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A Comparison of Kid Goat Lipase and Microbial Lipase on the Development of Cheddar Cheese Flavor

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Master of Science

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ABSTRACT

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on the Development of Cheddar Cheese Flavor

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Background: Manufacturers continue to look for methods to save time in aging cheese. Complex reactions occur during cheese ripening that produce characteristic flavors and aromas. The addition of exogenous lipase enzymes is one accelerated ripening method that has been studied. Our objective was to document and compare the flavor profiles of cheddar cheeses created with either microbial lipase or animal lipase.

Materials and Methods: The experiment followed a conventional formulation to create experimental batches of cheddar cheese. Kid goat lipase and three microbial lipase treatments were compared against a control. Other animal lipases were evaluated in preliminary research but were omitted in the final experiment due to bitter and unfavorable flavor development. Objective cheese parameters evaluated were texture, moisture, volatile, and free fatty acid (FFA) analysis. We observed subjective sensory properties through quantitative descriptive analysis.

Results: The results of the current study showed that the addition of exogenous lipases caused minimal changes in cheese moisture and a reduction in firmness in cheese samples. Various levels of microbial and animal lipase in Cheddar cheese showed significant differences in the FFA profile of the aged cheese. Microbial lipases tended to liberate more medium and long-chain fatty acids while animal lipases tended to liberate more short-chain fatty acids. *Mucor javanicus* lipase was an exception among microbial lipases and liberated relatively greater amounts of short-chain fatty acids. The addition of exogenous lipase had significant impacts on cheese volatiles. Acetic acid levels decreased in the lipase-treated samples and there were changes in odd-numbered FFA. In terms of sensory analysis, the lipase-treated cheeses showed an increase in FFA flavor notes.

Conclusions: If a microbial lipase were to replace kid goat lipase in the production of Cheddar cheese, the results of the current study show that *Mucor javanicus* microbial lipase would result in the closest free fatty acid profile and sensory characteristics to that of kid goat gastric lipase. Future studies could investigate the addition of animal lipases in cheese that traditionally makes use of exogenous lipases, such as Blue cheese, Emmental Parmesan, Romano, Provolone, and Feta cheeses.

Keywords: cheddar cheese, animal lipase, microbial lipase, textural analysis, QDA

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Development of flavor profiles of Cheddar cheese with the addition of animal and microbial lipase

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INTRODUCTION

Although the precise origins of cheese are uncertain, one account suggests it was made accidentally by an Arabian merchant who put his supply of milk into a pouch made from a sheep's stomach as he set out on a day's journey across the desert. The rennet in the lining of the pouch, combined with the heat of the sun, caused the milk to separate into curd and whey (History of Cheese, 2023). The process of cheesemaking and the scale of production have vastly changed over the centuries. Cheesemaking is now viewed as both an art and a science and has become a valuable international industry.

Many of the textural, functional, and sensory attributes of cheese are developed during cheese ripening. Milk enzymes, starter bacteria, coagulant, and secondary bacteria interact through complex biochemical reactions in the curd during ripening to produce changes in flavor of the maturing cheese (McSweeney and Sousa, 2000, Sousa et al., 2001, McSweeney, 2004). As a result of these complex biochemical interactions, the elastic and rubbery curd is converted into a smooth-bodied and fully flavored cheese.

Ripening can be a slow and expensive process. Lengthy ripening times result in additional costs to manufacturers and identifying strategies to accelerate ripening and subsequently flavor development, would benefit manufacturers significantly. Shortened storage times may increase the utilization capacities of ripening rooms, thereby increasing production capacities, reducing production costs, and providing significant economic benefits to producers.

Many methods have been used to accelerate ripening which include exogenous enzymes, modified starters, use of cheese slurries, adjunct cultures, attenuated starters, and elevated temperatures (Wilkinson, 1993, Azarnia et al., 2006, Murtaza et al., 2014) Each method has its associated advantages and disadvantages. Modified starters are easy to incorporate and the

natural enzyme balance can be retained; however, the modification of starters, either by physical or genetic means, is complex and costly. Adjunct cultures may have the potential to accelerate ripening and, to date, their use has been confined to the limited availability of suitable strains such as *Leuconostoc mesenteroides* ssp *cremoris*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus curvatus*, and *Lactobacillus rhamnosus*. Ripening at elevated temperatures poses a risk in terms of the growth of unwanted microbial contaminants; however, it may be used when cheese is made from pasteurized milk under good manufacturing practices (Law, 2001, Azarnia et al., 2006). Exogenous enzymes are relatively cheap, have specific action, and have been developed to give a choice of flavor options. The use of enzymes does not come without its downfalls. As the choice of useful enzymes is sometimes limited, there is a risk of over-ripening, and there are difficulties with uniform incorporation.

Since volatile short-chain fatty acid production during ripening is important to cheese flavor, exogenous lipases are often added to accelerate ripening. Lipases are enzymes that hydrolyze triglycerides into mono and diglycerides, glycerol, and free fatty acids (FFA) (Sharma et al., 2001, Barron et al., 2004, Kumar et al., 2013). Lipases are produced by plants, animals, and microbes. Of the various sources of lipases, microbial sources are of high commercial interest due to their lower relative cost, ease of production, and the predictability and controllability of their enzymes (Kumar et al., 2013). Animal pregastric lipases, such as kid or goat lipase, release short-chain fatty acids, particularly butanoic acid (Arnold et al., 1975, Nelson et al., 1977). These animal lipases are typically used in the cheese industry to give cheese a distinctive flavor and to accelerate ripening. Taste and flavor are influenced by the chain-length of the fatty acid released during lipolysis. Short-chain fatty acids (C4-C8) produce a cheesy

flavor while high concentrations of long-chain fatty acids (C14-C18) produce a soapy flavor (Peng et al., 2014).

The products of lipase activity contribute directly to typical cheese flavor production, especially when properly balanced with products of proteolysis and other reactions (Yilmaz et al., 2005). FFAs act as precursor molecules for a series of catabolic reactions leading to the production of flavor and aroma compounds such as methyl ketones, lactones, esters, alkanes, and secondary alcohols (Gripon et al., 1991, Fox and Wallace, 1997, McSweeney and Sousa, 2000).

While the development of flavor from exogenous enzymes allows for greater variety and innovation in cheese production, addition of lipases derived from slaughtered animals is viewed as unfavorable by many consumers. Demand for 'free-from' foods is on the rise as consumers become better educated and more fearful about allergies and additives. "Their choices to rule out certain foods and ingredients have deep roots in special diets and ideological lifestyles such as vegetarianism and veganism" (Bingle, 2017). Market research has shown that consumers are willing to go the extra mile when it comes to changing their consumption habits and choosing healthier options (Bingle, 2017). While cheese itself does not fit into a vegan lifestyle, as it is made from bovine milk, the replacement of animal pregastric lipase with a non-animal lipase makes the resulting product more appropriate for vegetarians.

De Felice et al. (1991) produced cheese using animal (kid, lamb, and calf) and microbial lipases to aid in the development of flavor. They found that the fatty acid composition differed depending on the origin of the lipase used. The addition of animal lipase tended to liberate short chain fatty acids, mainly $C_{4:0}$ and $C_{6:0}$, while the addition of microbial lipase did not show any preference for low or high molecular weight fatty acids. They concluded that the use of microbial

lipase could be used to produce a product with a milder aroma than cheese produced with animal lipase.

There have been very few studies directly comparing the effects of animal and microbial lipase addition in cheese. The focus of this study was to evaluate the texture, flavor, and compositional profile of cheddar cheese made with the addition of animal and microbial exogenous lipase. Because cheese is a complex matrix and there are numerous reactions that occur during ripening, our aim was to document the lipase effects in this complex matrix rather than in a simpler matrix, like oil. We chose to evaluate the addition of exogenous lipase in Cheddar cheese due to its relatively short ripening period as compared to that of Parmesan cheese where exogenous lipase would more likely be used.

MATERIALS AND METHODS

Experimental Design

Three microbial lipases and one animal lipase were tested against a control (no exogenous lipase) and evaluated at two different levels in Cheddar cheese against a control void of exogenous lipase, to determine their effects on the flavor profile and textural attributes of the finished cheese. Quantitative Descriptive Analysis (QDA) allowed us to determine flavor notes and textural characteristics using trained panelists. The QDA flavor data was compared with the FFA profile obtained using gas chromatography and the QDA texture data was compared with instrumental texture and moisture data. This data was used to evaluate the flavor and texture profiles obtained from using these specific exogenous lipases in Cheddar cheese.

Lipase Enzymes

In preliminary research, multiple microbial lipase enzymes were sourced and tested. Some enzymes produced cheese that was wholly unappealing with strong butanoic acid and

bitter notes. From our preliminary research, we selected three viable microbial lipase enzymes: *Candida cylindracea* Lipase (CC) (Creative Enzymes, Shirley, NY), *Aspergillus niger* Lipase (AN) (Creative Enzymes, Shirley, NY), and *Mucor javanicus* Enzeco Esterase/Lipase (MJ) (Enzyme Development Corporation, New York, NY). These were compared against a kid goat animal lipase (KGL): Lipase Powder 300 (Danisco, Copenhagen, Denmark). Lipases that were omitted due to adverse taste were *Rhizopus oryzae* Enzeco Esterase/Lipase RO2 and Enzeco Candida Lipase 400 (Enzyme Development Corporation, New York, NY).

Lipase assay

Because various lipase powders have differing activity levels, we decided to standardize the addition of lipase by activity level instead of powder weight. Based on preliminary research, we decided to test two levels of lipase addition (30U and 75U) to better understand what effect the lipase addition would have on the cheese. A titrimetric lipase assay was used to determine enzyme activity according to the method described by Kumar et al. (2013). Briefly, olive oil (5% v/v) was emulsified with gum Arabic (5% w/v) in 50 mM, pH 7.0 potassium phosphate buffer. The suitably diluted enzyme was incubated with the emulsified substrate and the reaction was carried out at 37 °C for 1 h. The reaction was stopped by the addition of acetone:ethanol (1:1 v/v) solution. The amount of fatty acid liberated by lipase action was estimated by titrating with 0.05 M NaOH to pH 10.5 (Jensen, 1983). One unit of lipase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of fatty acids per minute at 37°C, pH 7.0.

Cheese making

Pasteurized, non-homogenized whole milk (3.8% butterfat) was sourced from a local commercial manufacturer (Rosehill Dairy, Morgan, UT). For each batch of cheese, 11.35 liters of milk were heated to 88°C before the starter and adjunct cultures were added. Starter culture

DVS 850 was used along with adjunct cultures DVS LH-32 and DVS CR-213 (Chr. Hansen, Hoersholm, Denmark). Milk was allowed to acidify for 1 hour or until the pH dropped 0.1 units from the initial pH. Microbial lipase treatments were added at two levels (30U and 75U) in separate batches, followed by the addition of calcium chloride (Earthborn Elements, Beaverton, OR), annatto (CodeX-ing Biotech Ingredients, Miami FL), and rennet (Microlant Supreme 750 NB, Chr. Hansen, Hoersholm, Denmark). Milk was allowed to coagulate for 60 minutes until a firm curd had set. After coagulation, the curds were cut into 1 cm cubes and allowed to rest undisturbed for five minutes. The temperature was increased 1°C every seven minutes until a final cook temperature of 39°C was reached. Once the final cook temperature was achieved, the curds were held for 30-45 min or until pH was 6.1-6.3. Upon completion, whey was drained off and the curds were allowed to cheddar for 1 hour and the pH further decreased to 5.4-5.5. The slab was cut into cubes and salted at a rate of 2.68%. The curds were molded and an 18kg weight was applied for 16 hours, after which the cheese was vacuum sealed and stored for aging. The cheese was allowed to age for 90 days at 5°C during the entire ripening period.

Moisture and Texture analysis

Cheese samples from all treatments were analyzed for various parameters at different time periods (2, 30, 60, and 90 days) during the ripening process. Moisture content was determined following AOAC 926.08.

A Warner-Bratzler test on a TA-XT2 Texture Analyzer (Texture Technologies Co., Scarsdale, NY) was used to measure the firmness of cheese samples. Performed in triplicate, each sample (2.5 x 1 x 1 cm) was sheared under the following experimental conditions: Load cell 50 kg, cross head speed 50 mm/min (Rani and Jagtap, 2019). Applications software (Texture

Exponent 32, V6.1.13.0, Stable Micro Systems Ltd., Godalming, Surrey, UK), and system macros were applied without further modification.

Determination of FFA Profile

Freeze-dried cheese samples were ground in liquid N2 and 200mg of cheese was solvent extracted with 2:1:0.016 n-propanol:H2O:HCl (v/v/v). Biphasic separation was achieved by adding 1mL of methylene chloride, vigorously mixing, and centrifuging at 18,000 x g for 2 min. The bottom organic layer was collected and adsorbed on a polymeric adsorbent using vapor phase extraction (Schmelz et al., 2011) and eluted into GC inserts using methylene chloride. Samples were analyzed on an Agilent 7890B EI-GC/MS with a DB-FATWAX UI, 30 m x 0.25 mm, 0.25µm column in split mode at 50:1, 40 cm^3/s, with an inlet temperature of 280ºC. Helium was used as a carrier gas at a constant flow rate of 40 cm^3/s. GC oven temperatures were ramped from 100ºC to 250ºC for 10 min, then 250ºC-260ºC for 10 min, with the flame ionization detector maintained at 200ºC. Quantification of butanoic acid, hexanoic acid, octanoic acid, n-decanoic acid, dodecanoic acid, tetradecanoic acid, n-hexadecanoic acid, and octadecanoic acid was performed via comparison to a standard curve generated from a mixture of analytical standards at 0.5, 1.0, 10.0, and 30.0 µg/µL (Sigma-Aldrich, St. Louis, MO, USA).

Determination of Volatile Components

The determination of volatile components was performed by solid-phase microextraction coupled with gas chromatography–mass spectrometry (SPME–GC-MS). Prior to analysis, cheese samples were sliced, frozen in liquid nitrogen, and then pulverized into small granules and stored at −20°C. Three grams of each cheese was then placed in a 15-mL vial and allowed to equilibrate at 40°C for 30 min. Extraction of the volatiles in the sample headspace was carried out by injecting a carboxen-polydimethylsiloxane fiber into the vial and exposing it to the headspace for

30 min at 40°C. Samples were desorbed onto an Agilent FFAP column, 50 m × 0.2 mm × 0.33 µm (Agilent Technologies, Inc., Milan, Italy). The instrument was held at 40°C for 2 min, then increased at 5°C per minute to 70°C, where it was held for 2 min. The temperature was then increased at 10°C per minute to 240°C and held to give a run time of 35 min. Mass spectrometer settings were programed to record 33 to 450 amu (threshold 1,000) at a sampling rate of 1.11 scans per second. The components were identified and a database was set up to quantify relative amounts of each. The database was constructed using selected ion monitoring as the parameter to determine the amount of each component.

Quantitative Descriptive Analysis

Cheeses were cut into 4 oz lidded soufflé cups with 3 digit codes for descriptive sensory analysis. The cheeses were tempered at 10°C for one hour and were served at this temperature for flavor and texture profiling with spring water and unsalted crackers for palate cleansing. Paper ballots were used. Descriptive analysis of flavor used a 0 to 15 point universal intensity scale with the Spectrum™ method (Meilgaard et al., 1999, Drake and Civille, 2003) and a previously established Cheddar cheese flavor sensory language. Descriptive analysis of texture was conducted in separate sessions and involved an established lexicon and a 0 to 15 point product-specific scale (Brown et al., 2003). A descriptive sensory panel (n=7, 7 females, ages 22 – 46 y) with more than 200 h experience with the descriptive analysis of Cheddar cheese flavor and texture evaluated the cheeses. Consistent with Spectrum™ descriptive analysis training, panelists were presented with reference solutions of sweet, sour, salty, and bitter tastes to learn to consistently use the universal intensity scale (Meilgaard et al., 1999, Drake and Civille, 2003). Following consistent use of the Spectrum ™ scale with basic tastes, panelists learned to identify and scale flavor descriptors using the same intensity scale through presentation and discussion of

flavor definitions, references, and a wide array of cheeses. Discussion and evaluation of a wide array of cheeses was also conducted during training to enable panelists to consistently differentiate and replicate samples. Analysis of data collected from training sessions confirmed that panel results were consistent and that terms were not redundant, consistent with previous use of the developed language (Drake et al., 2001, Drake et al., 2005). Each panelist evaluated each product in duplicate.

Statistical Analysis

Moisture, texture, volatile, and FFA profile statistics of lipase-treated cheese were run on JMP Pro16 software (SAS) using a Tukey-Kramer HSD test for significance, with an alpha of 0.05. All tests were performed in triplicate. The Quantitative Descriptive Analysis data were analyzed using SAS version 9.4, SAS Institute, Inc., Cary, NC. For each of the dependent variables, a mixed models Analysis of Covariance was performed. Each analysis was blocked by panelist and a post-hoc pairwise analysis was done for the three-way interaction of lipase treatment, treatment level, and age of cheese.

RESULTS AND DISCUSSION

Moisture

Results obtained from moisture analysis can be found in Table 1. The lower lipase cheeses (30U) showed some slight variability at the 2-day mark, but as the cheese aged, there were no statistical differences in moisture content at days 30, 60, and 90. Studies in Swiss and Lighvan cheeses found that microbial lipase did not significantly affect cheese moisture, which was consistent with our findings (Aminifar and Emam-Djomeh, 2014, Rani and Jagtap, 2019). However, among the higher lipase cheeses (75U), MJ had a higher moisture content throughout

the entire aging process as compared to the other treatments. Kheadr et al. (2002) did identify that encapsulated exogenous lipase increased moisture levels in finished Cheddar cheese.

Texture

The firmness of Cheddar cheese at different points in the aging process is shown in Table 2. Firmness is defined as the maximum force required to cut through the cheese samples. Our results showed there were no changes in firmness among the different lipase treatments at all four of the time periods sampled. Seyed-Moslemi et al. (2021) found that in Quark cheese, there was no increase in the firmness of their enzyme-treated cheese as compared to a control. Kheadr et al. (2002) found that the firmness and hardness of Cheddar cheese significantly increased with the addition of exogenous lipase. These differences could have been attributed to dynamics around an encapsulation technology they employed for their lipase. Additionally, Aminifar and Emam-Djomeh (2014) and Rani and Jagtap (2019) found that microbial lipase treatments significantly increased the hardness of Lighvan and Swiss cheeses respectively. These textural changes in other cheeses could be due to multiple reasons including different fat contents of milk sources, varying rates of hydrolysis in the cheese, and varying interactions such as cross-linking between the casein matrix and fat phase which can cause a plasticizing effect (Aminifar and Emam-Djomeh, 2014).

FFA Profile

The Total FFA content for each of the cheeses are shown in Table 3. At the lower treatment level (30U), both MJ and KGL produced higher amounts of FFA as compared to the control cheese. At the higher lipase treatment level (75U), CC, MJ, and KGL produced higher amounts of FFA as compared to the control cheese.

Correlated changes in FFA composition through gas chromatography of Cheddar cheese treated with different levels of lipase during ripening, are shown in Figure 1(a-d). Each lipase treatment produced a unique profile of individual FFA which can be grouped into three categories: short-chain fatty acids (SCFA) ($C_{4:0-6:0}$), medium-chain fatty acids (MCFA) ($C_{8:0-12:0}$), and long-chain fatty acids (LCFA) ($C_{14:0-18:0}$).

The cheese produced with 30U of lipase produced some changes in the FFA profile. At day 30, treatment with MJ caused an increase in MCFA and LCFA, and treatment with KGL caused an increase in SCFA and MCFA. At day 60, treatment with both MJ and KGL caused an increase in SCFA, MCFA, and LCFA. Between days 30 and 60, AN and MJ saw an increase in MCFA, and CC saw an increase in SCFA and LCFA.

The cheese produced with 75U of lipase saw greater increases in FFA as compared to the control cheese. At day 30, treatment with CC caused an increase in LCFA, treatment with MJ caused an increase in SCFA, MCFA, and LCFA, and treatment with KGL caused an increase in SCFA and MCFA. At day 60, treatment with CC caused an increase in MCFA and LCFA, and treatment with MJ and KGL caused an increase in SCFA, MCFA, and LCFA. Between days 30 and 60, KGL saw an increase in SCFA and MCFA. CC saw an increase in SCFA, MCFA, and LCFA.

When all FFA data are evaluated together, some trends appear. The AN treatment did not produce any significant increase and any FFA as compared to the control cheese. The CC treatment did not produce any significant changes at the lower treatment level (30U), however, it did cause MCFA and LCFA to increase at the higher treatment level (75U). Both the MJ and KGL treatment caused increases in SCFA, MCFA, and LCFA at both the lower and higher treatment levels.

De Felice et al. (1991) discovered similar results using animal (kid, lamb, and calf) and microbial lipases to aid in the development of flavor during cheese aging. They found that the fatty acid composition differed depending on the origin of the lipase used. The addition of animal lipase tended to liberate SCFA, mainly $C_{4:0}$ and $C_{6:0}$, while the addition of microbial lipase did not show any preference for low or high-molecular-weight fatty acids. Yilmaz et al. (2005) found that LCFA dominated in their microbial lipase-treated Tulum cheese. Aminifar and Emam-Djomeh (2014) also found that at the end of ripening, MCFA and LCFA were the most abundant in their microbial lipase treated-Lighvan cheese. These higher concentrations of MCFA and LCFA are seen as a good quality in these cheeses. Other cheeses where higher concentrations of these higher molecular weight fatty acids would be welcomed include Blue cheese, Emmental Parmesan, Romano, Provolone, and Feta cheeses. Sowa et al. (2022) found that the addition of a novel microbial lipase to feta cheese proved to be competitive to the use of pregastric lipase and could therefore replace the products of animal origin.

Lipase enzymes display various types of specificity: the type of substrate they preferentially hydrolyze, the FA preferentially released, and the position (*sn*-1,2,3,) of the ester bond preferentially hydrolyzed on the glycerol backbone (Dherbecourt et al., 2010). Each of the lipases used in this study poses unique specificity and produces a unique composition of FFA in Cheddar cheese. If one of the tested microbial lipases were to be chosen to replace the animal lipase KGL, MJ would likely be the best option as is the only microbial lipase to produce a significant increase in SCFA, which are important to Cheddar cheese flavor.

Volatiles

The changes in volatile compounds, as determined by GC-MS SPME are shown in Figure 2(a-d). The compounds that showed statistical significance were acetic acid, benzaldehyde,

benzoic acid, butanoic acid, heptanoic acid, octanoic acid, and decanoic acid. Other compounds that were detected but showed no statistical difference included, 2-heptanone, pentanoic acid, hexanoic acid, nonanoic acid, and dodecanoic acid. The butanoic, octanoic, and decanoic acid levels are similar to the levels found in the GCMS FFA analysis, except for KGL which showed lower levels than what was measured in the FFA analysis. For many of the volatile fatty acids that were measured, KGL showed smaller amounts than what was measured in the FFA analysis. This could be because our volatile analysis was quantified on a relative basis. The KGL-treated cheese samples had more volatile compounds that were detected and thus the volatile fatty acids that were measured became a smaller percentage of all the compounds detected. With the number of replicates that we performed and with the equipment available to us, we were unable to detect any significant differences in volatile compounds as they aged from 30 to 60 days. In order to detect these differences more replications would need to be performed on more sensitive equipment.

Quantitative Descriptive Analysis

QDA evaluation results are presented in Figure 3. Flavoring attributes that showed the most variation between experimental treatments were Diacetyl, FFA, and Brothy. Diacetyl, among other mild flavors such as cooked, whey, and milkfat, predominates in young or mild cheeses while the flavor FFA is usually indicative of an aged cheese (Drake et al., 2001). At the higher treatment level, all four lipase treatments showed increased FFA flavor over the control, while at the lower treatment level, only the MJ and KGL showed an increase in FFA flavor. Kheadr et al. (2002) similarly found that flavor intensity of Cheddar cheese increased with the addition of exogenous microbial lipase. They also found that cheeses subjected to high lipase treatments developed soapy off-flavors. They attributed these off flavors to the accumulation of

higher quantities of medium and long-chain fatty acids ($C_{10:0}$-$C_{18:0}$). Rani and Jagtap (2019) found that in Swiss cheese, the addition of exogenous lipases increased the sharpness, flavor intensity, and overall acceptability of the cheese as scored by panelists.

In the sensory analysis, butanoic acid was selected as the chemical reference for the term free fatty acid. The QDA FFA results match closely with the results obtained from gas chromatography FFA analysis. In many cases, the Diacetyl flavor decreased as the cheese aged and the FFA flavor increased as the cheese aged. CC, MJ, and KGL all showed an increase in Brothy flavor at the higher treatment level as compared to the control. According to Drake et al. (2001), FFA and Brothy flavor notes are correlated.

The panelists also conducted sensory analysis on the hand firmness and mouth firmness of the cheeses. All lipase treatments showed a significant decrease in firmness as compared to the control at both 30 and 60 days. This contrasts with our instrumental firmness data.

CONCLUSIONS

The results of the current study showed that the addition of exogenous lipases caused minimal changes in cheese moisture and a reduction in firmness in cheese samples. Various levels of microbial and animal lipase in Cheddar cheese showed significant differences in the FFA profile of the aged cheese. Microbial lipases tended to liberate more medium and long-chain fatty acids while animal lipases tended to liberate more short-chain fatty acids. MJ was an exception among microbial lipases and liberated relatively greater amounts of short-chain fatty acids. The addition of exogenous lipase had significant impacts on cheese volatiles. Acetic acid levels decreased in the lipase-treated samples and there were changes in odd-numbered FFA. In terms of sensory analysis, the lipase-treated cheeses showed an increase in FFA flavor notes. While it is uncommon to add exogenous lipase to Cheddar cheese, if a microbial lipase were to

be recommended to replace animal lipase, the results of the current study show that MJ would have the closest FFA profile to that of KGL. Future studies could investigate the addition of animal lipases in cheese that traditionally makes use of exogenous lipases, such as Blue cheese, Emmental Parmesan, Romano, Provolone, and Feta cheeses.

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CONFLICT OF INTERESTS

The authors of this article certify that they have *no* affiliations or involvement with any organization or entity related to the subject matter or materials discussed in this manuscript.

Table 1. Moisture Results Experimental Cheddar cheese percent moisture compared to a control with no exogenous lipase. CC, C. cylindracea lipase; AN, A. niger lipase; MJ, M. javanicus lipase; KGL, kid goat lipase. Values with a ± represent the standard deviation for the value directly above. Different uppercase superscripts in a row represent significant differences between means within a row. Different lowercase superscripts in a column represent significant differences between the means of 2, 30, 60, and 90-day aged cheese. (n=3, $p<0.05$).

Lipase Level	Attribute (Unit)	Days Aged	Control	CC	AN	MJ	KGL
30 U	Moisture (%)	2	43.2^{Bab} ±0.21	41.8^{Ca} ±0.32	43.3^{Bab} ±0.25	44.8^{Aa} ±0.28	41.3^{Ca} ±0.21
		30	45.1^{Aa} ±0.50	42.9^{Aa} ±0.53	43.7^{Aab} ±1.56	43.2^{Aab} ±0.76	41.7^{Aa} ±0.55
		60	43.9^{Aab} ±1.18	43.3^{Aa} ±0.82	44.4^{Aa} ±0.62	44.0^{Aab} ±1.29	41.8^{Aa} ±1.33
		90	41.8^{Ab} ±1.12	41.4^{Aa} ±1.95	41.3^{Ab} ±1.02	42.5^{Ab} ±0.57	39.8^{Aa} ±1.05
75 U	Moisture (%)	2	42.3^{Ca} ±0.24	41.2^{Cb} ±0.61	44.8^{Bab} ±0.89	48.7^{Aa} ±0.43	44.2^{Ba} ±0.91
		30	43.3^{Aa} ±0.68	42.3^{Ab} ±0.25	42.6^{Ab} ±0.57	45.3^{Aab} ±2.45	43.0^{Aa} ±2.24
		60	43.4^{Ba} ±0.67	44.2^{Ba} ±0.68	45.2^{ABa} ±0.54	46.9^{Aab} ±0.94	45.1^{ABa} ±1.55
		90	41.9^{Ba} ±1.33	42.7^{ABab} ±0.52	43.5^{ABab} ±0.86	45.4^{Ab} ±0.96	44.6^{ABa} ±1.62

Table 2. Texture Results Experimental Cheddar cheese textural properties compared to a control with no exogenous lipase. CC, C. cylindracea lipase; AN, A. niger lipase; MJ, M. javanicus lipase; KGL, kid goat lipase. Values with a ± represent the standard deviation for the value directly above. Different uppercase superscripts in a row represent significant differences between means within a row. Different lowercase superscripts in a column represent significant differences between the means of 2, 30, 60, and 90-day aged cheese. (n=3, $p<0.05$).

Lipase Level	Attribute (Unit)	Days Aged	Control	CC	AN	MJ	KGL
30 U	Firmness (N)	2	4.9^{Ab} ±0.69	5.5^{Ab} ±0.48	6.6^{Aa} ±1.21	4.6^{Ab} ±0.29	8.4^{Aa} ±0.48
		30	10.0^{Aa} ±1.37	9.3^{Aa} ±0.57	9.9^{Aa} ±1.55	8.5^{Aa} ±1.08	10.5^{Aa} ±0.90
		60	6.2^{Aab} ±2.96	4.5^{Ab} ±0.23	7.3^{Aa} ±1.41	7.2^{Aa} ±0.56	6.9^{Aa} ±1.50
		90	7.1^{Aab} ±0.13	6.6^{Ab} ±1.73	7.3^{Aa} ±0.77	6.8^{Aa} ±0.69	5.9^{Aa} ±3.07
75 U	Firmness (N)	2	4.4^{Ab} ±0.07	5.3^{Ab} ±0.92	4.7^{Ab} ±0.83	4.6^{Ac} ±0.64	5.9^{Ab} ±0.71
		30	9.7^{Aa} ±1.68	8.8^{Aa} ±1.63	9.1^{Aa} ±0.43	8.1^{Aa} ±0.46	8.8^{Aa} ±1.83
		60	7.6^{Aa} ±0.64	7.1^{Aab} ±0.83	6.2^{Ab} ±0.65	6.0^{Ab} ±0.37	7.6^{Aab} ±0.41
		90	$6..9^{Aab}$ ±1.42	6.5^{Aab} ±0.79	8.5^{Aa} ±0.44	7.0^{Aab} ±0.35	6.8^{Aab} ±0.92

Firmness is defined as the maximum force required to cut through the cheese samples.

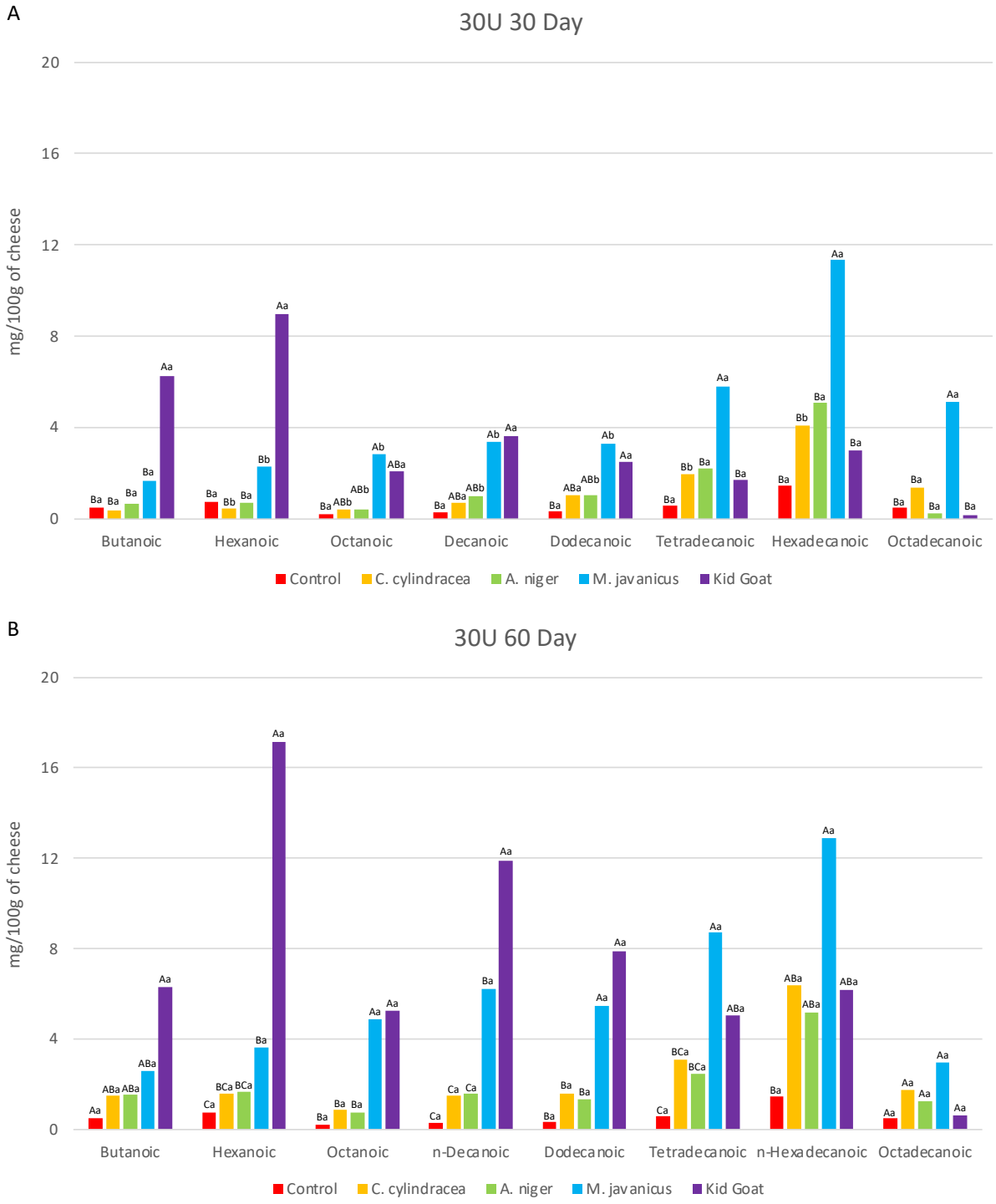
Table 3. Total Free Fatty Acids: Experimental Cheddar cheese total free fatty acids compared to a control with no exogenous lipase. CC, C. cylindracea lipase; AN, A. niger lipase; MJ, M. javanicus lipase; KGL, kid goat lipase. Values with a ± represent the standard deviation for the value directly above. Different uppercase superscripts in a row represent significant differences between means within a row. Different lowercase superscripts in a column represent significant differences between the means of 30 and 60-day aged cheese. (n=3, $p<0.05$).

Lipase Level	Days Aged	Control	CC	AN	MJ	KGL
30 U	30	4.4^{Ba} ±1.55	10.3^{Bb} ±8.01	11.2^{Ba} ±0.95	35.6^{Ab} ±7.83	28.2^{Aa} ±10.81
	60	6.4^{Ba} ±1.76	18.1^{Ba} ±6.24	15.6^{Ba} ±9.10	47.3^{Aa} ±22.43	60.2^{Aa} ±13.69
75 U	30	4.4^{Ca} ±1.55	37.6^{ABa} ±16.42	17.8^{BCa} ±4.03	68.1^{Aa} ±7.46	47.1^{ABa} ±29.15
	60	6.4^{Ca} ±1.76	67.6^{Aa} ±33.50	18.0^{BCa} ±12.27	67.1^{ABa} ±21.48	106.9^{Aa} ±29.83

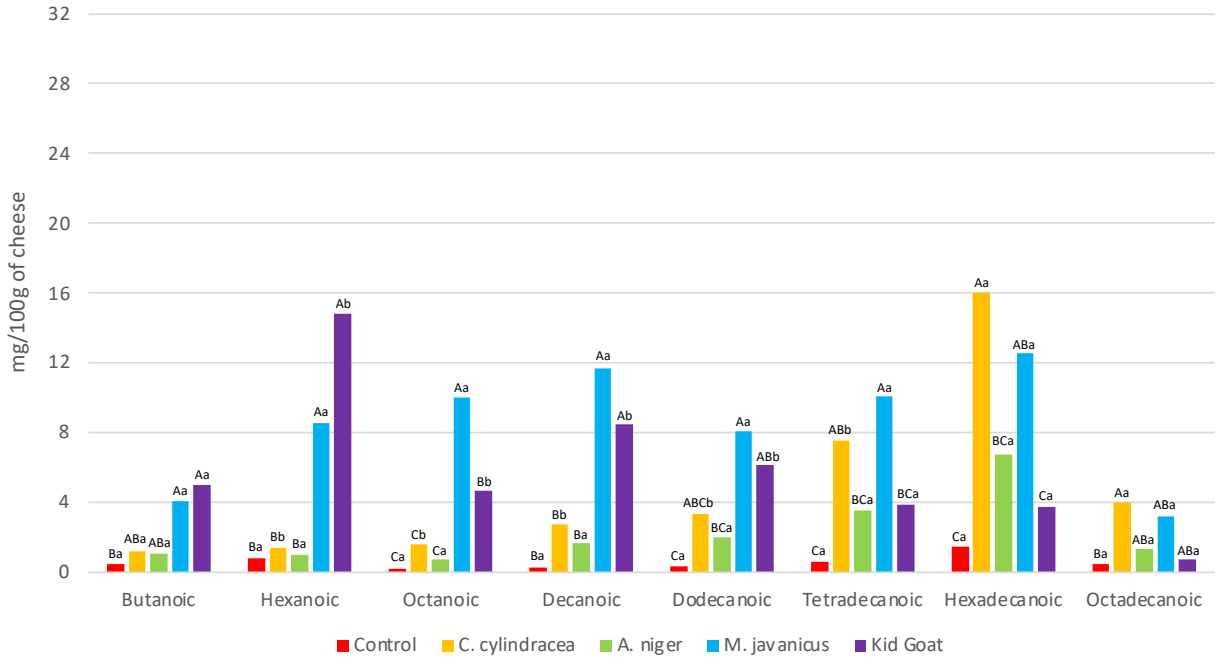
Table 4. Quantitative Descriptive Analysis Texture Results: Experimental Cheddar cheese texture compared to a control with no exogenous lipase as determined by our QDA panel. CC, C. cylindracea lipase; AN, A. niger lipase; MJ, M. javanicus lipase; KGL, kid goat lipase. Values with a ± represent the standard deviation for the value directly above. Different superscripts in a row represent significant differences between means (n=7, $p<0.05$).

Lipase Level	Attribute (Unit)	Days Aged	Control	CC	AN	MJ	KGL
30 U	Hand Firmness	30	9.7^{A} ±0.47	8.8^{B} ±0.47	8.9^{AB} ±0.81	8.7^{B} ±0.71	9.1^{AB} ±0.49
		60	10.9^{A} ±0.20	9.6^{B} ±0.36	9.8^{B} ±0.65	8.8^{C} ±0.57	9.5^{BC} ±0.37
30 U	Mouth Firmness	30	8.2^{A} ±0.77	7.2^{A} ±1.14	7.0^{A} ±1.05	7.1^{A} ±1.09	7.0^{A} ±0.80
		60	9.4^{A} ±0.35	9.2^{A} ±0.47	7.8^{BC} ±0.47	7.4^{C} ±0.42	8.4^{B} ±0.43
75 U	Hand Firmness	30	9.2^{A} ±0.70	8.3^{A} ±0.37	8.3^{A} ±0.71	8.4^{A} ±0.44	9.0^{A} ±0.56
		60	9.8^{A} ±0.30	9.4^{AB} ±0.45	8.6^{B} ±0.43	8.8^{B} ±0.72	9.4^{AB} ±0.50
75 U	Mouth Firmness	30	7.6^{A} ±0.56	6.7^{BC} ±0.67	6.7^{BC} ±0.52	6.4^{C} ±0.35	7.3^{AB} ±0.61
		60	8.4^{A} ±0.31	7.0^{C} ±0.47	7.8^{AB} ±0.51	6.7^{C} ±0.27	7.7^{B} ±0.44

Figure 1. Free Fatty Acid Composition: Experimental Cheddar cheese FFA composition as determined by gas chromatography-mass spectroscopy compared to a control with no exogenous lipase. Different uppercase superscripts above a bar represent significant differences between means within an individual FFA. Different lowercase superscripts above a fatty acid represent significant differences between 30U FFA means at 30 and 60 days or 75U FFA means at 30 and 60 days. (n=3, $p<0.05$).



C


75U 30 Day
mg/100g of cheese
32
28
24
20
16
12
8
4
0
Butanoic
Hexanoic
Octanoic
Decanoic
Dodecanoic
Tetradecanoic
Hexadecanoic
Octadecanoic
Control
C. cylindracea
A. niger
M. javanicus
Kid Goat

D

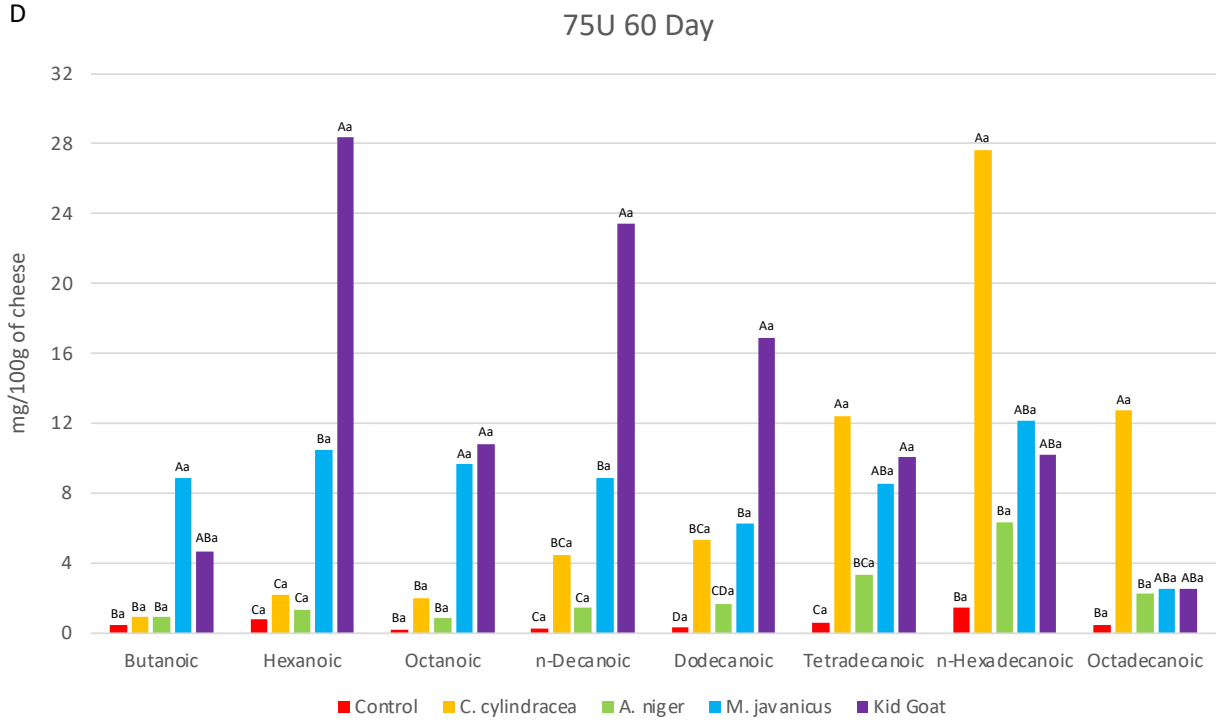
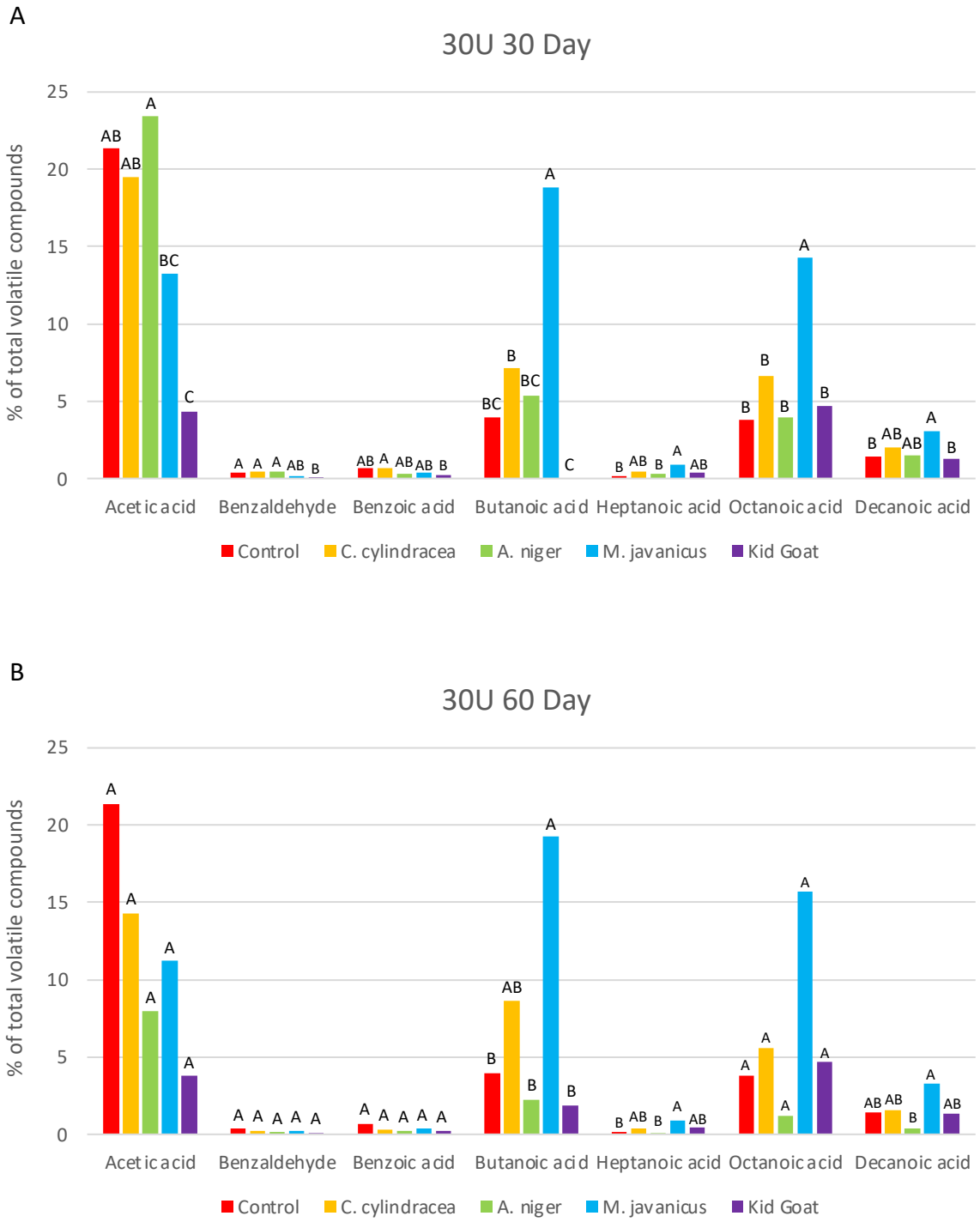
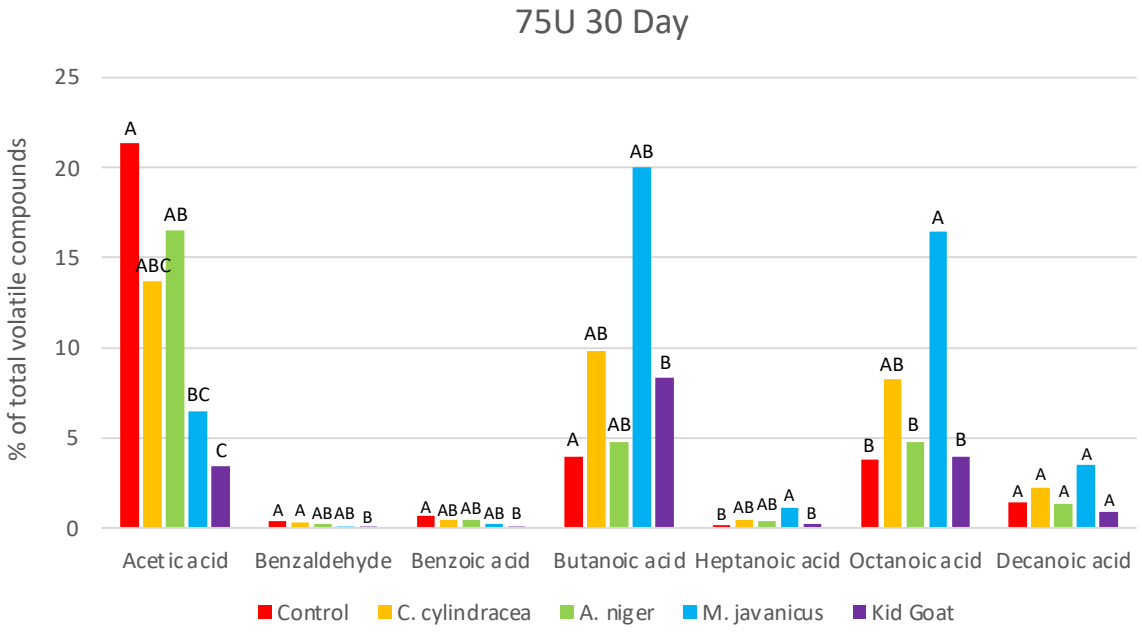

75U 60 Day
mg/100g of cheese
32
28
24
20
16
12
8
4
0
Butanoic
Hexanoic
Octanoic
n-Decanoic
Dodecanoic
Tetradecanoic
n-Hexadecanoic
Octadecanoic
Control
C. cylindracea
A. niger
M. javanicus
Kid Goat

Figure 2. Volatile Composition: Experimental Cheddar cheese volatile composition compared to a control with no exogenous lipase. Different superscripts above a bar represent significant differences between means. Compounds with no superscripts did not show any significant differences. (n=3, $p<0.05$).



C


75U 30 Day
% of total volatile compounds
25
20
15
10
5
0
A
ABC
AB
BC
C
A A AB AB B
A AB AB AB B
A AB AB AB B
B AB AB A B
B AB AB A B
A A A A A
Acetic acid
Benzaldehyde
Benzoic acid
Butanoic acid
Heptanoic acid
Octanoic acid
Decanoic acid
Control
C. cylindracea
A. niger
M. javanicus
Kid Goat

D

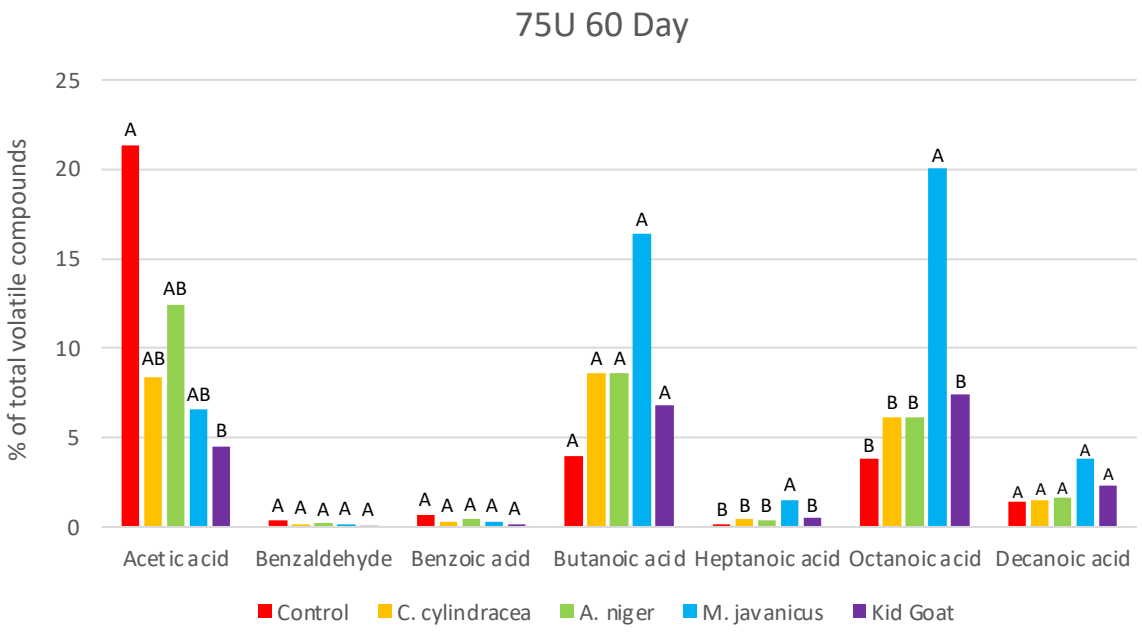
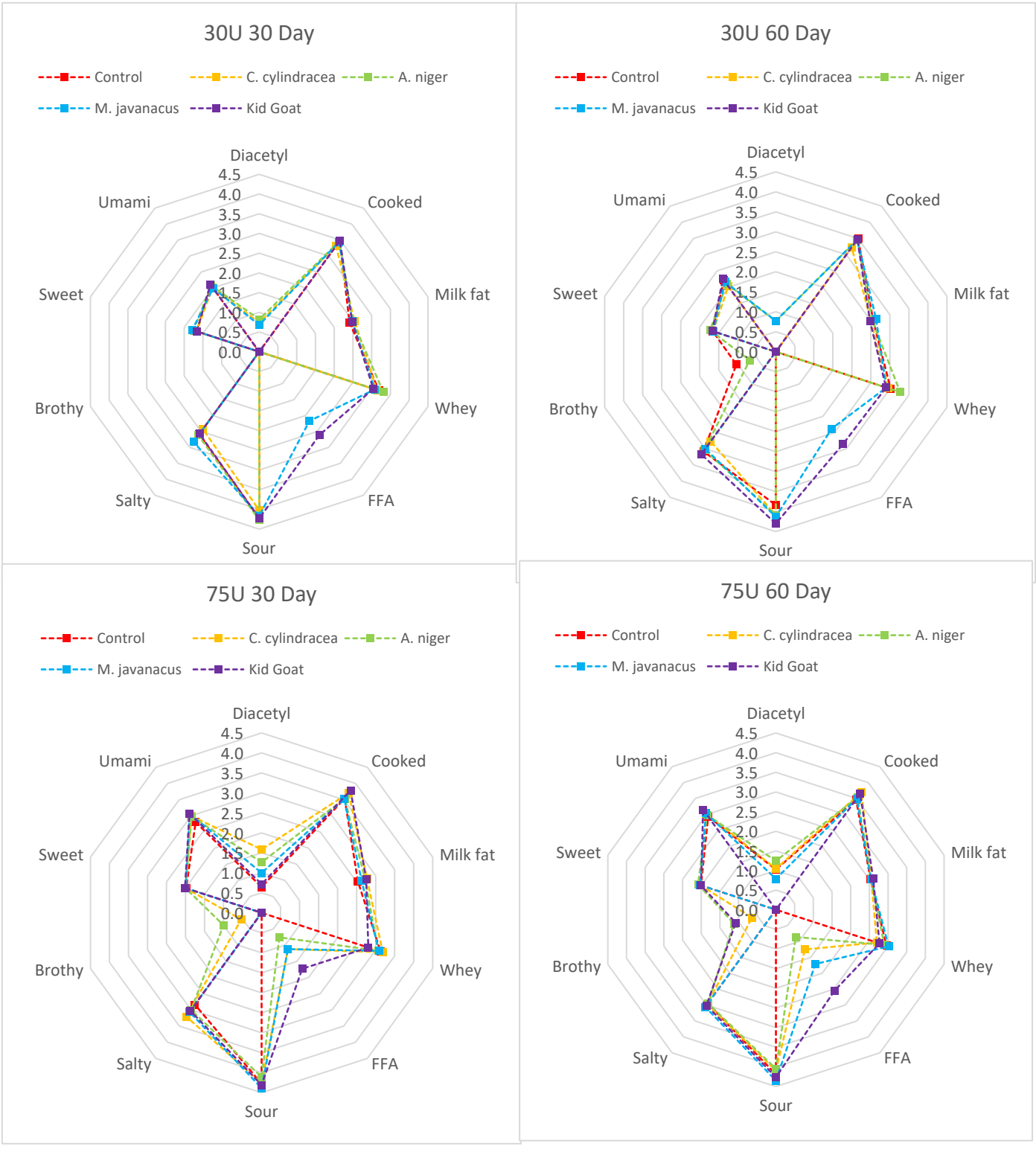

75U 60 Day
% of total volatile compounds
25
20
15
10
5
0
A
AB
AB
AB
B
A A A A A
A A A A A
A A A A A
B B B A B
B B B A B
A A A A A
Acetic acid
Benzaldehyde
Benzoic acid
Butanoic acid
Heptanoic acid
Octanoic acid
Decanoic acid
Control
C. cylindracea
A. niger
M. javanicus
Kid Goat

Figure 3. Quantitative Descriptive Analysis Flavor Properties: Experimental Cheddar cheese sensory properties compared to a control with no exogenous lipase. Data collected using trained panelists in a QDA sensory panel. (n=7).



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APPENDIX

Literature Review

Abstract

Food producers are currently looking for more impactful flavors, with consumer-friendly labeling that appeals to growing diet trends including gluten-free, vegetarianism, and veganism. Additionally, the ethical use of animal ingredients has come under more scrutiny and alternative ingredients are being considered. Animal lipases, specifically goat/kid lipase, have historically been used to accentuate flavor in some cheese. Many studies have looked at the effect of lipases on Cheddar cheese including increased ripening, flavor profile, and free fatty acid profile; however, there have been no studies that directly compare the effects of animal and non-animal lipases on Cheddar cheese flavor development. The goal of this study is to discover functional alternates to animal lipases in cheese, thereby giving food producers unique cheese flavorings with more animal-friendly labels. This study will use various types of non-animal lipases to create accelerated flavoring in cheeses. Physical and organoleptic properties of cheeses will be examined at multiple time points in the aging process using various types of non-animal lipases with emphasis on creating accelerated flavoring profiles in cheese.

Cheese making Process

Although the actual time and origins of cheese are unknown, according to an ancient legend, it was made accidentally by an Arabian merchant who put his supply of milk into a pouch made from a sheep's stomach as he set out on a day's journey across the desert. The rennet in the lining of the pouch, combined with the heat of the sun, caused the milk to separate into curd and whey (IFDA, 2020). The process of cheesemaking and the scale of production have vastly changed

over the centuries. Cheesemaking is now viewed as both an art and a science, holding an estimated market value of approximately $69.8 billion USD in 2019.

Multiple factors influence the final cheese product yield and quality including milk composition, starter bacterial strains, ripening temperature, coagulation time, curd size, and fermentation time. Casein is the protein that is used in the cheesemaking process while most of the whey proteins, which constitute about 20% of the proteins, will be lost with the whey during cheesemaking (Murtaza et al., 2013). Because of variations in milk composition that arise during lactation, milk used for cheesemaking is generally standardized for fat and casein values. This process involves the separation of whole milk into skim milk and cream and then blending to the desired fat percentage.

One of the primary events in the cheesemaking process is the acidification of milk, which occurs when lactose is converted to lactic acid by lactic acid bacteria (LAB). The most commonly used bacteria in cheesemaking are *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *lactis* (Michel and Martley, 2001). In Cheddar type cheese, most acid is produced before moulding, while in most other varieties, it occurs mainly after moulding. This acidification of the milk greatly affects the composition, texture, and flavor of the cheese. Acidity also has a significant effect on the activity of the coagulant, the level of proteolysis, and other reactions in the resulting cheese (Rehman et al., 2004).

The role of pH in cheese texture is particularly important, as changes in pH are related directly to chemical changes in the protein network of the cheese curd. As the pH of the cheese curd decreases, there is a loss of colloidal calcium phosphate from the casein micelles. At pH 5.5 or lower, a gradual dissociation of the sub-micelles into smaller aggregates occurs (Lawrence et al., 1987). A desired texture can be obtained between pH 5.3 and 5.1 since a wide range of casein

aggregates are present. The solubilization of colloidal calcium phosphate, among other factors, affects curd texture, stretchability, and meltability.

After the milk has been acidified, it is then coagulated by the addition of rennet. The molecular components in casein are αS1-, αS2-, β-, and κ-casein differing in amino acid composition, phosphorylation, and glycosylation (Walstra et al., 2006). Rennet coagulation of milk is a two-step process. The first step involves the enzymatic hydrolysis of κ-casein, and the second involves the coagulation of casein by Ca2+ at temperatures >20°C. Chymosin in rennet specifically cleaves κ-casein between phenylalanine and methionine, at positions 105 and 106 respectively, which leads to the release of the hydrophilic portion of κ-casein located at the surface of the casein micelles. When intact, the micelles are kept colloidally dispersed in milk by steric and electrostatic repulsion involving the negatively charged portion of κ-casein. The casein micelles become unstable following the removal of these hydrophilic peptides; at an appropriate temperature (e.g., 30°C), the milk coagulates under the influence of Ca2+ in the medium (Dalgleish, 1993). Coagulation time depends on the protein content and enzyme concentration. A minimum protein level of 2.5–3.0% is necessary for gel formation in cheese manufacturing as an increase in milk protein (>3%) will result in an increase in coagulation time. (Fox et al., 2000).

The rennet milk gel is quite stable, however, once it is cut or broken syneresis occurs rapidly, leading to whey being released from the curd. Cutting the curd into smaller pieces gives faster syneresis which is proportional to the area of the surface exhibiting syneresis. The rate and extent of syneresis are influenced primarily by milk calcium level and casein concentration, pH of the whey, cooking temperature, rate of stirring of the curd-whey mixture, and time (Walstra et al., 1987).

After the curd is cut and the whey is removed, the curds are cheddared. This cheddaring process involves piling the curds together into slabs and turning and stacking the slabs every fifteen minutes for about an hour and a half. This process allows the curds to knit together and further ripen and ferment while expelling additional whey from the curd. Afterward, the curd is cut into small pieces (milling) and salted. The addition of salt aids in the production of organic acids, directly modifies flavor, promotes curd syneresis, reduces water activity, and influences the activity of rennet, LAB, and endogenous milk enzymes (Murtaza et al., 2012). Salt also controls the metabolism of lactose and the pH of the fresh cheese, which in turn affects the rate of maturation and cheese quality (Fox, 1987).

The last step in Cheddar manufacturing is moulding and pressing. Moulding is the process of forming the salted curds into a shape by the use of metal, plastic, or wooden molds. During moulding, the curds are allowed to form into a continuous mass. Pressing the moulded cheese aids in the final expulsion of whey, leading to a close-textured, well-shaped cheese.

Ripening

Textural, functional, and sensory attributes of cheese are further developed during ripening. Milk enzymes, starter bacteria, coagulant, and secondary bacteria interact through complex biochemical reactions in the curd during ripening to produce changes in flavor of the maturing cheese (McSweeney, 2000; McSweeney, 2004; Sousa et al. 2001). As a result of these complex biochemical interactions, the elastic and rubbery curd is converted into a smooth-bodied and fully flavored cheese. Depending on cheese variety and desired flavor profiles, the ripening process can take from 2 months for a mild cheddar cheese to 24 months for a sharp Parmesan. Glycolysis, proteolysis, and lipolysis are the main reactions that occur during ripening.

Glycolysis

As cheese is a fermented product, an important feature of its manufacture is glycolysis or the metabolism of lactose to lactate by selected cultures of LAB (McSweeney, 2004). Lactose degradation influences cheese flavor as a number of flavor compounds are produced including diacetyl, acetic acid, and propionic acid (Forde and Fitzgerald, 2000). In Cheddar cheese, glycolysis usually ends early in the manufacturing process once the available lactose has been metabolized. While lactose metabolism is decreased by the addition of salt during Cheddar cheese manufacture, most of the lactic acid is produced in the cheese before salting and moulding.

Proteolysis

Proteolysis is the most complex primary event that occurs during cheese ripening and plays a vital role in the development of texture and flavor (Forde and Fitzgerald, 2000; Sousa et al., 2001; Smit et al., 2005). Coagulating proteases, plasmin, and microbial proteases influence proteolysis during ripening. These proteolytic enzymes provide a mixture of small peptides and amino acids which directly change the taste of cheese (Law, 2001). Plasmin, the principal endogenous milk proteinase, is mainly responsible for the relatively limited proteolysis of α-casein in Cheddar cheese (Visser, 1993). The final pH, moisture, salt in moisture (S/M), temperature, and duration of ripening, to a large extent, control the proteolysis in cheese. The primary proteolysis results from the actions of chymosin and plasmin (Forde and Fitzgerald, 2000). Hydrolysis of caseins leads to the formation of large and intermediate-sized peptides, which are then broken down to smaller peptides by enzymes derived from starter and non-starter bacteria. Intracellular bacterial peptidases further degrade the small peptides into free amino acids.

Amino acids are degraded into flavoring compounds such as amines, aldehydes, alcohols, and ammonia (Fox et al., 1996; Law, 2001). The total amount and composition of the amino acid mixture in cheese has been used as an index of cheese ripening (Fox et al., 1995). In Cheddar cheese, it has been found that the correlation between amino acid concentration and ripening time provided an indication of cheese maturity, although the age of a cheese sample could not be predicted accurately (Grierson, 1985). If proteolysis occurs at elevated rates, it can produce heightened levels of bitter compounds and produce unsatisfactory flavors.

Accelerated Ripening

Ripening is a slow and expensive process. Lengthy ripening times cause additional cost to manufacturers, and identifying strategies to accelerate ripening and thus flavor development would benefit manufacturers significantly. Shortened storage times may increase utilization capacities of ripening rooms, increase production capacities, reduce production costs, and provide significant economic contributions to producers.

Many methods have been used to accelerate ripening which include exogenous enzymes, modified starters, use of cheese slurries, adjunct cultures, attenuated starters, and elevated temperatures (Wilkinson, 1993; Azarnia et al., 2006). Each method has its associated advantages and disadvantages. Exogenous enzymes are relatively cheap, have specific action, and have been developed to give a choice of flavor options. The use of enzymes does not come without its downfalls, as the choice of useful enzymes is rather limited, there is a risk of over-ripening, and there are difficulties with uniform incorporation. Modified starters are easy to incorporate and the natural enzyme balance can be retained; however, the modification of starters, either by physical or genetic means, is complex and costly. Adjunct cultures may have the potential to accelerate ripening, and to date, their use has been confined to the limited availability of suitable

strains such as *Leuconostoc mesenteroides* ssp *cremoris*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus curvatus*, and *Lactobacillus rhamnosus*. Ripening at elevated temperatures poses a risk in terms of the growth of unwanted microbial contaminants; however, it may be used when cheese is made from pasteurized milk under good manufacturing practices (Law, 2001; Azarnia et al., 2006).

When it comes to the addition of exogenous enzymes, exogenous lipase has proven most successful to date, especially in the ripening of hard cooked cheese (Wilkinson and Kilcawley 2005). Exogenous proteases have also been explored, but they have proven unfruitful as shifts in typical cheese proteolysis result in defects, such as bitterness and unbalanced textural and unsavory flavor profiles (Wilkinson and Kilcawley 2005).

Lipolysis

Lipases are enzymes that hydrolyze triglycerides into mono and diglycerides, glycerol, and free fatty acids (FFAs) (Sharma et al. 2001; Barron et al. 2004; Kumar et al. 2013). FFAs also act as precursor molecules for a series of catabolic reactions leading to the production of flavor and aroma compounds such as methyl ketones, lactones, esters, alkanes, and secondary alcohols (Yvonne et al., 2003).

Lipases are produced by plants, animals, and microbes. Of the various sources of lipases, microbial sources are of high commercial interest due to their relative cheapness, ease of production, and the predictability and controllability of their enzyme contents (Kumar et al 2013). Taste and flavor depend on the type of fatty acid chain released during lipolysis. Short-chain fatty acids (C4-C8) produce a cheesy flavor while high concentrations of long-chain fatty acids produce a soapy flavor (Peng et al. 2014). Animal pregastric lipase, such as kid or goat lipase, releases short-chain fatty acids, particularly butanoic acid (Arnold et al. 1975; Nelson et

al. 1977). These animal lipases are typically used in the cheese industry to give cheese a distinctive flavor and to accelerate ripening.

Lipases of LAB tend to be the principal lipolytic agents in Cheddar cheese made from pasteurized milk (Fox et al., 2000). LAB possess lipolytic enzymes capable of hydrolyzing a range of FFA, tri-, di-, and monoglyceride substrates (Holland & Coolbear, 1996; Chich et al., 1997; Fox & Wallace, 1997; Liu, Holland, & Crow, 2001). *Lactococcus* and *Lactobacillus* spp. are generally considered to be weakly lipolytic in comparison to species such as *Pseudomonas, Acinetobacter,* and *Flavobacterium* (Stadhouders & Veringa, 1973; Fox et al., 1993; Chich et al., 1997). LAB are considered to be responsible for the liberation of significant levels of FFA in cheddar cheese because they are present in high quantity during the ripening period.

Non-Animal Lipase

Demand for 'free-from' foods is on the rise as consumers become better educated and more fearful about allergies and additives. Their choices to rule out certain foods and ingredients have deep roots in special diets and ideological lifestyles such as vegetarianism and veganism (Mintel, 2020). Market research has shown that consumers are willing to go the extra mile when it comes to changing their consumption habits and choosing healthier options (Mintel, 2020). While cheese itself does not fit into a vegan lifestyle, as it is made from bovine milk, the replacement of animal pregastric lipase with a non-animal lipase is appropriate for vegetarians.

Jolly & Kosikowski (1975) studied the effect of animal and microbial lipase preparations on carbonyl concentrations in Blue cheese. They found that two of the ten *Aspergillus* lipase preparations produced Blue cheese with excellent flavor and highly acceptable texture. They concluded that the addition of animal and microbial lipase preparations increased proteolysis and lipolysis, which reduced the overall ripening time. Jolly & Kosikowski also noted that a

fragmentary knowledge existed as to the behavior of their lipases and subsequent end products and further research would be needed to understand the accelerated ripening.

De Felice et al. (1991) produced cheese using animal (kid, lamb, and calf) and microbial lipases to aid in the development of flavor. They found that the fatty acid composition differed depending on the origin of the lipase used. The addition of animal lipase tended to liberate short chain fatty acids, mainly $C_{4:0}$ and $C_{6:0}$, while the addition of microbial lipase did not show any preference for low or high molecular weight fatty acids. They concluded that the use of microbial lipase could be used to produce a product with a milder aroma compared to the untreated sample.

Both of these studies commented on the possible protease contamination of the lipase used in their research. They also did not include detailed flavor profiles or volatile analysis. My research would build upon these studies and provide further insights into the functionality and feasibility of using exogenous microbial and fugal lipase to accelerate ripening in Cheddar cheese.

Objectives

- Identify/reconfirm standards of milk used for cheese making (protein, lipid, and carbohydrates)
- Manufacture smaller batches of cheese with identical parameters while manipulating the variable of exogenous lipase
- Age cheese to various time points (2, 30, 45, 60, and 90 days)
- Determine analytical profile of cheese and evaluate the difference in fatty acid profile, volatiles, moisture, fat, and texture between the various samples
- Run QDA sensory evaluation on variations and report taste difference between processing parameters (including statistical significance). General preference ranking
- Compare results with any available published data

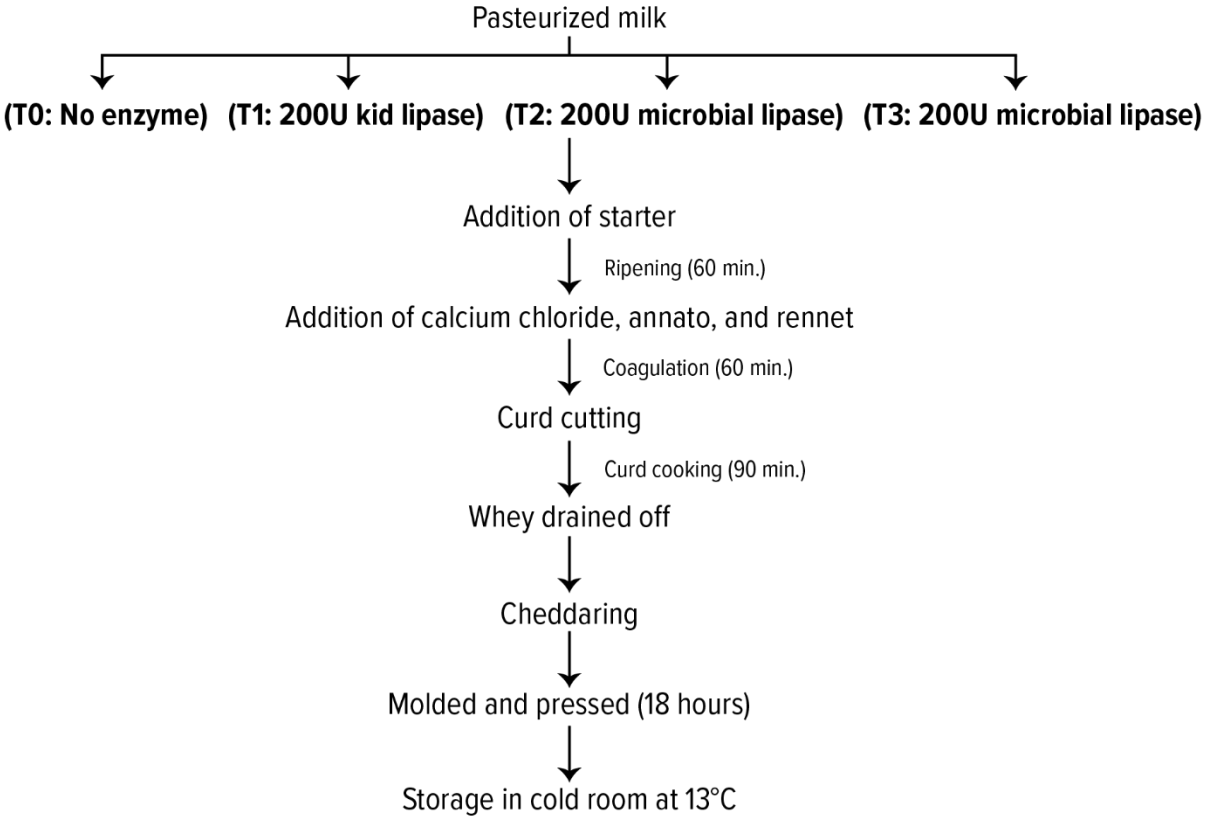
Proposed Methods

Lipase Activity

A Sigma-Aldrich Lipase Activity Assay Kit will be used to determine lipase activity. The kit will provide a simple and direct procedure for measuring lipase activity. Lipase activity will be determined using a coupled enzyme reaction, which will result in a colorimetric (570 nm) product proportional to the enzymatic activity present. One unit of Lipase is the amount of enzyme that will generate 1.0 µmole of glycerol from triglycerides per minute at 37°C.

Cheese Making

Figure 1: Experimental Plan



For the experimental plan (see Figure 1), 11.35-liter batches of cheese will be produced. All parameters will be held constant except for the addition of exogenous lipase. After the lipase has been added to the milk, the started cultures will be added and allowed to ripen and ferment. After

sufficient lowering of the pH, the calcium chloride, annatto, and rennet will be added to the milk. The mixture will then be allowed to coagulate for 90 minutes or until a clean break has formed. The curd will then be cut and cooked for approximately 90 minutes. The whey will be drained off and the curds will be cheddared for approximately 60 minutes. The curds will then be salted and moulded to further extract whey for 18 hours.

Moisture

Two to three grams of cheese will be weighed into a round, flat-bottom metal dish with a tight-fit, slip-in cover. The loosely covered dish will be placed in a vacuum oven and kept at 100°C. It will be dried to constant weight (ca 4 h) under pressure ≤100 mm Hg (13.3 kPa). During drying, a slow current of air will be admitted into the oven. When drying is complete, the dish will be removed from the oven, cooled in a desiccator, and weighed. (AOAC 926.08)

Fat

One gram of cheese will be weighed into an extraction flask and 9 mL H2O will be added. The mixture will then be warmed at low heat until casein is well softened. Ten mL HCl and a few glass beads will be added, and the mixture will boil gently for 5 min. The solution will be cooled and transferred to a fat-extraction flask or tube; rinsed successively with 10 mL alcohol, 25 mL ether, and 25 mL petroleum ether (boiling range 30°–60°C); and transferred to flask. The mixture will then be centrifuged to obtain a clear separation of phases. The ether solution will be decanted, and two more extractions will take place. After the solvent has been evaporated, the extracted fat will be dried in a forced-air oven and weighed. (AOAC 933.05)

Texture

A Warner–Bratzler Blade will be used to shear the cheese samples. The texture will be analyzed by using a Texture Analyzer. One cheese sample will be sheared under the following experimental conditions: Load cell 50 kg, cross head speed 50 mm/min.

Microstructure

Cheese samples of 2, 30, 45 and 60 days of ripening will be lyophilized to remove the water content, set in an osmium tetroxide vapor, and coated in gold for microstructure analysis using a Scanning Electron Microscope (SEM) to observe the casein matrices, fat globules, aggregated fat globules and non-fat globules. (Drake et al., 1996; Madadlou et al., 2005)

Trained Sensory Panel

A trained panel will be used to evaluate key sensory properties of the cheese. Participants of the trained panel will evaluate flavor (odor and taste), body and texture, mouth feeling, saltiness, sharpness, and overall likability along with other attributes determined from a developed cheese lexicon. The panel will be trained in multiple hour-long training sessions so they can uniformly rate each attribute. Cheddar cheese flavor language developed by Drake et al. (2001) will be used.

Fatty Acid Analysis

Lactic acid will be removed by a partition pre-column followed by isolation of FFA on a modified silicic acid–potassium hydroxide arrestant column. FFA will then be separated in formic acid- mobilized elutes on a glass column packed with diethylene glycol succinate (DEGS-PS) by GC. This GC procedure will enable rapid separation and quantification of FFA (Woo et al., 1982).

GC Volatiles

The determination of volatile components will be performed by solid-phase microextraction coupled with gas chromatography–mass spectrometry (SPME–GC-MS). Prior to analysis, cheese samples will be sliced, frozen in liquid nitrogen, and then pulverized into small granules and stored at −20°C. Three grams of each cheese will then be placed in a 15 mL vial and allowed to equilibrate at 40°C for 30 min. Extraction of the volatiles will be carried out by injecting a carboxen-polydimethylsiloxane fiber into the vial and exposing it to the headspace for 30 min at 40°C. Samples will be desorbed onto an Agilent FFAP column, 50 m × 0.2 mm × 0.33 μm. The oven will be held at 40°C for 2 min, then increased at 5°C per minute to 70°C, where it will be held for 2 min. The temperature will then be increased at 10°C per minute to 240°C and held to give a run time of 35 min. The mass spectrometer will be set to record 33 to 450 amu (threshold 1,000) at a sampling rate of 1.11 scans per second. The components will be identified and a database will be set up to quantify relative amounts of each (Coda et al., 2006).

Meltability

A Rapid Visco Analyzer (RVA) will be used to measure the meltability of the cheese. This machine will slowly heat and cool the cheese while measuring and recording the apparent viscosity.

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Table 5. Quantitative Descriptive Analysis Raw Data. Flavor attribute intensities of experimental Cheddar compared to a control with no exogenous lipase. A Tukey's HSD Test* determined the significance of observed differences. Different superscripts in a row represent significant differences between means (n=7, $p<0.005$).

Attribute	Lipase Level	Days Aged	Control	CC	AN	MJ	KGL
Cooked	30 U	30	3.5^{A} ±0.04	3.3^{B} ±0.07	3.5^{A} ±0.03	3.4^{A} ±0.09	3.5^{A} ±0.04
		60	3.5^{A} ±0.06	3.2^{B} ±0.10	3.5^{A} ±0.04	3.5^{A} ±0.04	3.5^{A} ±0.06
	75 U	30	3.5^{A} ±0.06	3.7^{B} ±0.10	3.5^{A} ±0.05	3.5^{A} ±0.04	3.8^{B} ±0.04
		60	3.5^{A} ±0.05	3.7^{B} ±0.08	3.5^{A} ±0.05	3.5^{A} ±0.00	3.7^{B} ±0.12
Whey	30 U	30	3.2^{AB} ±0.07	3.1^{AC} ±0.12	3.3^{B} ±0.05	3.1^{AC} ±0.09	3.0^{C} ±0.05
		60	3.0^{A} ±0.04	3.0^{A} ±0.22	3.3^{B} ±0.09	2.9^{A} ±0.16	2.9^{A} ±0.08
	75 U	30	3.1^{A} ±0.05	3.2^{A} ±0.05	3.1^{A} ±0.09	3.1^{A} ±0.09	2.8^{B} ±0.06
		60	3.0^{A} ±0.08	2.7^{B} ±0.14	2.9^{AC} ±0.11	3.0^{A} ±0.02	2.8^{BC} ±0.05
Diacetyl	30 U	30	0.0^{A} ±0.00	0.7^{B} ±0.16	0.8^{B} ±0.13	0.7^{B} ±0.19	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.8^{B} ±0.15	0.8^{B} ±0.21	0.0^{A} ±0.00
	75 U	30	0.6^{A} ±0.20	1.6^{B} ±0.19	1.3^{C} ±0.10	1.0^{D} ±0.13	0.7^{A} ±0.17
		60	1.0^{A} ±0.08	1.0^{AB} ±0.05	1.2^{B} ±0.20	0.8^{C} ±0.17	0.0^{D} ±0.00
Milk fat	30 U	30	2.4^{A} ±0.08	2.5^{A} ±0.04	2.5^{A} ±0.02	2.5^{A} ±0.02	2.5^{A} ±0.04
		60	2.6^{A} ±0.12	2.5^{A} ±0.03	2.5^{A} ±0.04	2.6^{A} ±0.07	2.5^{A} ±0.04
	75 U	30	2.5^{A} ±0.05	2.8^{B} ±0.04	2.7^{B} ±0.07	2.6^{C} ±0.06	2.8^{B} ±0.05
		60	2.5^{A} ±0.04	2.6^{A} ±0.06	2.5^{A} ±0.05	2.5^{A} ±0.05	2.6^{A} ±0.06
Fruity	30 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
	75 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00

Sulfur	30 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
	75 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
FFA	30 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	2.2^{B} ±0.10	2.6^{C} ±0.15
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	2.4^{B} ±0.28	2.9^{C} ±0.15
	75 U	30	0.0^{A} ±0.00	1.1^{B} ±0.09	0.8^{C} ±0.02	1.1^{B} ±0.13	1.7^{D} ±0.21
		60	0.0^{A} ±0.00	1.3^{B} ±0.31	0.9^{C} ±0.28	1.7^{D} ±0.19	2.5^{E} ±0.12
Brothy	30 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	1.0^{A} ±0.13	0.0^{B} ±0.00	0.7^{C} ±0.23	0.0^{B} ±0.00	0.0^{B} ±0.00
	75 U	30	0.0^{A} ±0.00	0.5^{B} ±0.09	1.0^{C} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.6^{B} ±0.20	1.1^{C} ±0.08	0.0^{A} ±0.00	1.1^{C} ±0.13
Nutty	30 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
	75 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
Sour	30 U	30	4.2^{A} ±0.07	4.0^{B} ±0.11	4.3^{A} ±0.05	4.2^{A} ±0.07	4.2^{A} ±0.06
		60	3.8^{A} ±0.05	4.1^{B} ±0.09	4.1^{B} ±0.07	4.1^{B} ±0.11	4.3^{C} ±0.05
	75 U	30	4.3^{AB} ±0.07	4.3^{A} ±0.06	4.1^{C} ±0.06	4.4^{B} ±0.06	4.3^{AB} ±0.07
		60	4.2^{AB} ±0.05	4.1^{AC} ±0.07	4.1^{C} ±0.11	4.4^{D} ±0.05	4.3^{BD} ±0.04
Bitter	30 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
	75 U	30	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
		60	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00	0.0^{A} ±0.00
Salty	30 U	30	2.6^{AB} ±0.30	2.4^{A} ±0.20	2.6^{AB} ±0.28	2.8^{B} ±0.23	2.6^{AB} ±0.41

		60	3.1^{A} ±0.09	2.8^{B} ±0.17	3.0^{AB} ±0.13	3.0^{AB} ±0.00	3.2^{A} ±0.14
	75 U	30	2.8^{A} ±0.09	3.2^{B} ±0.06	3.0^{AB} ±0.00	3.1^{AB} ±0.07	3.0^{AB} ±0.04
		60	3.0^{A} ±0.08	3.0^{A} ±0.16	2.9^{A} ±0.08	3.1^{A} ±0.07	3.0^{A} ±0.05
Sweet	30 U	30	1.7^{A} ±0.19	1.7^{A} ±0.25	1.8^{A} ±0.15	1.8^{A} ±0.22	1.7^{A} ±0.33
		60	1.7^{A} ±0.17	1.7^{A} ±0.17	1.7^{A} ±0.08	1.6^{A} ±0.13	1.7^{A} ±0.11
	75 U	30	2.0^{A} ±0.07	2.0^{A} ±0.00	2.0^{A} ±0.00	2.0^{A} ±0.00	2.0^{A} ±0.04
		60	2.0^{A} ±0.06	2.0^{A} ±0.06	2.1^{A} ±0.11	2.0^{A} ±0.04	2.0^{A} ±0.24
Umami	30 U	30	2.1^{A} ±0.09	2.0^{A} ±0.04	2.1^{A} ±0.09	2.0^{A} ±0.18	2.1^{A} ±0.08
		60	2.2^{AB} ±0.04	2.0^{C} ±0.04	2.1^{AC} ±0.10	2.2^{ABC} ±0.07	2.3^{B} ±0.05
	75 U	30	2.8^{A} ±0.08	3.0^{B} ±0.02	3.0^{B} ±0.06	3.1^{B} ±0.06	3.1^{B} ±0.10
		60	2.9^{A} ±0.04	3.0^{A} ±0.04	3.0^{A} ±0.04	3.0^{A} ±0.05	3.2^{B} ±0.06