. Prior to testing, the vials were placed in a 300ºC oven overnight to drive off any contamination. A 3-phase DVB/Carboxen/PDMS (Sigma Aldrich, St. Louis, MO) was inserted into the HS and the extraction of volatiles took place for 45 minutes at a temperature of 45ºC. After extraction the fiber was inserted into the injection port

(250ºC), and was left for 10 minutes to fully desorb the metabolites. In all cases the gas chromatography apparatus used was a GC-2010 Shimadzu (Kyoto, Japan) with a DB-5ms column (length 30m, thickness 0.5µm, and diameter 0.25µm) (Agilent Technologies, Santa Clara, Ca) using splitless injection. Carrier gas was helium with a flow rate of 25 mL/min. Oven temperature program was as follows: hold at 40ºC for 3 minutes, increase temperature at a rate of 10ºC/min until 90ºC, increase temperature at a rate of 5ºC/min until 200ºC hold at 200ºC for 10 minutes, increase temperature at a rate of 20ºC/min until 250ºC hold at 250ºC for 10 minutes.

Mass spectrometry (MS) analysis was carried out using a QP 2010S Shimadzu MS (Kyoto, Japan). Electron impact ionization mode was used with the ionization voltage set at 70 eV. The ion source temperature was 220ºC. Data acquisition was performed in both scan and SIM mode 10 times per second alternating between the 2 methods. The scan method scanned between the range of 33-200 m/z. The target ions of 43, 57, 83, 114, and 142 m/z were used because they represent the target masses for aroma compounds associated with LF cheese.

The analysis of the GC-MS data was performed using the deconvolution software AMDIS. The spectra of peaks were compared to the NIST database to identify the compounds analyzed and the retention indices were cross referenced to those in the literature to further verify compound identity. The program SpectConnect was used to conserve unidentified compounds across replicates and conditions. The peak areas were normalized to that of the internal standard 2-methyl-3-heptanone.

A variable reduction was performed to the high number of compounds detected in the HS analysis. The reduction was accomplished by comparing the average

concentration of each compound of the treatments to their respective controls. If the values did not fall in their respective standard error that compound was retained. This served as a method of eliminating the compounds that were not produced in the presence starter culture.

Metabolomic Methods

Metabolomic methods were employed to monitor changes in the concentration of small molecules within both the CCE and the bacteria. To measure the metabolites in the bacteria the following procedure was followed: A 12 ml sample of CCE was collected from each batch run at days 0, 1, 7, and 14. Each sample was centrifuged for 10 minutes at 3000 X G at a temperature of 4°C. The liquid was reserved for HS and CCE metabolite analysis. A 10 ml aliquot of 50 mM phosphoric buffer was then added to the pellet and vortexed. After a centrifugation under the same conditions as the previous centrifugation, the supernatant was discarded and intracellular components of the bacteria extracted by adding 1.5 ml methanol at -45ºC, 0.75 ml chilled distilled water and 1.5 ml of -45ºC chloroform to the pellet. An internal standard of ribotol was added to the sample followed by vortexing and centrifugation. The supernatant of methanol and water was collected and frozen at -80ºC and dried. The dried sample was derivitized by adding 40 µl of MOX reagent (Thermo Fisher Scientific, Waltham, MA) and incubating for 90 min at 40ºC. Next 70 µl of MSTFA (Thermo Fisher Scientific, Waltham, MA) was added to the sample and incubated for 50 min at 40ºC.

In order to measure the metabolites in the CCE media the following procedures were followed: 100 µl of the reserved sample liquid was taken and the same extraction, drying, and derivitization process conducted during the bacterial metabolomics method.

One µl of sample was injected into the GC-MS apparatus described previously under the following conditions: The injection port was maintained at a temperature of 280ºC and was splitless. The column was the same DB-5ms column used in the HS analysis. The carrier gas was helium with a column flow rate of 0.85 ml/min. The oven temperature program began at 70ºC for 5 min and increased up to 280ºC at a rate of 5ºC/min. Electron impact ionization mode was used with the ionization voltage set at 70 eV. The ion source temperature was set at 200ºC. Data acquisition was performed in the full scan mode (m/z 40-600).

The raw GC-MS data was both enumerated (i.e., distinguish "true" peaks from noise in a chromatogram) and spectrally deconvoluted (i.e., obtain putative pure spectra from two overlapping peaks) by use of a freely available program AMDIS (Styczynski et al 2007). Peaks were identified by standards where indicated and by NIST spectral database library. The program SpectConnect was used to conserve unidentified compounds across replicates and conditions. The peak areas were normalized to the ribotol standard. The peak areas obtained from each condition were compared for similarities and differences using various statistical methods.

Statistical Analysis

All statistical analyses were performed using SAS (v 9.1, Statistical Analysis Software, Cary, NC). All missing values for each analysis (HS and metabolomics) were

replaced with half of the lowest value found in their respective data set (Xia and others 2009). The principal component analysis (PCA) was performed using Proc Princomp, while further analysis of compounds was accomplished via repeated measures ANOVA using Proc Mixed. Replicate, treatment, day, and treatment*day were included in the model as fixed effects with day as the repeated measure. The fixed effects were considered significant if p-values were below 0.05. Covariance structure was autoregressive (1)[AR(1)]. The means were then compared using differences of least square means. Differences in the means were determined significant if the Tukey adjusted p-value were less than 0.05.

RESULTS AND DISCUSSION

Cell Recovery and Enumeration

Figure 6 shows the averaged growth curve of the bacteria in both the LF and FF CCE from day 0 to 14. The growth during the fermentation process was minimal with a slight peak at day 7 for both the FF and LF samples. The bacterial counts in the CCE during the fermentation process are similar to the counts in Cheddar cheese during the ripening process.

The growth of bacteria in the LF sample was expected to be higher than the FF sample due to the difference in the salt content. Like most model systems the CCE model system is not perfect and is lacking the protein and fat matrix found in cheese. During the make procedure of CCE, water soluble compounds that could be of interest to bacterial growth and the aroma HS could have remained with the retentate during the ultrafiltering process. The results found represent the bacterial metabolism within CCE and may or may not represent the bacterial metabolism in LF and FF Cheddar cheese.

The ripening of Cheddar cheese can take anywhere from 3 months to 2 years typically at a temperature of 8 to 10°C (Singh and others 2003). The temperature of incubation was increased to 30°C in this study to accelerate the activity of the bacteria and enable the visualization of trends in aroma and metabolite profiles in the media. This rise in temperature could cause a shift in the aroma and metabolite profiles away from Cheddar cheese.

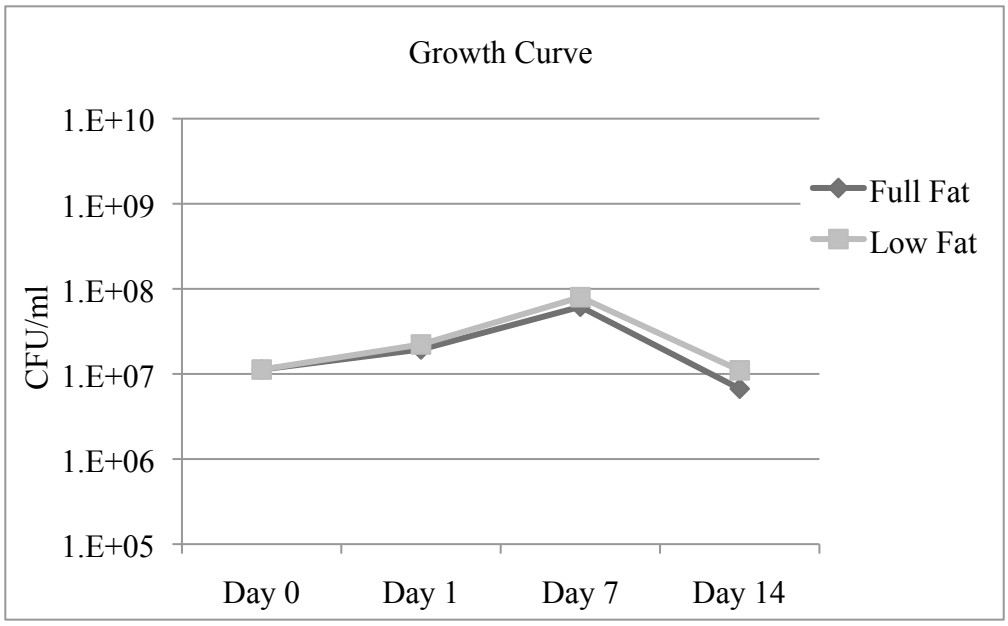


Figure 6. Average growth curve during fermentation (n=3)

Headspace Solid Phase Micro-Extraction Gas Chromatography Mass Spectrometry

Initial output contained 109 potential compounds that were detected in the HS of the samples. The variable reduction procedure reduced the compounds down to 14. Table 7 shows those compounds along with the CAS identification number, RI and the odor generally associated with each compound. Figure 7 shows the molecular structure of the retained compounds. Figure 8 shows an example of the chromatogram of a LFT and FFT run at day 14. There is little difference between the FFT and the LFT chromatogram. Tables A1-4 contain the average concentration as well as the standard deviation of each compound.

Table 7. Retained compounds from headspace analysis

	CAS Number	RI	Odor[ab]
Propan-2-one	67-64-1	552	Wood pulp, hay[a]
3-Methylbutanal	590-86-3	653	Green, malty[a]
3-Methylbutanol	123-51-3	737	Green, alcoholic[a]
Heptan-2-one	110-43-0	890	Fruity, fatty, spicy[a]
Heptanal	111-71-7	902	Soapy[a]
2,6-Dimethylheptan-4-ol	108-82-7	949	mild fresh ethereal fermented yeasty[b]
Benzaldehyde	100-52-7	960	strong sharp sweet bitter almond cherry[b]
Dimethyl trisulfide	3658-80-8	968	Sulfurous, cabbage[a]
2-Ethylhexan-1-ol	104-76-7	1025	citrus fresh floral oily sweet[b]
p-Cymene	99-87-6	1027	Fresh citrus terpene woody spice[b]
2-Phenylacetaldehyde	122-78-1	1042	Rosey, stirene[a]
Nonan-2-one	821-55-6	1090	Malty, fruity[a]
Isothiocyanato cyclohexane	1122-82-3	1238	No info
Undecan-2-one	112-12-9	1294	Floral[a]

[a]Odor descriptions from Curioni and Bosset (2002) in cheese using GC-olfactometry
[b]Odor descriptions were taken from the website www.thegoodscentscompany.com

Repeated measures ANOVA

Repeated measures ANOVA showed all of the compounds had a significant Treatment and Treatment*Day effect with a p-value less than 0.05 (Table 8). 3-Methylbutanal, nonan-2-one, and undecan-2-one had an insignificant p-value for the Day effect. The interactions are characterized in the concentration versus time graphs for each compound in Figures 9-12. Tables 9-11 show the least square mean values at each time period contrasting FFC and FFT, LFC and LFT, and FFT and LFT. Tables B1-4 show

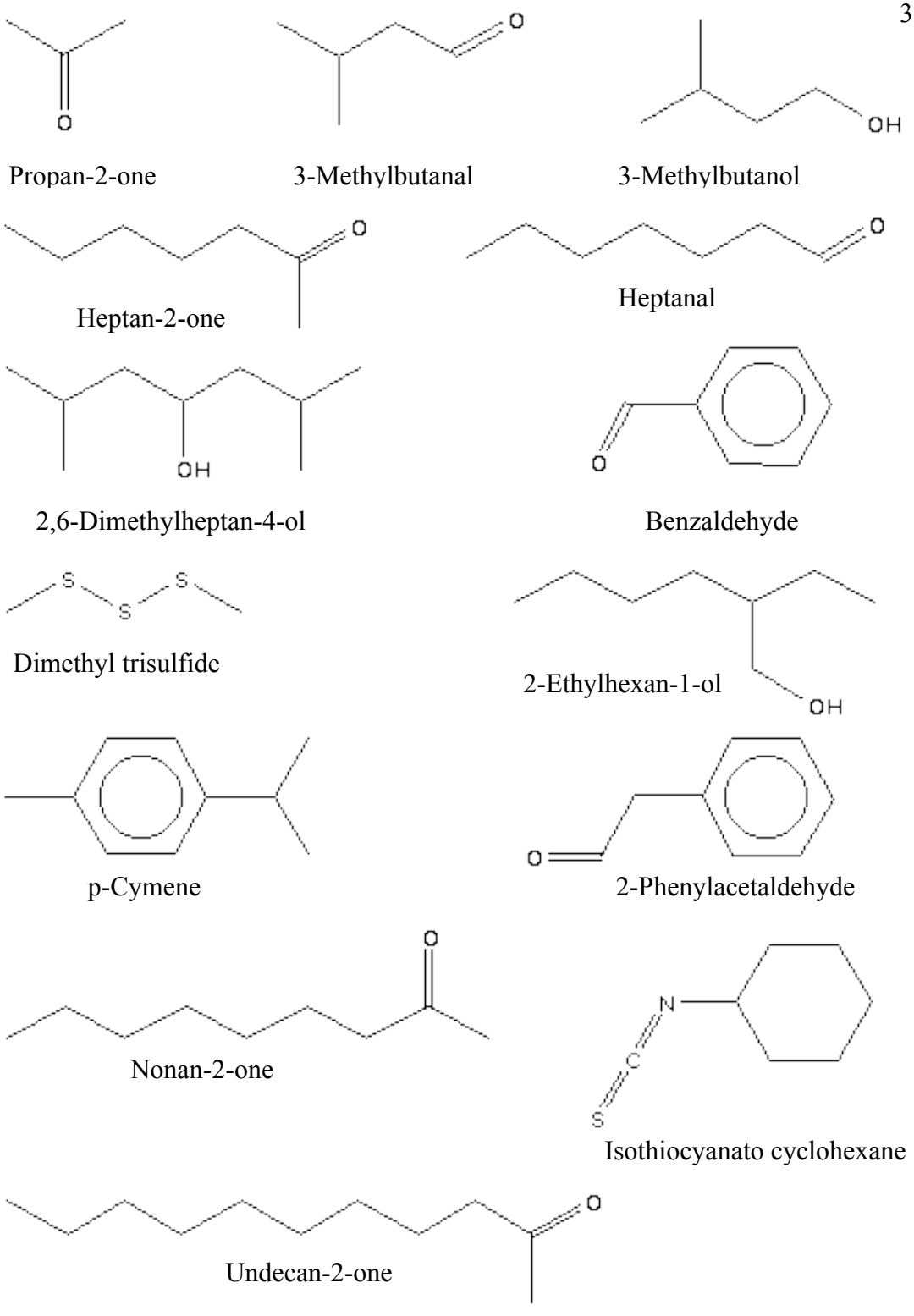


Figure 7. Molecular structure of retained compounds

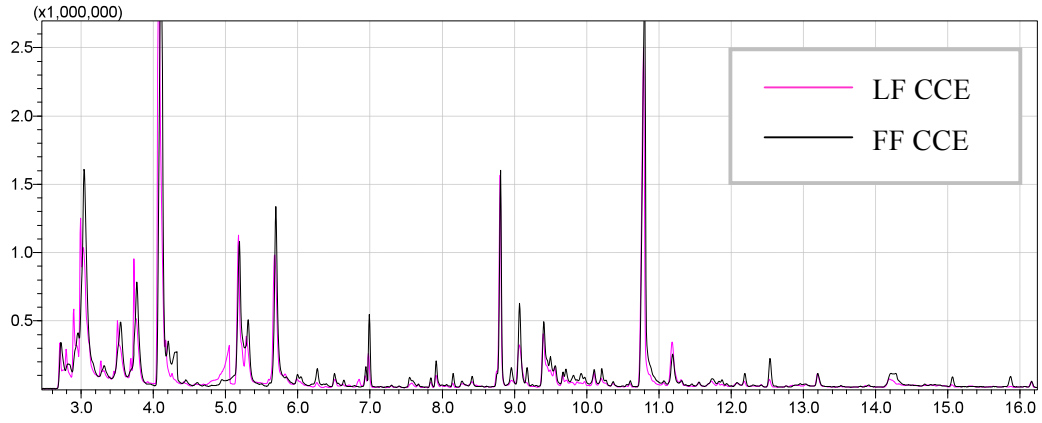


Figure 8. Chromatogram of a low-fat and full-fat treatment run at day 14

the least square mean values for each treatment contrasting the separate time points. The compared means were considered significantly different if the Tukey adjusted p-value was less than 0.05.

Ketones

Propan-2-one, heptan-2-one, nonan-2-one, and undecan-2-one are ketones that were retained in Table 7. Figure 1 shows that fatty acid β-oxidation could be a potential source of these ketones. The graphs of concentration versus time in Figure 9 indicate that the fat level in the samples may be a contributing factor in the compound concentration for heptan-2-one and nonan-2-one due to the increased concentration in FFC and FFT when compared to LFC and LFT. Each of these compounds has been found in Cheddar cheese (Curioni and Bosset 2002).

Propan-2-one imparts a high wood pulp and hay odor. The propan-2-one graph within Figure 9 shows propan-2-one levels starting out the same for all controls and treatments at day 0, as time passed the treatments stayed constant and the controls

Table 8. Headspace repeated measures ANOVA p-values from type 3 tests for fixed effects

	Treatment	Day	Treatment*Day
Propan-2-one	0.002	<.001	0.004
3-Methylbutanal	<.001	0.546	0.004
3-Methylbutanol	0.032	0.001	0.005
Heptan-2-one	<.001	0.018	<.001
Heptanal	0.001	<.001	<.001
2,6-Dimethylheptan-4-ol	<.001	<.001	0.0001
Benzaldehyde	<.001	<.001	0.0022
Dimethyl trisulfide	<.001	0.009	0.016
2-Ethylhexan-1-ol	0.001	<.001	<.001
p-Cymene	<.001	<.001	0.008
2-Phenylacetaldehyde	<.001	<.001	0.020
Nonan-2-one	0.001	0.516	0.001
Isothiocyanato cyclohexane	<.001	<.001	<.001
Undecan-2-one	0.016	0.882	0.032

decreased. The propan-2-one could be reacting with other compounds in the HS, in the treatment samples the propan-2-one levels are being replaced by action of the starter culture while in the controls there is no propan-2-one production causing a decrease in the propan-2-one levels. Examining the least square mean values for propan-2-one at each time point, there is a significant difference in the propan-2-one levels of FFC and FFT sample at day 14 as well as for LFC and LFT at day 7. There does not appear to be a statistical difference between the FFT and LFT samples. The least square mean values for each treatment indicates that there is a change over time in propan-2-one for the FFC and LFC samples while there is no significant change in the FFT and LFT over time.

Table 9. Headspace tukey adjusted p-values comparing the full-fat control and full-fat treatment least square means at each time point

	FFC0 FFT0	FFC1 FFT1	FFC7 FFT7	FFC14 FFT14
Propan-2-one	1.000	1.000	0.071	0.024
3-Methylbutanal	0.639	0.045	0.015	<.001
3-Methylbutanol	1.000	1.000	0.196	0.164
Heptan-2-one	0.676	0.999	<.001	<.001
Heptanal	0.998	1.000	0.845	<.001
2,6-Dimethylheptan-4-ol	1.000	0.015	<.001	<.001
Benzaldehyde	1.000	0.899	0.195	0.002
Dimethyl trisulfide	1.000	0.021	0.004	0.008
2-Ethylhexan-1-ol	1.000	1.000	0.016	<.001
p-Cymene	0.999	1.000	1.000	1.000
2-Phenylacetaldehyde	1.000	0.584	0.993	0.732
Nonan-2-one	0.975	1.000	0.0852	<.001
Isothiocyanato cyclohexane	1.000	1.000	1.000	0.975
Undecan-2-one	0.869	1.000	1.000	0.083

For each column, the heading indicates which time and treatment are being compared

Heptan-2-one imparts a high fruity and fatty odor. The heptan-2-one graph within Figure 9 shows heptan-2-one levels for the FFC and FFT samples starting at a slightly higher concentration than the LFC and LFT samples. As time passes the controls decrease while the treatments increase. The least square mean values for heptan-2-one at each time point indicates that there is a difference between the FFC and FFT samples and the LFC and LFT samples at both day 7 and 14. There also was a difference in the FFT and LFT at day 0. The least square means values for each treatment indicates a difference in the FFC, FFT, and LFT samples overtime, while no difference is apparent in

Table 10. Headspace tukey adjusted p-values comparing the low-fat control and low-fat treatment least square means at each time point

	LFC0 LFT0	LFC1 LFT1	LFC7 LFT7	LFC14 LFT14
Propan-2-one	1.000	0.992	0.032	0.139
3-Methylbutanal	0.963	0.108	<.001	<.001
3-Methylbutanol	1.000	1.000	0.011	0.054
Heptan-2-one	1.000	0.985	<.001	<.001
Heptanal	1.000	1.000	1.000	0.691
2,6-Dimethylheptan-4-ol	0.085	<.001	<.001	<.001
Benzaldehyde	1.000	0.311	0.075	<.001
Dimethyl trisulfide	1.000	0.061	0.470	0.002
2-Ethylhexan-1-ol	1.000	0.999	<.001	<.001
p-Cymene	1.000	1.000	1.000	1.000
2-Phenylacetaldehyde	1.000	0.168	0.035	0.004
Nonan-2-one	1.000	1.000	0.238	0.232
Isothiocyanato cyclohexane	1.000	<.001	<.001	<.001
Undecan-2-one	0.999	1.000	1.000	1.000

For each column, the heading indicates which time and treatment are being compared the LFC sample.

Nonan-2-one imparts a medium malty and fruity odor. The nonan-2-one graph within Figure 9 shows a similar trend as the heptan-2-one graph. The least square mean values for nonan-2-one at each time point shows a difference between the FFC and the FFT samples at day 14, with no difference found between the LFC and LFT and the FFT and LFT samples at any time point. The least square means values for each treatment showed no difference in the treatments and controls over time with exception of the FFC,

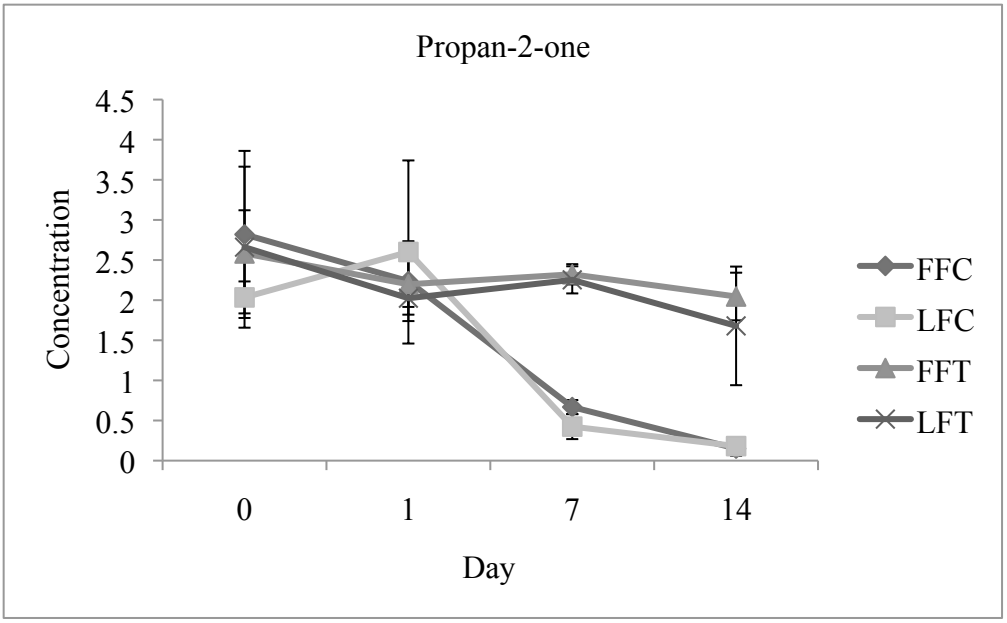
Table 11. Headspace tukey adjusted p-values comparing the full-fat treatment and low-fat treatment least square means at each time point

	FFT0 LFT0	FFT1 LFT1	FFT7 LFT7	FFT14 LFT14
Propan-2-one	1.000	1.000	1.000	1.000
3-Methylbutanal	1.000	1.000	1.000	1.000
3-Methylbutanol	1.000	1.000	0.986	1.000
Heptan-2-one	0.022	0.069	0.289	0.101
Heptanal	0.160	0.023	0.715	<.001
2,6-Dimethylheptan-4-ol	1.000	1.000	1.000	1.000
Benzaldehyde	1.000	1.000	1.000	0.999
Dimethyl trisulfide	1.000	1.000	1.000	1.000
2-Ethylhexan-1-ol	1.000	1.000	0.018	0.618
p-Cymene	<.001	0.004	0.279	0.852
2-Phenylacetaldehyde	1.000	1.000	0.495	0.856
Nonan-2-one	0.084	0.476	0.619	0.196
Isothiocyanato cyclohexane	1.000	<.001	<.001	<.001
Undecan-2-one	0.898	0.505	0.231	0.083

For each column, the heading indicates which time and treatment are being compared

which showed a difference between the Nonan-2-one concentrations between day 0 and 14.

Undecan-2-one imparts a medium floral odor. The undecan-2-one graph within Figure 9 shows a similar trend as the heptan-2-one and Nonan-2-one levels. Examining the least square mean values for Undecan-2-one at each time point and for each treatment over time, there were not any interesting significant differences to report.



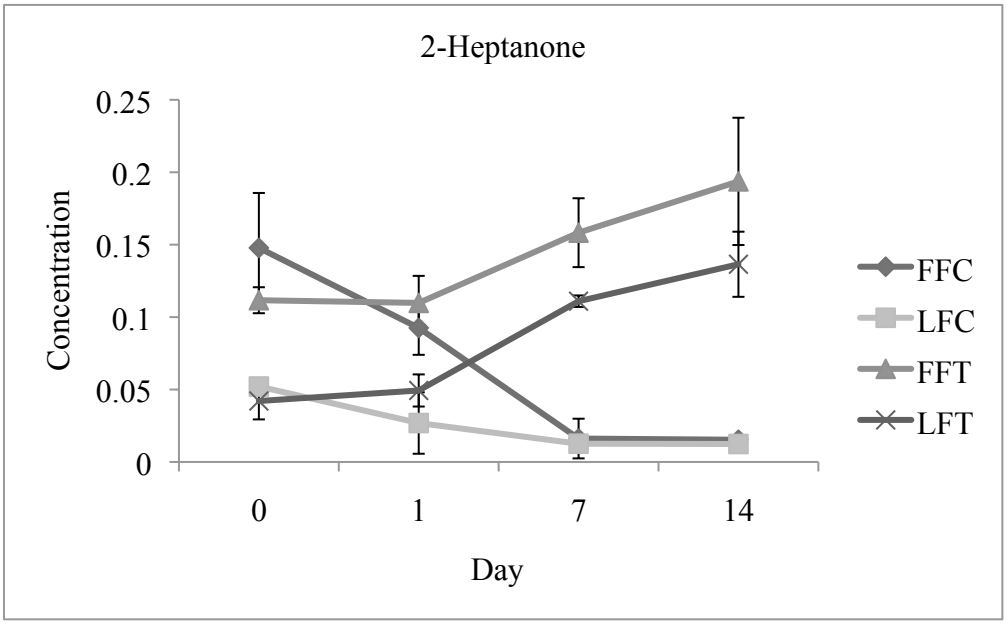
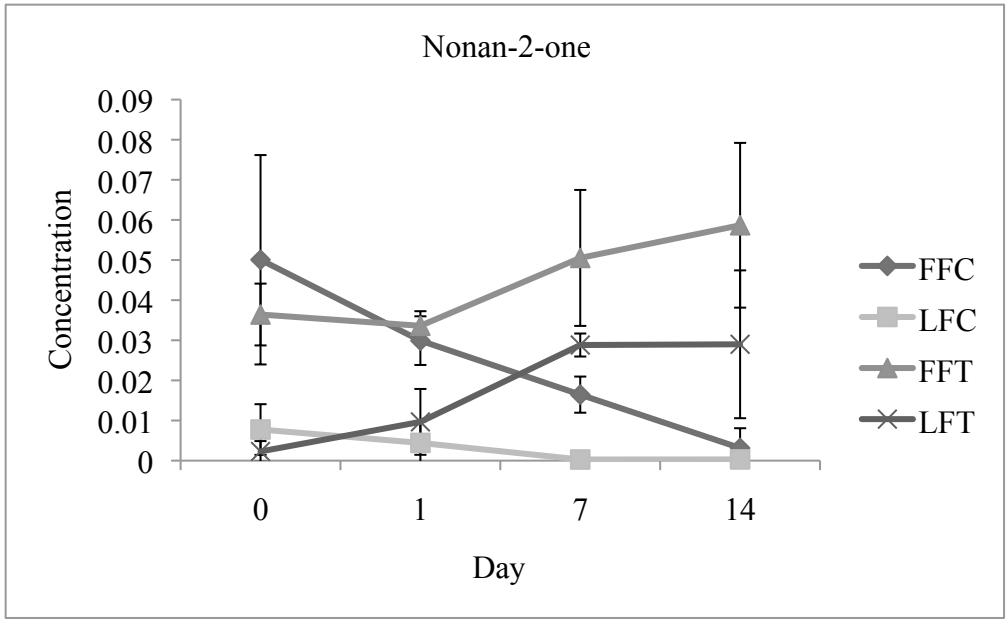


Figure 9. Graphs of concentration versus day for ketone compounds (propan-2-one, heptan-2-one, nonan-2-one, and undecan-2-one)



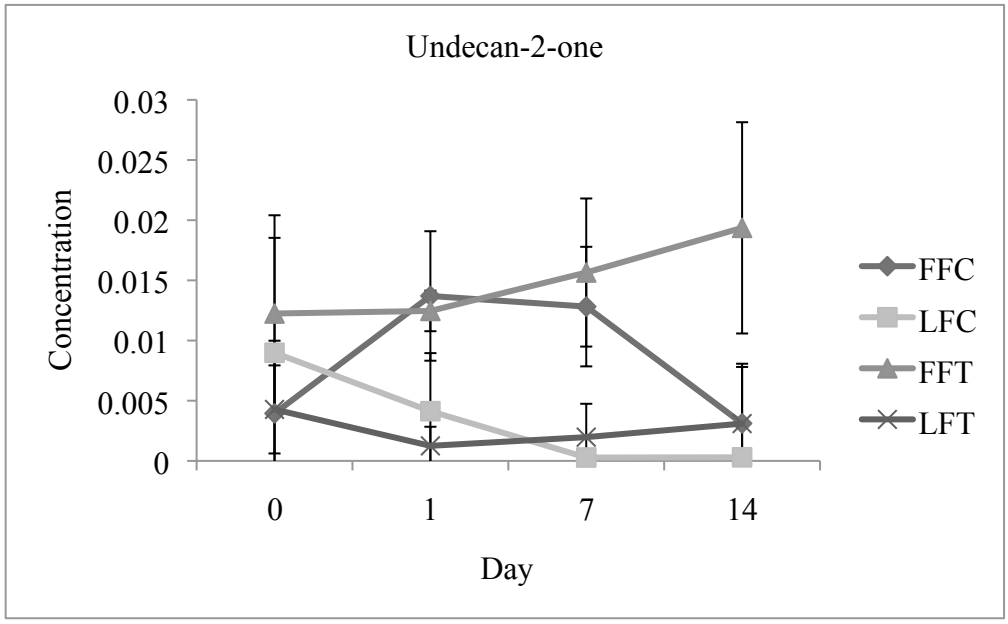


Figure 9. Continued

Aldehydes

3-Methylbutanal, heptanal, benzaldehyde, and 2-phenylacetaldehyde are aldehydes that were that were retained in Table 7. Fatty acids and amino acids are potential sources for these compounds. Figure 1 shows aldehyde production from unsaturated fatty acids as a result of hydroperoxide lyase. 3-methylbutanal could originate as a result of Strecker reactions between α-amino acids and α-keto acids (McSweeney and Sousa 1999). While the compounds benzaldehyde and 2-phenylacetaldehyde could originate from the amino acid phenylalanine (McSweeney and Sousa 1999). Each of these compounds has been found in Cheddar cheese (Curioni and Bosset 2002). The graphs in Figure 10 show an upward trend as time passes in the aldehyde concentrations in the LF and FF treatments while the controls stay at a lower concentration throughout time.

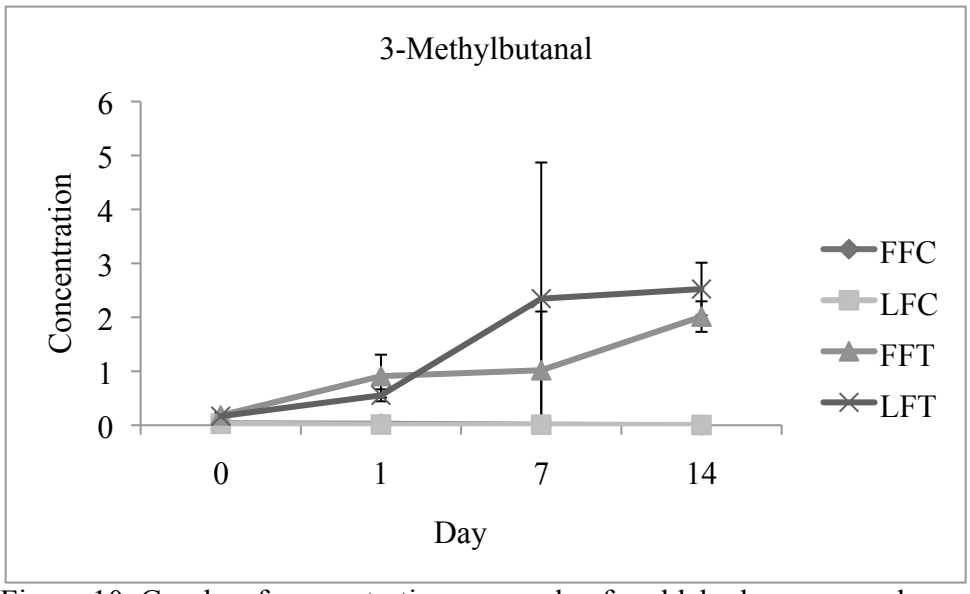
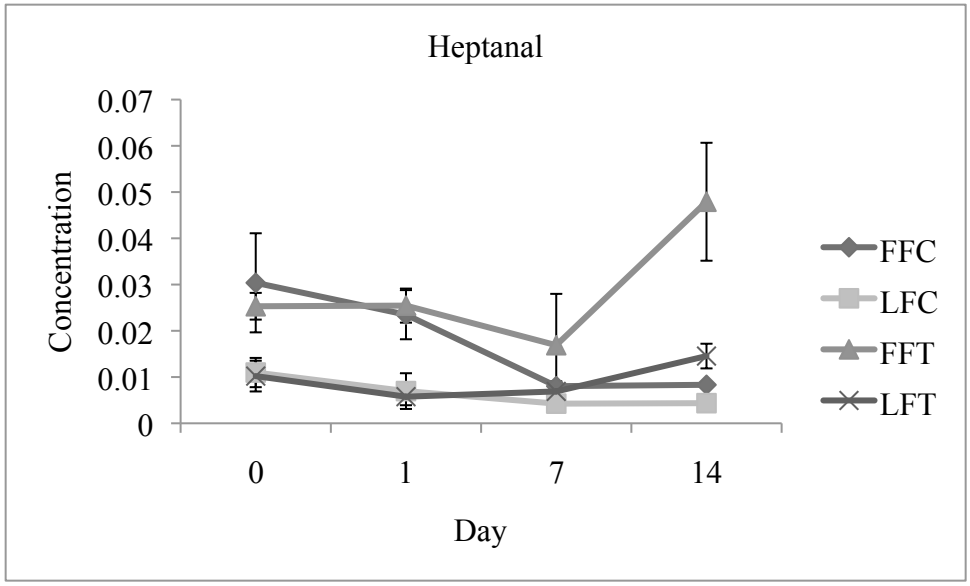


Figure 10. Graphs of concentration versus day for aldehyde compounds (3-methylbutanal, heptanal, benzaldehyde, and 2-phenylacetaldehyde)



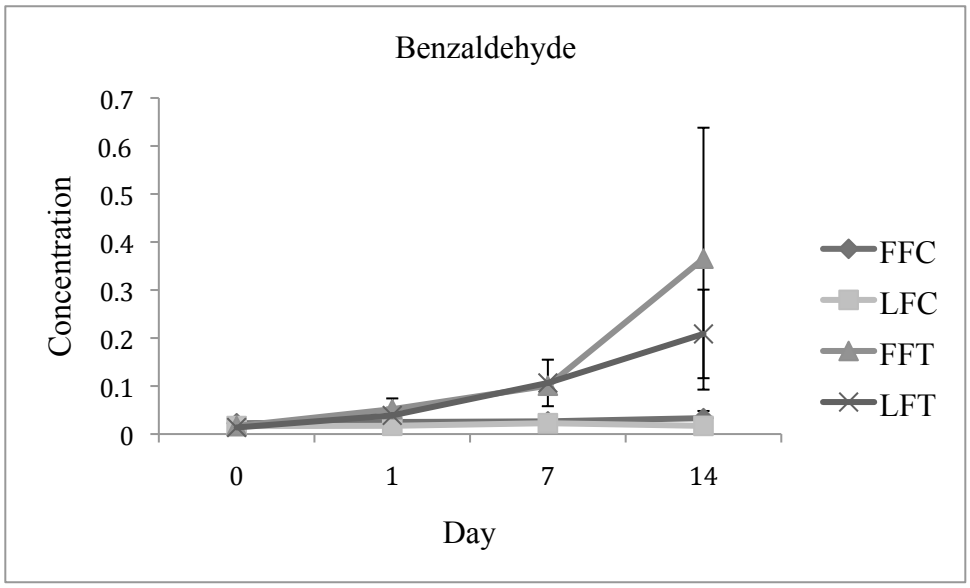


Figure 10. Continued

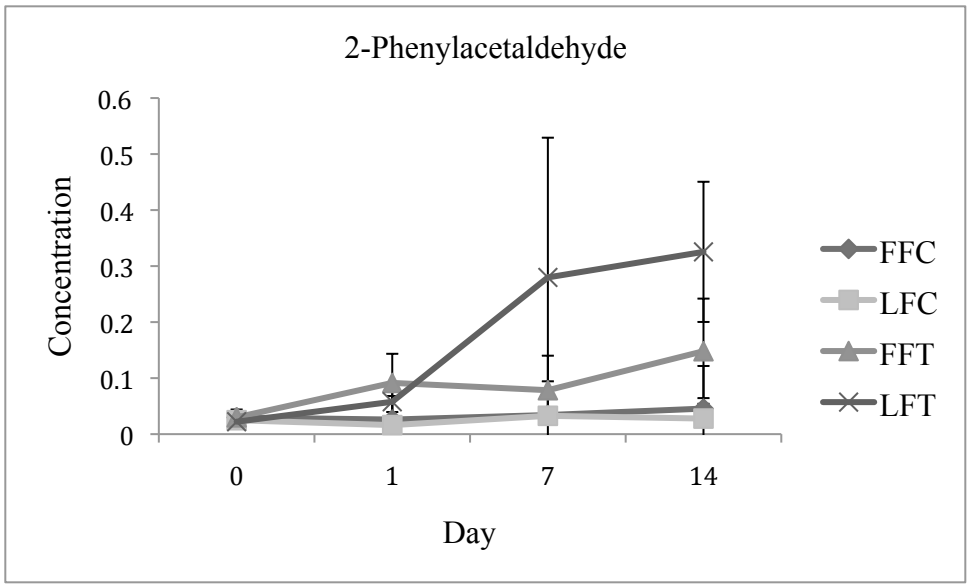


Figure 10. Continued

3-Methylbutanal imparts a high green and malty odor. The 3-methylbutanal graph within Figure 10 shows an increase in the concentration of 3-methylbutanal in the treatments and a near zero level in the controls over time. The least square means values at each time point indicate a difference between FFC and FFT at days 1, 7, and 14, with differences in LFC and LFT at days 7 and 14, and no differences apparent between FFT and LFT at any time points. The least square means values for each treatment showed no difference in each of the individual treatments and controls over time.

Heptanal imparts a high soapy odor. The heptanal graph within Figure 10 shows an initial difference in concentration at day 0 with an increase over time in the FFT and a decrease in the FFC, while the LFC and LFT remain at a low concentration. The least square means values for each time point shows a difference in the FFC and FFT as well as the FFT and LFT all at day 14. The least square means values for each treatment over time showed a change in the FFC and FFT and no change for the LFC and LFT.

Benzaldehyde imparts a high sharp, sweet, bitter, almond, and cherry odor. The benzaldehyde graph within Figure 10 shows an increase in the concentration of FFT and LFT samples while the FFC and LFC benzaldehyde levels remained near zero. The least square mean values for each time point indicates a difference in the FFC and FFT and the LFC and LFT both at day 14 and no difference between the FFT and LFT samples. The least square means values for each treatment over time showed a change in the FFT and LFT and no change for the FFC and LFC.

2-Phenylacetaldehyde imparts a high rosey stirene odor. The 2-phenylacetaldehyde graph within Figure 10 shows an increase in the FFT and LFT levels over time with a higher amount found in the LFT sample. The FFC and the LFC levels stay at a low level. The least square mean values for each time point indicates a difference between the LFC and LFT samples at days 7 and 14 and no difference between FFC and FFT and FFT and LFT. The least square mean value for each treatment over time showed a difference in the LFT 2-phenylacetaldehyde concentration and no difference in the FFC, LFC, and FFT overtime.

Alcohols

3-Methylbutanol, 2,6-dimethylheptan-4-ol, and 2-ethylhexan-1-ol are alcohols that were retained in Table 7. Fatty acids and amino acids are potential starting substrates for the production of alcohol, see Figure 1 and Table 4. Each of these compounds has been found in Cheddar cheese (Curioni and Bosset 2002). The trends in the graphs of concentration versus time in Figure 11 for the alcohol compounds show an increase in the FFT and LFT samples while the FFC and LFC samples stayed at or near zero.

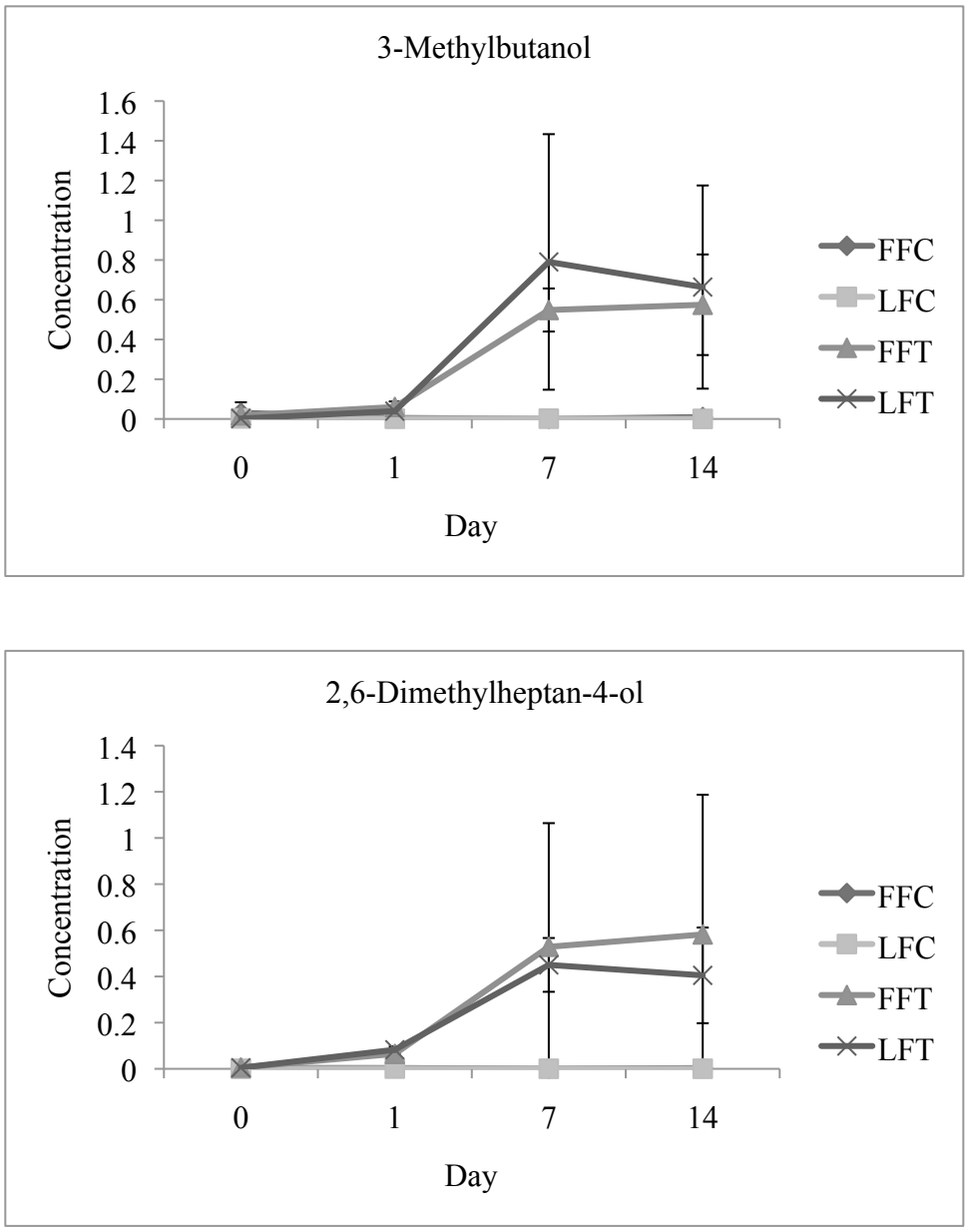


Figure 11. Graphs of Concentration versus Day for alcohol compounds (3-Methylbutanol, 2, 6-Dimethylheptan-4-ol, and 2-Ethylhexan-1-ol)

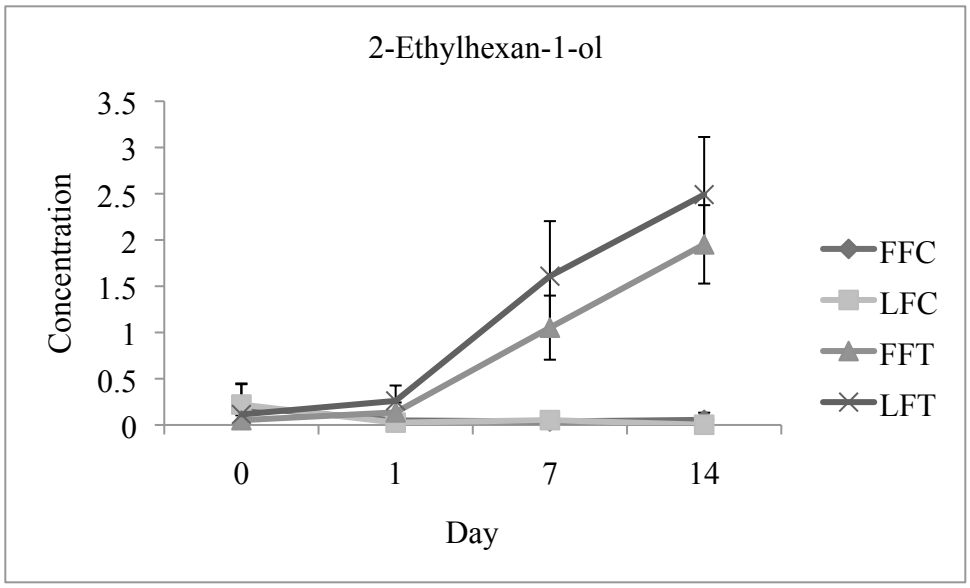


Figure 11. Continued

3-Methylbutanol imparts green and alcoholic odor. The concentration of the FFT and LFT increase while the FFC and LFC remain constant overtime. The least square means p-values for 3-methylbutanol at each time point indicates no difference between the FFC and FFT, LFC and LFT, and FFT and LFT samples. The least square means of each treatment shows a difference in the FFC, FFT, and LFT samples over time.

2,6-Dimethylheptan-4-ol imparts a mild fresh, ethereal, fermented, and yeasty odor. The concentration of the FFT and LFT increase while the FFC and LFC remain constant over time. The least square means p-values for 2,6-Dimethylheptan-4-ol at each time point indicate differences between the FFC and FFT and the LFC and LFT both at days 7 and 14. The least square means of each treatment show a difference in the FFT and LFT samples over time.

2-Ethylhexan-1-ol imparts a medium citrus, fresh, floral, oily, and sweet odor. The concentration of the FFT and LFT increase while the FFC and LFC remain constant

over time. The least square means p-values for 2-ethylhexan-1-ol at each time point indicate differences between the FFC and FFT and the LFC and LFT both at days 7 and 14, as well as between FFT and LFT at day 7. The least square means of each treatment show a difference in the FFT and LFT samples over time.

Others

Dimethyl trisulfide, p-cymene, and isothiocyanato cyclohexane were categorized in the other group of compounds that were retained in Table 7. The origins of p-cymene and isothiocyanato cyclohexane are unknown. Their ring structure could indicate an aromatic amino acid such as phenylalanine as a potential source. Dimethyl trisulfide is believed to be a byproduct of methionine metabolism (McSweeney and Sousa 1999). Dimethyl trisulfide is a well-known flavor compound found in cheese while there is no known source identifying p-cymene and isothiocyanato cyclohexane in Cheddar cheese.

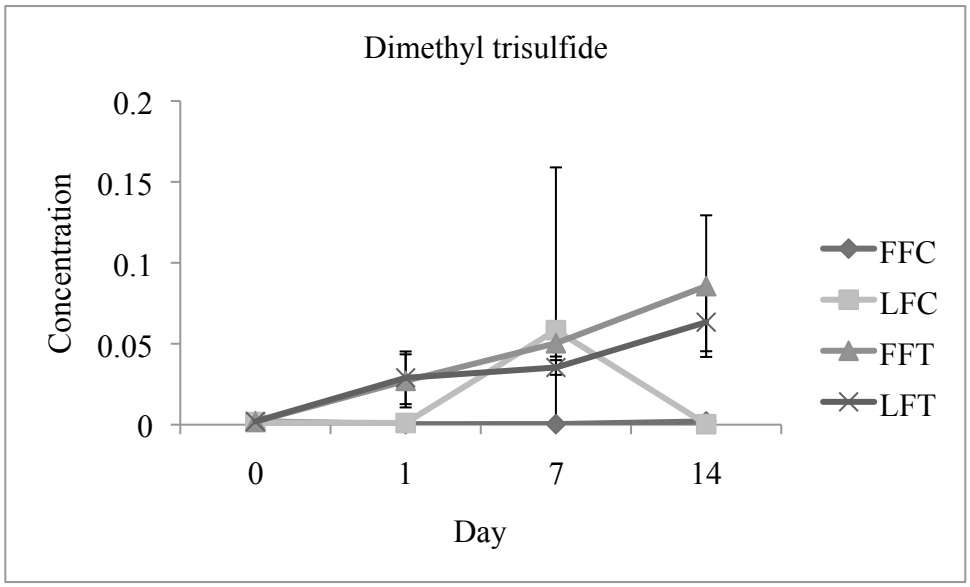
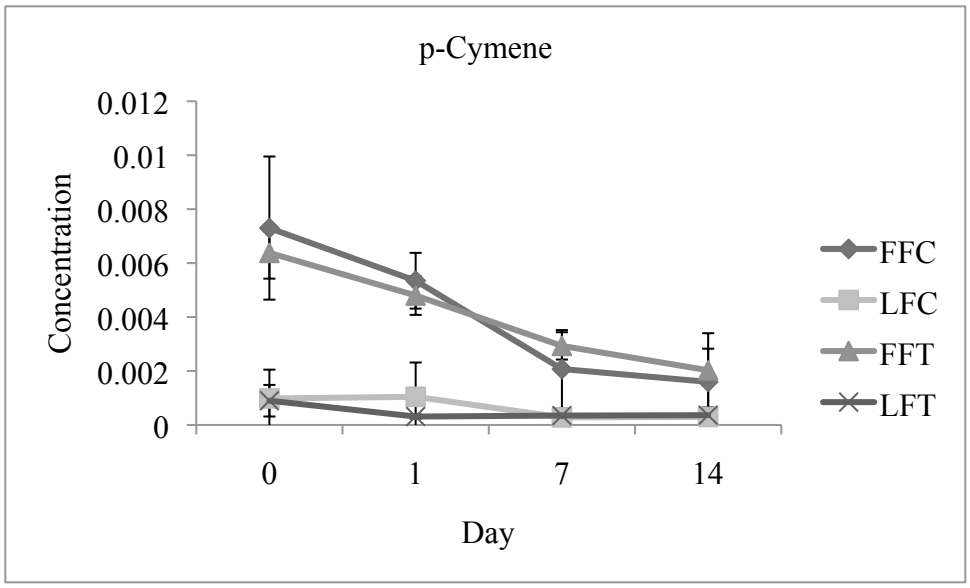


Figure 12. Graphs of Concentration versus Day for other compounds (dimethyl trisulfide, p-cymene, and isothiocyanato cyclohexane)



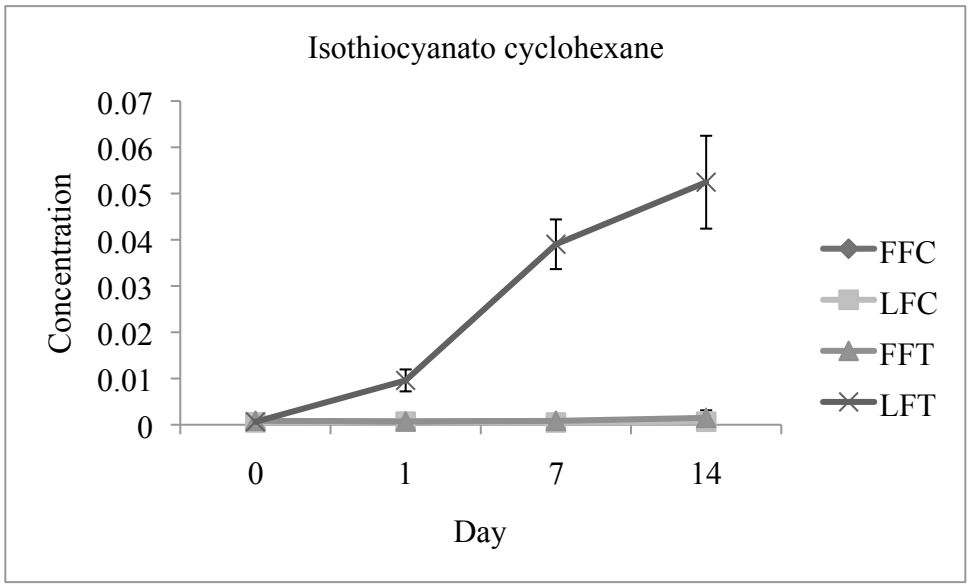


Figure 12. Continued

Dimethyl trisulfide imparts a high sulfurous and cabbage odor. The dimethyl trisulfide graph within Figure 12 shows an increase in the FFT and LFT levels overtime while the FFC and the LFC levels stayed at a low level with the exception of LFC at day 7. The least square mean values for each time point indicates a difference between the FFC and FFT samples at days 1, 7, and 14 and LFC and LFT at day 14. The least square mean value for each treatment over time showed a difference in the FFT dimethyl trisulfide concentration over time and no difference in the FFC, LFC, and LFT overtime.

P-cymene imparts a high fresh, citrus, terpene, woody, and spice odor. The p-cymene graph within Figure 12 shows a constant low concentration in the LFC and LFT samples while the FFC and FFT samples decrease overtime. This indicates that the p-cymene could be coming from the MFGM and may be a product of bacterial metabolism in the media. The least square mean values for each time point indicates a difference between the FFT and LFT samples at days 0 and 1. The least square mean value for each treatment over time showed a difference in the FFC and FFT p-cymene concentration over time and no difference in the LFC and LFT samples overtime.

The odor imparted by isothiocyanato cyclohexane is unknown. According to Rapior and others (1997) isothiocyanato cyclohexane was found in garlic and was hypothesized to play contributing part in the garlic odor and taste. The isothiocyanato cyclohexane graph within Figure 12 shows an increase in the LFT isothiocyanato cyclohexane concentrations while the FFC, FFT, and LFC samples indicated concentrations near zero throughout time. The least square mean values for each time point indicates a difference between the LFC and LFT and the FFT and the LFT samples

at days 1, 7, and 14. The least square mean values for each treatment overtime showed a difference in only the LFT sample isothiocyanato cyclohexane concentrations.

Headspace principal component analysis

Using the 14 compounds in Table 7 a PCA was performed. The eigenvalues of the correlation matrix in Table 12, as well as the eigenvalue plot in Figure 13 were used to determine that the first two principal components were sufficient to explain 67.4% of the variation in the data.

Table 12. Eigenvalues of the correlation matrix for the headspace principal component analysis

	Eigenvalue	Difference	Proportion	Cumulative
1	6.53995003	3.63820478	0.4671	0.4671
2	2.90174525	1.73388887	0.2073	0.6744
3	1.16785638	0.32391118	0.0834	0.7578
4	0.84394520	0.08752693	0.0603	0.8181
5	0.75641826	0.21303167	0.0540	0.8721
6	0.54338659	0.11761922	0.0388	0.9110
7	0.42576737	0.14615792	0.0304	0.9414
8	0.27960945	0.04702536	0.0200	0.9613
9	0.23258409	0.11072012	0.0166	0.9779
10	0.12186397	0.05637399	0.0087	0.9867
11	0.06548998	0.01437088	0.0047	0.9913
12	0.05111910	0.00997367	0.0037	0.9950
13	0.04114544	0.01202656	0.0029	0.9979
14	0.02911888		0.0021	1.000

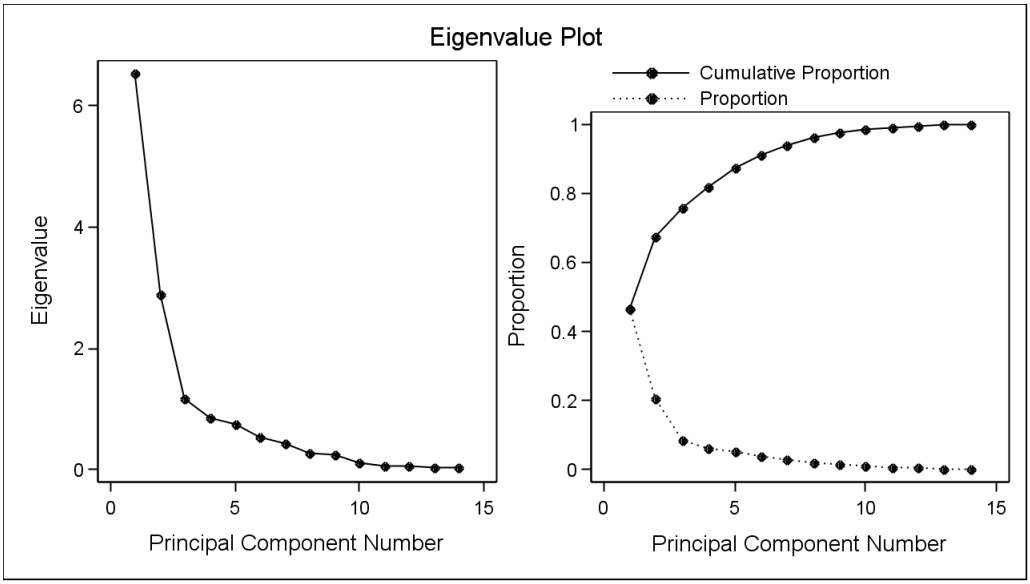


Figure 13. Eigenvalue plot for the headspace principal component analysis

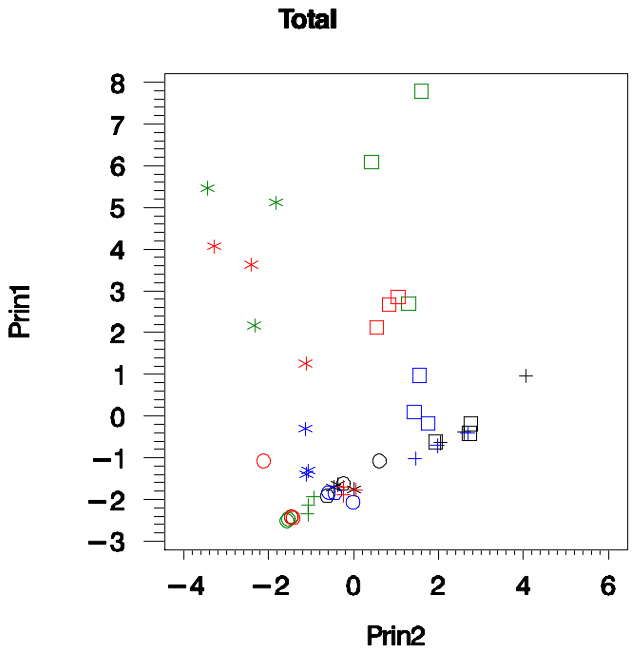


Figure 14. Score plots of every day combined for the headspace principal component analysis; FFC: plus sign; LFC: circle; FFT: square; LFT: asterisk
Day 0: black; Day 1: blue; Day 7: red; Day 14: green

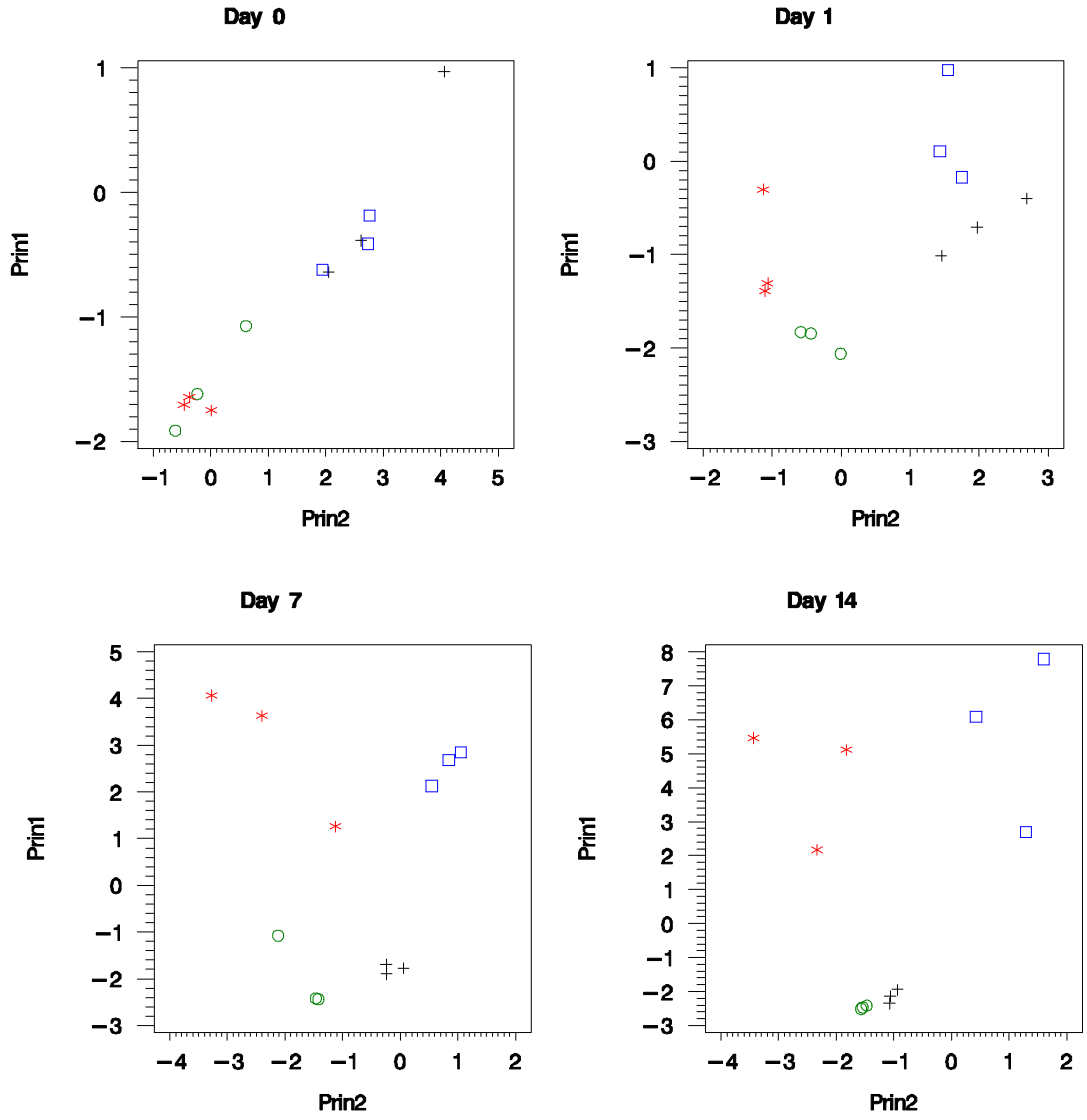


Figure 15. Score plots of days 0, 1, 7, 14 for the headspace principal component analysis; FFC: black plus sign; LFC: green circle; FFT: blue square; LFT: red asterisk

The score plots in Figure 14 and 15 show little discrimination between days 0 and 1 but days 7 and 14 there is a clear discrimination between the treatments. At day 14, Prin1 separates the treatments from the controls and Prin2 separates the LFT and FFT samples. The eigenvectors in Table 10 for Prin1 indicate that 3-methylbutanal, heptan-2-one, benzaldehyde, 2-ethylhexan-1-ol, 2,6-dimethylheptan-4-ol, and 3-methylbutanol, using 0.3 as a cut off, play the largest role in separating the treatments from the controls. The eigenvectors from the same table for Prin2 indicate that heptanal, p-cymene, nonan-2-one, and undecan-2-one, using 0.3 as a cut off, play the largest role in separating the LF from the FF samples.

Table 13. Eigenvectors of principal component 1 and principal component 2 for the headspace principal component analysis

	Prin1	Prin2
Propan-2-one	0.119028	0.269622
3-Methylbutanal	0.306599	-.184472
Heptan-2-one	0.331138	0.253856
Heptanal	0.217257	0.398993
Benzaldehyde	0.316766	-.017690
p-Cymene	0.013619	0.520334
2-Ethylhexan-1-ol	0.349095	-.182013
2-Phenylacetaldehyde	0.274115	-.264580
Nonan-2-one	0.289992	0.337198
2,6-Dimethylheptan-4-ol	0.313748	-.069826
3-Methylbutanol	0.303569	-.144666
Dimethyl trisulfide	0.254344	-.110392
Undecan-2-one	0.145318	0.309860
Isothiocyanato cyclohexane	0.277395	-.218491

Metabolomics

Cheddar cheese extract principal component analysis

The metabolite analysis of the CCE produced 23 compounds that were significantly different between the LF and FF samples. A PCA analysis was conducted to reduce the variables and reveal potential trends in the data. The eigenvalues of the correlation matrix in Table 14, as well as the eigenvalue plot in Figure 16 were used to determine that the first three principal components were sufficient to explain 65.4% of the variation in the data.

The score plots in Figure 17 do not show a clear separation of the FF and LF samples. It could be argued that there is a slight separation of the FF and LF samples along the Prin3 axis in the Prin2 versus Prin3 graph.

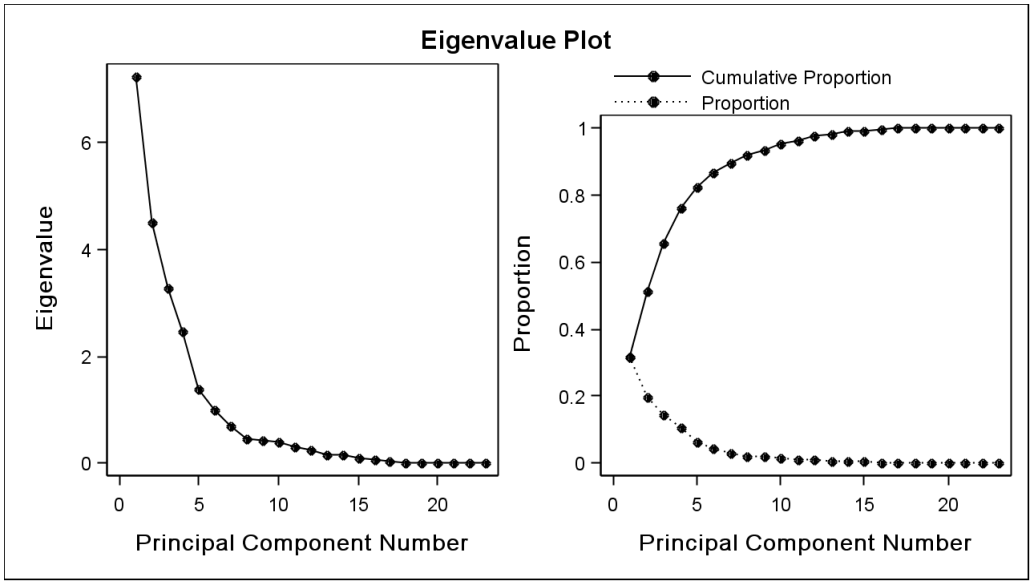


Figure 16. Eigenvalue plot for the Cheddar cheese extract metabolites principal component analysis

Table 14. Eigenvalues of the correlation matrix for the Cheddar cheese extract metabolites principal component analysis

	Eigenvalue	Difference	Proportion	Cumulative
1	7.23750326	2.71812069	0.3147	0.3147
2	4.51938256	1.23421507	0.1965	0.5112
3	3.28516749	0.81702165	0.1428	0.6540
4	2.46814585	1.06742925	0.1073	0.7613
5	1.40071660	0.39029526	0.0609	0.8222
6	1.01042134	0.31526829	0.0439	0.8661
7	0.69515305	0.23750313	0.0302	0.8964
8	0.45764992	0.04618574	0.0199	0.9163
9	0.41146418	0.02222579	0.0179	0.9342
10	0.38923839	0.09195315	0.0169	0.9511
11	0.29728524	0.05914323	0.0129	0.9640
12	0.23814201	0.06819771	0.0104	0.9744
13	0.16994430	0.02501507	0.0074	0.9817
14	0.14492923	0.04173826	0.0063	0.9880
15	0.10319097	0.03511190	0.0045	0.9925
16	0.06807906	0.01800842	0.0030	0.9955
17	0.05007064	0.03132476	0.0022	0.9977
18	0.01874588	0.00232734	0.0008	0.9985
19	0.01641854	0.00613542	0.0007	0.9992
20	0.01028312	0.00519889	0.0004	0.9996
21	0.00508423	0.00284011	0.0002	0.9999
22	0.00224412	0.00150410	0.0001	1.000
23	0.00074002		0.0000	1.000

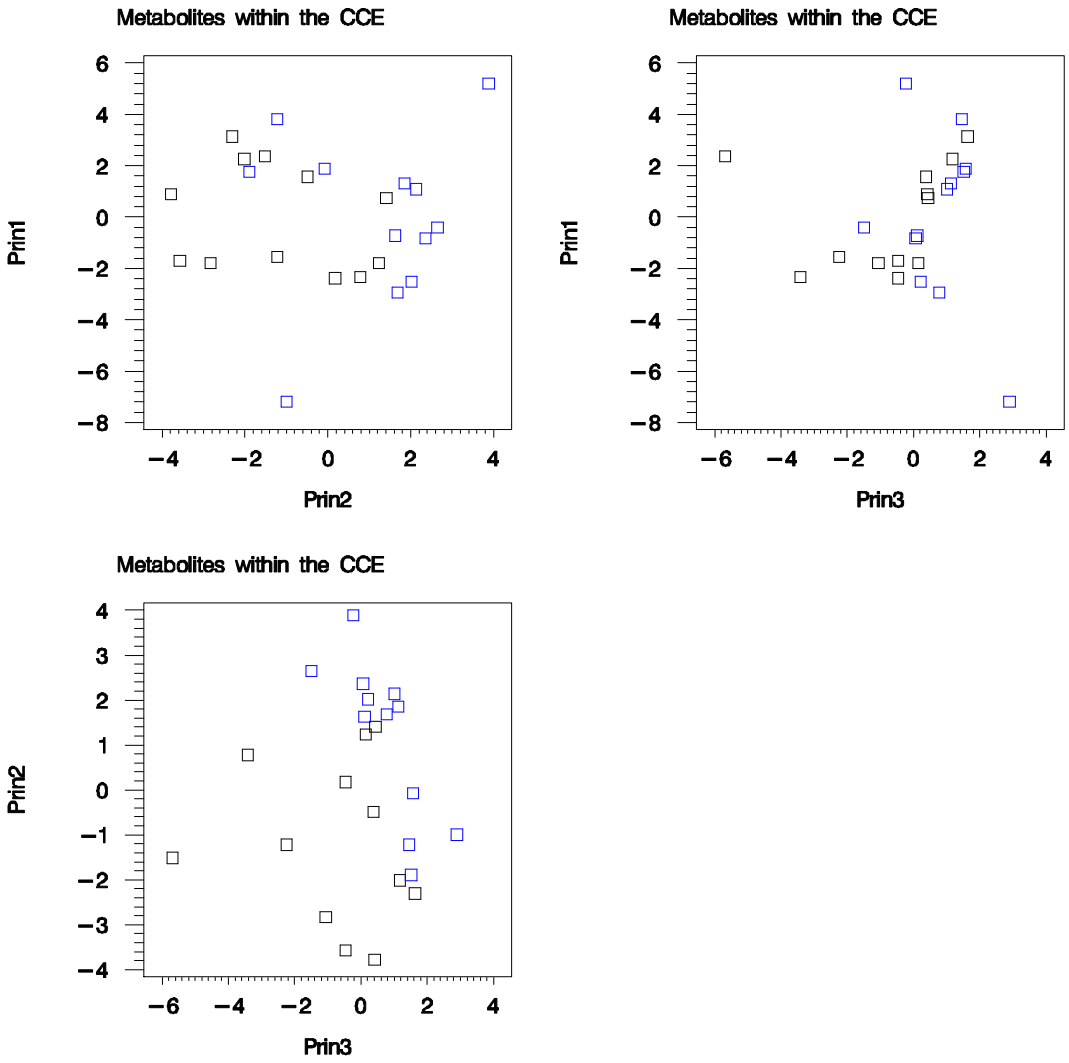


Figure 17. Score plots of every day combined for the Cheddar cheese extract metabolites principal component analysis; full-fat: blue square, low-fat: black square

Bacterial principal component analysis

The metabolite analysis of the bacteria produced 9 compounds that were significantly different between the LF and FF samples. A PCA analysis was conducted to reduce the variables and reveal potential trends in the data. The eigenvalues of the correlation matrix in Table 16, as well as the eigenvalue plot in Figure 18 were used to

determine that the first two principal components were sufficient to explain 75.7% of the variation in the data.

Table 15. Eigenvectors of principal component 1, principal component 2, and principal component 3 for the Cheddar cheese extract metabolites principal component analysis

	Prin1	Prin2	Prin3
Unknown	0.320069	-.153259	-.034325
Unknown	0.015528	-.245981	-.263845
L-Aspartic acid (3TMS)	0.116906	-.058386	-.277402
Unknown	0.195215	-.024408	-.387903
L-Asparagine, N,N2-bis(trimethylsilyl)	0.317572	0.096230	-.202650
L-Glycerol-3-phosphate (4TMS)	0.230566	0.066331	-.283791
L-Glutamine (3TMS)	0.318834	0.012027	-.055434
Unknown	0.203090	0.148706	0.260753
Unknown	0.224152	-.263635	0.229715
L-Arginine (5TMS)	0.258672	-.266747	-.012258
d-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)	0.143895	-.301076	0.204022
Unknown	0.117400	0.293783	0.043884
L-Tyrosine, N,O-bis(trimethylsilyl)	0.303161	0.125219	-.215036
Unknown	0.290680	-.060093	0.247129
Unknown	0.147292	0.210464	0.260091
Unknown	0.128212	0.258309	0.192428
Lactose methoxyamine	0.154922	-.273628	0.194209
Unknown	-.201651	0.123962	0.204847
L-Isoleucine	0.074924	0.138292	0.025714
Unknown	-.034735	0.324171	-.154462
L-Threonine (3TMS)	0.222303	0.203308	0.283653
Unknown	0.022732	0.358250	-.121234
Unknown	0.247036	0.209839	0.036093

Table 16. Eigenvalues of the correlation matrix for the bacterial metabolites principal component analysis

	Eigenvalue	Difference	Proportion	Cumulative
1	5.33848144	3.86044966	0.5932	0.5932
2	1.47803178	0.40695880	0.1642	0.7574
3	1.07107298	0.53707734	0.1190	0.8764
4	0.53399564	0.13072195	0.0593	0.9357
5	0.40327369	0.29771860	0.0448	0.9805
6	0.10555509	0.05344991	0.0117	0.9923
7	0.05210518	0.03869025	0.0058	0.9981
8	0.01341493	0.00934564	0.0015	0.9995
9	0.00406929		0.0005	1.000

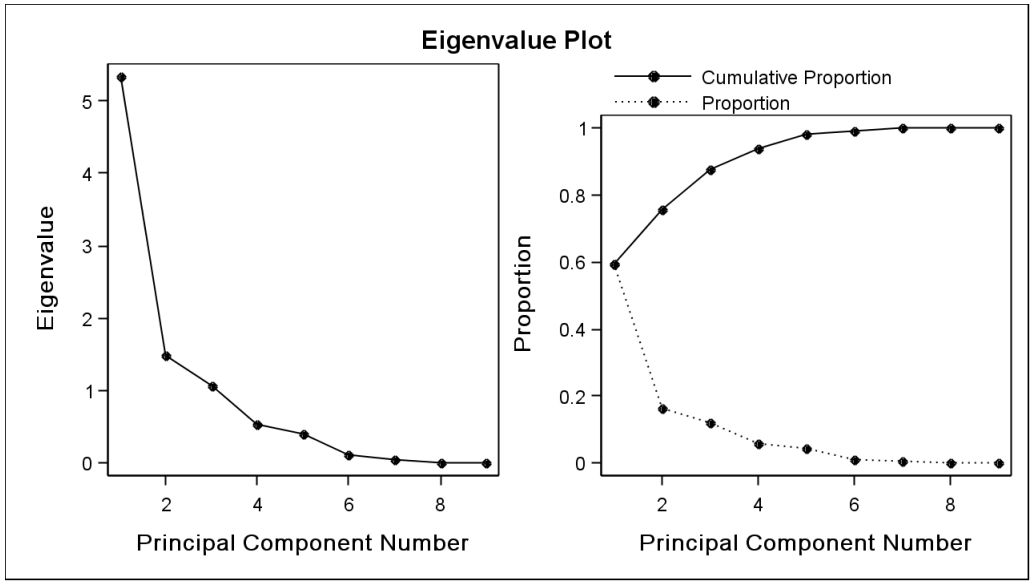


Figure 18. Eigenvalue plot for the bacterial metabolites principal component analysis

The score plot of Figure 19 does not show a complete separation between the FF and LF samples. Prin1 does slightly separate the FF and LF samples. Using 0.40 as a cutoff, Hexadecanoic acid (1TMS), Oleic acid trimethylsilyl ester, and Octadecanoic acid

trimethylsilyl ester give the largest contribution to the variation of the LF and FF bacterial metabolites from Table 17.

Table 17. Eigenvectors of principal 1 and principal 2 for the bacterial metabolites principal component analysis

	Prin1	Prin2
Pyroglutamic acid (2TMS)	0.307438	0.431379
Alanine, phenyl-trimethylsilyl ester	0.214843	0.466161
Citric acid (4TMS)	0.337256	0.457391
Hexadecanoic acid (1TMS)	0.406653	-.204536
Oleic acid, trimethylsilyl ester	0.410859	-.145816
Octadecanoic acid, trimethylsilyl ester	0.402848	-.256275
Glycine (2TMS)	0.136623	0.224317
Unknown	0.397103	-.135434
Unknown	0.269756	-.435849

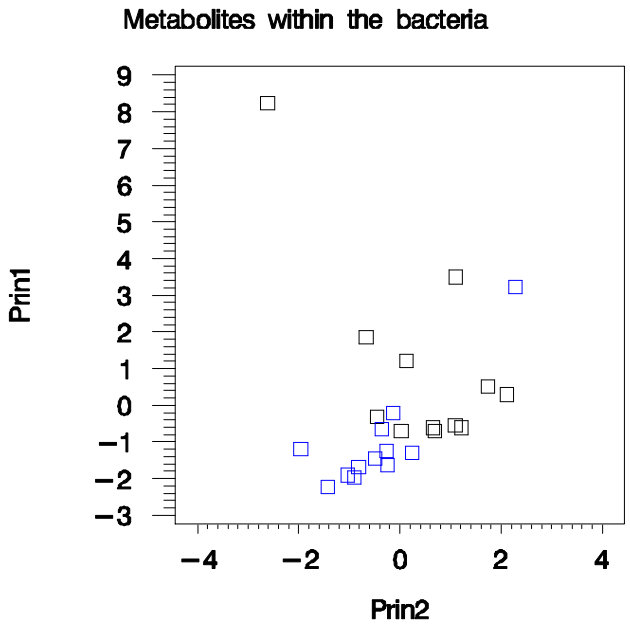


Figure 19. Score plots of every day combined for the bacterial metabolites principal component analysis; full-fat: blue square, low-fat: black square

CONCLUSIONS

The bacterial growth during the fermentation was minimal. The cell counts resembled the counts found in ripening Cheddar cheese of around 10^8 CFU per g or ml. The lack of growth could be attributed to the lack of growth substrates as well as the salt concentrations found in both the LF and FF CCE. The lack of growth indicates that the conditions within the CCE successfully mirrored ripening cheese.

Propan-2-one, heptan-2-one, 3-methylbutanal, heptanal, benzaldehyde, 2-ethylhexan-1-ol, and dimethyl trisulfide were present at a higher concentration in the FFT and LFT when compared to their respective controls. Thus, the presence of these compounds can be attributed to the action of the starter cultures in the samples. This shows that the metabolic action of the starter culture *Lactococcus lactis* does alter the HS profile in CCE and potentially Cheddar cheese.

Differences between the FF and LF samples were seen in the heptan-2-one and p-cymene concentrations early in the fermentation process with a higher concentration found in the FF compared to the LF. The early difference indicates that this difference could be due to the FF and LF conditions in the CCE media, primarily the fat or MFGM level. Other differences in the heptanal, 2-ethylhexan-1-ol, and cyclohexyl isothicyanate concentrations, between the FFT and LFT samples, were detected later in the fermentation process. This indicates that differences were brought about by the growth of starter cultures in there respective medias. The media conditions of FF and LF affects the heptanal, 2-ethylhexan-1-ol, and cyclohexyl isothiocyanate levels as a result of starter culture metabolism. These findings further substantiate the credibility of the second LF

theory that states the microbial physiology itself, and thus overall metabolism, is altered by differences in the physico-chemical environment in ways that affect the production of flavor and aroma active metabolites. These compounds could play a contributing role in the off flavors found in LF Cheddar cheese.

The HS PCA provided a separation of the FFC, LFC, FFT, and LFT based on both FF versus LF and control versus treatment. The compounds found to be most responsible for the variation between FF and LF samples are heptanal, p-cymene, Nonan-2-one, and Undecan-2-one. Examining the graphs of concentration versus time for each of these compounds, at day 0 the FF samples have a higher concentration of these compounds when compared to the LF samples. The compounds found to be the most responsible for the variation between the control and treatment samples are 3-methylbutanal, heptan-2-one, benzaldehyde, 2-Ethylhexan-1-ol, 2,6-Dimethylheptan-4-ol, and 3-methylbutanol. An explanation of this separation could be due to the significant differences found between the treatments and controls in all these compounds except 2,6-Dimethylheptan-4-ol.

The PCA metabolomic results for both the bacteria and CCE metabolites did not produce a clear separation between the FF and LF samples. This could be due to the sensitivity of the method or the metabolites in the samples are not sufficiently different enough to produce a separation. Perhaps a larger sample size would have produced a better separation of the treatments or the addition of another variable like different strain of starter culture or another incubation temperature. The two-week incubation time could be too short and a longer time maybe needed to produce a separation between the FF and LF samples.

Differences in the HS of the FF and LF samples occurred as a result of the metabolism of the starter cultures at 30°C, further tests are needed to verify if similar differences occur at ripening temperatures. Other future tests could involve sensory analysis of the compounds found to be different in the FFT and the LFT in a Cheddar cheese sample to verify if the differences in concentration can be detected.

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APPENDICES

Appendix A: Headspace Solid Phase Micro-Extraction Means and Standard Deviations

Table A1. Headspace full-fat control means and standard deviation

	FFC 0		FFC 1		FFC 7		FFC 14	
Propan-2-one	2.8193	1.0412	2.2373	0.4978	0.6677	0.0879	0.1503	0.0901
3-Methylbutanal	0.0348	0.0340	0.0269	0.0235	0.0088	0.0074	0.0003	0.0001
3-Methylbutanol	0.0311	0.0530	0.0051	0.0082	0.0004	0.0001	0.0095	0.0159
Heptan-2-one	0.1478	0.0379	0.0926	0.0186	0.0162	0.0137	0.0153	0.0010
Heptanal	0.0304	0.0107	0.0235	0.0053	0.0080	0.0010	0.0083	0.0006
2,6-Dimethylheptan-4-ol	0.0039	0.0030	0.0034	0.0027	0.0004	0.0001	0.0032	0.0026
Benzaldehyde	0.0225	0.0095	0.0251	0.0140	0.0265	0.0028	0.0332	0.0149
Dimethyl trisulfide	0.0021	0.0028	0.0003	0.0001	0.0004	0.0001	0.0020	0.0030
2-Ethylhexan-1-ol	0.1940	0.2550	0.0493	0.0535	0.0380	0.0396	0.0553	0.0775
p-Cymene	0.0073	0.0027	0.0053	0.0010	0.0021	0.0014	0.0016	0.0012
2-Phenylacetaldehyde	0.0298	0.0142	0.0260	0.0038	0.0341	0.0050	0.0456	0.0187
Nonan-2-one	0.0501	0.0261	0.0299	0.0061	0.0165	0.0045	0.0031	0.0049
Isothiocyanato cyclohexane	0.0008	0.0003	0.0007	0.0002	0.0008	0.0003	0.0006	0.0002
Undecan-2-one	0.0039	0.0060	0.0137	0.0054	0.0128	0.0050	0.0031	0.0050

Table A2. Headspace low-fat control means and standard deviation

	LFC 0		LFC 1		LFC 7		LFC 14	
Propan-2-one	2.0343	0.1991	2.6004	1.1410	0.4241	0.1558	0.1811	0.0577
3-Methylbutanal	0.0275	0.0146	0.0129	0.0092	0.0094	0.0158	0.0050	0.0081
3-Methylbutanol	0.0027	0.0041	0.0010	0.0013	0.0014	0.0018	0.0003	0.0000
Heptan-2-one	0.0521	0.0046	0.0269	0.0212	0.0126	0.0052	0.0124	0.0019
Heptanal	0.0110	0.0032	0.0070	0.0039	0.0043	0.0015	0.0044	0.0013
2,6-Dimethylheptan-4-ol	0.0003	0.0001	0.0010	0.0013	0.0003	0.0001	0.0003	0.0000
Benzaldehyde	0.0166	0.0079	0.0172	0.0157	0.0226	0.0121	0.0171	0.0015
Dimethyl trisulfide	0.0015	0.0020	0.0010	0.0013	0.0584	0.1006	0.0003	0.0000
2-Ethylhexan-1-ol	0.2207	0.2198	0.0253	0.0414	0.0524	0.0617	0.0023	0.0034
p-Cymene	0.0010	0.0011	0.0010	0.0013	0.0003	0.0001	0.0003	0.0000
2-Phenylacetaldehyde	0.0249	0.0150	0.0161	0.0118	0.0327	0.0139	0.0278	0.0039
Nonan-2-one	0.0078	0.0063	0.0044	0.0053	0.0003	0.0001	0.0003	0.0000
Isothiocyanato cyclohexane	0.0005	$6.3\ E^{-6}$	0.0007	0.0002	0.0005	$5.6\ E^{-6}$	0.0007	0.0002
Undecan-2-one	0.0090	0.0095	0.0041	0.0048	0.0003	0.0001	0.0003	0.0000

Table A3. Headspace full-fat treatment means and standard deviation

	FFT 0		FFT 1		FFT 7		FFT 14	
Propan-2-one	2.5820	0.5384	2.1981	0.3788	2.3223	0.1265	2.0456	0.2968
3-Methylbutanal	0.1819	0.0396	0.9097	0.3972	1.0187	1.0887	2.0133	0.2830
3-Methylbutanol	0.0178	0.0151	0.0603	0.0278	0.5484	0.1084	0.5748	0.2532
Heptan-2-one	0.1117	0.0090	0.1098	0.0187	0.1583	0.0238	0.1937	0.0439
Heptanal	0.0253	0.0029	0.0254	0.0037	0.0169	0.0111	0.0479	0.0128
2,6-Dimethylheptan-4-ol	0.0053	0.0043	0.0647	0.0153	0.5286	0.5357	0.5821	0.6052
Benzaldehyde	0.0168	0.0037	0.0518	0.0224	0.1007	0.0050	0.3653	0.2727
Dimethyl trisulfide	0.0019	0.0016	0.0271	0.0164	0.0503	0.0082	0.0856	0.0437
2-Ethylhexan-1-ol	0.0520	0.0521	0.1361	0.1067	1.0521	0.3465	1.9527	0.4239
p-Cymene	0.0064	0.0010	0.0048	0.0007	0.0029	0.0005	0.0020	0.0014
2-Phenylacetaldehyde	0.0300	0.0009	0.0915	0.0520	0.0784	0.0616	0.1481	0.0939
Nonan-2-one	0.0364	0.0077	0.0336	0.0036	0.0505	0.0169	0.0587	0.0205
Isothiocyanato cyclohexane	0.0009	0.0003	0.0007	0.0002	0.0008	0.0005	0.0015	0.0016
Undecan-2-one	0.0122	0.0082	0.0125	0.0017	0.0157	0.0062	0.0194	0.0088

Table A4. Headspace low-fat treatment means and standard deviation

	LFC 0		LFC 1		LFC 7		LFC 14	
Propan-2-one	2.6604	1.0035	2.0242	0.1085	2.2532	0.1690	1.6794	0.7391
3-Methylbutanal	0.1672	0.0686	0.5540	0.1110	2.3460	2.5234	2.5241	0.4880
3-Methylbutanol	0.0021	0.0023	0.0393	0.0137	0.7903	0.6432	0.6638	0.5114
Heptan-2-one	0.0421	0.0127	0.0494	0.0111	0.1110	0.0040	0.1365	0.0225
Heptanal	0.0102	0.0033	0.0058	0.0018	0.0069	0.0010	0.0145	0.0027
2,6-Dimethylheptan-4-ol	0.0047	0.0006	0.0827	0.0145	0.4501	0.1165	0.4048	0.2072
Benzaldehyde	0.0137	0.0039	0.0389	0.0159	0.1065	0.0484	0.2086	0.0922
Dimethyl trisulfide	0.0020	0.0009	0.0290	0.0163	0.0353	0.0045	0.0633	0.0179
2-Ethylhexan-1-ol	0.1139	0.1440	0.2606	0.1660	1.6088	0.5951	2.4907	0.6229
p-Cymene	0.0009	0.0006	0.0003	0.0001	0.0003	0.0000	0.0004	0.0001
2-Phenylacetaldehyde	0.0222	0.0079	0.0578	0.0321	0.2798	0.2494	0.3254	0.1251
Nonan-2-one	0.0023	0.0026	0.0097	0.0082	0.0288	0.0029	0.0290	0.0184
Isothiocyanato cyclohexane	0.0006	0.0002	0.0096	0.0024	0.0390	0.0054	0.0524	0.0100
Undecan-2-one	0.0043	0.0037	0.0013	0.0016	0.0020	0.0028	0.0031	0.0047

Appendix B: Headspace Comparison of Least Square Means Overtime

Table B1. Headspace tukey adjusted p-values comparing full-fat control least square means overtime

	FFC0 FFC1	FFC0 FFC7	FFC0 FFC14	FFC1 FFC7	FFC1 FFC14	FFC7 FFC14
Propan-2-one	0.993	0.007	0.001	0.117	0.009	0.998
3-Methylbutanal	1.000	1.000	0.163	1.000	1.000	0.770
3-Methylbutanol	1.000	1.000	1.000	0.001	1.000	1.000
Heptan-2-one	0.024	<.001	<.001	1.000	0.004	1.000
Heptanal	0.830	0.002	0.005	0.018	0.086	1.000
2,6-Dimethylheptan-4-ol	1.000	0.413	1.000	0.551	1.000	0.608
Benzaldehyde	1.000	1.000	1.000	1.000	1.000	1.000
Dimethyl trisulfide	0.999	0.999	1.000	1.000	1.000	1.000
2-Ethylhexan-1-ol	0.997	1.000	1.000	1.000	1.000	1.000
p-Cymene	0.501	0.001	<.001	0.027	0.019	1.000
2-Phenylacetaldehyde	1.000	1.000	1.000	1.000	0.998	1.000
Nonan-2-one	0.407	0.058	0.004	0.893	0.234	0.900
Isothiocyanato cyclohexane	1.000	0.998	1.000	1.000	1.000	1.000
Undecan-2-one	0.284	0.681	1.000	1.000	0.426	0.290

For each column, the heading indicates which time and treatment are being compared.

Table B2. Headspace tukey adjusted p-values comparing low-fat control least square means overtime

	LFC0 LFC1	LFC0 LFC7	LFC0 LFC14	LFC1 LFC7	LFC1 LFC14	LFC7 LFC14
Propan-2-one	0.995	0.086	0.029	0.007	0.002	1.000
3-Methylbutanal	1.000	0.541	0.396	0.980	0.860	1.000
3-Methylbutanol	1.000	1.000	1.000	1.000	1.000	1.000
Heptan-2-one	0.831	0.445	0.506	1.000	0.998	1.000
Heptanal	0.998	0.999	1.000	1.000	1.000	1.000
2,6-Dimethylheptan-4-ol	0.998	1.000	1.000	0.996	0.999	1.000
Benzaldehyde	0.999	1.000	1.000	0.905	0.999	1.000
Dimethyl trisulfide	1.000	0.996	0.999	0.983	1.000	0.736
2-Ethylhexan-1-ol	0.961	0.999	0.997	1.000	1.000	1.000
p-Cymene	1.000	0.999	1.000	0.999	0.999	1.000
2-Phenylacetaldehyde	0.968	1.000	1.000	0.609	0.843	1.000
Nonan-2-one	1.000	0.999	0.999	1.000	1.000	1.000
Isothiocyanato cyclohexane	0.996	1.000	0.999	0.985	1.000	0.983
Undecan-2-one	0.976	0.707	0.784	0.997	0.999	1.000

For each column, the heading indicates which time and treatment are being compared.

Table B3. Headspace tukey adjusted p-values comparing full-fat treatment least square means overtime

	FFT0 FFT1	FFT0 FFT7	FFT0 FFT14	FFT1 FFT7	FFT1 FFT14	FFT7 FFT14
Propan-2-one	0.999	1.000	0.996	1.000	1.000	1.000
3-Methylbutanal	0.996	0.999	0.789	1.000	1.000	0.999
3-Methylbutanol	1.000	0.080	0.105	0.027	0.099	1.000
Heptan-2-one	1.000	0.221	0.003	0.067	0.002	0.988
Heptanal	1.000	0.803	0.004	0.559	0.002	<.001
2,6-Dimethylheptan-4-ol	0.020	<.001	<.001	0.590	0.655	1.000
Benzaldehyde	0.250	0.014	<.001	0.813	0.013	0.230
Dimethyl trisulfide	0.348	0.073	0.028	1.000	0.996	1.000
2-Ethylhexan-1-ol	1.000	<.001	<.001	<.001	<.001	<.001
p-Cymene	0.783	0.039	0.005	0.567	0.173	0.997
2-Phenylacetaldehyde	0.639	0.963	0.290	1.000	0.999	0.971
Nonan-2-one	1.000	0.947	0.550	0.659	0.316	0.999
Isothiocyanato cyclohexane	1.000	1.000	1.000	1.000	1.000	1.000
Undecan-2-one	0.999	1.000	1.000	1.000	0.958	0.963

For each column, the heading indicates which time and treatment are being compared.

Table B4. Headspace tukey adjusted p-values comparing low-fat treatment least square means overtime

	LFT0 LFT1	LFT0 LFT7	LFT0 LFT14	LFT1 LFT7	LFT1 LFT14	LFT7 LFT14
Propan-2-one	0.984	0.999	0.699	1.000	1.000	0.994
3-Methylbutanal	0.999	0.814	0.616	0.999	0.992	1.000
3-Methylbutanol	1.000	0.002	0.027	<.001	0.021	0.999
Heptan-2-one	1.000	0.013	0.001	0.008	0.001	0.820
Heptanal	0.994	1.000	0.999	1.000	0.758	0.716
2,6-Dimethylheptan-4-ol	0.032	0.001	0.001	0.569	0.821	1.000
Benzaldehyde	0.329	0.005	<.001	0.392	0.031	0.861
Dimethyl trisulfide	0.483	0.272	0.099	1.000	0.999	1.000
2-Ethylhexan-1-ol	0.997	<.001	<.001	<.001	<.001	<.001
p-Cymene	1.000	1.000	1.000	1.000	1.000	1.000
2-Phenylacetaldehyde	0.769	0.005	0.001	0.175	0.066	1.000
Nonan-2-one	0.999	0.245	0.291	0.481	0.684	1.000
Isothiocyanato cyclohexane	<.001	<.001	<.001	<.001	<.001	0.966
Undecan-2-one	0.999	1.000	1.000	1.000	1.000	1.000

For each column, the heading indicates which time and treatment are being compared.