Nutritional and Volatile Characterisation of Milk Inoculated with Thermo-Tolerant *Lactobacillus bulgaricus* Developed from Adaptive Laboratory Evolution

Jiahui (Hedy) Liang

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List of abbreviations

ALE: Adaptive Laboratory Evolution

ANOVA: One-Way Analysis of Variance

ATP: Adenosine triphosphate

E.coli*: Escherichia coli*

EMP: Embden-Meyerhof-Parnas glycolytic pathway

EPS: Exopolysaccharides

FAAs: Free amino acids

FSANZ: Food Standard Australia New Zealand

FtsZ: Filamenting Temperature-Sensitive Mutant Z

GC-MS: Gas Chromatography-Mass Spectrometry

HTS: High-throughput sequencing

LAB: lactic acid bacteria

L. bulgaricus*: Lactobacillus delbrueckii subsp. bulgaricus*

LCMS: Liquid Chromatography-Mass Spectrometry

LMRS: De Man, Rogosa and Sharpe agar with 2% lactose

MRS: De Man, Rogosa and Sharpe agar

NADH: nicotinamide adenine dinucleotide

OAV: Odour Threshold and relative Odour Activity Value

PIG: phenotypically important genes

S. cerevisiae*: Saccharomyces cerevisiae*

SPME: Solid Phase Microextraction

SNP: single-nucleotide polymorphism

S. thermophilus*: Streptococcus thermophilus*

VOCs: Volatile organic compounds

WHC: Water holding capacity

WGS: whole-genome sequencing

Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, 'Nutritional and volatile characterisation of yoghurt developed from Adaptive Laboratory Evolution of *Lactobacillus bulgaricus*', contains no material previously published or written by another person (expect where explicitly defined in the acknowledgements) nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Name: Jiahui (Hedy) Liang

Signed:

Date: 03/11/2021

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Abstract

Lactic acid bacteria (LAB) has been studied excessively to create positive changes in nutritional and sensorial properties in yoghurt products. The aim of this approach is to meet market demand for novel dairy products and increase cost efficiency and productivity. Creating new LAB strains with distinct functional properties such as stress tolerance and molecule production would be one of the most efficient ways to meet the requirement motioned above. A mutant strain of *Lactobacillus bulgaricus (L.bulgaricus)* was developed by increasing temperature gradually with Adaptive Laboratory Evolution (ALE). A viable count of the control strain on De Man, Rogosa and Sharpe agar (MRS) agar with 2% lactose (LMRS) was 4.66 ± 0.59 log cfu/mL. The colony count of mutant strain was 1.87 ± 0.98 log cfu/mL. The morphology of the mutant changed became more elongated when compared to the wild type strain as the incubation temperature increased to 52°C. This morphology change remained stable after storing the samples at -80°C for 3 weeks. When mutant *L.bulgaricus* was used to produce yoghurt, lactic acid was absent, resulting in a pH of 6.84± 0.13 compared to the control (pH 4.55 ± 0.04). The low acid production has caused a weakened protein network, and shrinkage of the milk gel structure, resulting with a significantly lower water holding capacity of 37.1 ± 0.35% compared to the control (98.10 ± 0.60). Free amino acids analysis by LC-MS showed high proteolytic activity. Arginine and methionine were found 115 and 275 folds higher than the control, (237.24 ± 5.94, 98.83 ± 1.78 μg/100g), which changed the aroma of the yoghurt to be more like cheese. VOCs analysis through SPME-GC-MS discovered both control (313.62 ± 0.20 μg/L) and mutant yoghurt samples (844.79 ±0.13 μg/L) showed high diacetyl production. In the mutant yoghurt samples, esters such as ethyl butanoate, ethyl butanoate, and ethyl decanoate were higher than the control strain, contributing to a more cheesy-like

flavour. These suggest that applying ALE to probiotics produce new peptides and free amino acids to increase the nutritional value of dairy products.

Chapter 1. Introduction

Lactic acid bacteria (LAB) use lactose in milk to produce lactic acid during the fermentation process in the production of yoghurt. The pH is reduced from 6.8 to 4.5 during the fermentation process, resulting in the formation of the milk gel network due to changes in the casein protein structures. During fermentation, factors such as LAB strain type, incubation time, and temperature all play a significant role in stabilising the interactions of casein micelles and forming a three-dimensional gel network (Puvanenthiran, Stevovitch-Rykner, McCann, & Day, 2014). During incubation, exopolysaccharides (EPS) produced by the LAB bind with proteins, resulting in a variety of textures. Some LAB strains used in the production of yoghurt are considered probiotics because they may benefit the gut microbiome and overall health. As a result, the consumer preference for probiotic dairy products has grown (Hekmat & Reid, 2006). The most common probiotic strains are the genera *Lactobacillus* and *Bifidobacterium* (Heller, 2001). Different countries have different requirements in using LAB in yoghurt production. For example, in Australia and New Zealand, Food standard Australia New Zealand (FSANZ) (2015) requires dairy product fermentation with two particular bacteria: *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus (L. bulgaricus*).

L.bulgaricus is facultative culture but prefers an anaerobic environment that uses the Embden-Meyerhof-Parnas glycolytic pathway (EMP) (Panesar, Kennedy, Gandhi, & Bunko, 2007). *L. bulgaricus* produces hydrogen peroxide during lactic acid fermentation by oxidation of reduced NADH to NAD+, which results from the conversion of pyruvate to lactate. As a homofermentative lactic acid bacteria, it produces lactic acid as the primary by-product, as well as proteolytic and diacetyl, EPS, which contribute to the acidity and thickness of yoghurt, and organic acid and aromatic compounds (Hatti-Kaul, Chen, Dishisha, & Enshasy, 2018).

The proteolytic and lipolytic properties of LAB are critical in the degradation of proteins and lipids. During proteolysis, the enzyme uses intracellular and extracellular enzymes to break down the milk protein network, release free amino acids, and reduce sapid compounds. Lactate is the primary product of LAB fermentation, other flavour compounds such as diacetyl, acetoin, acetaldehyde, or acetic acid create distinct flavour profiles for *Streptococcus thermophilus and L. bulgaricus* yoghurts.

Diacetyl is widely used as a flavouring agent in food and beverages. Diacetyl formation is one of the most important flavour compounds in yoghurt and can be detected by consumers at low concentrations. Diacetyl can be identified by its distinctive buttery aroma in amounts as low as 1 mg/kg. and in commercial yoghurt products, the range of diacetyl in yoghurt products ranges from 0.2 to 3mg/kg for a balanced buttery flavour (Chen et al., 2017; Guerra, Hernández, Estepa, & Rivas, 1995).

Recently, the use of various LAB has been tested in order to accelerate acidification and improve the health benefits of yoghurt (Bintsis, 2018). This approach has also contributed to favourable changes in nutritional and sensorial properties in yoghurt products, in order to meet the market demand for novel dairy products.A growing number of studies have demonstrated the selection of new LAB strains with distinct functional properties, such as stress tolerance and molecule production (enzymes, polysaccharides, aromatic compounds) (Liu, Chan, Chen, Solem, & Jensen, 2019; Papadimitriou et al., 2016).

Temperature, pH, osmotic stress, and other variations during fermentation are all examples of stress tolerance (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). When fermentation first begins, the osmotic pressure changes due to the high concentration of solutes in the medium, which may cause cell exposition, resulting in water efflux from the cell and inactivation of the critical enzymes of microbial metabolism (Mitchell et al., 2009). pH and temperature change later in the fermentation

process was due to acid products and incubation temperatures. The culture concentration and temperature, in particular, have a significant impact on the physicochemical properties of the end products in yoghurt (Aldaw Ibrahim, Naufalin, Erminawati, & Dwiyanti, 2019). *Acetobacter rancens, A. lovanienis, A. xylinum,* and *Lactobacillus plantarum* have demonstrated pH resistance by changing membrane composition and increasing proton efflux and amino acids, catabolism, and the formation of DNA repair enzymes (Gullo, Verzelloni, & Canonico, 2014; Hayek & Ibrahim, 2013; Larsen, Werner, Vogensen, & Jespersen, 2015; Narendranath & Power, 2005; Panesar et al., 2007). Based on the limitations on yoghurt production, obstacles such as maintaining its viability during the processing,storage, cost efficacy, and resistance to the physicochemical processing must be considered (GuhanNath, Sam Aaron, Raj, & Ranganathan, 2014). These limitations can be overcome by genetic engineering the strains. However, careful consideration of use is required to ensure that the developed strains are appropriate for the intended purpose and safe for consumption.

Adaptive Laboratory Evolution (ALE) has been used in several studies to control evolution and create strains with distinct characteristics. However, in the early 1950s, there were disagreements about the accuracy of continuous culturing due to a lack of a generally accepted theoretical foundation, difficulty maintaining sterility during long runs, and the possibility of mutations (Herbert, Elsworth, & Telling, 1956). ALE. is believed to be methodologically straightforward in order to investigate the effect of evolutionary forces on strain phenotypes, performance, and stability (Sandberg, Salazar, Weng, Palsson, & Feist, 2019). The growth rate and production of metabolites such as lactic acid and other desirable flavour compounds would be used to evaluate LAB's fitness level. The primary parameter in replicating the dynamics of the surrounding environment is changing in the level of fitness of the samples. With the quantification of

the samples, these factors have become the long-time goal for evolutionary theory (Eyre-Walker & Keightley, 2007). ALE is thought to be a simple and straightforward method for investigating the effect of evolutionary forces on strain phenotypes, performance, and stability (Sandberg et al., 2019). Examples such as ALE has been used to create new brewing yeast for ale and lager to improve the brewing efficiency and beer quality; application on *Escherichia coli* (*E.coli*) for developing freeze-thaw for baker's yeast and *Lactobacillus rhamnosus* through freeze-thaw to select tolerant strains (Aguilera, Andreu, Randez-Gil, & Prieto, 2010; Gibson et al., 2020; Kwon, Bae, Kim, & Han, 2018). Applying ALE to *L.bulgaricus* in yoghurt production is a novel approach. Previous studies on ALE have proved promising results in terms of reducing obstacles and increasing the productivity and quality of various products. Furthermore, the future application of ALE does not only apply to lactobacillus but also to wild starter strains that have not yet been refined by ALE or any genetic modification but may still be of interest in terms of yoghurt fermentation.

The aim of the present study was to use ALE on *L.bulgaricus* to create a mutant strain by gradually increasing the incubation temperature. The study also aimed to use the mutated strain to make yoghurt and investigate any changes in the microbiological, physicochemical, sensorial, and nutritional properties of the yoghurt.

Chapter 2 Literature review

2.1.Metabolic pathways of Lactic acid bacteria

Heterofermentation and homofermentation are the two primary fermentation pathways for LAB. Homofermentative metabolism of carbohydrates results in having lactate as the sole and the primary product of the metabolism (Figure 1). This metabolic pathway is primarily anaerobic and aerobic, as long as the fermentable carbohydrates are rich. Most of the fermentation in the food industry is related to glycolysis pathways, also known as Embden-Meyerhof-Parnas glycolytic pathway (EMP) (Hatti-Kaul et al., 2018). Glycolysis is the metabolic pathway that converts glucose into pyruvate with nicotinamide adenine dinucleotide (NADH) and Adenosine triphosphate (ATP) with the end products such as lactic acid, ethanol, acetic acid. In lactic acid fermentation, strains like *Lactobacilli* produce hydrogen peroxide through oxidation of reduced NADH to NAD^+, resulting from the conversion of pyruvate into lactate. When lactic acid is the only product from fermentation, the process is known as homolactic fermentation. *L. delbrueckii ssp. Bulgaricus* is used to produce lactic acid, proteolytic and diacetyl, exopolysaccharide for products' acidity and thickness texture.

In heterofermentative metabolism, metabolites such as ethanol, carbon dioxide or aroma compounds use the phosphoketolase pathway (PKP) using glucose as the carbon source (Årsköld et al., 2008).

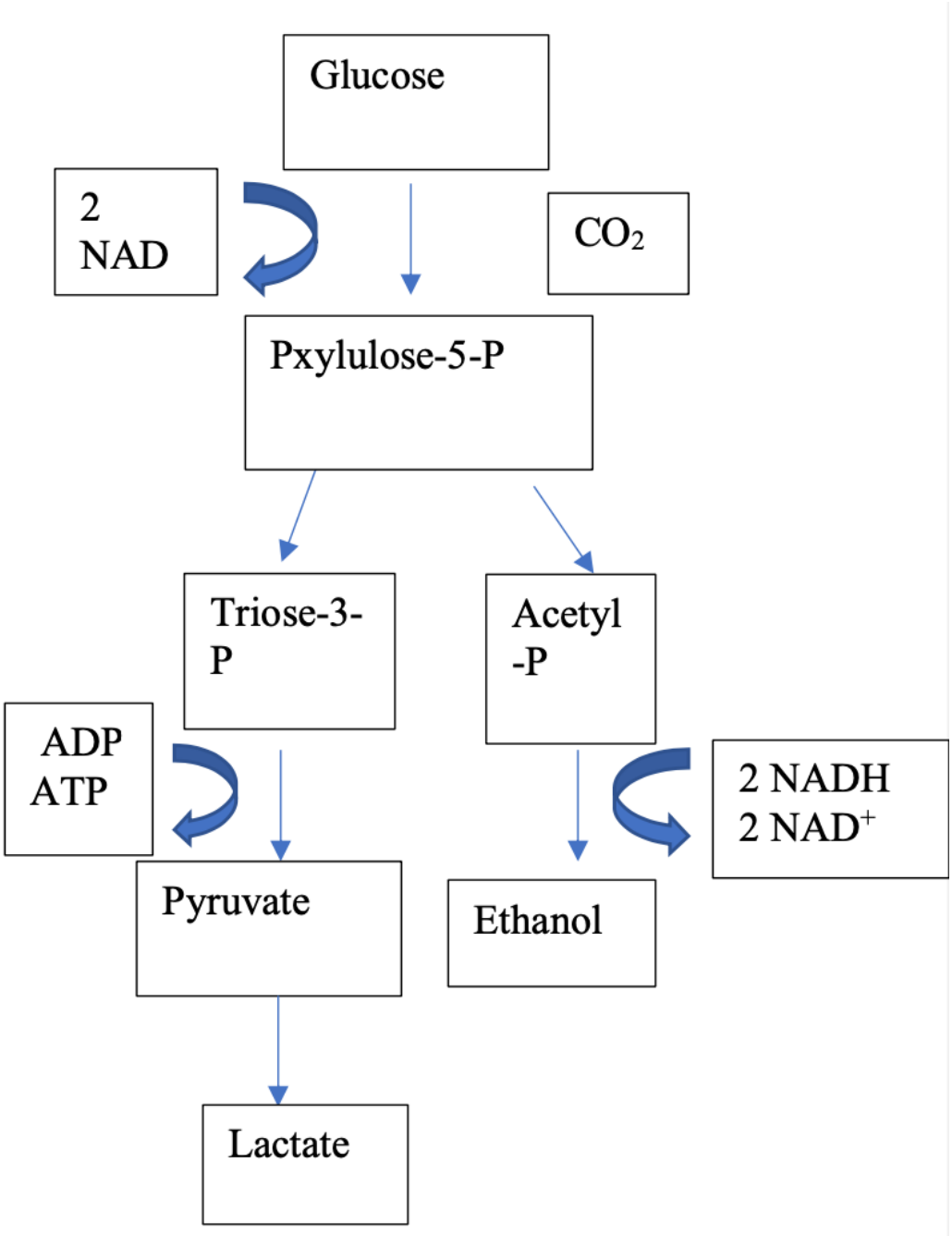


Figure 1: Heterofermentative metabolism of hexose through phosphoketolase pathway. Modified from (Papagianni, 2012b). ADP: Adenosine diphosphate; ATP: Adenosine Triphosphate; NAD: Nicotinamide adenine dinucleotide; NADH: nicotinamide adenine dinucleotide (NAD) + hydrogen (H).

2.2. Natural selection and evolution of microorganisms

In nature, evaluation has many forms and can be contributed by adaptation, driven by the principles of natural selection, genetic drift, or maladaptive (Brady et al., 2019) Bajic and Sanchez (2020) suggested that the nutrient preference and co-utilisation pattern define the metabolic strategy, determining species coexistence or exclusion. Similar results suggested that wild yeast strains have different growth responses to different sugar environments, resulting in variations in evolutionary pressures (New et al., 2014).

One crucial finding regarding the growth rate of microorganisms is transcriptional reprogramming. The result suggested that the gene expression and growth to changing carbon source of some wild yeast strains can adapt rapidly and uniformly. In contrast, the lag phase of the slow-switching strain of the *Saccharomyces cerevisiae (S. cerevisiae)* mutant in the experiment was shortened by repeatedly alternating the strain between carbon sources. For LAB transcriptional reprogramming, despite the number of studies on this topic, the understanding of adaptive strategies on the final expressions of genomic information is still unclear. Although through thousands of years, people have been improving the use of LAB by backslopping methods, which is adding old fermented food into a new batch of fermentation, resulting in adapting the microbial with better yield, improved properties (Dimidi, Cox, Rossi, & Whelan, 2019). Backslopping can be somehow viewed as the ancient way of reprogramming through adaptation. The amount and the depth of this topic have been reached compared to microbes such as *Escherichia coli* (*E. coli)*, where it has been used and developed in many other categories. DNA sequence data can help identify these mutations. The mutation accumulation experiment is used to investigate the rates and effects of spontaneous mutations. The majority of mutations appear to have an impact

that is too small to detect in the laboratory (Dubernet, Desmasures, & Guéguen, 2002).

2.3. Microbial domestication

Yeast is the most well-known domesticated microbe that are commonly used in beer, wine and bread making. In the past 13,000 years of human history, plant and animal domestication plays a significant role in the development of civilization and transformed global demography (Diamond, 2002). The two main sets of unpredictable consequences from domestication are changes in plants and animals, and changes in human behaviour are two sets of changes. For instance, hunters in the past selected, gathered and brought back wild plants and manage wild animals for a very long time. However, until such time as the end of the Pleistocene, hunter and gatherers' behaviour has changed for to the fact that the different factors that increased the risk of unpredictable variation in the food supply. The result of the changes have led to more productive habitats and intentional cultivation, and led to what we have now an agriculture society (Diamond, 2002; Hillman & Davies, 1990).

Microbial domestication is a genetic modification of a species. The modification aims to enhance the utility to humans by isolating and breeding a particular species from its ancestral population (Diamond, 2002). The human diet is stable, abundant, simplified and less competitive, using an artificial selection that leads to microbial genome optimisation to suit human digestion (Gibbons & Rinker, 2015). The idea of microbial domestication in the food industrial bacteria, such as LAB, can be considered a competition of "level of fitness" among the different microbial under a controlled environment, such as chemically modified medium, and monitored growth temperature. Microbial diversity as one of the significant products of domestication due to the reason that the fact that the requirement of different manufactured environments created over time. After thousand years of domestication, the LAB strains have new factorable

changes which benefit the modern industrial needs, such as high yielding of pure cultures with higher survival rates.

2.3.1. Criteria for the starter culture

Selection criteria for starters differ depending on the purpose and requirements of the products. Microorganisms chosen for use as starter cultures are expected to have characteristics such as adaptability to raw materials and processes, sensory quality development, elongating shelf life, and the efficacy of processing time and energy consumption during production (Corsetti, Perpetuini, Schirone, Tofalo, & Suzzi, 2012). Before selecting microorganisms in fermentation, several tests have to be performed to identify possible candidates. These tests include screening for stressful fermentation conditions, identifying crucial metabolite producers, and evolving technological parameters (Vinicius De Melo Pereira et al., 2020). Different variations of factors such as temperature, pH and osmotic stress are some of the major stress tolerances during fermentation. When fermentation starts, the osmotic pressure changes the reason that the fact that high concentration of solutes in the medium, may lead to cell exposition, causing water efflux from the cell, causing inactivation of the critical enzymes of microbial metabolism (Mitchell et al., 2009). Later on, pH and temperature change for the reason that acid products and incubation temperatures during the fermentation process. For yoghurt, the culture concentration and temperature affect the physicochemical properties of the end products. Knowing the desired metabolic products such as secondary metabolites, ethanol, acetic acid, and lactic acid from the microorganism determines the outcome of the final product, such as alcoholic beverages, vinegar, or yoghurt.

2.3.2. Bacterial genome responses under different stress

Genes in bacteria that change expression in response to environmental stressors are frequently assumed to be phenotypically important. However, according to different studies, differentially expressed genes are rarely phenotypically significant (Birrell et al., 2002; Giaever et al., 2002; Jensen, Zhu, & van Opijnen, 2017).

Jensen et al. (2017) further explained that the type of stressor impacts whether transcriptional patterns may be used to predict phenotypically essential genes (PIG). Transcriptional profiles of stressors, such as food deprivation for a metabolic network, identify differentially expressed genes presumably connected to a nearby PIG. After an incoherent stimulus (such as antibiotics), differentially expressed (metabolic) genes may not be related to any PIGs or pathways.

Genetic interaction analysis enables the linking of genes and the expansion of knowledge of such networks on a genome-wide scale. Tests on genetic interactions and the fitness effects of gene mutations must be evaluated. Food regulatory and public health agencies are using whole-genome sequencing (WGS) to help detect, investigate, and control foodborne outbreaks and food-related regulations (Brown, Dessai, McGarry, & Gerner-Smidt, 2019). WGS generates the most comprehensive data set on an individual's genome, provides a broader range of coverage of the exome, detects intronic variants, and creates calling all structural variants, including single exon deletions (Gilissen et al., 2014; Sun et al., 2015). Analytical post-sequencing approaches such as base by base single-nucleotide polymorphism (SNP) analysis. SNP is a nucleotide difference that occurs due to a genetic mutation event at a specific position in the genome of a test strain relative to the reference strain sequence (Brown et al., 2019; Stucki & Gagneux, 2013). However, the capture kit used, sequencing design, bioinformatic pipeline, and selected gene panel all significantly impact using WGS and SNP to identify stress response of bacterial mutation.

2.4. LAB genome engineering

In the past, genome engineering for LAB was limited to *S. cerevisiae* and *E. coli.* In 2002, extensive sequencing of the genomes of LAB was announced by the Lactic Acid Bacteria Genome Sequencing Consortium (Klaenhammer et al., (2002). The program had a breakthrough with expanding the number of completed genome sequences. The rage was increased from 19 complete genomes of different strains of five streptococci species only to 18 more complete genome sequences of 14 species from the *Lactobacillales* by 2006 (Makarova & Koonin, 2007).

The genome engineering for LAB is mainly focused on food-grade because of the restrictive legislation and consumers' preference of non-genetically modified organisms in food. However, ALE has been considered a non- Genetically modified organism (GMOs) as the random mutagenesis for strain improvement and the natural selection aspects. As mentioned before, for that aspect of the random mutagenesis of LAB genome engineering, advanced technologies such as high-throughput sequencing (HTS) have been developments of genome editing tools concentrated on speed, targeting, and a more stable genomic modification (Walsh, Crispie, Claesson, & Cotter, 2017). However, studies and experiments on LAB is a new discipline, especially in the application in the food industry. Much more work is required to explore the possibilities for the improvement of LAB genome engineering.

2.4.1. Mutations and selection (ALE)

Natural selection and evolution are preferred techniques for strain improvements with causal mutations occurring within the microorganisms (Derkx et al., 2014; Johansen, 2018). In the early 1950s, controversies on the accuracy of continuous culturing have surfaced: lacking a generally accepted theoretical background and difficulty maintaining

sterility during long runs and the probability of mutations (Herbert et al., 1956). ALE is considered simple to investigate the effect of evolutionary forces on strain phenotypes, performance, and stability (Sandberg et al., 2019). The activity measurement would indicate growth rate, production of metabolites (such as lactic acid in LAB), and antibiotic resistance. Changes in the level of fitness of the samples are the primary parameter in replicating the dynamics of the surrounding environment. With the quantification of the samples, these factors have become the long-time goal for evolutionary theory (Eyre-Walker & Keightley, 2007).

Although ALE is evolutionary engineering that can modify bacteria strain by imitating natural evolution, the metabolic reaction is that microorganisms have complex interactions, resulting from different outcomes such as low product yield and growth rate. Factors such as pH, the concentration of nutrients, metabolic products and oxygen level remain constant and may be independently controlled by researchers (Table1).

2.5. Ingredients and manufacturing process of yoghurt

2.5.1 Ingredients for yoghurt manufacture

Pasteurization, fermentation, processing, and storage alter the underlying flavour of yoghurt, which is mainly derived from the volatile compounds in cow's milk. Milk is the primary producer of many volatile chemical compounds found in yoghurt (Routray & Mishra, 2011). The high temperature of raw milk before starter inoculation helps to destroy the potential competition for starter bacteria also improve the firmness of yoghurt gel, as whey protein denatured through high temperature (Lee & Lucey, 2003). Homogenization helps prevent cream separation during fermentation and cooling periods and improves the consistency and smoothness of yoghurt (Massoud, Belgheisi, & Massoud, 2016). The following ingredients skim milk, casein, cream, and whey proteins may be used in yoghurt production to provide rheological properties, such as mouthfeel,

viscosity, and consistency (Fazilah, Ariff, Khayat, Rios-Solis, & Halim, 2018; Vinderola, Costa, Regenhardt, & Reinheimer, 2002). The flow properties of the yoghurt samples were favoured by higher fat levels, creating more stable viscoelastic gel networks. Sensory features were valued for samples with a higher fat content (Vasilean, Aprodu, & Patrascu, 2015). However, yoghurts with lower fat content (0.2%) were found to produce volatiles faster and with greater intensity, but with less persistence, than yoghurts with higher fat content (3.5 and 10%) (Brauss, Linforth, Cayeux, Harvey, & Taylor, 1999).

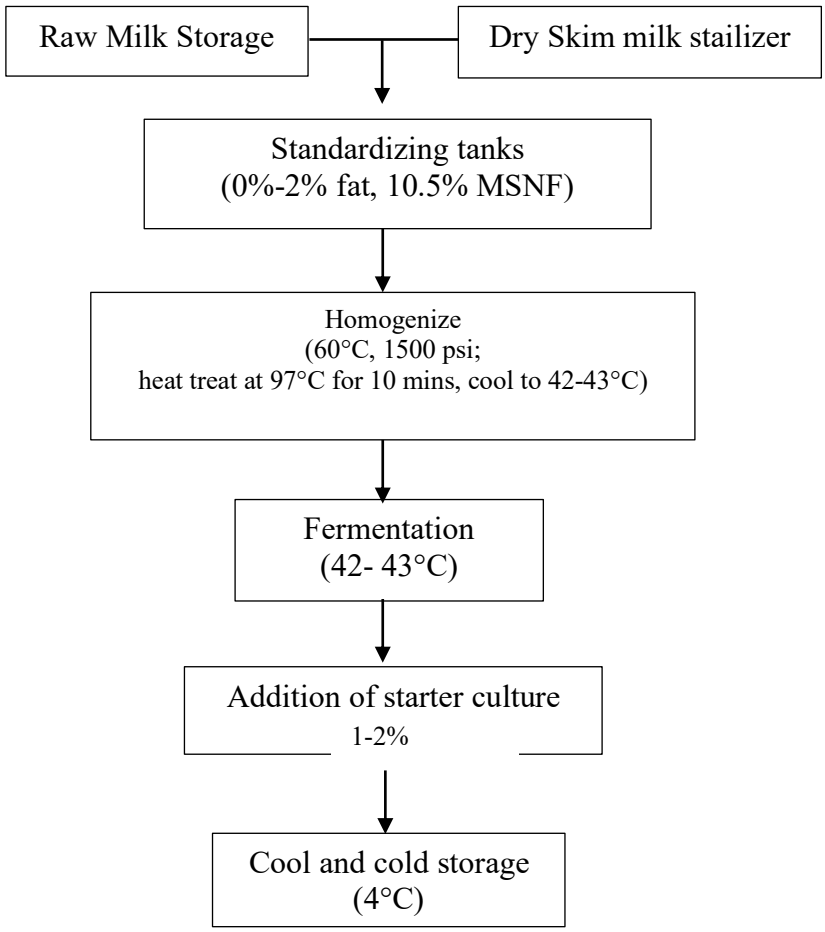


Figure 2: Major manufacturing steps in set yoghurt production. (Massoud et al., 2016)

2.5.2. Incubation and fermentation of yoghurt

The primary role of starter culture is to promote a faster, more consistent fermentation of the final product (Vinicius De Melo Pereira et al., 2020). *Saccharomyces* and *Lactobacillus* are the two groups of bacteria most commonly used in fermented food products. Most of the LAB are under the *Lactobacillales*, which is known as nonsporulating, gram-positive bacteria, with a few species, belong to the *Actinobacteria* Phylum (Makarova & Koonin, 2007).

L. bulgaricus is a common microorganism found in yoghurt with a growth temperature range from 42 to 43°C. Commercial yoghurt is usually stored in a refrigerated environment at 4°C to ensure the quality of the yoghurt; however, the critical control of storing temperature has also led to a potential problem such as stabilizing probiotics with

a higher survival rate during the cool transportations. In recent years, the mixture of different starter bacteria has been to fasten the acidification during fermentation. The requirements of using mixed cultures have been advanced since there are more desired probiotics are added to improve the health benefits of the yogurt and not necessarily for fermentation. Manufacturing processes used to manufacture dairy products need to be modified to meet the new requirement (Dragosits & Mattanovich, 2013; Høier et al., 2010). There have been difficulties in the differential enumeration of probiotics and yoghurt starter due to the microorganisms' overlapping biochemical profiles and growth requirements (Tabasco, Paarup, Janer, Peláez, & Requena, 2007). Novel bateria types, growth mechanisms, and applications are currently being investigated, and they have the potential to change scientific understanding as well as the nutritional and healthcare applications of these interventions (Cunningham et al., 2021).

2.5.3. Texture and flavour perception affected by LAB

The proteolytic and lipolytic properties of LAB play essential roles in protein and lipid degradation. During proteolysis, the enzyme breaks down the milk protein network and releases free amino acids, this reduces sapid compounds with intracellular and extracellular enzymes of LAB. Resulting attributes to the texture of the final product. Lipolysis release fatty acid, which creates the flavour profile of cheese (Heller, 2001; Karaca & Güven, 2018). Lactate is the primary product of LAB fermentation, other flavour compounds such as diacetyl, acetoin, acetaldehyde, acetic acid create specific flavour profiles for yoghurts *S. thermophilus* and *L. bulgaricus*.

Gel formation is significant in yoghurt qualities. Factors such as strains of starter culture and incubation time and temperature during fermentation play a considerable role in stabilizing and reassuring the interactions of casein micelles in forming a three-dimensional gel network (Puvanenthiran et al., 2014). Spontaneous whey separation

may be linked to the rearrangement of the gel matrix due to pH change. In the experiment, High-speed centrifugation is commonly used to measure whey expulsion from yogurt, followed by discharging whey through a sieve to monitor whey separation from yogurt (Aswal, 2012).

EPS produced during incubation binds with proteins creating different textures. Some strains *S. thermophilus* can form a firmer structure of gel at pH 4.4 and by producing EPS in yoghurt sample (Beal, Skokanova, Latrille, Martin, & Corrieu, 1999). Furthermore, the same study suggested the effect of texture on fermentation temperature can be related to acidification. Which stated that the lower the temperature, the slower the acidification, resulting in high viscosity (Beal et al., 1999; Heller, 2001). Although much has been done for increasing the flavour enhancement, some reviews have summarised the pathway for flavour-forming of LAB (Liu et al., 2016; Smid & Hugenholtz, 2010). Especially the enzymatic degradation of caseinsin. particular results in the formation of key-flavour components that contribute to the sensory perception of dairy products. Furthermore, caseins degrade into peptides and amino acids, the latter of which are important precursors for volatile aroma compounds (Smit, Smit, & Engels, 2005).

2.5.4.Chemical, physical, microbiological analysis of yoghurt

Microbial quality testing is time-sensitive as storage time, and environment can affect coliform production. Thus, once the yoghurt is produced, yoghurt needs to be tested immediately for microbial testing such as viable counts. Yoghurt stability testing (pH, viscosity, texture) usually occurs after the yoghurt is made (Lucey, 2001). Rheology tests measure yoghurt quality by applying a small amount of stress to detect changes in yoghurt structure (Sharma, 2013). Yield stress is defined as the point when the shear stress begins to decrease, is one of the rheological

characteristics that can be determined via physical deformation testing: a greater yield value implies a robust yoghurt gel network. A greater yield value suggests a stronger robust yoghurt gel network, whereas the lower yield suggests a weak or short-skin yoghurt gel (Lee & Lucey, 2003). The characteristics of yoghurt gel yield consist of different factors, such as the strength of the protein bond, the number of bonds in each chain's cross-section and the network bond's relaxation time, and the chain's orientation in the matrix (Ozcan, 2013).

LAB Species	Factors affecting metabolic activities of LAB	Effects on LAB
Lactococcus lactis. lactis *Lactobacillus delbrueckii*, *Lactococcus lactis* *Lacticaseibacillus casei*	metabolic activity: - Carbohydrates Metabolism - Protein Metabolism - Lipid Metabolism	- resulting in a variety of end-products. - Proteinases and peptidases degrade proteolysis into polypeptides, amino acids, and peptides. - Lipid metabolism is the breakdown of lipids into fatty acids and glycerol by lipases.
Lactococcus lactis. lactis *Lactococcus lactis. Cremoris* *Lactobacillus acidophilus*	Biochemical Environment : - Carbohydrates - Amino Acids and Peptides - Vitamins	- With the addition element in the growing environment, LAB growth were enhanced and stable metabolic activities.
Lactococcus lactis. lactis *Lactococcus lactis. Cremoris*	pH	- medium pH decreased from 5.5 to 4.0 - a decrease in LAB growth rate and in the amount of lactic acid produced - the function of its enzymes and the transport of nutrients into the cell. - limit the synthesis of metabolic enzymes responsible for protoplasm synthesis.

Table 1. l Inhabitations and challenges with lactic acid bacteria. (Narendranath & Power, 2005) (Gullo et al., 2014; Hayek & Ibrahim, 2013; Larsen et al., 2015; Panesar et al., 2007)

2.6. Application of adaptive evolution for starter culture development

Although fermentation is widely used in different industries, productivity and cost are also considered as the primary concern. One issue that has been rising is the unbalanced redox, which affects the metabolic activity of the microorganisms during fermentation (Civelek Yoruklu, Oguz Koroglu, Demir, & Ozkaya, 2019). In the food industry, LAB is used as a starter culture for fermented food and other products based on preventing spoilage and flavour forming. For increasing the requirement of starter culture in different industries, creating a new starter culture is to satisfy various food commodities in another sector. More studies have demonstrated the selection of new strains of LAB with other and specific functional properties, such as stress tolerance, production of particular molecules (enzymes, polysaccharides, aromatic compounds) (Liu et al., 2019; Torino, Font de Valdez, & Mozzi, 2015). In dairy products, LAB that produce EPS is used to improve milk products' texture and functional properties; bacteriocin-producing LAB are used in meat fermentation to inhibit the growth of pathogenic bacteria and increase the shelf life. In contrast, bacteria survival in sufficient numbers and their physical and genetic stability during storage is crucial and needs to be guaranteed (Behera, Ray, & Zdolec, 2018)

Chapter 3 Materials and methods

3.1. Isolation of *L. bulgaricus*

A commercial yoghurt starter, which consisted of S. *thermophilus* and *Lactobacillus delbrueckii* (Yoflex Express 1.1 powder, CHR Hansen, Denmark) was used in this project. The starter culture of 0.1% (w/v) was added to 10 mL MRS broth made according to the manufacturer's instructions (Difco Laboratories, Francisco Soria Melguizo, Madrid, Spain). Serial dilutions were performed from 10^{-1} to 10^{-7} with MRS broth and each of the diluted sample was plated on MRS agar to calculate the viasle cells (cfu/mL). *L. bulgaricus* was isolated and grown on selective media MRS agar (Difco Laboratories, Francisco Soria Melguizo, Madrid, Spain) with additional 2% of lactose as a source of energy (LMRS), which was sterilised at 121°C, 15 p.s.i for pressure for 15 minutes. *L.bulgaricus* Plates were incubated anaerobically with BD GasPac Anaerobe Container System (Becton Dickinson Microbiology Systems) at 42°C for 48 hours in the incubator (Thermo Scientific™ Heracell™ VIOS CO_2 incubator). Colony was randomly selected from the plate, and morphology was observed by Gram staining. The process was repeated by sub-culturing the colony three times until such time as the gram staining appeared to be all gram-positive and rod-shaped, to validate for collecting pure culture of *L. bulgaricus*. Once the culture was confirmed as pure *L.bulgaricus*, spread plating on LMRS was performed in triplicate. The pure culture was then divided into 1 mL stock, containing 0.5 mL of pure culture in peptone water and 0.5 mL of 40% (w/v) glycerol water, and stored at -80°C for up to 3 weeks for characterisation of bacterial morphology with respect to change in growth temperature and freeze-thaw stability (Figure 3).

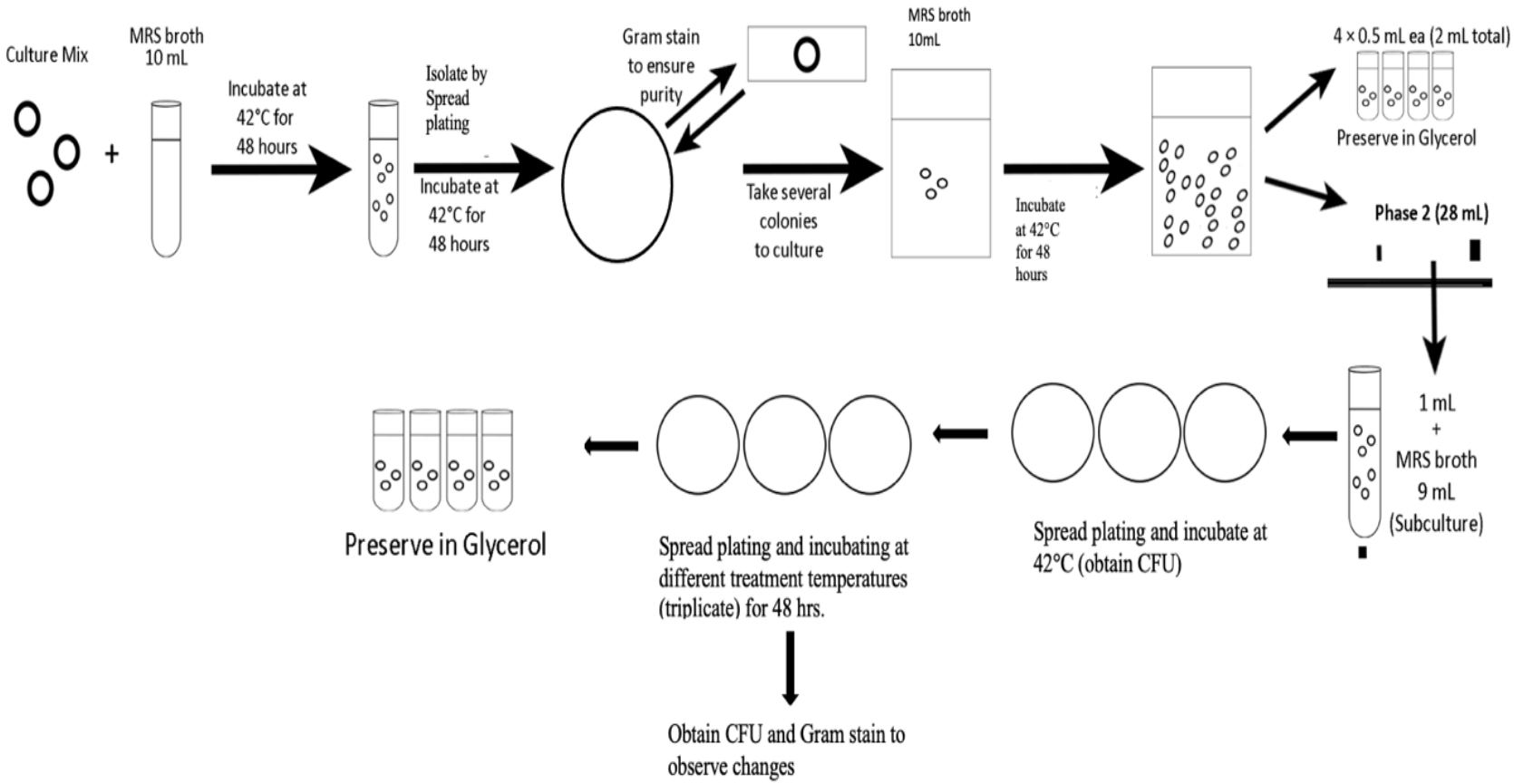


Figure 3: Flow diagram of isolation of *L. bulgaricus* pure culture at 42°C and spontaneous *L. bulgaricus* mutant induction at 52 °C.

3.2. Application of adaptive laboratory evolution to generate mutant strain

The laboratory evolution experiments were modified based on the protocol by Pena-Miller et al. (2013). ALE was applied to the purified *L. bulgaricus* by exposing them to a gradual change in growth temperature in repeated batch cultivations. The morphology changes of the strains were observed every 48 hours. Using standard spread plate technique on LMRS agar plate, each of the purified sub-culture from section 2.1 was firstly Gram stained to check for morphology under a light microscope (Celestron Labs CB2000CF Compound Microscope) at 1000x with paraffin oil. Then the sub-culture was plated and incubated at 42°C in anaerobic condition with a GasPac system (BD GasPac Anaerobe Container System (Becton Dickinson Microbiology Systems) and an anaerobic chamber for 48 hours. *L. bulgaricus* collected was harvested in peptone, and serially diluted with 0.1% of peptone water. Then 0.1 mL of each serial dilution was inoculated by spread plating onto new LMRS plate in triplicate. After 48 hours, the culture was sub-cultured onto new LMRS plates in triplicate and the growth temperature was increased by 1.0°C, under the same incubation condition. The procedure was repeated and data were collected per 1.0°C temperature increase until such time as no viable counts were achieved. The viable counts were presented as means of the colony-forming unit log (CFU) g/mL. The subculture was then harvested and the colonies from the plate were harvested to 1mL of peptone water, then centrifugated at 3000 rpm for 5 minutes (Eppendorf Centrifuge 5430 R) at 25°C. After removing the supernatant, the culture pellet was preserved in glycerol, following the same protocol in section 2.1, for further work on characterisation of bacterial morphology with respect to change in growth temperature and freeze-thaw stability.

3.2.1. Level of fitness of heat-tolerant *L.bulgaricus*

To measure the level of fitness of heat-tolerant *L.bulgaricus*, the amount of total lactic acid produced was quantified by using L-/D- Lactic acid assay (Megazyme International Ireland, Bray, Wicklow, Ireland) following the manufacturer's instructions. A mixture of 1.60 mL of distilled water with solutions and suspension provided according to the manufacturer's guideline was used as blank (MegazymeLtd, 2018).1 g yoghurt sample and 60 mL of distilled water were added to a 100 mL volumetric flask. 2 mL of Carrez I solution, 2 mL of Carrez II solution and 4mL of NaOH solution were added into the yoghurt mixture and the volume was adjusted to 100mL with distilled water. 1.5 mL of the yoghurt solution mixture was analysed according to the instructions of the kit (MegazymeLtd, 2018).

As solution A1 was the sample mixture, solution A2 was solution A1 with suspension 5 from the kit. A spectrophotometer (Spectrophotometer UV-VIS Model UV-1280, Shimadzu, Tokyo, Japan) was used to measure the absorbance of both d- and l-Lactic acid contents at 340 nm. The lactic acid concentration was calculated using the equation below. After 3 minutes, the absorbances of solution A1 were absorbed, and the absorbances of solution A2 were absorbed after 5 minutes of reaction time with A1. All samples were examined in triplicate.

Equation 1:

$$\text{C [g/L]} = (V \times MW/\varepsilon \times \text{d} \times \text{v}) \times {}^{\Delta\text{A}}\text{ D-lactic acid}$$

where

V= final volume (mL)

MW = molecular weight of D-lactic acid or L-lactic acid(g/mol)

ε = extinction coefficient of NADH at 340 nm

d= light path (cm)

v= sample volume (mL)

ΔA = the absorbance difference (A2 − A1)

3.3. Inoculated milk production

Starter culture consisted of YC-380 Yo-Flex®-encoded *S. thermophilus* and *L. bulgaricus* from the company Chr.Hansen. Starter culture was made by inoculating the pure stock culture (control, grown at 42°C) or the mutant culture (grown at 52°C) to 100mL of Anchor™ Trim Milk of 0.1% fat sourced from a local market. The concentration of both inoculated milk sample with control and mutated *L.bulgaricus* strain of 0.01% (w/v). The milk was incubated in anaerobic condition with GasPac system (BD GasPac Anaerobe Container System (Becton Dickinson Microbiology Systems) and anaerobic chamber in a sealed and sterilised glass jar with a lid at the temperature of 42°C for both control and mutant cultures for 24 hrs. Yoghurt was immediately transferred to a refrigerator and stored at 4 °C until such time as further analysis. The described yoghurt production was repeated three times.

3.4. Chemical analysis of yoghurt

3.4.1 Determination of pH and viscosity

A pH meter with a glass electrode (Thermo Scientific Orion Star A211e) was calibrated and then measurements were taken from 10 g of yoghurt samples. Brookfield viscometer (RST-CC Coaxial Cylinder) with Bob 40 mm Brookfield Ametek spindle (CCT-40) measuring system was used to measure the viscosity. Both control and

mutant yoghurt samples were tested in triplicates. Each of the yoghurt sample filled up to the maximum level marked in the sample holder (approximately 68 mL). The viscometer was then operated at a speed of 400 rotations per second for 2 minutes. The RST Rheometer was programmed using Rheo3000 to control the shear stress or shear rate, and to calculate yield and average viscosity. All viscosity measurements for each yoghurt sample were performed in triplicate.

3.4.2. Water Holding Capacity (WHC)

Method used for determining the Water holding capacity (WHC) was modified from Remeuf et al. (2003). 10 g of each inoculated milk sample were placed in 15mL centrifuge tube and centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant of the samples were collected and weighed measurements were taken in triplicates. The following equation was used to determent the WHC value of equation 2.

Equation 2:

WHC (%) = (weight of 10 g of yoghurt sample – weight of supernatant) / weight of 10g of yoghurt Sample × 100

3.4.3. Determination of free amino acids with Liquid Chromatography-Mass Spectrometry (LCMS)

Preparation and analysis methods used by the same research group of Diep et al. (2020) were followed. Each yoghurt sample was analysed three times.

A 40 µL of supernatant of yoghurt sample was placed in a 1.5 mL polypropylene microcentrifuge tube, mixed with 40 µL of methanol containing 10 mg/L of d4-alanine as internal standard-spiked methanol. LCMS (Agilent 1260 Infinity Quaternary LC System, Santa Clara, CA95051 USA) equipped with Kinetex Evo C18 (150mm x

2.1mm x 1.7 µm. Phenomenex, USA) column was used at 25 °C with the flow rate of 0.25 mL/min. With at mobile phase A, the condition was 0.1% formic acid in MilliQ water, where mobile phase B was 0.1% formic acid in Acetonitrile. The total run time was 29 minutes.

At the following gradient rate:

0 minutes – 5 %B
3 minutes – 5 %B

8 minutes – 10 %B

13 minutes – 15 %B

16.5 minutes – 80 %B

17 minutes – 80 %B

18.5 minutes – 5%B

The R^2 value was calculated from the linear calibration curves of each FAA (plotted as concentrations (µm) vs relative responses). The correlation coefficient (r) was calculated for each amino acid and considered as acceptable when the coefficient of determination (r^2) ≥ 0.95 (Gu, Liu, Wang, Aubry, & Arnold, 2014) shown in table 2.

Amino acid	R^2
Essential FAA	
Lysine	0.999 ± 0.03
Phenylalanine	0.997 ± 0.41
Threonine	0.999 ± 0.65
Histidine	0.990 ± 0.52
Valine	0.997 ± 0.73
Leucine	0.997 ± 0.11
Isoleucine	0.997 ± 0.64
Tryptophan	0.996 ± 0.09
Methionine	0.997 ± 0,21
Non-essential amino acid	
Glutamic_acid	0.999 ± 0.45
Proline	0.997 ± 0.12
Ethanolamine	0.998 ± 0.76

Alanine	0.999 ± 0.54
Glycine	0.997 ± 0.05
Taurine	0.998 ± 0.07
Serine	0.998 ± 0.21
Aspartic_acid	0.999 ± 0.03
Tyrosine	0.997 ± 0.06
Arginine	0.997 ± 0.12
Ornithine	0.998 ± 0.43
Citrulline	0.996 ± 0.26
Alanine	0.999 ± 0.11
L_ α_Amino_n_butyric_acid	0.999 ± 0.12
δ_Hydroxylysine	0.999 ± 0.05
Hydroxy_L_Proline	0.997 ± 0.04
γ_Amino_n_butyric_acid	0.998 ± 0.53
Sarcosine	0.999 ± 0.57
DL_ β_Aminoisobutryic_acid	0.999 ± 0.12

Table 2: Average R^2 values of compounds:

LC-MS analyses were conducted using an Agilent 1260 Infinity Quaternary LC System (Santa Clara, CA95051 USA), and consisted of the following parts: 1260 quaternary pump (model number: G1311B), 1260 infinity ALS sampler (model number: G1329B), 1260 infinity TCC column component (model number: G1316A), 1260 infinity diode array detector (DAD) (model number: G4212B), connected to a 6420 triple quadrupole LC/MS system with electrospray ionisation (ESI) source (model number: G1948B). The MS ionisation source conditions were capillary voltage of 4 kV, drying gas temperature of 300 °C, drying gas flow of 10 L/min, nebulizer pressure of 30 psi. The positive ion mode was performed with multiple reaction monitoring (MRM) for quantitative analysis.

3.4.4 Determination of volatile compounds with Solid Phase Microextraction - Gas Chromatography

Volatile components of the yoghurt samples were analysed according to the methods used by Diep et al. (2021) with slight modifications. A sample of approximately 1g of

yoghurt sample was quickly introduced to 10 mL headspace vial, followed by addition of 4 µL of internal standard (2-chloro-phenol). It was then sealed with a polytetrafluoroethylene silicon septum. The vials were then incubated at 50°C in a thermal block for 15 minutes with agitation. The Solid Phase Microextraction (SPME) fibre of 50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS), StableFlex fiber 24 ga, with length of 10 mm (Supelco, Bellefonte, PA, USA) was exposed to the sample headspace for 5 minutes. The volatile compounds were separated on a capillary column (Zebron Guardian Capillary GC Column ZB-1701) with Helium as a carrier gas at a constant flow rate of 1.1 mL/min, using Gas Chromatography-Mass Spectrometry (GC-MS) (Agilent Technologies 7890B GC-System & 5977B MSD).

The injection mode was splitless, and the injection port inlet temperature was set to 250 °C, with a split flow rate of 45 mL/min and a splitless time of 2 minutes. The oven was set to 40 °C for 2 minutes, then raised to 280 °C at a rate of 8 °C/min for 1 minute. The total running time was 33 minutes. The MS was run in electron impact mode at 230 °C, 150 °C for the quadrupole, 70 eV for the ionising voltage, and 250 °C for the transfer line. The mass spectrometer scanned masses ranging from 38 to 450 m/z. Samples were analysed in at least triplicate. The concentration of volatile compounds in the yogurt samples were calculated using equation 3:

Equation 3:

$$[P] = (AP/AI\ S) \times [IS]$$

Where:

P (µg/L) = concentration of the product

IS (µg/L) = concentration of internal standard

AP = peak area of the product

AIS = peak area of the internal standard

3.4.5 Determination of Odour Threshold and relative Odour Activity Value (OAV)

The odour threshold values (OAV) of yoghurt samples were calculated using the OAV published by Leffingwell et al. (2020). OAV = c/t, where c is the total compound concentration in the yoghurt and t is the odour threshold value, was used to calculate relative OAV for each volatile compound. The lowest value was used to calculate the relative OAV for compounds with a range of odour thresholds rather than a single number (e.g., 1-Octanol, odour threshold 110-130). Compounds with relative OAVs greater than 1 were considered as they contribute to yoghurt aroma.

Odour descriptions of volatiles were adapted form Cheng (2010) and Thierry et al. (2015).

3.5. Preliminary study

3.5.1. Chemically modified medium

Medium preparation and methods analysis were conducted using Talwalkar & Kailasapathy (2004) method, howeverwith slight modification. The defined medium was MRS with 2% D-glucose (GMRS), and another defined medium was MRS with 2% lactose (LMRS) as a carbon source. Modified MRS agar plates (LMRS and GMRS) were incubated anaerobically with the GasPac system and anaerobic chamber from 42 to 52°C for 48 hours. Cells from single colonies were subculture at 1:10 (v/v) dilution into new modified MPS medium plates. Serial dilution was performed from 10^{-1} to 10^{-7} to calculate the colony-forming unit (cfu/mL). For a detailed method, see the isolation of *L. bulgaricus* from the technique and material above.

3.5.2. Growth curve observation of heat-tolerant *L.bulgaricus*

Preparation and analysis methods used with modifications of Piliizota & Shaevitz (2014). Control and heat-tolerant *L. bulgaricus* were re-suspended then diluted in LMRS broth to make a pure culture with 0.01% Micro method. In a Micro method Plate (96 wells), five wells were loaded with 0.2 mL of the control *L.bulgaricus* resuspension, and five wells were loaded with 0.2 mL of the heat-tolerant resuspension. Along with five wells loaded with pure LMRS broth, serving as a blank. The plate was sealed with LightCycler® 480 Sealing Foil and a drop of paraffin oil to each well to create the anaerobic condition. The growth curve was monitored using FLUROstar® Omega microplate reader at the respective testing temperature for 24 hours from 42°C to 52°C, depending on the incubation temperature of the strains. The micro reader was set to shake at 100 rpm, measure Optical Density (O.D) at 600 nm every 12 minutes, and calibrated internally using the path correction option and the blank.

3.5.3. DNA extraction of mutant *L.bulgaricus*

3.5.3.1.DNeasy Blood and Tissue Kits:

DNA was extracted using DNeasy blood and tissue kit (Qiagen) as the manufacturer's instruction recommended (QIAGEN, 2006). Pretreatment for gram-positive *L. bulgaricus* was required. *L.bulgaricus* cells for control (42°C) and mutant strain were harvested from LMRS agar plate strains (control 4.8×10^6; Mutant 2.5×10^5 cfu/mL) by centrifugating 2 mL Microcentrifuge tubes for 10 min at 7500 rpm. The supernatant was discarded, then the sample was resuspended in 180 µl enzymatic lysis buffer, incubated for 1 hour at 37°C in the heating block. 25 µl Proteinase K and 200 µl Buffer AL (without ethanol) were added into the suspension and mixed thoroughly by vortexing and incubated in the heating block at 56°C for 30 minutes.) 200 µl of 100% ethanol was added to the sample and mixed thoroughly by vortex for a homogeneous solution.

For purification, the suspension was transferred into the DNeasy Mini spin column, placed in a 2 mL collection tube, and centrifuged at 8000 rpm for 1 minute. 500 µl Buffer AW2 were then added and centrifuge for 3 min at 14,000 rpm to dry the DNeasy membrane. Buffer AE (200µl) was pipetted onto the DNeasy membrane (mini spin column with microcentrifuge tube) and incubated at 25°C for 1 minute, and then centrifuged for 1 minute at 8000 rpm. This step was repeated to maximize DNA yield. All tests were done in triplicates for the control and the thermo-treated *L.bulgaricus*.

3.5.3.2.Trizol reagent (DNA isolation):

DNA was isolated using TRIzol reagent as recommended and modified of Hummon et al. (2007). To lyse and separate the DNA of *L. bulgaricus* samples, 0.75 mL of TRIzol reagent was added to 0.5 mL of sample strains (control 4.2×10^6; Mutant 3.6×10^5 cfu/mL) was harvested from LMRS agar (same method as shown in DNeasy Blood& Tissue kit). The solution was homogenized by piper the lysate up and down 5 times. The suspension was then incubated for 5 minutes at room temperature (25°C) to dissociate the nucleoprotein complex. Chloroform (0.2 mL) was added to the suspension and incubated for 3 minutes at room temperature, then centrifuged for 15 minutes at 12000 x g at 4 °C to form layers. The upper colourless aqueous phase containing RNA was discarded, then continued with the interphase containing DNA and added 0.3 mL of 100% ethanol. The samples were mixed by inverting in a capped tube and incubated for 3 minutes at room temperature. The sample was centrifuged for 5 minutes at 2000 x g at 4°C to pellet the DNA and transfer the phenol-ethanol supernatant to a new tube.

Wash the DNA by resuspending the pellet in 1mL of 0.1 M sodium citrate in 10% ethanol and incubated for 30 minutes at room temperature, occasionally mixing by gentle inversion. Later, centrifuge the sample for 5 minutes at 2000 mg at 4°C and discard the supernatant. The above steps were repeated three times to remove any

chemicals remaining in the samples. The sample was then resuspended in 2 mL of 75% ethanol and incubated for 20 minutes. Then centrifuged for another 5 minutes at 2000 mg at 4°C, discarded the supernatant, and air-dried the DNA pellet for 20 minutes.

To solubilize the DNA, the pellet was resuspended from the above steps in 0.6 mL of 8mM NaOH and pipet up and down 5 times to mix them thoroughly.

The pellet was then centrifuged for 10 minutes at 12,000 x g at 4°C to remove insoluble material. The pellet was then stored at 4°C overnight before determining the DNA yield of the sample. Immediately before use, add lysozyme to 20 mg/ml.

To determine the DNA yield of the samples, the dsDNA was quantified by using the Qubit dsDNA Assay kit (Invitrogen, Massachusetts, USA) according to the kit's instructions. Fluorescence selectively measured the intact DNA in the sample without having protein or other contaminants in the sample. All tests were done in triplicates for both control and thermo- treated *L.bulgaricus.*

3.5.3.3. PCR amplify methods and condition:

PCR method used for both DNA extraction methods were adapted from Dubernet et al. (2002). A 21-mer polymerase chain reaction (PCR) primer was designed on this site and called LbLMA1-rev (5′-CTC AAA ACT AAA CAA AGT TTC-3′). A second primer R16–1 (5′-CTT GTA CAC ACC GCC CGT CA-3′) (Dubernet et al., 2002; Piuri, Sanchez-Rivas, & Ruzal, 2005), The flanking terminal sequence of the 16S rRNA gene was used, which is conserved among lactobacilli.

For amplification, a PTC 200 thermal cycler was used (MJC research, Waltham, USA). 25 pmol of each primer, 0.2 mM of each deoxyribonucleotide triphosphate 1 PCR buffer without MgCl2, 1.5, 2.0, 2.5, or 3.0 mM MgCl2, 50–100 ng of bacterial DNA, and 2.5 U of Taq DNA Polymerase were included in the reaction mixture (Qbiogene, Illkirch, France). The conditions of PCR were follows as:

Initial denaturation at 95°C for 5 minutes, followed by 20 or 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 7 minutes. The samples were stored at 4°C until they were analysed.

3.6. Statistical Analysis

Data were analysed using Rstudio (Version 1.3.1093) and presented as mean ± standard deviation. All tests were carried out in triplicate and analyzed by using One-Way Analysis of Variance (ANOVA). Fisher's (LSD) multiple comparison tests were used to differentiate the differences between the means. A P value < 0.05 was considered statistically significant. Unpaired T-test was used to differentiate the differences between the means in free amino acid profile of samples. A p value <0.001 was considered statistically significant.

Chapter 4. Preliminary study

This chapter consists of 5 sections as follows:

Section 4.1: describes the purpose and background of prior research and gave a constructive view on how ALE affects different bacteria strains and the main objective of the preliminary study.

Section 4.2 .1 describes and discusses the different energy sources used to chemically modify the MRS agar plate to create a nutritious growing environment for the control and mutated *L.bulgaricus.*

Section 4.2.2. describes and discusses the process of obtaining growth curves for *L.bulgaricus* strains.

Section 4.2.3.. describes and discusses different DNA extraction methods and PCR used and the purity and quantity of the DNA of the samples.

Section 4.3. concludes the methods and results that were used in this preliminary study.

4.1. Introduction

Applying ALE on *L. bulgaricus* and using the mutant strain in yoghurt production is a novel approach in the industry. Although studies mentioned above have shown the possibilities and approaches in applying ALE on different bacteria strains, due to the spontaneous mutation that occurs to the bacteria strains, there are no certainties the outcome would be positive or anything that could be predicted.

Firstly, the concentration of the mutated *L. bulgaricus* was important for this study. DNA detection and extraction methods have a minimum requirement depending on the manufacturer's guidelines. To ensure other parts of this study could be carried on, it was essential to create an environment that could protect and stabilize the *L.bulgaricus* during the gradual increase in temperature to their lethal temperature range (~52°C).

Secondly, to characterize the mutations that might occur within the mutant *L.bulgaricus*, whole-genome sequencing was required to analyze entire genomes using purified DNA of the strain.

Carrying out a preliminary study helped collect data and results generated by prior research. It gave a constructive view of how ALE affects different bacteria strains and the already known challenges this study would face later. Hence, this preliminary study aimed to modify the existing MRS agar plate to increase the survival rate of *L. bulgaricus* and test the current DNA extraction methods of the *L. bulgaricus* samples.

4.2. Result and Discussion

4.2.1 Chemically Modified medium

The energy source has been an essential factor in increasing or stabilizing the fitness level for bacteria growth. This study aimed to grow *L.bulgaricus* up to an extreme temperature (52°C), using ALE. From present studies, researchers have suggested that with the medium containing different carbon sources such as glucose, lactose would result in additional production of end products (Kato et al., 2018; Stumpf, Pértile, Rambo, & Porto, 2013). For example, a study conducted in Turkey suggested that increasing glucose concentration (30 g/L) in the medium stimulates the growth and EPS production of *L. bulgaricus* (Grobben et al., 1998; Yuksekdag & Aslim, 2008). However, the results of this study showed the opposite effect. The growth rate on the GMRS plate has been inconstant, even for control strains at 42°C, there was no growth most of the time. With lactose as the carbon source, studies agree that lactose growth was faster than growing on agar with glucose supplied. However, lactose concentration under 0.4% would play an opposite effect, which would limit the growth of the bacteria (Chervaux, Ehrlich, & Maguin, 2000; Grobben, Sikkema, Smith, & de Bont, 1995).

Figure 4, showed that there was apparent growth of *L. bulgaricus* cells on the LMRS plate, and no growth was obtained on the MRS plate. LMRS resulted in extended survival, indicating that the lactose added into the medium has shown certain additives are effective in protecting *L. bulgaricus* throughout storage at -80°C (Carvalho et al., 2004). The reason behind this finding is believed to be that sugars can replace structural water molecules in membranes after dehydration. This results in the prevention of unfolding and aggregation of proteins by hydrogen bonding with polar groups of proteins (Costa, Usall, Teixidó, Garcia, & Viñas, 2000). Lactose in fermentation systems play a role as a supplementary energy source for the microbes. During the growth process, lactose was hydrolysis hydrolys into glucose and galactose by lactase. For lactose-consuming microbes, simultaneous phosphorylation and translocation of lactose across the cell membrane could occur due to at least two alternative mechanisms (lactose-proton symporter and lactose-galactose antiporter) (Domingues, Guimarães, & Oliveira, 2010).

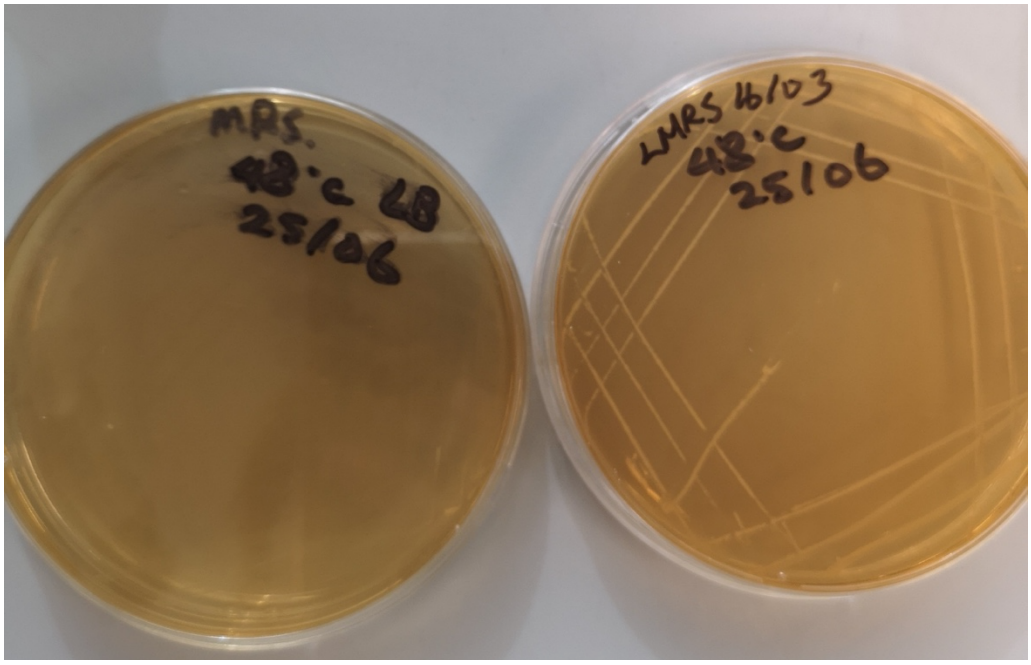


Figure 4: *L.bulgaricus* growth on MRS agar plate compared to LMRS plate incubated at 48°C. LMRS plate showed growth whereas no growth observed on MRS plate.

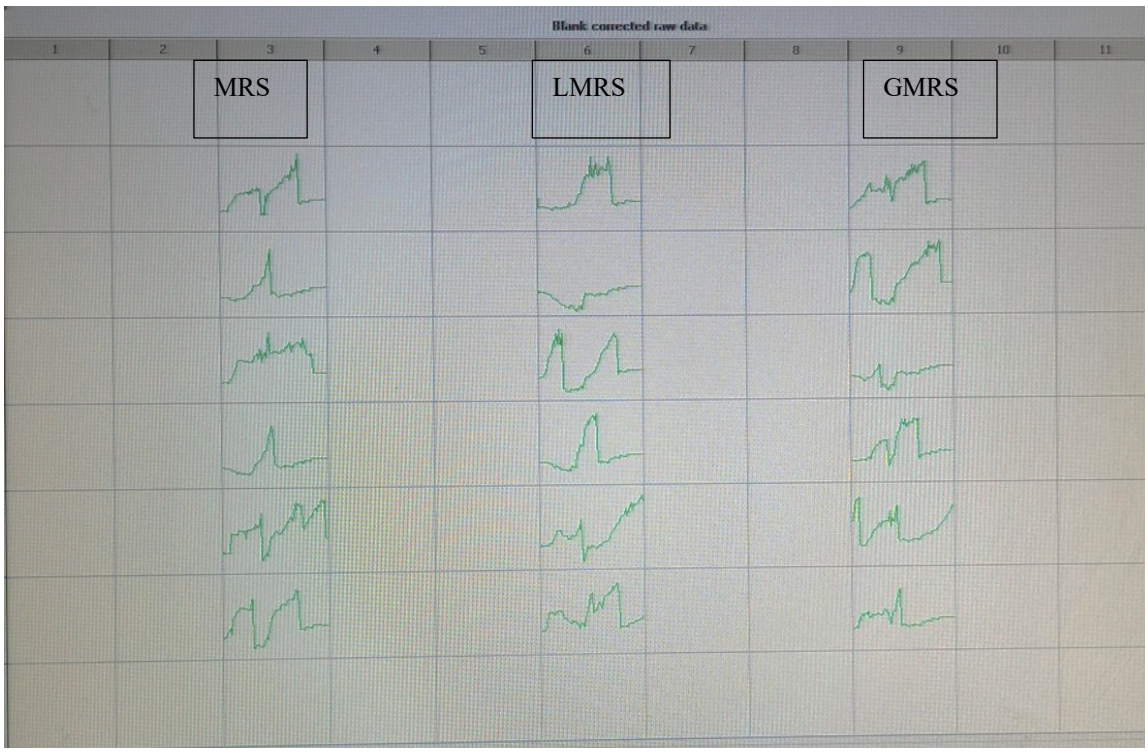
4.2.2. Growth curve of heat-tolerant *L. bulgaricus*

there was no growth curve for both control and mutant *L.bulgaricus.* As shown in figure 5a, the control strain (at 42°C) also failed to obtain a standard growth curve with lag, exponential (log), stationary, and death phases of *L.bulgaricus*. From the growth curves shown, both *L. bulgaricus* strains showed a high absorbance reading as the starting point. However, as time passed, the curves for mutated *L.bulgaricus* strains all tended to go downward, indicating the growth of the strains were decreasing (Figure 5b).

Incubating *L. bulgaricus* in the microplate reader was aimed to maintain an anaerobic environment as the sealing foil and the paraffin oil may not be enough to block out all the oxygen in the surrounding. Since growth curves were unobtainable, harvesting cultures during the log phase could not be confirmed. Generally, inoculation with higher concentration (10^6 cfu/mL or higher) is recommended.

Another possible explanation would be growing the heat-tolerant strain does not grow well in LMRS broth. When testing for a different type of medium, different MRS broth was tested alongside MRS agar plates. However, using MRS broth failed as no growth was observed as the incubation temperature increased to 48°C.

a.



b.

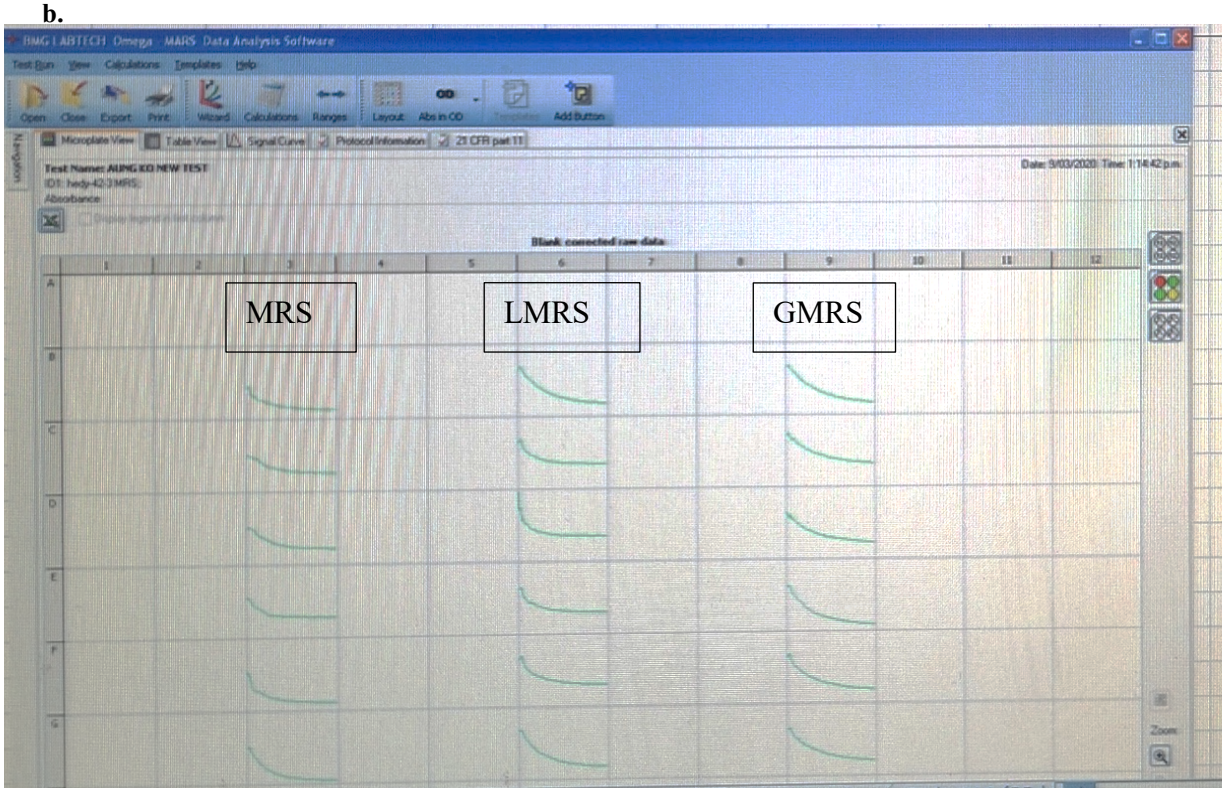


Figure 5. Growth curves obtained FLUROstar. a) Three set of *L.bulgaricus* on different modified medium (MRS, LMRS, GMRS) analysed at 42°C. b) Three set of bacteria on different modified medium analysed at 52°C

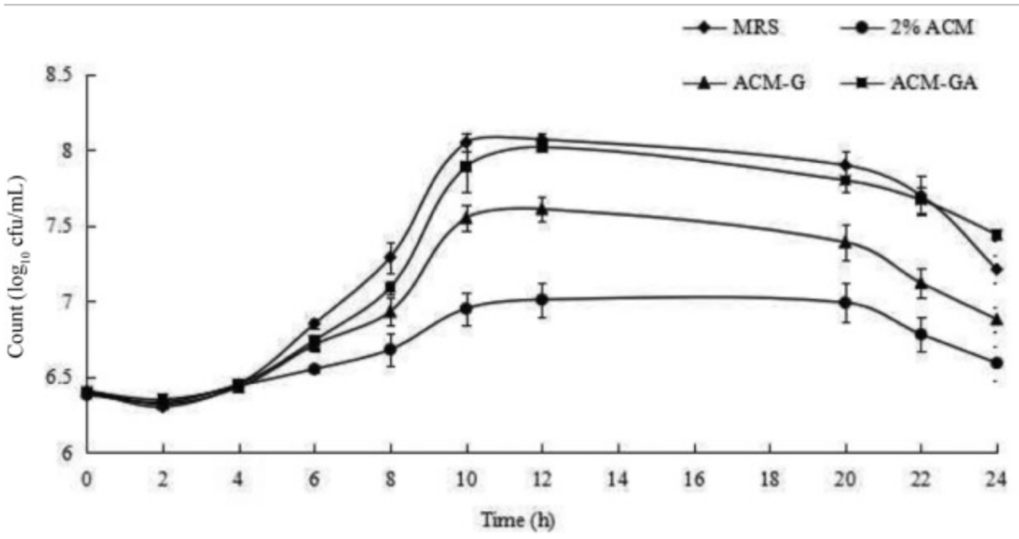


Figure 6: The expected growth curve of *L.bulgaricus* with different growth medium. ACM-G = 2% ACM containing 1.9% glucose; ACM-GA = 2% ACM containing 1.9% glucose and 2 g/L AA mixture; MRS = de Man, Rogosa, and Sharpe.
Retrieved from (Li et al., 2016).

4.2.3. DNA extraction of mutant *L.bulgaricus*

The aim of using the blood & Tissue kit was to optimize the effect of direct cell lysis then selective binding of DNA to the DNeasy membrane(QIAGEN, 2020). The centrifugation removed contaminants and enzyme inhibitors in the suspension increase purified DNA content for *L.vbulgaricus* samples. However, the Blood and Tissue kit was unsuccessful at extracting the required amounts of DNA from *L. bulgaricus*, as shown in the lack of genomic bands on an agarose gel (Figure 7b).

L.bulgaricus, as a gram-positive bacteria, which is a highly resistant bacteria, it was expected to be challenging to achieve cell lysis with lysozyme and develop successful procedures for DNA isolation (Chassy & Giuffrida, 1980; Klaenhammer, 1984; Nagaoka et al., 1990). The bacterium cell wall is a murein layer, which is a thick peptidoglycan layer providing shape and stability and viability (Piuri et al., 2005). Different methods have been used to lysis lactobacilli species using the chemical and mechanical protocol (Keer & Birch, 2003). Lysozyme is one of the chemical methods that lysed the peptidoglycan. However, high resistance to lysozyme is observed in several lactobacilli species (Piuri et al., 2005), which also was observed to the *L.bulgaricus* strains in this study.

Trizol reagent were able to extract DNA from thermo-treated *L.bulgaricus* strains. However, when calculating the ratio of the readings at 260 nm and 280 nm (*A*260/*A*280), the ratio of the best out of all samples was 1.2, where the pure DNA had an *A*260/*A*280 ratio of 1.8-2.0. Lower pH might have resulted in a lower *A*260/*A*280 ratio and reduced sensitivity due to protein contamination. Qubit (Qubit™ dsDNA HS and BR Assay Kits) testing was also done to determine the DNA quantity with the Qubit Fluorometer. 0.9 μg/μl, which was lower than the recommended quantity for submitting whole-genome sequencing according to the instruction within the Qubit handbook. Qubit fluorometer was used to check DNA quality before sequencing as it

measured intact dsDNA (Nakayama, Yamaguchi, Einaga, & Esumi, 2016). Although a clear band was formed on the agarose gel, the *A*260/*A*280 and Qubit values were lower than the submission line for whole-genome sequencing. The result indicated that the DNA in the strains had not been separated from other macromolecules to form a complete library. Figure 7a showed a picture of the extracted DNA of the mutated *L.bulgaricus* strain after washing three times.

a)

b)

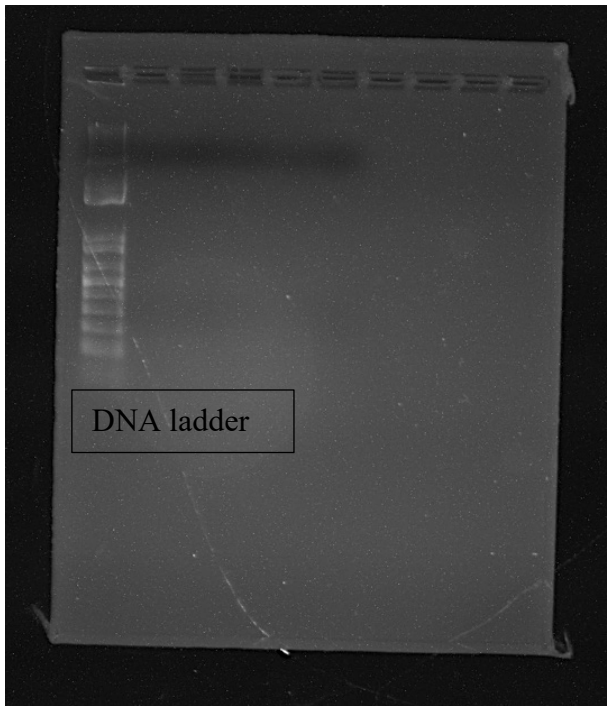


Figure 7: a) Extracted and purified DNA using Trizol reagent. the DNA has been washed three times with the procedure above. b) An agarose gel of mutant *L.bulgaricus* that was fialed to obtained after the PCR amplification showing no DNA information. The visible band showed was the DNA ladder .

4.3. Conclusion

This preliminary study aimed to use glucose and lactose as the carbon source to increase the survival rate of *L.bulgaricus* during the experiment to create a stable and effective chemically modified medium for this study. Meanwhile, research on other extraction methods and kits is needed in the future, as from the Trizol result, it is possible to get purified DNA of the samples for sequencing, but quantity and quality of the DNA needs to be improved. Chassy & Guiffrida (1980) suggested that, it is easier to lyse bacteria strains isolated from log-phase than stationary phase. Another critical issue is the quality of extracted plasmids from bacterial isolates, which affects the sensitivity and utility of biological analyses like PCR.

Chapter 5 Result and Discussion

5.1.Generation of thermo-tolerant strains of *L. bulgaricus*

High temperature was used as an environmental stimulus to induce ALE for an isolated pure culture of *L. bulgaricus* grown at 42°C. Over the course of 20 days, LAB that survived the temperature increase were sub-cultured, and the bacterial cells were examined for morphology under a light microscope at each temperature point. As shown in Figure 8a, initially, the control, *L. bulgaricus* grown at 42°C had short, rod-shaped cells, with 7.62 log CFU/mL on average when grown on LMRS. The temperature was increased to 52°C while maintaining 4.66 log CFU/mL (Table 2). Starting at 47°C, the survival rate of *L. bulgaricus* is known to decrease due to the loss of its polysaccharide production ability (Malaka, Maruddin, Dwyana, & Vargas, 2020). As a result, adding 2% lactose to MRS supported the development of a mutant strain. As given in Figure 8b, a mutant strain changed its bacterial morphology to elongated cells in response to environmental stimuli, as explained by Young (2006). The ability of bacteria to survive after being exposed to a lethal temperature has been linked to the production of heat-shock proteins (Carvalho et al., 2004; Lindquist & Craig, 1988). Filamenting Temperature-Sensitive Mutant Z (FtsZ) is the first tubulin-like protein to respond during cell division, recruiting other proteins that produce a new cell wall between diving cells and form filaments with smooth morphology (de Boer, Crossley, & Rothfield, 1992). As mutated bacteria lacking FtsZ do not divide but elongate into filaments (Mercier, Kawai, & Errington, 2016).

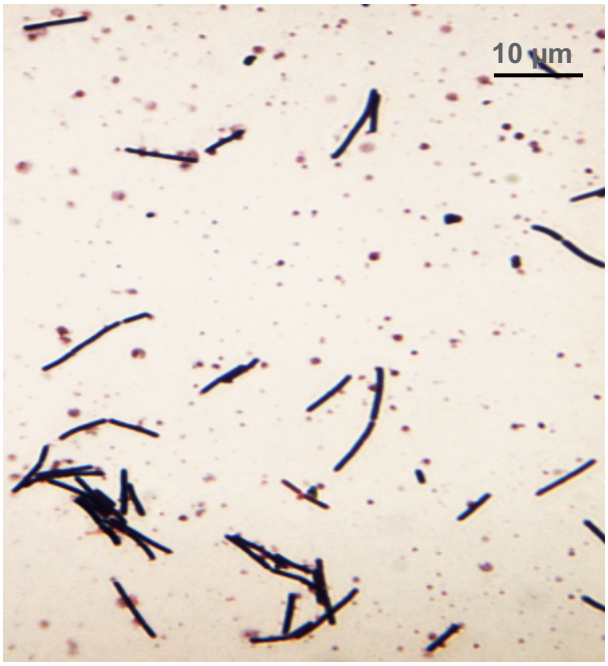
When both the control and mutant strains were reserved in glycerol at -80°C for three weeks to test for freeze-thaw stability, another morphological change was observed, but only in the thermo-tolerant strain. For 48 hours, this strain was re-incubated and sub-cultured on LMRS agar. On the first subculture, morphology changed to cocci, as shown in Figure 8c. After three rounds of sub-culturing (48 hours per sub-culture), more

rod-shaped cells were converted into cocci with irregular edges (Figure 8d) (Figure 8e). The observations can be explained as a heat stress adaptation mechanism of *L.bulgaricus* by taking appropriate molecular responses to reduce the extreme effects and restore and survive (Papadimitriou et al., 2016). The observed change in morphology may be caused by mutation occurring in mreB, which encodes an actin-like cytoskeletal protein found in most rod-shaped bacteria. Previously, transition from growing as a rod-shaped cell to coccus has been observed in *E. coli, Arthrobacter, Acinetobacter, and Rhodococcus equi* (Bean et al., 2009; Bendezú & de Boer, 2008; Takacs et al., 2010).

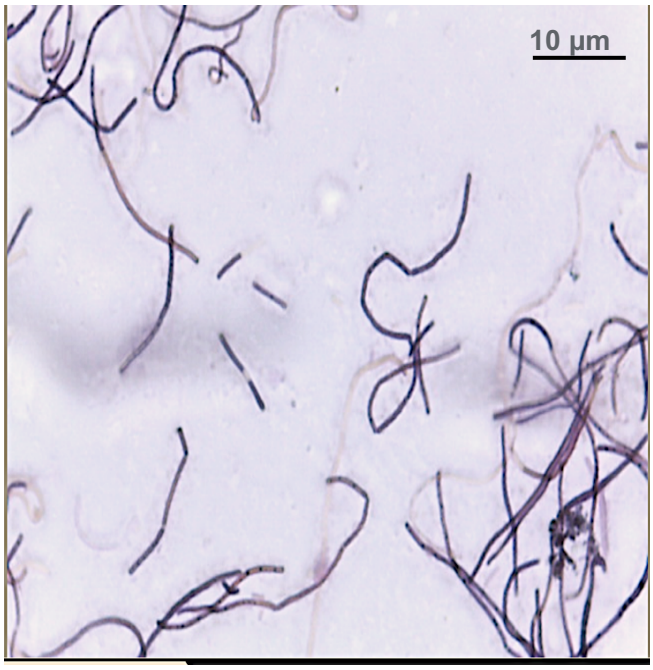
There is a chance that new microbial communities made up of rod and coccus shapes formed in a layered structure, with a group of cocci layering on top of the rod shape cells. as proposed by Smith et al, (2017). In this study, *L.bulgaricus* was sub-cultured on a new LMRS plate every 48 hours. The nutrient was delivered from the plate's bottom, which favoured the rod-shaped cells in the layered structure. This would have affected the cocci's fitness at the expense of rod-shaped cells. With a favourable position in the biofilm. *L.bulgaricus* strains formed white larger colonies with rough edges, with stronger rod-shaped cells at the bottom and small cocci at the top. Similar observations have also been reported in multispecies biofilms (Christensen, Haagensen, Heydorn, & Molin, 2002; Hansen, Rainey, Haagensen, & Molin, 2007).

Carvalho et al. (2014) also suggested that lactose supplementation to the growth medium (LMRS) provided a protective effect during *L.bulgaricus* freezing storage as sugars can replace structural water molecules in membranes after dehydration to prevent protein unfolding and aggregation by hydrogen bonding with protein polar groups (Carvalho et al., 2004; Costa et al., 2000).

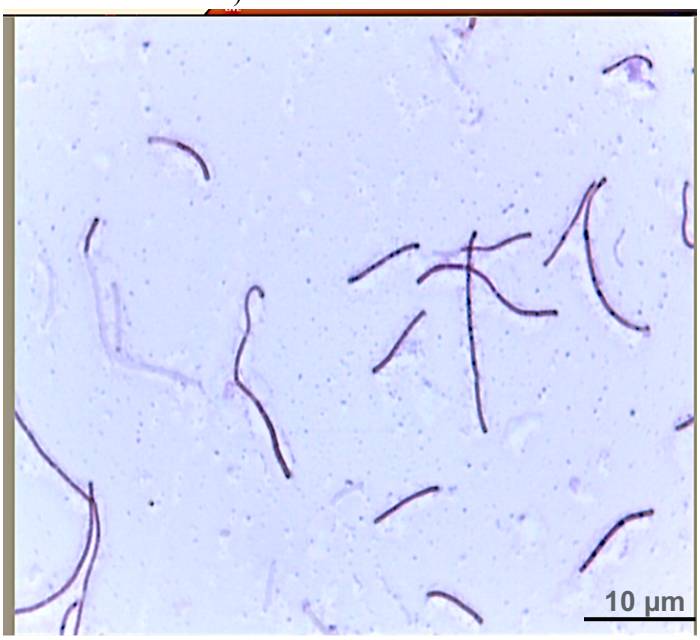
a)



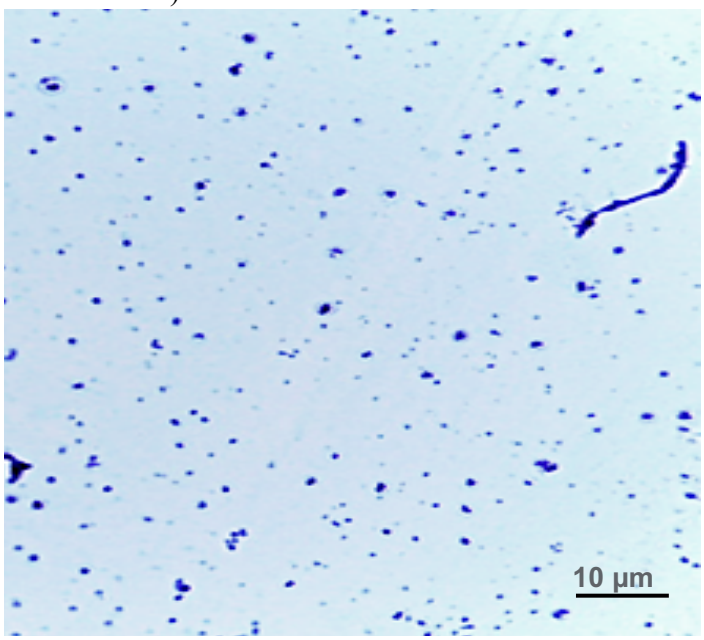
b)



c)



d)



e)

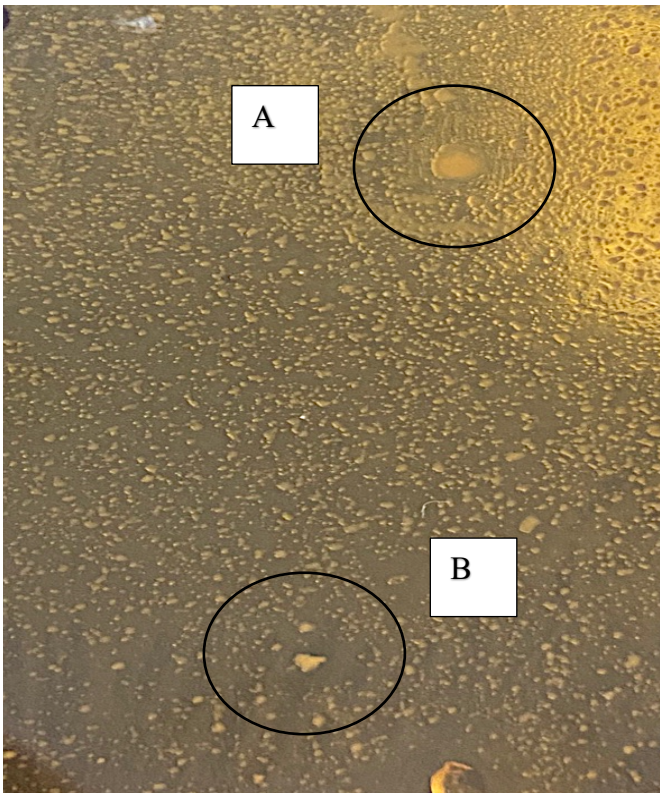


Figure 8: Microscope images (x1000 magnification) of gram stained *L.bulgaricus*.

a) Pure *L.bulgaricus* strain incubated at 42°C, showing short and long rod shapes; b) Mutant strain of *L.bulgaricus* incubated at 52°C, showing elongated shape; c) First incubation at 52°C after storing at -80°C for 3 weeks, showing truncation in elongated cells and formation of cocci; d) Post to three rounds of sub-culturing at 52°C, showing further transformation of truncated elongated rod-shaped cells (from 3c) into cocci; e) Colony A and B are examples of *L.bulgaricus* showing irregular edges at 52°C

Incubation Temp.	LMRS (log CFU/mL)	Yogurt (log CFU/mL)
Control (42°C)	7.62 ± 0.23	7.41 ± 0.36
Mutant (52°C)	4.66 ± 0.59	1.87 ± 0.98

Table 3: Viable counts of control and mutant *L.bulgaricus* growing on LMRS plate vs after incubated as yogurt.

5.2. Level of fitness for heat-tolerant *L.bulgarius* and pH

Lactic acid and other acids contribute to yogurt acidity. Lactic acid, in particular, is the primary source of yogurt refreshing tartness and functionality. During fermentation, approximately 20-40% of the lactose is converted into lactic acid, resulting in approximately 0.9 percent of the lactic acid with a pH of around 4.4 in yoghurt (Cheng, 2010). However, based on the findings of this study, the concentrations of L-lactic acid and D-lactic acids is the contributor to yogurt acidity. The predominant metabolite formed during milk fermentation is L-lactic acid, with small amounts of D-lactic acid found in yoghurt, which isomer reduces cell metabolism and causes acidosis. The pH and lactic acid concentrations of the control and mutant inoculated milk samples were significantly different in this study as the incubation temperatures were increased. L-lactic acid is the most common metabolite formed during milk fermentation. D-lactic is an acidosis-causing isomer found in small amounts in yoghurt (Slapkauskaite et al., 2016). Both L- and D- lactic acid isomers were found in the control inoculated milk samples; 5.40 ± 0.20 and 2.13 ± 0.80 g/L, respectively, where neither of the lactic acid isomers were found from milk inoculated with the mutant strain. The absence of lactate dehydrogenase, the primary enzyme responsible for lactic acid production during metabolism, may explain the result. It is possible that the interconversion of pyruvate and lactate using NADH and NAD+ had decreased as proposed by Gaspar and others (2014). Another possible explanation could be that by-product (ethanol or acetic acid) formation may have interfered with the lactic acid production (Zhou, Shanmugam, & Ingram, 2003).

Apart from the lack of L-lactate dehydrogenase and D-lactate dehydrogenase, studies have revealed other limitations, such as low yield, because of the formation of by-products (ethanol production or acetic acid production) (Zhou et al., 2003). Later in this

study's free amino acids (FAAs) and volatile part would demonstrate the relationship between by-product and lactic acid production of the bacteria strains.

The pH of the inoculated milk samples made from the two different strains differed significantly ($p < 0.05$). The average pH of samples made from the thermo-tolerant strain was 6.84 ± 0.13, which was much higher than the average pH of yoghurt in general, 4.2 to 4.6, and 4.55 ± 0.04 when compared to the control in the study. Low pH in inoculated milk is caused by casein precipitation and coagulation, resulting in a dense texture. In the absence of lactic acid in thermo-tolerant strain-inoculated milk, a low level of casein crosslinking would have occurred to form the gel network. Lucey (2001) suggested claims that using a high incubation temperature for yoghurt production accelerates acidification, the concept was not applicable to using bacteria grown at a higher temperature as a starter culture. Despite the higher pH, the texture of the inoculated milk after fermentation remained firm, which could be explained by the bacteria's production of EPS. More testing of the EPS production process will be required to validate this explanation.

5.3. Water Holding Capacity of inoculated milk samples

The amount of water that were retained by protein matrix in inoculated milk were significantly different ($P < 0.05$) between the two inoculated milk samples (control $98.10\% \pm 0.60$ and mutant $37.1\% \pm 0.35$). This parameter indicated a possible presence of larger pores in the gel network of the inoculated milk samples. Whey protein denatured and junction in the gel network resulted in possible rearrangements of casein particles and the gel network (Lucey, 2001). In general, there are two main types of water held in a protein structure: 1) bonding parts to the molecules has lost its ability, which can be defined as the absorbed water and 2) the other part trapped in the protein matrix of polysaccharide or fat matrix as the retained water in yoghurt (Kneifel, Paquin,

Abert, & Richard, 1991). WHC is a measurement the combined ability of absorbing and retaining water in yogurt.

Studies have suggested that factors such as high incubation temperatures, fast rates of acidification increase whey separation (Lucey, 2001).

The pH of the 52°C samples was 6.84 ± 0.13, which was higher than the yogurt pH range of 4.2 to 4.6. The product's typical dense texture is thought to be due to casein precipitation and coagulation, which results in the low pH. Lactic acid was absent from the yogurt samples, leading to a low level of casein crosslinking, that further formed the gel network. Given the increased pH of the samples, the texture of the 52°C samples was hard. Production of EPS could explain the firmness of the mutant inoculated milk samples. Validation of this explanation will require analytical testing of the EPS manufacturing process.

Sample (Incubation Temp)	WHC (%)	pH	L-Lactic acid (g/L)	D- Lactic acid (g/L)
Control (42°C)	98.10 ± 0.60^{a}	4.55 ± 0.04^{a}	5.40 ± 0.20	2.13 ± 0.80
Mutant52°C	37.1 ± 0.35^{b}	6.84 ± 0.13^{b}	ND	ND

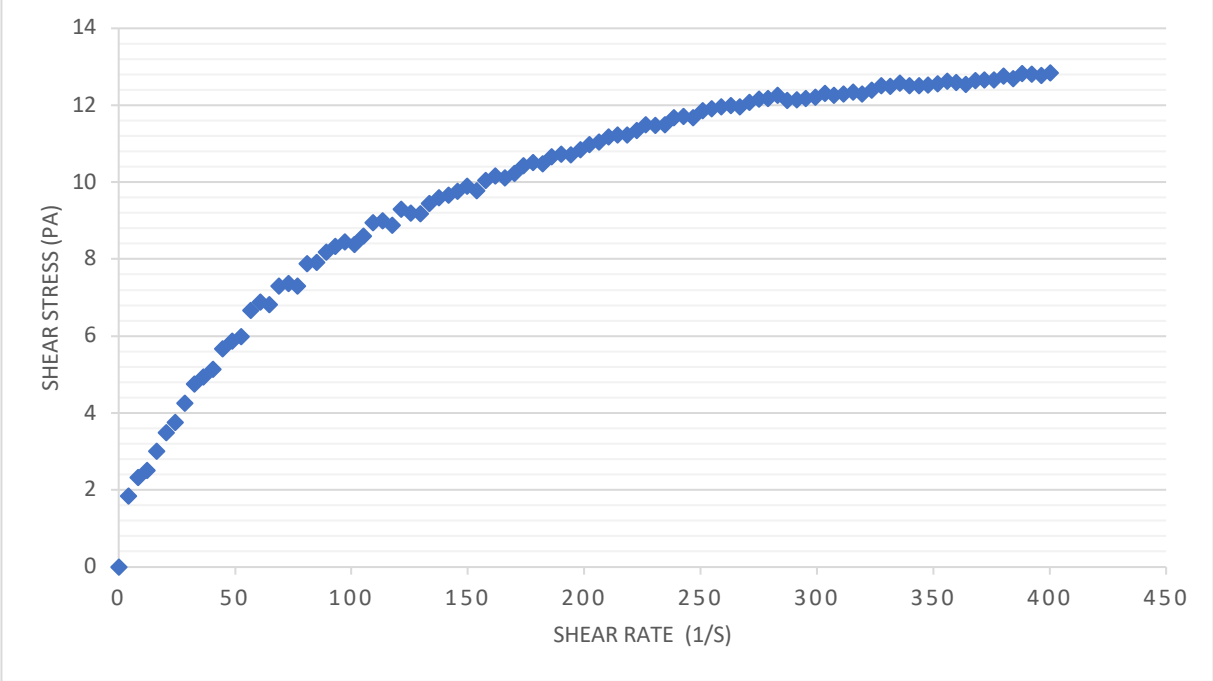
Table 4: Physico-chemical properties of control and mutant inoculated milk samples. Means are different at $P < 0.05$. Letters (a, b) indicate statistical difference ($P < 0.05$) across each column.
ND = Not detectable

5.4. Shear viscosity of inoculated milk samples

Yield stress is associated with the presence of a cross-linked yogurt structure, which is used to characterise yogurt (Lobato-Calleros, Ramírez-Santiago, Vernon-Carter, & Alvarez-Ramirez, 2014). The aggregation of casein particles influences the rheological behaviour of yogurt as a cross-linked structure or the interactive (Lucey, 2001; Yu,

Wang, & McCarthy, 2016). Usually, the use of a single strain of *Lactobacilli* is known to produce the lowest firmness and curd tension in yogurt production (Hassan, Frank, Schmidt, & Shalabi, 1996), which has a weaker ability to hold onto water, causing syneresis. In order to stable texture and viscosity and minimise syneresis in yogurt samples, both the control and mutant strain inoculated milk samples were incubated at 42°C for 24 hours. The control inoculated milk sample required approximate 2 Pa of yield stress applied before the flow. The entangled molecules in the milk gel structure started to untangle (from stress rate 0 to 72.72 s^{-1}) and became less resistant to flow, which demonstrated a shear-thinning behaviour. Mutant inoculated milk samples showed a lower yield stress of 0.5 Pa. Compared to the control sample, the flow curve of mutant inoculated milk samples appeared to be more linear, indicating the shear-thinning behaviour could be explained by the weak alignment of the biopolymer-biopolymer molecules interactions or biopolymer molecules with the shear field (Lobato-Calleros et al., 2014). High yield stress prevents the yogurt from undergoing the separation phase or breaking down (Saleh et al., 2020), which is linked to the result of WHC of the samples, where mutant inoculated milk samples appeared to be thinner with production of whey (Figure 9a and 9b). Yogurt gel with homogeneous networks in which cells can grow and stay in linked chains, implying that bacteria are evenly distributed throughout the gel system with solid attachments between EPS and protein (Teggatz & Morris, 1990).

a)



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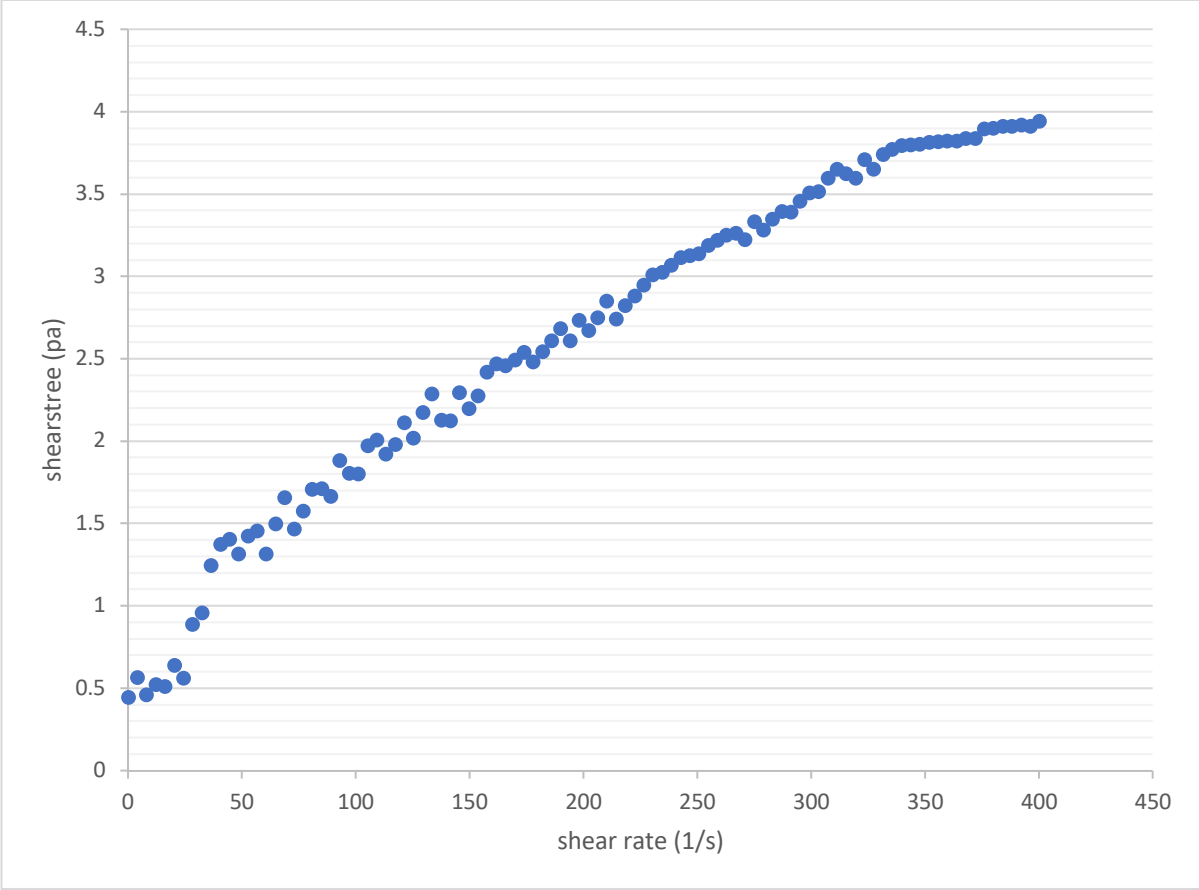


Figure 9: Shear stress versus shear rate relationships for the control inoculated milk sample (42°C). b). Shear stress versus shear rate relationships for mutant inoculated milk sample (52°C).

5.5. Free Amino Acid Profile of Yogurt Samples

A total of 27 free amino acids (FAAs) were identified in the study, with a total amino acid content of 225.25 ± 37.45 µg/100g in control inoculated milk and 1747.42 ± 34.69 µg/100g, which is 7.76 times higher than control inoculated milk samples. Literature using *L.bulgaricus* as a starter showed a big range of variation of total amino acid content. From 24.98 mg/g with 5% *L.bulgaricus* (Rao, Pulusani, & Rao, 1982); 76.22 mg/100g with 1% (v/v) of *L.bulgaricus* (Beshkova, Simova, Frengova, Simov, & Adilov, 1998). As shown in Table 5, most of the individual FAAs detected in the current study were significantly different between those made from the control and the thermo-tolerant strain ($p < 0.001$). Significantly higher proteolytic activity, measured from the release of FAAs (Donkor, Henriksson, Vasiljevic, & Shah, 2007), in both total and individual FAAs amounts, was found in inoculated milk produced from the thermo-tolerant strain. Inoculated milk samples of mutant strain highest proteolytic activity released the highest amount of free amino groups (Donkor et al., 2007). In comparison to the control inoculated milk sample, the concentration of the following amino acid increased in mutant inoculated milk samples. Histidine (19.95 times), arginine (115.73 times), threonine (10.31 times), alanine(3.59 times), ornithine (55.64 times), methionine (274.52 times), valine (19.27times), lysine (7.69 times), tyrosine (37.27 times), isoleucine (14.99 times), leucine (47.67 times), phenylalanine (21.51 times) and tryptophan (19.63 times) (Table 4). with mutant inoculated milk sample recorded the highest concentration of glutamic acid (265.88 ± 5.08 ug/100g), which is 4.8 times higher than the concentration of glutamic acid found in control inoculated milk sample (55.27 ± 9.67 ug/100g). Furthermore, glutamic acid shown the highest concentration rise among all the FAAs. With NAD+ or NADP+ as a co-factor, glutamate dehydrogenases catalyse the reversible deamination of glutamate and free NH_4+ to create glutamic acid (Sawada, Koyano, Yamamoto, & Yamada, 2021). Increased amino

acid levels could be associated with an increased biosynthetic activity, which allows exopeptidases to hydrolyse the polypeptide chain of storage proteins (Asano, Nakamura, Kawai, Miwa, & Nio, 2010). *L. bulgaricus* is mainly responsible for the hydrolysis of milk protein. Exopeptidases hydrolysed the peptide bond and continued the reaction to a higher degree of hydrolysis in mutant inoculated milk samples as the number of amino acids increased, indicating that in mutant inoculated milk samples, exopeptidases hydrolysed the peptide bond and continued the reaction to a higher degree of hydrolysis than in control samples (Gu et al., 2021; Mann, Athira, Sharma, & Bajaj, 2017). Moreover, as a result, it forms more amino acids, amino acids and oligopeptides (such as alcohols, aldehydes, acids and esters). Non- essential arginine (control stain 2.05 ± 0.35 ug/100g and mutant strain 237.24 ± 5.94 ug/100g) and essential amino acid methionine 0.36 ± 0.07 ug/100g, 98.83 ± 1.78 ug/100g) have shown the concentrations increased over 100 folds and more. Functional amino acids, such as arginine and methionine, influence growth and health via regulating immune defence and an antioxidant system (Castro & Kim, 2020).

As mentioned before, glutamic acid in mutant inoculated milk has the highest yield, the pH of the mutant inoculated milk, the sample was at 6.86 ± 0.13, there are all desirable factors for arginine formation (Hwang & Lee, 2018; Utagawa, 2004). As a result, it's critical to find a balance between the production of peptides and the degree of breakdown into free amino acids.

L.bulgaricus has enzyme systems that use amino acids in their metabolism and form flavour compounds in food. The flavour compounds are formed by the biochemical and chemical conversion of the amino acid. L-methionine catabolism generated methanethiol and developed cheesy flavour compound in mutant yogurt sample (Papagianni, 2012a).

As seen in this study, *L.bulgaricus* strains modified by ALE were employed to isolate amino acid overproducers. Mutant strains were thought to be deficient in analogues, synthesising too much methionine and releasing it into mutant inoculated milk samples (Kase & Nakayama, 1975). Although amino acids do not contribute to the flavour and aroma characteristic of yogurt, numerous reactions in yogurt are caused by carbonyl molecules. For example, 1-methoxy-2-propanol (Valine), 2-methyl-1-butanol (Leucine), and sulfur compounds methionine) (Ardö, 2006; Simova et al., 2006; Smit et al., 2005). Which was relevant to the flavour and aroma compounds are discussed later.

	Control (*µg/100g)*	Thermo-tolerant *(µg/100g)*
Essential amino acid		
Lysine	22.73 ± 3.22 [a]	174.79 ± 1.90 [b]
Phenylalanine	6.091 ± 1.05[a]	137.45 ± 2.25 [b]
Threonine	4.80± 0.78 [a]	49.49 ± 1.17 [b]
Histidine	4.63 ± 0.23[a]	92.36 ± 2.4[b]
Valine	4.62 ± 0.87 [a]	89.02 ±2.05 [b]
Leucine	4.59 ± 0.77 [a]	218.79 ±4.36 [b]
Isoleucine	2.78 ± 0.46 [a]	41.66 ± 1.12 [b]
Tryptophan	1.20 ± 0.20 [a]	23.55 ± 0.31 [b]
Methionine	0.36 ± 0.07 [a]	98.83 ± 1.78 [b]
Non-essential amino acid		
Glutamic acid	55.27 ± 9.67 [a]	265.88 ± 5.08 [b]
Proline	40.76 ± 7.19 [a]	17.52 ± 0.42 [b]
Ethanolamine	22.72 ± 3.93 [a]	4.07 ± 0.14 [a]
Alanine	22.29 ± 4.02 [a]	79.98 ± 2.63 [b]
Glycine	9.57 ± 1.58 [a]	14.31 ± 0.34 [b]
Taurine	8.34 ± 1.44 [a]	6.52 ± 0.26 [a]
Serine	2.65 ± 0.32 [a]	0.28 ± 0.04 [b]
Aspartic acid	2.59 ± 0.47 [a]	1.11 ± 0.05 [b]
Tyrosine	2.35 ± 0.35 [a]	87.59 ± 2.89[b]
Arginine	2.05 ± 0.35 [a]	237.24 ± 5.94 [b]
Ornithine	1.86 ± 0.16 [a]	103.49 ± 2.09 [b]
Citrulline	1.03 ± 0.10 [a]	2.30 ± 0.04 [b]

Alanine	0.59 ± 0.09 [a]	0.19 ± 2.63 [a]
L-α-Aminon-butyric acid	0.57 ± 0.11 [a]	0.26 ± 0.02 [a]
δ-Hydroxylysine	0.31 ± 0.08 [a]	Not detected
Hydroxy-L-Proline	0.19 ± 0.03 [a]	0.18 ± 0.01 [a]
Γ-Amino-n-butyric acid	0.19 ± 0.03 [a]	0.42 ± 0.02 [a]
Sarcosine	0.07 ± 0.01 [a]	0.04 ± 0.01 [a]
DL-β-Aminoisobutryic acid	0.03 ± 0.01 [a]	0.11 ± 0.01 [b]
Total free amino acids	225.25 ± 37.45	1747.42 ± 34.69

Table 5: FAAs profile of inoculated milk samples made from the control and mutant strain of *L.bulgaricus* using LCMS. Data are presented as means ± SD, from the highest to the lowest quantity. Letters (a and b) indicate statistical difference ($P < 0.001$) in each row, n=2.

5.6. Volatile organic compounds (VOCs) and non-volatile compounds (non-VOCs) in inoculated milk samples

SPME-GC-MS detected a total of 56 chemicals in both control and mutant inoculated milk samples. In yogurt fermentation, a single starter strain of *S.thermophilus* produced 53 volatile compounds (Zhang, Mi, Liu, Sang, & Wang, 2020), At the same time, 31 volatile compounds were found in cow milk and 34 volatile compounds in goat milk (Erkaya & ŞEngÜL, 2011). As shown in Table 6, these were divided into chemical groups: 10 carboxylic acids, 8 aldehydes, 11 ketones, 17 alcohols, and 10 esters. The major compounds found in yogurt are usually acetaldehyde, ethanol, acetone, diacetyl, and 2-butanone have a significant impact on the flavouring (Cheng, 2010; Gyosheva, 1985; Tamime & Robinson, 2007). However, acetaldehyde was not detected from either of the inoculated milk samples tested in this study. A similar finding was reported by Raya et al. (1986), suggesting that acetaldehyde had not formed from pyruvate. Due to the absence of α-carboxylase or aldehyde dehydrogenase, which generate acetaldehyde in *L.bulgaricus* Raya et al. (1986). All of the samples included 3-methyl-butanoic acid, which was likely generated from 3-methylbutanal by leucine catabolism, as seen in many dairy products, and contributed to the pleasant fresh cheese aroma (Curioni & Bosset, 2002; Smit, Engels, & Smit, 2009).

Both control (313.62 ± 0.20 µg/L) and mutant inoculated milk samples (844.79 ±0.13 µg/L) showed high diacetyl (2,3-butanediol) production, forming cheese-like aroma for the product. For LAB, nutrient conditions had an important impact on diacetyl production. In this study, the mutant strain that was grown on LMRS media may have led *L. bulgaricus* mutant strain to produce more diacetyl than the control strain due to the lactose supplementation. A similar result has confirmed the finding and suggested that a rich MRS media with glucose and citrate supplement boosted diacetyl production by more than threefold as compared to a minimum media (Choi, Yoon, Subbammal Kalichamy, Yoon, & Il Lee, 2016). Moreover, research on *L.lactis* utilising modified culture mediums

(potassium phosphate buffer with 0.5% glucose; reconstituted skim milk with 1% glucose) resulted in overexpressed NADH oxidase has been hypothesised to divert metabolic flux away from lactate formation and towards oxidised product during general citrate metabolic pathways, resulting in enhanced diacetyl production of 0.36 to 0.38 g/L (Bassit, Boquien, Picque, & Corrieu, 1993; Kleerebezemab, Hols, & Hugenholtz, 2000). Among all the ketone compounds, 2,3-butanedione (control:313.62 ± 0.20 ug/L, thermo-tolerant: 844.79± 0.13 ug/L); acetoin (control: 1619.33 ± 1.12 ug/L, thermo-tolerant: 1198.05 ± 2.13 ug/L), 2-heptanone (control: 126.81± 0.04 ug/L, thermo-tolerant: 2045.73 ± 0.35ug/L), and 2-nonanone (control: 50.58 ±0.27 ug/L, thermo-tolerant: 12.50 ±0.77) were the key compounds to providing the overall profile of the inoculated milk samples. Acetoin, which is formed by the reduction of diacetyl, contributes to the buttery, creamy flavour of yogurt, and lactate decarboxylase is also produced acetoin from a-acetolactate (Paul & Maria José, 2000)

Alcoholysis uses alcohol and fatty acyl-CoAs derived from the metabolism of fatty acids, amino acids, or carbohydrates in milk (Liu, Holland, & Crow, 2004). Alcoholysis promoted ester biosynthesis by lipase and LAB in fermented dairy products (Cristiani & Monnet, 2001). However, esterification is the process of forming an ester from alcohol and carboxylic acid.

Alcoholysis was one of the explanations for the lack of lactic acid in mutant inoculated milk samples, according to this finding. In the mutant inoculated milk samples, there was an increase in esterification and alcoholysis activity. VOCs discovered in the mutant inoculated milk samples were ethyl butanoate, ethyl butanoate, and ethyl decanoate, which are typical esters found in cheeses. These cheese flavour components contributed to the inoculated milk sample's fructify flavour and off-flavour development. Excessive production by-products, such as alcohol, can reduce lactic acid

production, as previously stated. The results in Table 6 show that among the several forms of alcohol produced, mutant inoculated milk had the highest concentration. The chemical reaction called Strecker corruption is a response where the corrosive amino gathering of amino corrosive with a α-decarbonyl. The response in escalated particularly under high temperature as the direct oxidative decarboxylation of amino corrosive (Dunn & Lindsay, 1985; Perpète & Collin, 2000). Strecker debasement of leucine generate 3-methylbutanal. Nonetheless, some other reviews proposed that the transformation of valine likewise generates aldehyde (Yaylayan & Keyhani, 2001). Also, the particular synthetic response produces dynamic flavour mixtures, which might add to the character arrangement in mutant inoculated milk flavouring. More exploration is wanted to decide the immediate connection between these compound responses under various maturation conditions.

Compound	m/z	RT (min)	RI	concentration (ug/L)	
				Control	Mutant
Carboxylic acid					
Oxalic acid	89.90	3.17	929.00	158.34 ± 0.19	7973.73 ± 3.45
Acetic acid	60.02	11.75	1443.27	1619.33 ± 1.12	1198.05 ± 2.13
Propanoic acid, 2-methyl-	88.05	13.66	1563.69	14.06 ± 0.33	130.81 ± 0.34
Butanoic acid	88.05	14.52	1620.85	4675.15 ± 3.76	3523.13 ±1.21
Pentanoic acid	102.07	16.18	1735.18	30.38 ± 0.61	18.97 ± 0.31
Hexanoic acid	116.08	17.60	1838.63	9195.56 ± 4.32	5026.78 ± 0.73
Octanoic acid	144.12	20.35	2053.31	4251.74 ± 1.11	2209.72 ± 2.13
Nonanoic acid	158.13	21.68	2164.51	0.76 ± 0.00	9.71 ± 0.20
Benzoic acid	122.04	24.62	2430.28	2304.57 ± 0.52	822.18 ± 0.78
Dodecanoic acid	200.18	25.14	2479.94	118.84 ± 0.94	177.05 ± 0.64
Aldehyde					
Pentanal	86.07	3.68	969.00	249.09 ± 0.83	69.44 ± 0.63
Hexanal	100.09	5.30	1073.77	107.97 ± 0.74	ND
Heptanal	114.10	7.13	1177.34	152.60 ± 0.37	26.64 ± 0.32
4-Heptenal, (Z)-	112.09	8.17	1235.22	12.37 ± 0.27	1.54.09 ± 0.62
Nonanal	142.14	10.87	1390.06	57.98 ± 0.08	33.29 ± 0.34
Methional	104.03	11.82	1447.77	12.02 ± 0,61	10.79 ±0.12
Benzaldehyde	106.04	12.92	1515.97	602.83 ±0.26	195.73 ± 0.12
2-Nonenal, (E)-	104.12	13.19	1533.35	50.58 ±0.27	12.50 ±0.77
Ketone					
Butanone	72.06	2.73	891.00	170.66 ± 0.51	459.19± 0.40
2,3-Butanedione	86.04	3.65	967.00	313.62 ± 0.20	844.79 ±0.13

2-Pentanone	86.07	3.66	968.16	313.62 ±0.63	845.02 ±0.13
2,3-Pentanedione	100.05	4.92	1051.51	75.49 ±0.12	8.27 ±0.32
3-Heptanone	114.10	6.55	1145.18	28.69 ± 0.01	9.11± 0.01
2-Heptanone	114.10	7.10	1175.84	126.81± 0.04	2045.73 ± 0.35
2-Nonanone	142.14	10.80	1385.67	98.23± 0.51	1881.56 ±0.87
3-Octen-2-one	126.10	11.11	1404.45	6.09 ±0.12	ND
1-Propanone, 1-(2-furanyl)-	124.05	13.71	1567.32	59.12± 0.68	18.65 ±0.76
2-Undecanone	107.17	14.17	1597.26	54.96 ± 0.32	222.63 ±0.49
2-Tridecanone	198.20	17.20	1809.13	12.67 ± 0.12	49.41± 0.77
Alcohol					
2-Butanol	74.07	4.48	1024.77	2.34 ± 0.15	16.94 ± 0.64
1-Propanol	60.06	4.69	1037.41	2.48 ± 0.01	61.96 ± 0.70
1-Butanol	74.07	6.69	1153.23	7.27 ± 0.34	33.59± 0.41
1-Pentanol	88.09	8.45	1250.99	380.14± 0.91	127.93± 0.18
2-Heptanol	116.12	9.69	1320.80	ND	419.95 ± 0.02
1-Hexanol	102.10	10.24	1353.39	159.70 ±0.73	181.99 ±0.91
1-Octen-3-ol	128.12	11.84	1448.93	45.45 ±0.82	19.08 ± 0. 19
1-Heptanol	116.12	11.94	1455.01	117.50 ± 0.38	58.30 ± 0.23
1-Hexanol, 2-ethyl-	130.14	12.49	1488.77	754.34± 0.94	1945.54 ± 0.76
2-Nonanol	144.15	12.97	1519.23	ND	189.36 ± 0.83
2,3-Butanediol	90.07	13.25	1537.43	24.49 ±0.42	6641.79 ± 0.18
Linalool	154.14	13.37	1545.42	46.18 ±0.85	10.41 ± 0.62
1-Octanol	130.14	13.56	1557.23	31.98 ± 0.76	158.46 ± 0.10

2-Undecanol	172.18	15.95	1718.99	21.66 ± 0.37	8.74 ± 0.99
1-Decanol	158.17	16.54	1761.46	6.05 ± 0.17	109.51± 0.57
Benzyl alcohol	108.06	18.01	1869.40	28.03± 0.82	38.95 ± 0.35
1-Dodecanol	186.20	19.26	1966.08	9.24 ± 0.74	9.10 ± 0.68
Ester					
Methyl butyrate	102.07	3.79	978.00	37.91± 0.27	8.19 ± 0.97
Ethyl butyrate	116.08	4.60	1031.81	51.60± 0.89	607.84 ± 0.47
Butanoic acid, 2-methylpropyl ester	144.12	6.64	1150.09	ND	26.88 ± 0.30
Ethyl hexanoate	144.12	8.03	1227.60	4.25 ± 0.51	168.82 ±0.63
Butanoic acid, 3-methylbutyl ester	158.13	8.62	1260.50	3.29 ± 0.58	50.87 ± 0.07
Ethyl octanoate	172.15	11.64	1436.68	55.99 ± 0.21	182.17 ± 0.16
Ethyl decanoate	200.18	14.78	1638.23	23.60 ± 0.74	313.61 ± 0.11
Ethyl dodecanoate	228.21	17.67	1844.22	112.40 ± 0.14	107.41± 0.28
Methyl hexadecanoate	270.26	22.29	2217.59	39.96 ± 0.47	30.74 ± 0.20
Ethyl hexadecanoate	284.27	22.72	2255.91	42.95 ± 0.71	40.25 ± 0.79

Table 6: Volatile compounds and non volatile compounds in inoculated milk samples analysed by SPME-GC-MS are shown. Data are presented as mean ± standard deviation. M/z refers to mass-to-charge ratio, RT refers to retention time, RI refers to retention index and ND refers to not detected.

5.7. Odour threshold and relative odour activity value (OAV) of inoculated milk samples

The ratio of the odour threshold concentration (OAV) to the volatile compounds, demonstrated how each compound contributes to the volatile profile of the sample. The overall odour is proportional to the OAV value—the greater the OAV value, the greater the overall odour. Only the inoculated milk samples' dominant odour/flavour profiles were collated in Table 6 due to the lack of information on the OAV in similar investigations. Values greater than their threshold concentrations (relative OAVs > 1) were identified as odour-active compounds. In the control inoculated milk sample, four carboxylic acids have shown relative OAVs greater than 1, with butanoic acid being the dominating compound (OAV of 19.47). For aldehydes, 2-Nonenal (E) was the highest with OAV of 632, followed by nonanal, heptanal and 4-Heptenal, (Z). For ketones, 2-Nonanone was the most dominant (OAV of 19.65), and for alcohols and esters, 1-often-3-ol (OAV 45.45) and ethyl butyrate (OAV 51.60) were the dominating compounds. Overall, the odour compound of the control samples appeared to be fruity with a mushroom note.

The relative OAVs of volatile compounds identified in inoculated milk made from the mutant strain, on the other hand, were similar to those reported in control. Butanoic acid was the most abundant compound in the carboxylic acid group, with an OAV value of 14.69. 2-Nonenal (E) was the most abundant compound in the aldehyde group (OAV 156.25) but significantly lower than the control. There were three major ketone compounds, the highest of which was 2-Nonanone (OAV 376.31), four dominant alcohol compounds, and two esters, the most significant of which were 2-Heptanol (OAV 139.98) and Ethyl butyrate (OAV 607.84). The inoculated milk made from the mutant strain was described as having a cheesy, greasy flavour with pineapple flavours.

Significant decreases in ethyl butyrate (by 7.7 times), heptanal (by 5.72 times), 2-nonenal (E) (by 4.05 times) and 4-heptenal (Z) (by 6.23 times) and significant increases in ethyl hexanoate (by 39.5 times) and 2-nonanone (by 19.15 times) were found in the OAVs, comparing the inoculated milk produced from the control and the mutant strain. 2-heptanol is an alcohol compound, was lacking in control but formed high value in the mutant inoculated milk. Compared to the control, these volatile compounds had a significant impact on the overall aroma of the inoculated milk, as shown in Table 5. FAAs such as methionine, phenylalanine, threonine, and branched-chain amino acids (leucine, isoleucine, and valine) (Smit et al., 2005) contribute to the cheese-like flavour is also significant (Table 4).

Compound	Odour description	Threshold in water (ppb)*	OAVs	
			Control	Thermo - tolerant
Carboxylic acid				
Acetic acid	Vinegar, pungent acidic	800	2.02	1.49
Propanoic acid	Vinegar, pungent, sour milk	20000	>1	>1
Butanoic acid	Fruity, dairy, cheesy	240	19.47	14.69
Hexanoic acid	Fatty, cheesy	3000	3.07	1.68
Octanoic acid	Fatty, cheesy	3000	1.41	>1
Nonanoic acid	Fatty, green	3000	>1	>1
Dodecanoic acid	Fatty, coconut, bay oil	10000	>1	>1
Aldehyde				
Pentanal	86.07	1500	>1	>1
Heptanal	Green, sweet	3	50.87	8.88
4-Heptenal, (Z)-	Cream and butter	0.8-10	15.46	2.48
Nonanal	Fatty, citrus, green	1	57.98	33.29
2-Nonenal, (E)-	Fatty, green, mushroom	0.08-0.1	632.25	156.25
Ketone				
2-Butanone	Varnish-like, sweet, fruity	50000	>1	>1
2-Pentanone	Fruity, acetone	70000	>1	>1
2-Heptanone	Banana-like, fruity	140-3000	>1	14.61
2-Nonanone	Fruity, cheesy, buttery	5-200	19.65	376.31
3-Octen-2-one	Mushroom, fruity	28	>1	ND
2-Undecanone	Floral, rose-like, herbaceous	14.17	3.87	15.71
Alcohol				

2-Butanol	wine	500	>1	>1
1-Propanol	Alcoholic, pungent	9000	>1	>1
1-Butanol	Fruity, alcoholic	500	>1	>1
1-Pentanol	Alcoholic, iodoform-like	4000	>1	>1
2-Heptanol	Earthy oily	3	ND	139.98
1-Octen-3-ol	Mushroom-like	1	45.45	19.08
1-Heptanol	Earthy, oily	3	39.17	19.43
1-Octanol	pungent	110-130	>1	1.44
Ester				
Methyl butyrate	Fruity, apple, pineapple	60-76	>1	>1
Ethyl butyrate	Pineapple-like	1	51.60	607.84
Ethyl hexanoate	Fruity, apple, banana	1	4.25	168.82
Ethyl hexadecanoate	Fruity, creamy, waxy	>2000	>1	>1

Table 7: Odour descriptors of selected volatile compounds, relative odour activity value (OAV) and odour threshold in water identified in inoculated milk samples are shown. Odour descriptions of volatiles were adapted from (Cheng, 2010; Thierry et al., 2015)

Chapter 6 Conclusion and Futures Prospects

For the first time, thermo-tolerant *L. bulgaricus* was created through spontaneous mutation using ALE. The thermo-tolerant strain survived repeated exposure to elevated growth temperature over time, albeit with a noticeable change in morphology and

reduced lactic acid production during fermentation. The molecular mechanisms underlying these observations are not fully understood and require additional research. When compared to the control, milk inoculated with the thermo-tolerant *L. bulgaricus* has higher proteolytic activity, producing more free amino acids, particularly essential amino acids. The milk inoculated with the thermo-tolerant *L. bulgaricus* produced significantly more volatile compounds, which contributed to the cheese-like aroma. ALE is a simple yet robust method to modified strain tolerance to the desired condition. A thermo-tolerant strain of *L.bulgaricus* was developed using ALE. Using a modified MRS agar plate with 2% lactose as the growth medium was able to protect *L. bulgaricus* at 52°C. The morphology, survival strategy, and level of fitness of the mutant strain were changed. Although the molecular mechanisms that caused the morphology changes in each of these examples were not precisely identified, this study's discovery was a significant step forwards. Nonetheless, because *L. bulgaricus* is used in the production of yogurt, the strain's spontaneous mutation produced unexpected results such as overproduction of amino acids using LCMS. This study attempted to explain the possible changes, as well as the metabolic pathways underlying each discovery. Many of the experiments (DNA profiling) were unable to be performed due to a lack of studies on a similar topic and the covid lockdown.

Stress caused by elevated growth temperature may have resulted in incomplete or a modified metabolism in *L. bulgaricus*, resulting in the production of organic acids. Competing microorganisms in the milk due to the lack of acidification may also have contributed to the production of these metabolites. When compared to the control, there was an increase in diacetyl formation, which contributed to the change in volatile profile to a cheese-like aroma. Further research into DNA profiling, metabolomics, and peptidomics will aid in understanding the mechanisms underlying the observed changes in the study. Measurements of EPS, antioxidant and ACE-inhibitory activities, as well

as the use of better imaging microscopy (e.g., FE-SEM), will aid in characterising the thermotolerant *L. bulgaricus*'s behaviour.

References

Aguilera, J., Andreu, P., Randez-Gil, F., & Prieto, J. A. (2010). Adaptive evolution of baker's yeast in a dough-like environment enhances freeze and salinity tolerance. *Microbial Biotechnology, 3*(2), 210-221. https://doi.org/https://doi.org/10.1111/j.1751-7915.2009.00136.x

Aldaw Ibrahim, I., Naufalin, R., Erminawati, & Dwiyanti, H. (2019, December 01, 2019). Effect of fermentation temperature and culture concentration on microbial and physicochemical properties of cow and goat milk yogurt Retrieved from https://ui.adsabs.harvard.edu/abs/2019E&ES..406a2009A https://doi.org/10.1088/1755-1315/406/1/012009

Ardö, Y. (2006). Flavour formation by amino acid catabolism. *Biotechnology Advances, 24*(2), 238-242. https://doi.org/https://doi.org/10.1016/j.biotechadv.2005.11.005

Årsköld, E., Lohmeier-Vogel, E., Cao, R., Roos, S., Rådström, P., & van Niel, E. W. J. (2008). Phosphoketolase Pathway Dominates in Lactobacillus reuteri ATCC 55730 Containing Dual Pathways for Glycolysis. *Journal of Bacteriology, 190*(1), 206. https://doi.org/10.1128/JB.01227-07

Asano, M., Nakamura, N., Kawai, M., Miwa, T., & Nio, N. (2010). Purification and Characterization of an N-Terminal Acidic Amino Acid-Specific Aminopeptidase from Soybean Cotyledons (Glycine max). *Bioscience, Biotechnology, and Biochemistry, 74*(1), 113-118. https://doi.org/10.1271/bbb.90617

Bassit, N., Boquien, C.-Y., Picque, D., & Corrieu, G. (1993). Effect of Initial Oxygen Concentration on Diacetyl and Acetoin Production by Lactococcus lactis subsp. lactis biovar diacetylactis. *Applied and Environmental Microbiology, 59*(6), 1893-1897. https://doi.org/10.1128/aem.59.6.1893-1897.1993

Beal, C., Skokanova, J., Latrille, E., Martin, N., & Corrieu, G. (1999). Combined Effects of Culture Conditions and Storage Time on Acidification and Viscosity of Stirred Yogurt. *Journal of Dairy Science, 82*(4), 673-681. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(99)75283-5

Bean, G. J., Flickinger, S. T., Westler, W. M., McCully, M. E., Sept, D., Weibel, D. B., & Amann, K. J. (2009). A22 disrupts the bacterial actin cytoskeleton by directly binding and inducing a low-affinity state in MreB. *Biochemistry, 48*(22), 4852-4857. https://doi.org/10.1021/bi900014d

Behera, S. S., Ray, R. C., & Zdolec, N. (2018). Lactobacillus plantarum with Functional Properties: An Approach to Increase Safety and Shelf-Life of Fermented Foods. *BioMed research international, 2018*, 9361614-9361614. https://doi.org/10.1155/2018/9361614

Bendezú, F. O., & de Boer, P. A. J. (2008). Conditional lethality, division defects, membrane involution, and endocytosis in mre and mrd shape mutants of Escherichia coli. *Journal of bacteriology, 190*(5), 1792-1811. https://doi.org/10.1128/JB.01322-07

Beshkova, D. M., Simova, E. D., Frengova, G. I., Simov, Z. I., & Adilov, E. F. (1998). Production of Amino Acids by Yogurt Bacteria. *Biotechnology Progress, 14*(6), 963-965. https://doi.org/https://doi.org/10.1021/bp980082j

Bintsis, T. (2018). Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. *AIMS microbiology, 4*(4), 665-684. https://doi.org/10.3934/microbiol.2018.4.665

Birrell, G. W., Brown, J. A., Wu, H. I., Giaever, G., Chu, A. M., Davis, R. W., & Brown, J. M. (2002). Transcriptional response of Saccharomyces cerevisiae to DNA-damaging agents does not identify the genes that protect against these agents. *Proceedings of the National Academy of Sciences, 99*(13), 8778. https://doi.org/10.1073/pnas.132275199

Brady, S. P., Bolnick, D. I., Angert, A. L., Gonzalez, A., Barrett, R. D. H., Crispo, E., . . . Hendry, A. P. (2019). Causes of maladaptation. *Evolutionary applications, 12*(7), 1229-1242. https://doi.org/10.1111/eva.12844

Brauss, M. S., Linforth, R. S., Cayeux, I., Harvey, B., & Taylor, A. J. (1999). Altering the fat content affects flavor release in a model yogurt system. *J Agric Food Chem, 47*(5), 2055-2059. https://doi.org/10.1021/jf9810719

Brown, E., Dessai, U., McGarry, S., & Gerner-Smidt, P. (2019). Use of Whole-Genome Sequencing for Food Safety and Public Health in the United States. *Foodborne Pathogens and Disease, 16*(7), 441-450. https://doi.org/10.1089/fpd.2019.2662

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., & Gibbs, P. (2004). Effects of Various Sugars Added to Growth and Drying Media upon Thermotolerance and Survival throughout Storage of Freeze-Dried lactobacillus delbrueckii ssp. bulgaricus. *Biotechnology Progress, 20*(1), 248-254. https://doi.org/https://doi.org/10.1021/bp034165y

Castro, F. L. d. S., & Kim, W. K. (2020). Secondary Functions of Arginine and Sulfur Amino Acids in Poultry Health: Review. *Animals : an open access journal from MDPI, 10*(11), 2106. https://doi.org/10.3390/ani10112106

Chassy, B. M., & Giuffrida, A. (1980). Method for the lysis of Gram-positive, asporogenous bacteria with lysozyme. *Appl Environ Microbiol, 39*(1), 153-158. https://doi.org/10.1128/aem.39.1.153-158.1980

Chen, C., Zhao, S., Hao, G., Yu, H., Tian, H., & Zhao, G. (2017). Role of lactic acid bacteria on the yogurt flavour: A review. *International Journal of Food Properties, 20*(sup1), S316-S330. https://doi.org/10.1080/10942912.2017.1295988

Cheng, H. (2010). Volatile Flavor Compounds in Yogurt: A Review. *Critical Reviews in Food Science and Nutrition, 50*(10), 938-950. https://doi.org/10.1080/10408390903044081

Chervaux, C., Ehrlich, S. D., & Maguin, E. (2000). Physiological study of Lactobacillus delbrueckii subsp. bulgaricus strains in a novel chemically defined medium. *Applied and Environmental Microbiology, 66*(12), 5306-5311. https://doi.org/10.1128/aem.66.12.5306-5311.2000

Choi, J. I., Yoon, K.-h., Subbammal Kalichamy, S., Yoon, S.-S., & Il Lee, J. (2016). A natural odor attraction between lactic acid bacteria and the nematode Caenorhabditis elegans. *The ISME Journal, 10*(3), 558-567. https://doi.org/10.1038/ismej.2015.134

Christensen, B. B., Haagensen, J. A. J., Heydorn, A., & Molin, S. (2002). Metabolic commensalism and competition in a two-species microbial consortium. *Applied and Environmental Microbiology, 68*(5), 2495-2502. https://doi.org/10.1128/AEM.68.5.2495-2502.2002

Civelek Yoruklu, H., Oguz Koroglu, E., Demir, A., & Ozkaya, B. (2019). Chapter 5.2 - The Electromotive-Induced Regulation of Anaerobic Fermentation: Electrofermentation. In S. V. Mohan, S. Varjani, & A. Pandey (Eds.), *Microbial Electrochemical Technology* (pp. 739-756): Elsevier. Retrieved from http://www.sciencedirect.com/science/article/pii/B9780444640529000303. https://doi.org/https://doi.org/10.1016/B978-0-444-64052-9.00030-3

Corsetti, A., Perpetuini, G., Schirone, M., Tofalo, R., & Suzzi, G. (2012). Application of starter cultures to table olive fermentation: an overview on the experimental studies. *Frontiers in Microbiology, 3*. https://doi.org/10.3389/fmicb.2012.00248

Costa, E., Usall, J., Teixidó, N., Garcia, N., & Viñas, I. (2000). Effect of protective agents, rehydration media and initial cell concentration on viability of Pantoea agglomerans strain CPA-2 subjected to freeze-drying. *Journal of Applied Microbiology, 89*(5), 793-800. https://doi.org/https://doi.org/10.1046/j.1365-2672.2000.01182.x

Cristiani, G., & Monnet, V. (2001). Food micro-organisms and aromatic ester synthesis. *Sciences des Aliments, 21*(3), 211-230. https://doi.org/10.3166/sda.21.211-230

Cunningham, M., Azcarate-Peril, M. A., Barnard, A., Benoit, V., Grimaldi, R., Guyonnet, D., . . . Gibson, G. R. (2021). Shaping the Future of Probiotics and Prebiotics.

Trends in Microbiology, 29(8), 667-685. https://doi.org/https://doi.org/10.1016/j.tim.2021.01.003

Curioni, P. M. G., & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography-olfactometry. *International Dairy Journal, 12*(12), 959-984. https://doi.org/https://doi.org/10.1016/S0958-6946(02)00124-3

de Boer, P., Crossley, R., & Rothfield, L. (1992). The essential bacterial cell-division protein FtsZ is a GTPase. *Nature, 359*(6392), 254-256. https://doi.org/10.1038/359254a0

Derkx, P. M. F., Janzen, T., Sørensen, K. I., Christensen, J. E., Stuer-Lauridsen, B., & Johansen, E. (2014). The art of strain improvement of industrial lactic acid bacteria without the use of recombinant DNA technology. *Microbial cell factories, 13 Suppl 1*(Suppl 1), S5-S5. https://doi.org/10.1186/1475-2859-13-S1-S5

Diamond, J. (2002). Evolution, consequences and future of plant and animal domestication. *Nature, 418*(6898), 700-707. https://doi.org/10.1038/nature01019

Diep, T. T., Pook, C., & Yoo, M. J. Y. (2020). Physicochemical properties and proximate composition of tamarillo (Solanum betaceum Cav.) fruits from New Zealand. *Journal of Food Composition and Analysis, 92*, 103563. https://doi.org/https://doi.org/10.1016/j.jfca.2020.103563

Diep, T. T., Yoo, M. J. Y., Pook, C., Sadooghy-Saraby, S., Gite, A., & Rush, E. (2021). Volatile Components and Preliminary Antibacterial Activity of Tamarillo (Solanum betaceum Cav.). *Foods (Basel, Switzerland), 10*(9), 2212. https://doi.org/10.3390/foods10092212

Dimidi, E., Cox, S. R., Rossi, M., & Whelan, K. (2019). Fermented Foods: Definitions and Characteristics, Impact on the Gut Microbiota and Effects on Gastrointestinal Health and Disease. *Nutrients, 11*(8), 1806. https://doi.org/10.3390/nu11081806

Domingues, L., Guimarães, P. M. R., & Oliveira, C. (2010). Metabolic engineering of Saccharomyces cerevisiae for lactose/whey fermentation. *Bioengineered bugs, 1*(3), 164-171. https://doi.org/10.4161/bbug.1.3.10619

Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2007). Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-converting enzyme inhibitory activity in fermented milk. *Lait, 87*(1), 21-38. https://doi.org/10.1051/lait:2006023

Dragosits, M., & Mattanovich, D. (2013). Adaptive laboratory evolution – principles and applications for biotechnology. *Microbial Cell Factories, 12*(1), 64. https://doi.org/10.1186/1475-2859-12-64

Dubernet, S., Desmasures, N., & Guéguen, M. (2002). A PCR-based method for identification of lactobacilli at the genus level. *FEMS Microbiology Letters, 214*(2), 271-275. https://doi.org/10.1111/j.1574-6968.2002.tb11358.x

Dunn, H. C., & Lindsay, R. C. (1985). Evaluation of the Role of Microbial Strecker-Derived Aroma Compounds in Unclean-Type Flavors of Cheddar Cheese1. *Journal of Dairy Science, 68*(11), 2859-2874. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(85)81179-6

Erkaya, T., & ŞEngÜL, M. (2011). Comparison of volatile compounds in yoghurts made from cows', buffaloes', ewes' and goats' milks. *International Journal of Dairy Technology, 64*(2), 240-246. https://doi.org/https://doi.org/10.1111/j.1471-0307.2010.00655.x

Eyre-Walker, A., & Keightley, P. D. (2007). The distribution of fitness effects of new mutations. *Nature Reviews Genetics, 8*(8), 610-618. https://doi.org/10.1038/nrg2146

Fazilah, N. F., Ariff, A. B., Khayat, M. E., Rios-Solis, L., & Halim, M. (2018). Influence of probiotics, prebiotics, synbiotics and bioactive phytochemicals on the

formulation of functional yogurt. *Journal of Functional Foods, 48*, 387-399. https://doi.org/https://doi.org/10.1016/j.jff.2018.07.039

Gaspar, P., Al-Bayati, F. A. Y., Andrew, P. W., Neves, A. R., & Yesilkaya, H. (2014). Lactate dehydrogenase is the key enzyme for pneumococcal pyruvate metabolism and pneumococcal survival in blood. *Infection and immunity, 82*(12), 5099-5109. https://doi.org/10.1128/IAI.02005-14

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Véronneau, S., . . . Johnston, M. (2002). Functional profiling of the Saccharomyces cerevisiae genome. *Nature, 418*(6896), 387-391. https://doi.org/10.1038/nature00935

Gibbons, J. G., & Rinker, D. C. (2015). The genomics of microbial domestication in the fermented food environment. *Current opinion in genetics & development, 35*, 1-8. https://doi.org/10.1016/j.gde.2015.07.003

Gibson, B., Dahabieh, M., Krogerus, K., Jouhten, P., Magalhães, F., Pereira, R., . . . Vidgren, V. (2020). Adaptive Laboratory Evolution of Ale and Lager Yeasts for Improved Brewing Efficiency and Beer Quality. *Annual Review of Food Science and Technology, 11*(1), 23-44. https://doi.org/10.1146/annurev-food-032519-051715

Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R., Powell, C. D., & Smart, K. A. (2007). Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Reviews, 31*(5), 535-569. https://doi.org/10.1111/j.1574-6976.2007.00076.x

Gilissen, C., Hehir-Kwa, J. Y., Thung, D. T., van de Vorst, M., van Bon, B. W. M., Willemsen, M. H., . . . Veltman, J. A. (2014). Genome sequencing identifies major causes of severe intellectual disability. *Nature, 511*(7509), 344-347. https://doi.org/10.1038/nature13394

Grobben, G. J., Chin-Joe, I., Kitzen, V. A., Boels, I. C., Boer, F., Sikkema, J., . . . de Bont, J. A. (1998). Enhancement of Exopolysaccharide Production by Lactobacillus delbrueckii subsp. bulgaricus NCFB 2772 with a Simplified Defined Medium. *Appl Environ Microbiol, 64*(4), 1333-1337. https://doi.org/10.1128/aem.64.4.1333-1337.1998

Grobben, G. J., Sikkema, J., Smith, M. R., & de Bont, J. A. M. (1995). Production of extracellular polysaccharides by Lactobacillus delbrueckii ssp. bulgaricus NCFB 2772 grown in a chemically defined medium. *Journal of Applied Bacteriology, 79*(1), 103-107. https://doi.org/10.1111/j.1365-2672.1995.tb03130.x

Gu, H., Liu, G., Wang, J., Aubry, A.-F., & Arnold, M. E. (2014). Selecting the Correct Weighting Factors for Linear and Quadratic Calibration Curves with Least-Squares Regression Algorithm in Bioanalytical LC-MS/MS Assays and Impacts of Using Incorrect Weighting Factors on Curve Stability, Data Quality, and Assay Performance. *Analytical Chemistry, 86*(18), 8959-8966. https://doi.org/10.1021/ac5018265

Gu, Y., Li, X., Chen, H., Guan, K., Qi, X., Yang, L., & Ma, Y. (2021). Evaluation of FAAs and FFAs in yogurts fermented with different starter cultures during storage. *Journal of Food Composition and Analysis, 96*, 103666. https://doi.org/https://doi.org/10.1016/j.jfca.2020.103666

Guerra, Hernández, E. J., Estepa, R. G., & Rivas, I. R. (1995). Analysis of diacetyl in yogurt by two new spectrophotometric and fluorimetric methods. *Food Chemistry, 53*(3), 315-319. https://doi.org/https://doi.org/10.1016/0308-8146(95)93939-O

GuhanNath, S., Sam Aaron, I., Raj, A. A. S., & Ranganathan, T. V. (2014). Recent innovations in nanotechnology in food processing and its various applications - A review. *International Journal of Pharmaceutical Sciences Review and Research, 29*(2), 116-124.

Gullo, M., Verzelloni, E., & Canonico, M. (2014). Aerobic submerged fermentation by acetic acid bacteria for vinegar production: Process and biotechnological aspects. *Process Biochemistry, 49*(10), 1571-1579. https://doi.org/https://doi.org/10.1016/j.procbio.2014.07.003

Gyosheva, B. (1985). Regression analysis applied to evaluation of aroma and flavour of Bulgarian sour milk. *Food / Nahrung, 29*(2), 185-190. https://doi.org/https://doi.org/10.1002/food.19850290214

Hansen, S. K., Rainey, P. B., Haagensen, J. A. J., & Molin, S. (2007). Evolution of species interactions in a biofilm community. *Nature, 445*(7127), 533-536. https://doi.org/10.1038/nature05514

Hassan, A. N., Frank, J. F., Schmidt, K. A., & Shalabi, S. I. (1996). Textural Properties of Yogurt Made with Encapsulated Nonropy Lactic Cultures. *Journal of Dairy Science, 79*(12), 2098-2103. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(96)76583-9

Hatti-Kaul, R., Chen, L., Dishisha, T., & Enshasy, H. E. (2018). Lactic acid bacteria: from starter cultures to producers of chemicals. *FEMS Microbiology Letters, 365*(20). https://doi.org/10.1093/femsle/fny213

Hayek, S. A., & Ibrahim, S. A. (2013). Current Limitations and Challenges with Lactic Acid Bacteria: A Review. *Food and Nutrition Sciences, Vol.04No.11*, 15. https://doi.org/10.4236/fns.2013.411A010

Hekmat, S., & Reid, G. (2006). Sensory properties of probiotic yogurt is comparable to standard yogurt. *Nutrition Research, 26*(4), 163-166. https://doi.org/https://doi.org/10.1016/j.nutres.2006.04.004

Heller, K. J. (2001). Probiotic bacteria in fermented foods: product characteristics and starter organisms. *The American Journal of Clinical Nutrition, 73*(2), 374s-379s. https://doi.org/10.1093/ajcn/73.2.374s

Herbert, D., Elsworth, R., & Telling, R. C. (1956). The Continuous Culture of Bacteria; a Theoretical and Experimental Study. *Microbiology, 14*(3), 601-622. https://doi.org/https://doi.org/10.1099/00221287-14-3-601

Hillman, G. C., & Davies, M. S. (1990). Measured domestication rates in wild wheats and barley under primitive cultivation, and their archaeological implications. *Journal of World Prehistory, 4*(2), 157-222. https://doi.org/10.1007/BF00974763

Høier, E., Janzen, T., Rattray, F., Sørensen, K., Børsting, M. W., Brockmann, E., & Johansen, E. (2010). The Production, Application and Action of Lactic Cheese Starter Cultures. In *Technology of Cheesemaking: Second Edition* (pp. 166-192). Retrieved from https://www.scopus.com/inward/record.uri?eid=2-s2.0-79954423271&doi=10.1002%2f9781444323740.ch5&partnerID=40&md5=8a1307740b831bc20c920b82430ed008. https://doi.org/10.1002/9781444323740.ch5

Hummon, A. B., Lim, S. R., Difilippantonio, M. J., & Ried, T. (2007). Isolation and solubilization of proteins after TRIzol® extraction of RNA and DNA from patient material following prolonged storage. *BioTechniques, 42*(4), 467-472. https://doi.org/10.2144/000112401

Hwang, H., & Lee, J.-H. (2018). Characterization of Arginine Catabolism by Lactic Acid Bacteria Isolated from Kimchi. *Molecules, 23*(11). https://doi.org/10.3390/molecules23113049

Jensen, P. A., Zhu, Z., & van Opijnen, T. (2017). Antibiotics Disrupt Coordination between Transcriptional and Phenotypic Stress Responses in Pathogenic Bacteria. *Cell Reports, 20*(7), 1705-1716. https://doi.org/https://doi.org/10.1016/j.celrep.2017.07.062

Johansen, E. (2018). Use of Natural Selection and Evolution to Develop New Starter Cultures for Fermented Foods. *Annu Rev Food Sci Technol, 9*, 411-428. https://doi.org/10.1146/annurev-food-030117-012450

Karaca, O. B., & Güven, M. (2018). Effects of Proteolytic and Lipolytic Enzyme Supplementations on Lipolysis and Proteolysis Characteristics of White Cheeses. *Foods, 7*(8), 125. https://doi.org/10.3390/foods7080125

Kato, S., Yamagishi, A., Daimon, S., Kawasaki, K., Tamaki, H., Kitagawa, W., . . . Kamagata, Y. (2018). Isolation of Previously Uncultured Slow-Growing Bacteria by Using a Simple Modification in the Preparation of Agar Media. *Applied and Environmental Microbiology, 84*(19), e00807-00818. https://doi.org/10.1128/AEM.00807-18

Keer, J. T., & Birch, L. (2003). Molecular methods for the assessment of bacterial viability. *J Microbiol Methods, 53*(2), 175-183. https://doi.org/10.1016/s0167-7012(03)00025-3

Klaenhammer, T., Altermann, E., Arigoni, F., Bolotin, A., Breidt, F., Broadbent, J., . . . Siezen, R. (2002). Discovering lactic acid bacteria by genomics. *Antonie van Leeuwenhoek, 82*(1), 29-58. https://doi.org/10.1023/A:1020638309912

Klaenhammer, T. R. (1984). A general method for plasmid isolation in lactobacilli. *Current Microbiology, 10*(1), 23-28. https://doi.org/10.1007/BF01576043

Kleerebezemab, M., Hols, P., & Hugenholtz, J. (2000). Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in Lactococcus lactis by metabolic engineering. *Enzyme and Microbial Technology, 26*(9), 840-848. https://doi.org/https://doi.org/10.1016/S0141-0229(00)00180-0

Kneifel, W., Paquin, P., Abert, T., & Richard, J. P. (1991). Water-Holding Capacity of Proteins with Special Regard to Milk Proteins and Methodological Aspects—A Review. *Journal of Dairy Science, 74*(7), 2027-2041. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(91)78373-2

Kwon, Y. W., Bae, J.-H., Kim, S.-A., & Han, N. S. (2018). Development of Freeze-Thaw Tolerant Lactobacillus rhamnosus GG by Adaptive Laboratory Evolution. *Frontiers in Microbiology, 9*(2781). https://doi.org/10.3389/fmicb.2018.02781

Larsen, N., Werner, B. B., Vogensen, F. K., & Jespersen, L. (2015). Effect of dissolved oxygen on redox potential and milk acidification by lactic acid bacteria isolated from a DL-starter culture. *J Dairy Sci, 98*(3), 1640-1651. https://doi.org/10.3168/jds.2014-8971

Lee, & Lucey, J. A. (2003). Rheological properties, whey separation, and microstructure in set-style yogurt: Effects of heating temperature and incubation temperature. *Journal of Texture Studies, 34*(5-6), 515-536. https://doi.org/10.1111/j.1745-4603.2003.tb01079.x

Li, C., Zhang, G. F., Mao, X., Wang, J. Y., Duan, C. Y., Wang, Z. J., & Liu, L. B. (2016). Growth and acid production of Lactobacillus delbrueckii ssp. bulgaricus ATCC 11842 in the fermentation of algal carcass. *Journal of Dairy Science, 99*(6), 4243-4250. https://doi.org/https://doi.org/10.3168/jds.2015-10700

Lindquist, S., & Craig, E. A. (1988). The heat-shock proteins. *Annu Rev Genet, 22*, 631-677. https://doi.org/10.1146/annurev.ge.22.120188.003215

Liu, J., Chan, S. H. J., Chen, J., Solem, C., & Jensen, P. R. (2019). Systems Biology – A Guide for Understanding and Developing Improved Strains of Lactic Acid Bacteria. *Frontiers in Microbiology, 10*(876). https://doi.org/10.3389/fmicb.2019.00876

Liu, S. Q., Holland, R., & Crow, V. L. (2004). Esters and their biosynthesis in fermented dairy products: a review. *International Dairy Journal, 14*(11), 923-945. https://doi.org/https://doi.org/10.1016/j.idairyj.2004.02.010

Liu, W., Yu, J., Sun, Z., Song, Y., Wang, X., Wang, H., . . . Heping, Z. (2016). Relationships between functional genes in Lactobacillus delbrueckii ssp. bulgaricus isolates and phenotypic characteristics associated with fermentation time and flavor production in yogurt elucidated using multilocus sequence typing.

Journal of Dairy Science, 99(1), 89-103. https://doi.org/https://doi.org/10.3168/jds.2015-10209

Lobato-Calleros, C., Ramírez-Santiago, C., Vernon-Carter, E. J., & Alvarez-Ramirez, J. (2014). Impact of native and chemically modified starches addition as fat replacers in the viscoelasticity of reduced-fat stirred yogurt. *Journal of Food Engineering, 131*, 110-115. https://doi.org/https://doi.org/10.1016/j.jfoodeng.2014.01.019

Lucey, J. A. (2001). The relationship between rheological parameters and whey separation in milk gels. *Food Hydrocolloids, 15*(4), 603-608. https://doi.org/https://doi.org/10.1016/S0268-005X(01)00043-1

Makarova, K. S., & Koonin, E. V. (2007). Evolutionary Genomics of Lactic Acid Bacteria. *Journal of Bacteriology, 189*(4), 1199. https://doi.org/10.1128/JB.01351-06

Malaka, R., Maruddin, F., Dwyana, Z., & Vargas, M. V. (2020). Assessment of exopolysaccharide production by Lactobacillus delbrueckii subsp. bulgaricus ropy strain in different substrate media. *Food science & nutrition, 8*(3), 1657-1664. https://doi.org/10.1002/fsn3.1452

Mann, B., Athira, S., Sharma, R., & Bajaj, R. (2017). Chapter 24 - Bioactive Peptides in Yogurt. In N. P. Shah (Ed.), *Yogurt in Health and Disease Prevention* (pp. 411-426): Academic Press. Retrieved from https://www.sciencedirect.com/science/article/pii/B9780128051344000249. https://doi.org/https://doi.org/10.1016/B978-0-12-805134-4.00024-9

Massoud, R., Belgheisi, S., & Massoud, A. M. (2016). Effect of High Pressure Homogenization on Improving the Quality of Milk and Sensory Properties of Yogurt: A Review. *International Journal of Chemical Engineering and Applications, 7*, 66-70.

MegazymeLtd. (2018). D-LACTIC ACID (D-LACTATE)(Rapid)andL-LACTIC ACID (L-LACTATE)ASSAY PROCEDURES. https://www.megazyme.com/documents/Assay_Protocol/K-DLATE_DATA.pdf

Mercier, R., Kawai, Y., & Errington, J. (2016). Wall proficient E. coli capable of sustained growth in the absence of the Z-ring division machine. *Nature Microbiology, 1*(8), 16091. https://doi.org/10.1038/nmicrobiol.2016.91

Mitchell, A., Romano, G. H., Groisman, B., Yona, A., Dekel, E., Kupiec, M., . . . Pilpel, Y. (2009). Adaptive prediction of environmental changes by microorganisms. *Nature, 460*(7252), 220-224. https://doi.org/10.1038/nature08112

Nagaoka, M., Muto, M., Nomoto, K., Matuzaki, T., Watanabe, T., & Yokokura, T. (1990). Structure of polysaccharide-peptidoglycan complex from the cell wall of Lactobacillus casei YIT9018. *J Biochem, 108*(4), 568-571. https://doi.org/10.1093/oxfordjournals.jbchem.a123243

Nakayama, Y., Yamaguchi, H., Einaga, N., & Esumi, M. (2016). Pitfalls of DNA Quantification Using DNA-Binding Fluorescent Dyes and Suggested Solutions. *PloS one, 11*(3), e0150528-e0150528. https://doi.org/10.1371/journal.pone.0150528

Narendranath, N. V., & Power, R. (2005). Relationship between pH and Medium Dissolved Solids in Terms of Growth and Metabolism of Lactobacilli and Saccharomyces cerevisiae during Ethanol Production. *Applied and Environmental Microbiology, 71*(5), 2239. https://doi.org/10.1128/AEM.71.5.2239-2243.2005

New, A. M., Cerulus, B., Govers, S. K., Perez-Samper, G., Zhu, B., Boogmans, S., . . . Verstrepen, K. J. (2014). Different levels of catabolite repression optimize growth in stable and variable environments. *PLoS biology, 12*(1), e1001764-e1001764. https://doi.org/10.1371/journal.pbio.1001764

Ozcan, T. (2013). Determination of yogurt quality by using rheological and textural parameters

Panesar, P. S., Kennedy, J. F., Gandhi, D. N., & Bunko, K. (2007). Bioutilisation of whey for lactic acid production. *Food Chemistry, 105*(1), 1-14. https://doi.org/https://doi.org/10.1016/j.foodchem.2007.03.035

Papadimitriou, K., Alegría, Á., Bron, P. A., de Angelis, M., Gobbetti, M., Kleerebezem, M., . . . Kok, J. (2016). Stress Physiology of Lactic Acid Bacteria. *Microbiology and molecular biology reviews : MMBR, 80*(3), 837-890. https://doi.org/10.1128/MMBR.00076-15

Papagianni, M. (2012a). Metabolic engineering of lactic acid bacteria for the production of industrially important compounds. *Computational and structural biotechnology journal, 3*, e201210003-e201210003. https://doi.org/10.5936/csbj.201210003

Papagianni, M. (2012b). METABOLIC ENGINEERING OF LACTIC ACID BACTERIA FOR THE PRODUCTION OF INDUSTRIALLY IMPORTANT COMPOUNDS. *Computational and Structural Biotechnology Journal, 3*(4), e201210003. https://doi.org/https://doi.org/10.5936/csbj.201210003

Paul, L. H. M., & Maria José, S. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Lait, 80*(3), 293-324.

Perpète, P., & Collin, S. (2000). Evidence of Strecker Aldehyde Excretion by Yeast in Cold Contact Fermentations. *Journal of Agricultural and Food Chemistry, 48*(6), 2384-2386. https://doi.org/10.1021/jf000071h

Pilizota, T., & Shaevitz, J. W. (2014). Origins of Escherichia coli growth rate and cell shape changes at high external osmolality. *Biophysical journal, 107*(8), 1962-1969. https://doi.org/10.1016/j.bpj.2014.08.025

Piuri, M., Sanchez-Rivas, C., & Ruzal, S. M. (2005). Cell wall modifications during osmotic stress in Lactobacillus casei. *J Appl Microbiol, 98*(1), 84-95. https://doi.org/10.1111/j.1365-2672.2004.02428.x

Puvanenthiran, A., Stevovitch-Rykner, C., McCann, T. H., & Day, L. (2014). Synergistic effect of milk solids and carrot cell wall particles on the rheology and texture of yoghurt gels. *Food Research International, 62*, 701-708. https://doi.org/https://doi.org/10.1016/j.foodres.2014.04.023

QIAGEN. (2020). DNeasy® Blood & Tissue Handbook.

Rao, D. R., Pulusani, S. R., & Rao, T. K. (1982). AMINO ACID COMPOSITION AND NUTRITIONAL IMPLICATIONS OF MILK FERMENTED BY VARIOUS LACTIC CULTURES. *Journal of Food Quality, 5*(3), 235-243. https://doi.org/https://doi.org/10.1111/j.1745-4557.1982.tb00746.x

Routray, W., & Mishra, H. N. (2011). Scientific and Technical Aspects of Yogurt Aroma and Taste: A Review. *Comprehensive Reviews in Food Science and Food Safety, 10*(4), 208-220. https://doi.org/https://doi.org/10.1111/j.1541-4337.2011.00151.x

Saleh, A., Mohamed, A. A., Alamri, M. S., Hussain, S., Qasem, A. A., & Ibraheem, M. A. (2020). Effect of Different Starches on the Rheological, Sensory and Storage Attributes of Non-fat Set Yogurt. *Foods, 9*(1). https://doi.org/10.3390/foods9010061

Sandberg, T. E., Salazar, M. J., Weng, L. L., Palsson, B. O., & Feist, A. M. (2019). The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. *Metabolic Engineering, 56*, 1-16. https://doi.org/https://doi.org/10.1016/j.ymben.2019.08.004

Sawada, K., Koyano, H., Yamamoto, N., & Yamada, T. (2021). The relationships between microbiota and the amino acids and organic acids in commercial vegetable pickle fermented in rice-bran beds. *Scientific Reports, 11*(1), 1791. https://doi.org/10.1038/s41598-021-81105-x

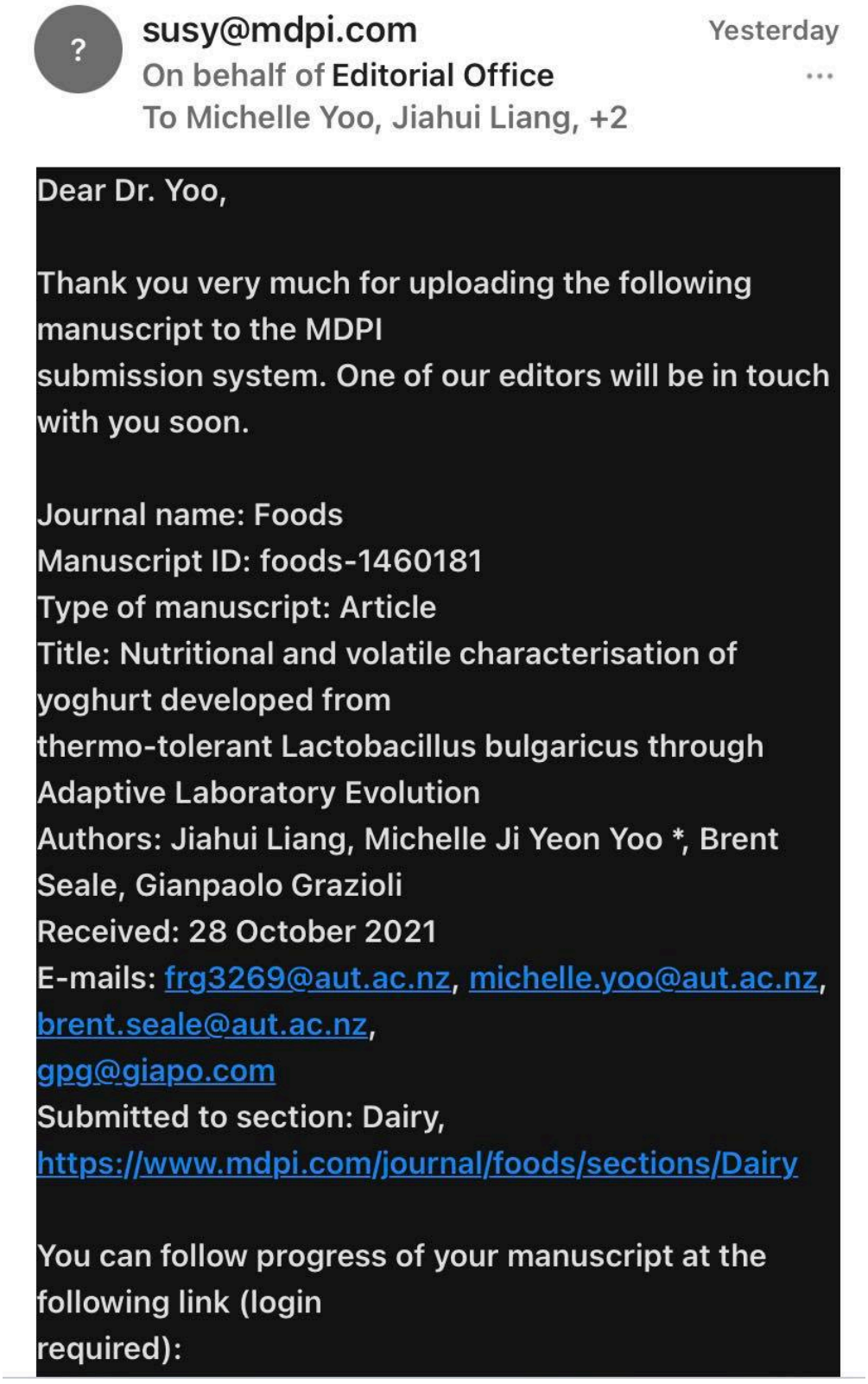
Sharma, R. (2013). Sensory Quality Aspects of Yoghurt. *Dairy innovation*.
Simova, E., Simov, Z., Beshkova, D., Frengova, G., Dimitrov, Z., & Spasov, Z. (2006). Amino acid profiles of lactic acid bacteria, isolated from kefir grains and kefir starter made from them. *International Journal of Food Microbiology, 107*(2), 112-123. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2005.08.020
Slapkauskaite, J., Sekmokiene, D., Kabasinskiene, A., Bartkiene, E., Juodeikiene, G., & Sarkinas, A. (2016). Influence of lactic acid bacteria–fermented Helianthus tuberosus L. and Lupinus luteus on quality of milk products. *CyTA - Journal of Food, 14*(3), 482-488. https://doi.org/10.1080/19476337.2015.1137355
Smid, E. J., & Hugenholtz, J. (2010). Functional Genomics for Food Fermentation Processes. *Annual Review of Food Science and Technology, 1*(1), 497-519. https://doi.org/10.1146/annurev.food.102308.124143
Smit, B. A., Engels, W. J. M., & Smit, G. (2009). Branched chain aldehydes: production and breakdown pathways and relevance for flavour in foods. *Applied Microbiology and Biotechnology, 81*(6), 987-999. https://doi.org/10.1007/s00253-008-1758-x
Smit, G., Smit, B. A., & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews, 29*(3), 591-610. https://doi.org/10.1016/j.fmrre.2005.04.002
Smith, W. P. J., Davit, Y., Osborne, J. M., Kim, W., Foster, K. R., & Pitt-Francis, J. M. (2017). Cell morphology drives spatial patterning in microbial communities. *Proceedings of the National Academy of Sciences, 114*(3), E280. https://doi.org/10.1073/pnas.1613007114
Stucki, D., & Gagneux, S. (2013). Single nucleotide polymorphisms in Mycobacterium tuberculosis and the need for a curated database. *Tuberculosis (Edinburgh, Scotland), 93*(1), 30-39. https://doi.org/10.1016/j.tube.2012.11.002
Stumpf, T. R., Pértile, R. A. N., Rambo, C. R., & Porto, L. M. (2013). Enriched glucose and dextrin mannitol-based media modulates fibroblast behavior on bacterial cellulose membranes. *Materials Science and Engineering: C, 33*(8), 4739-4745. https://doi.org/https://doi.org/10.1016/j.msec.2013.07.035
Sun, Y., Ruivenkamp, C. A. L., Hoffer, M. J. V., Vrijenhoek, T., Kriek, M., van Asperen, C. J., . . . Santen, G. W. E. (2015). Next-Generation Diagnostics: Gene Panel, Exome, or Whole Genome? *Human Mutation, 36*(6), 648-655. https://doi.org/https://doi.org/10.1002/humu.22783
Tabasco, R., Paarup, T., Janer, C., Peláez, C., & Requena, T. (2007). Selective enumeration and identification of mixed cultures of Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus, L. acidophilus, L. paracasei subsp. paracasei and Bifidobacterium lactis in fermented milk. *International Dairy Journal, 17*(9), 1107-1114. https://doi.org/https://doi.org/10.1016/j.idairyj.2007.01.010
Takacs, C. N., Poggio, S., Charbon, G., Pucheault, M., Vollmer, W., & Jacobs-Wagner, C. (2010). MreB drives de novo rod morphogenesis in Caulobacter crescentus via remodeling of the cell wall. *Journal of bacteriology, 192*(6), 1671-1684. https://doi.org/10.1128/JB.01311-09
Talwalkar, A., & Kailasapathy, K. (2004). Comparison of selective and differential media for the accurate enumeration of strains of Lactobacillus acidophilus, Bifidobacterium spp. and Lactobacillus casei complex from commercial yoghurts. *International Dairy Journal, 14*(2), 143-149. https://doi.org/https://doi.org/10.1016/S0958-6946(03)00172-9
Tamime, A. Y., & Robinson, R. K. (2007). 9 - Nutritional value of yoghurt. In A. Y. Tamime & R. K. Robinson (Eds.), *Tamime and Robinson's Yoghurt (Third Edition)* (pp. 646-684): Woodhead Publishing. Retrieved from

https://www.sciencedirect.com/science/article/pii/B9781845692131500090. https://doi.org/https://doi.org/10.1533/9781845692612.646
Teggatz, J. A., & Morris, H. A. (1990). CHANGES IN THE RHEOLOGY AND MICROSTRUCTURE OF ROPY YOGURT DURING SHEARING. *Food Structure, 9*(2), 133-138.
Thierry, A., Pogačić, T., Weber, M., & Lortal, S. (2015). Production of Flavor Compounds by Lactic Acid Bacteria in Fermented Foods. In *Biotechnology of Lactic Acid Bacteria* (pp. 314-340). Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1002/9781118868386.ch19. https://doi.org/https://doi.org/10.1002/9781118868386.ch19
Torino, M., Font de Valdez, G., & Mozzi, F. (2015). Biopolymers from lactic acid bacteria. Novel applications in foods and beverages. *Frontiers in Microbiology, 6*(834). https://doi.org/10.3389/fmicb.2015.00834
Utagawa, T. (2004). Production of Arginine by Fermentation. *The Journal of Nutrition, 134*(10), 2854S-2857S. https://doi.org/10.1093/jn/134.10.2854S
Vasilean, I., Aprodu, I., & Patrascu, L. (2015). Fat content in yoghurts versus non-fat fortifying–a rheological and sensorial approach. *Studia Universitatis Babes-Bolyai Chemia, 60*(2), 259-269.
Vinderola, C. G., Costa, G. A., Regenhardt, S., & Reinheimer, J. A. (2002). Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. *International Dairy Journal, 12*(7), 579-589. https://doi.org/https://doi.org/10.1016/S0958-6946(02)00046-8
Vinicius De Melo Pereira, G., De Carvalho Neto, D. P., Junqueira, A. C. D. O., Karp, S. G., Letti, L. A. J., Magalhães Júnior, A. I., & Soccol, C. R. (2020). A Review of Selection Criteria for Starter Culture Development in the Food Fermentation Industry. *Food Reviews International, 36*(2), 135-167. https://doi.org/10.1080/87559129.2019.1630636
Walsh, A. M., Crispie, F., Claesson, M. J., & Cotter, P. D. (2017). Translating Omics to Food Microbiology. *Annu Rev Food Sci Technol, 8*(1), 113-134. https://doi.org/10.1146/annurev-food-030216-025729
Yaylayan, V. A., & Keyhani, A. (2001). Carbohydrate and Amino Acid Degradation Pathways in l-Methionine/d-[13C] Glucose Model Systems. *Journal of Agricultural and Food Chemistry, 49*(2), 800-803. https://doi.org/10.1021/jf000986w
Young, K. D. (2006). The Selective Value of Bacterial Shape. *Microbiology and Molecular Biology Reviews, 70*(3), 660. https://doi.org/10.1128/MMBR.00001-06
Yu, H.-Y., Wang, L., & McCarthy, K. L. (2016). Characterization of yogurts made with milk solids nonfat by rheological behavior and nuclear magnetic resonance spectroscopy. *Journal of Food and Drug Analysis, 24*(4), 804-812. https://doi.org/https://doi.org/10.1016/j.jfda.2016.04.002
Yuksekdag, Z. N., & Aslim, B. (2008). Influence of different carbon sources on exopolysaccharide production by Lactobacillus delbrueckii subsp. bulgaricus (B3, G12) and Streptococcus thermophilus (W22). *Brazilian Archives of Biology and Technology, 51*, 581-585.
Zealand, F. S. A. N. (2015). FSANZ Consultation Paper on Completing the Review of Microbiological Criteria.
Zhang, L., Mi, S., Liu, R.-b., Sang, Y.-x., & Wang, X.-h. (2020). Evaluation of Volatile Compounds during the Fermentation Process of Yogurts by Streptococcus thermophilus Based on Odor Activity Value and Heat Map Analysis. *International Journal of Analytical Chemistry, 2020*, 3242854. https://doi.org/10.1155/2020/3242854

Zhou, S., Shanmugam, K. T., & Ingram, L. O. (2003). Functional replacement of the Escherichia coli D-(-)-lactate dehydrogenase gene (ldhA) with the L-(+)-lactate dehydrogenase gene (ldhL) from Pediococcus acidilactici. *Appl Environ Microbiol, 69*(4), 2237-2244. https://doi.org/10.1128/aem.69.4.2237-2244.2003

Appendix

1.Confirmation email from Food editorial office of MDPI



susy@mdpi.com
On behalf of Editorial Office
To Michelle Yoo, Jiahui Liang, +2

Yesterday

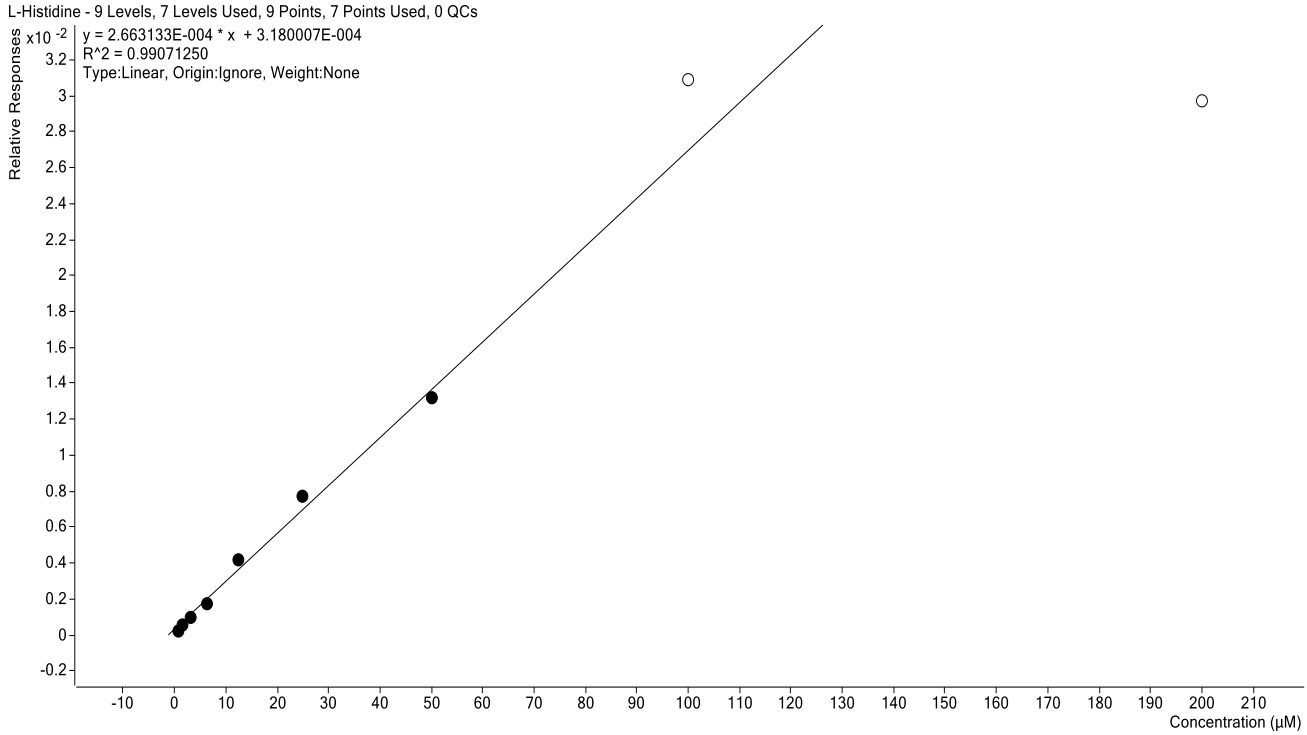
Dear Dr. Yoo,

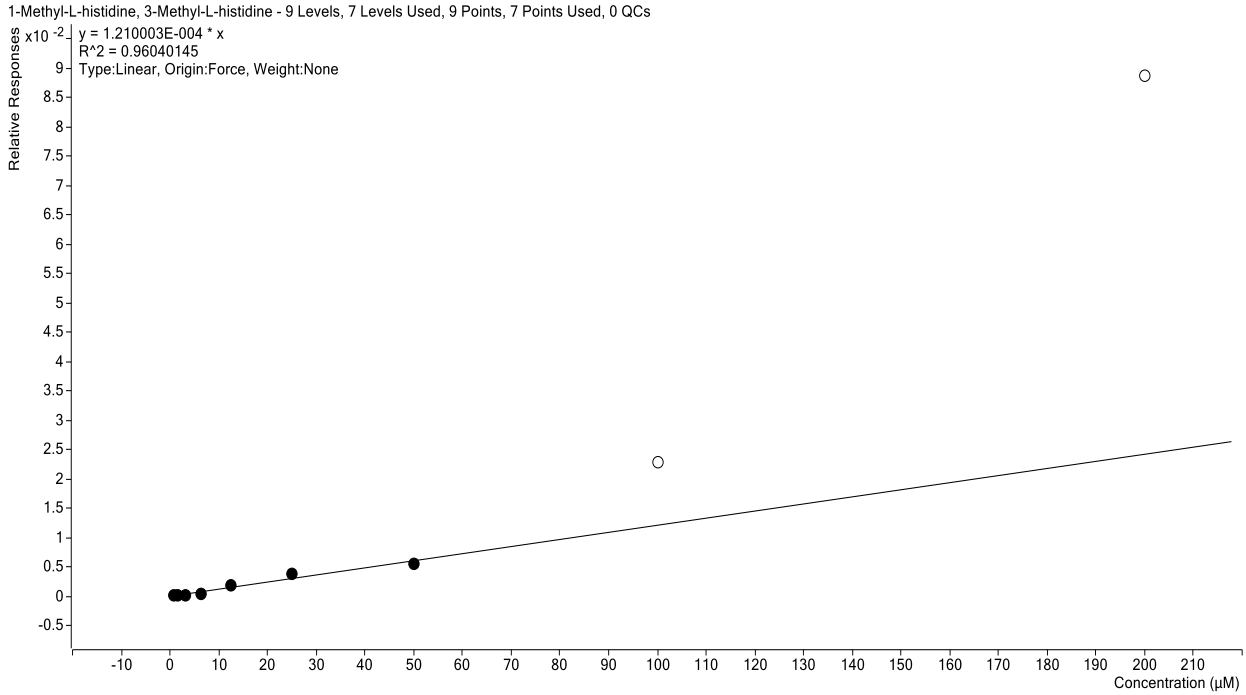
Thank you very much for uploading the following manuscript to the MDPI
submission system. One of our editors will be in touch with you soon.

Journal name: Foods
Manuscript ID: foods-1460181
Type of manuscript: Article
Title: Nutritional and volatile characterisation of yoghurt developed from
thermo-tolerant Lactobacillus bulgaricus through Adaptive Laboratory Evolution
Authors: Jiahui Liang, Michelle Ji Yeon Yoo *, Brent Seale, Gianpaolo Grazioli
Received: 28 October 2021
E-mails: frg3269@aut.ac.nz, michelle.yoo@aut.ac.nz, brent.seale@aut.ac.nz,
gpg@giapo.com
Submitted to section: Dairy,
https://www.mdpi.com/journal/foods/sections/Dairy

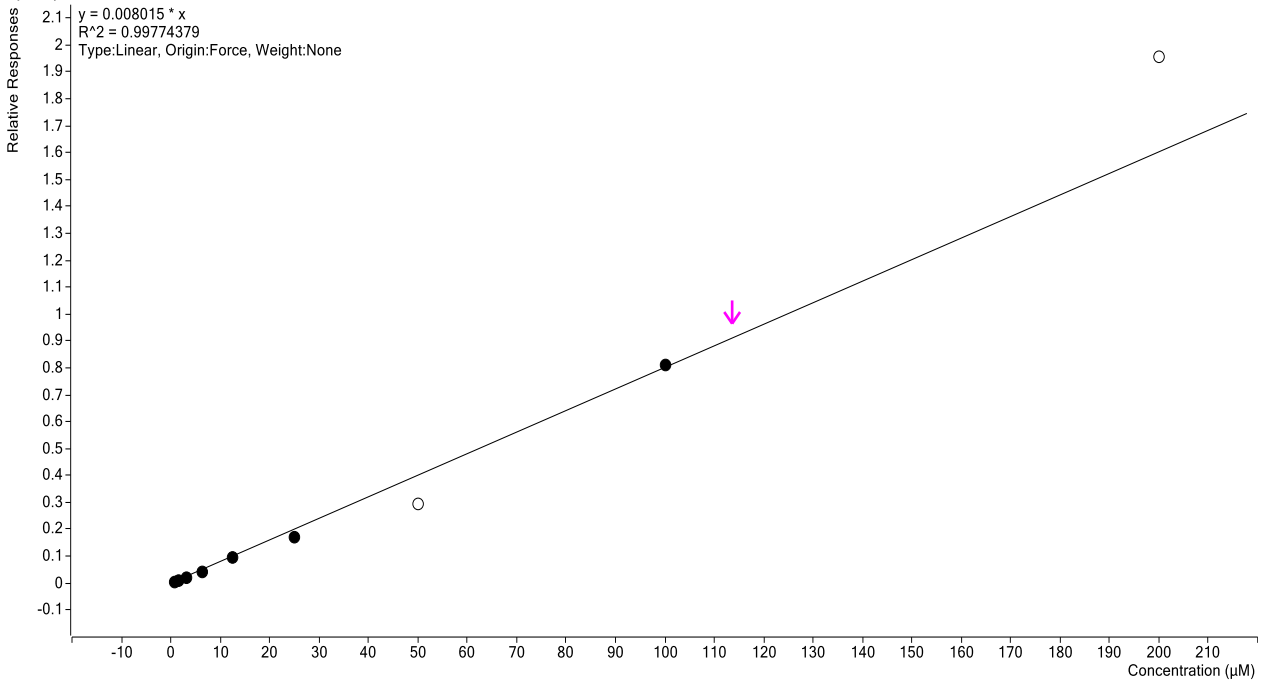
You can follow progress of your manuscript at the following link (login
required):

2. Calibration curves of FAAs by LCMS

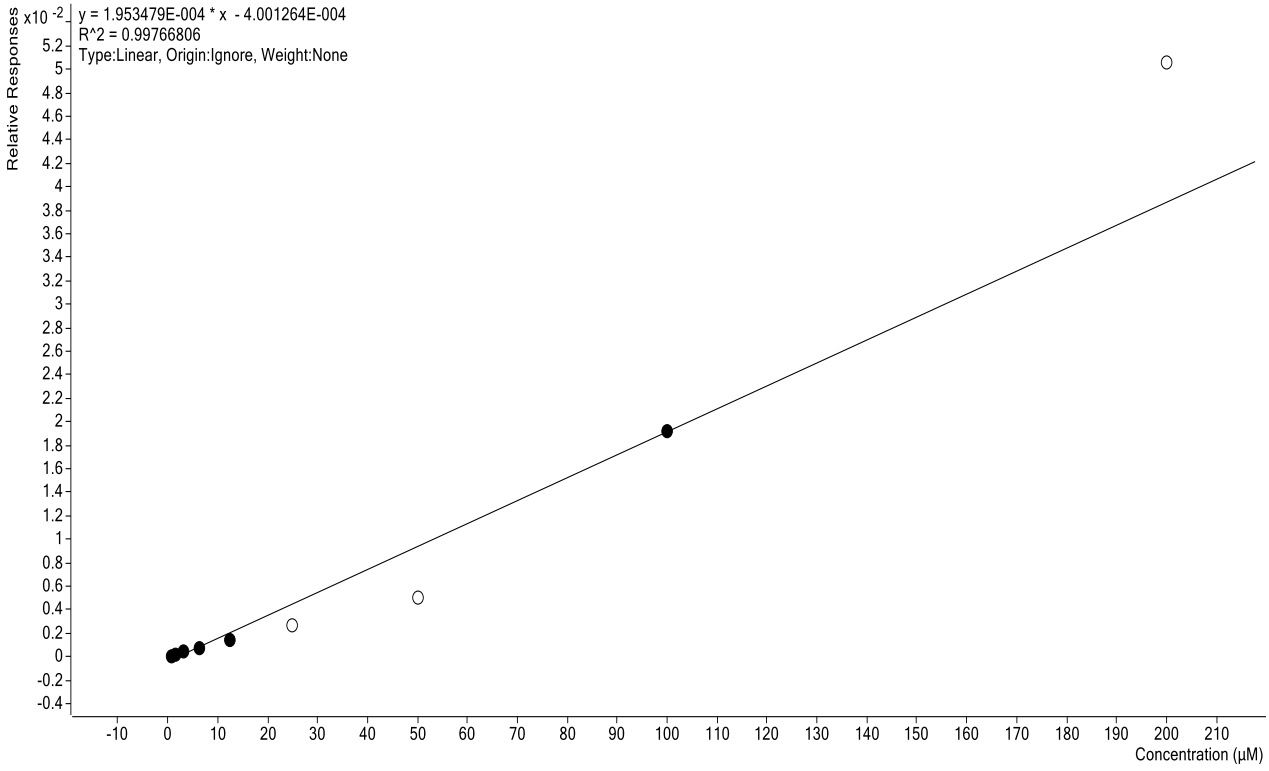


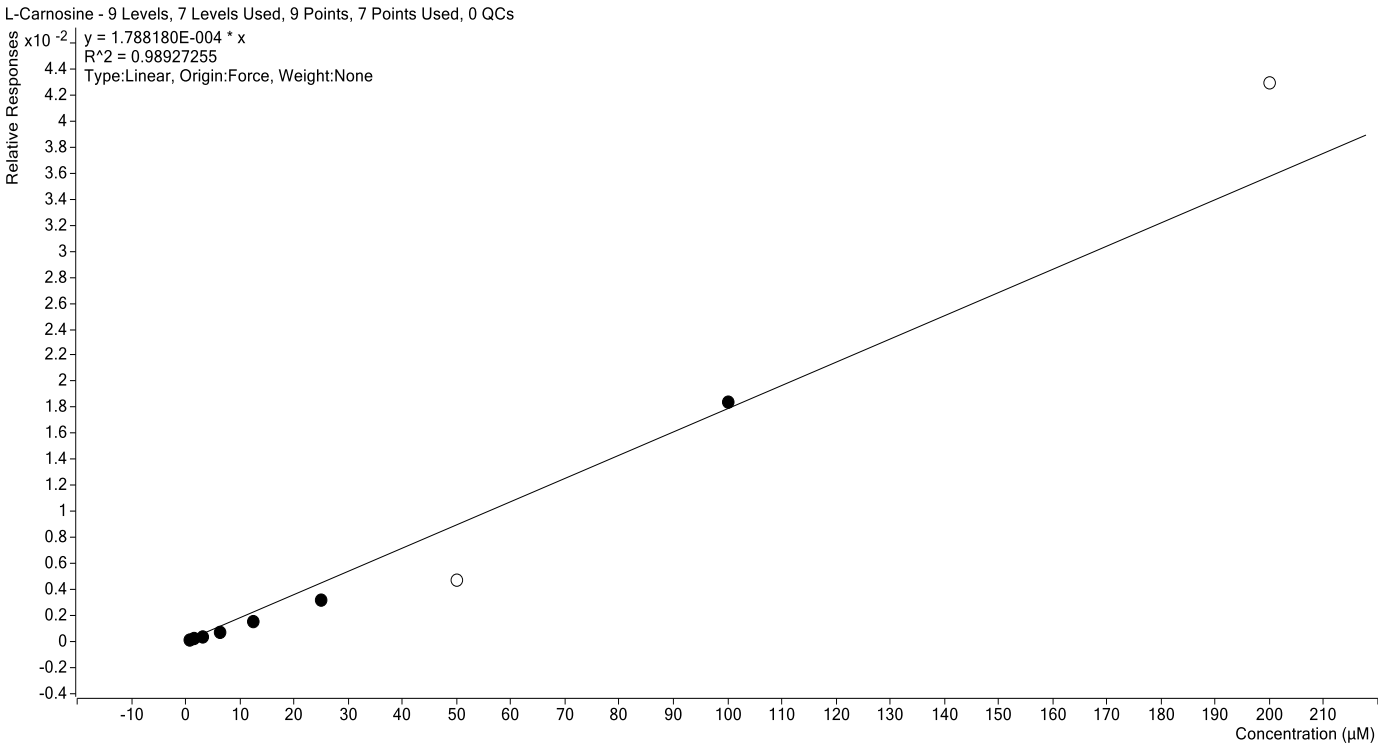


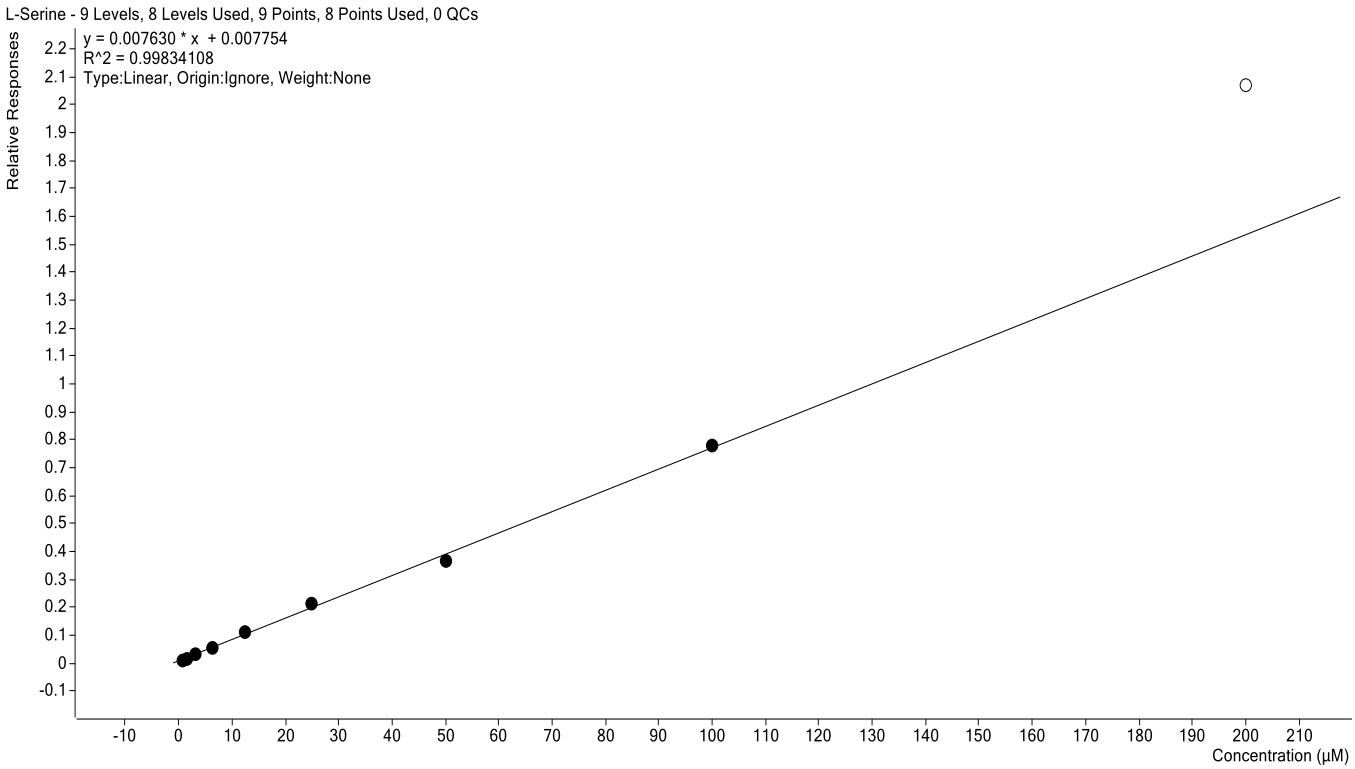
Hydroxy-L-Proline - 9 Levels, 7 Levels Used, 9 Points, 7 Points Used, 0 QCs

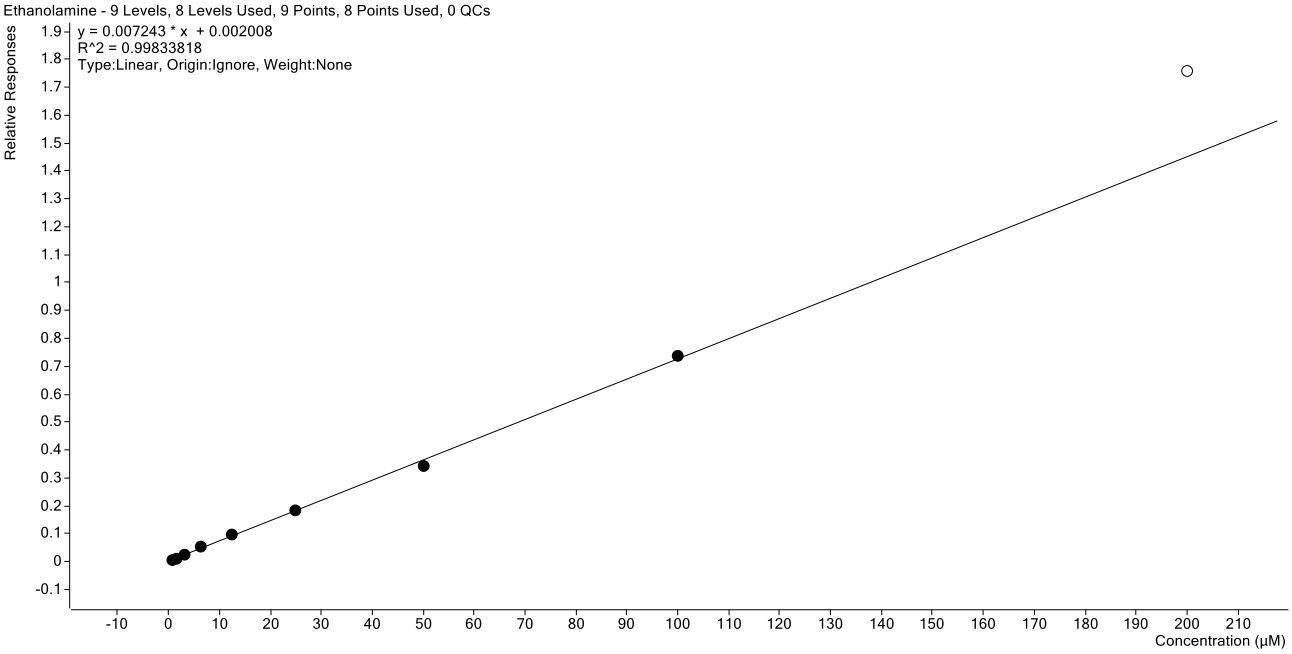


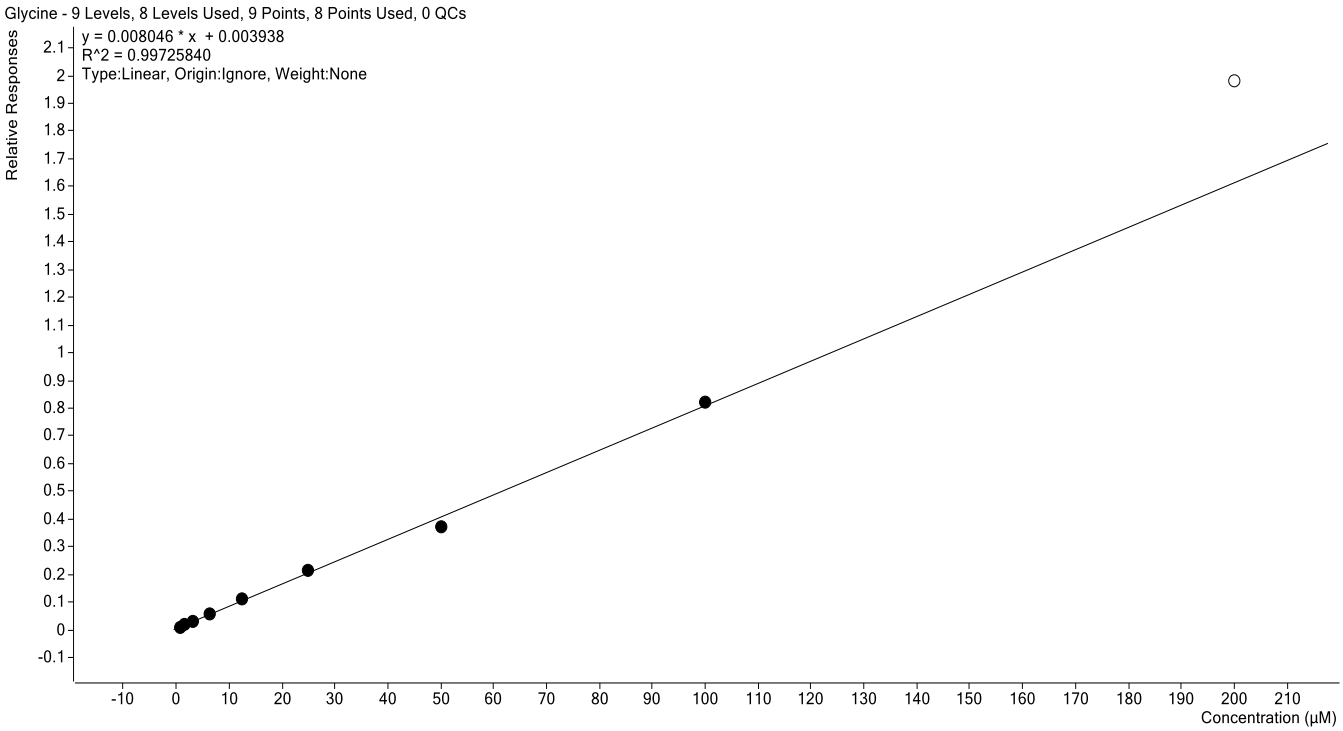
L-Arginine - 9 Levels, 6 Levels Used, 9 Points, 6 Points Used, 0 QCs

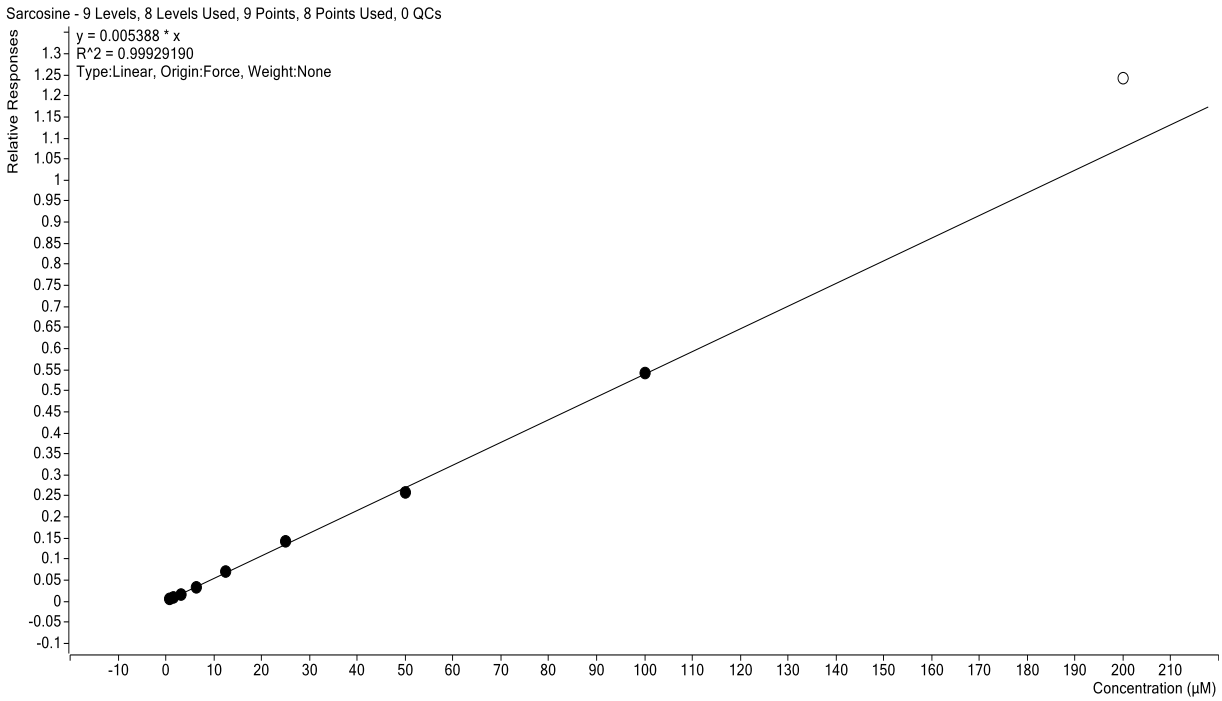


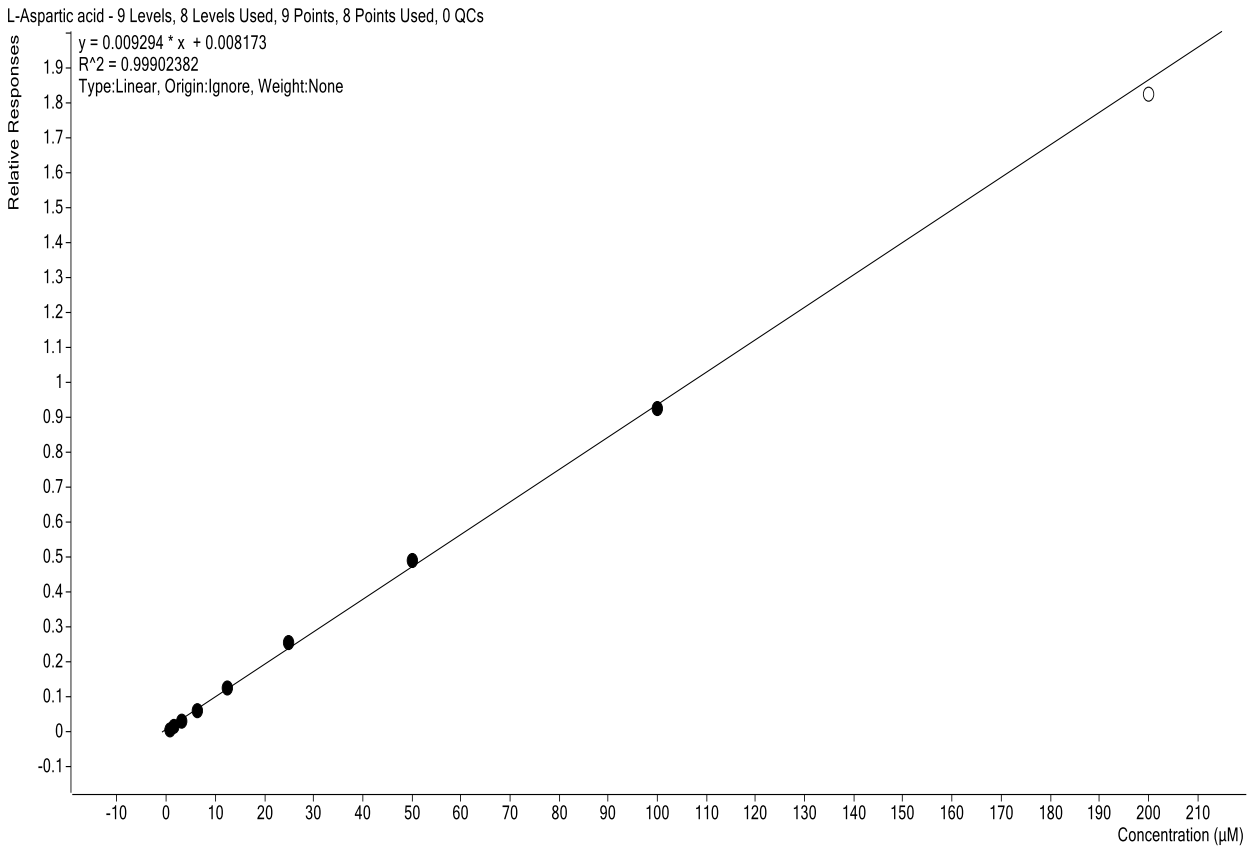

L-Carnosine - 9 Levels, 7 Levels Used, 9 Points, 7 Points Used, 0 QCs
y = 1.788180E-004 * x
R^2 = 0.98927255
Type:Linear, Origin:Force, Weight:None
Relative Responses
x10 -2
Concentration (µM)

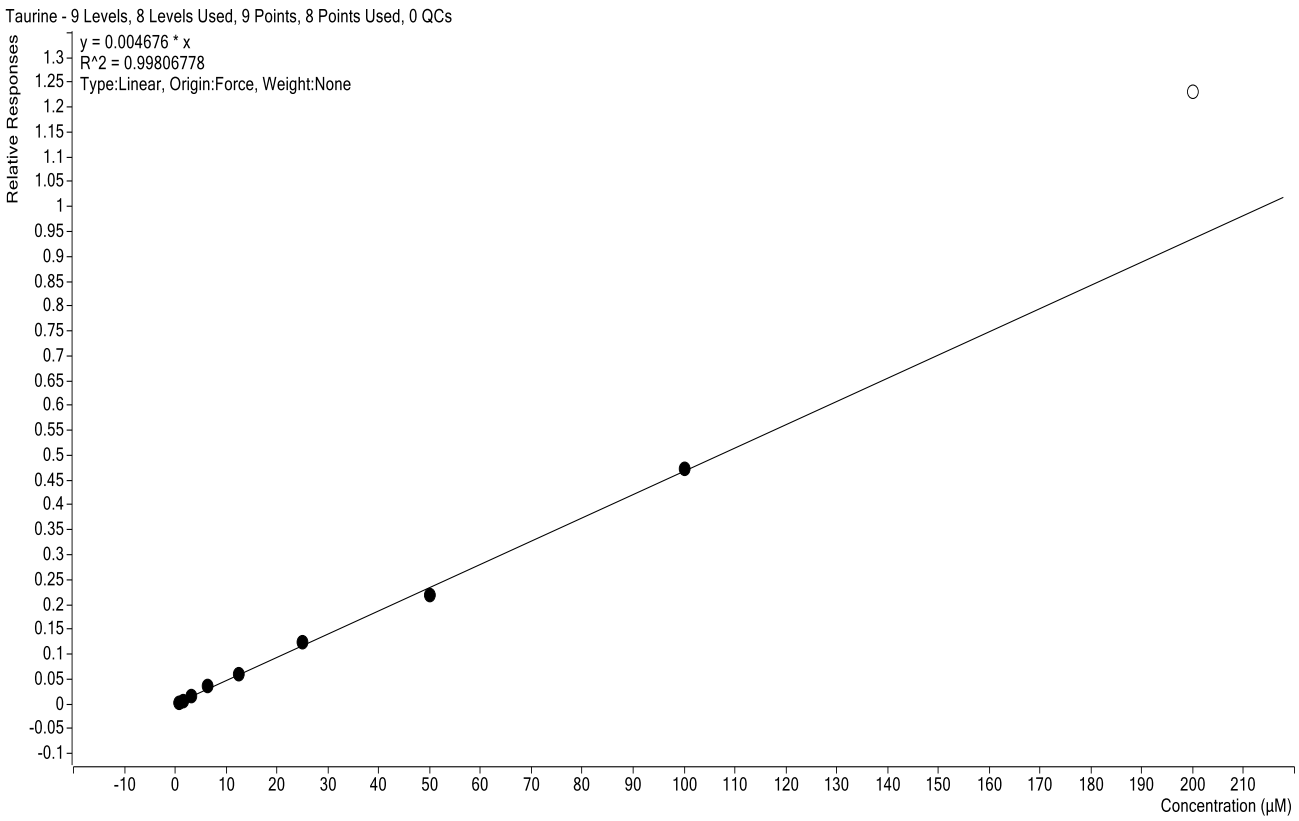

L-Serine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.007630 * x + 0.007754
R^2 = 0.99834108
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)


Ethanolamine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.007243 * x + 0.002008
R^2 = 0.99833818
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)


Glycine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.008046 * x + 0.003938
R^2 = 0.99725840
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)


Sarcosine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.005388 * x
R^2 = 0.99929190
Type:Linear, Origin:Force, Weight:None
Relative Responses
Concentration (µM)


L-Aspartic acid - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.009294 * x + 0.008173
R^2 = 0.99902382
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)


Taurine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.004676 * x
R^2 = 0.99806778
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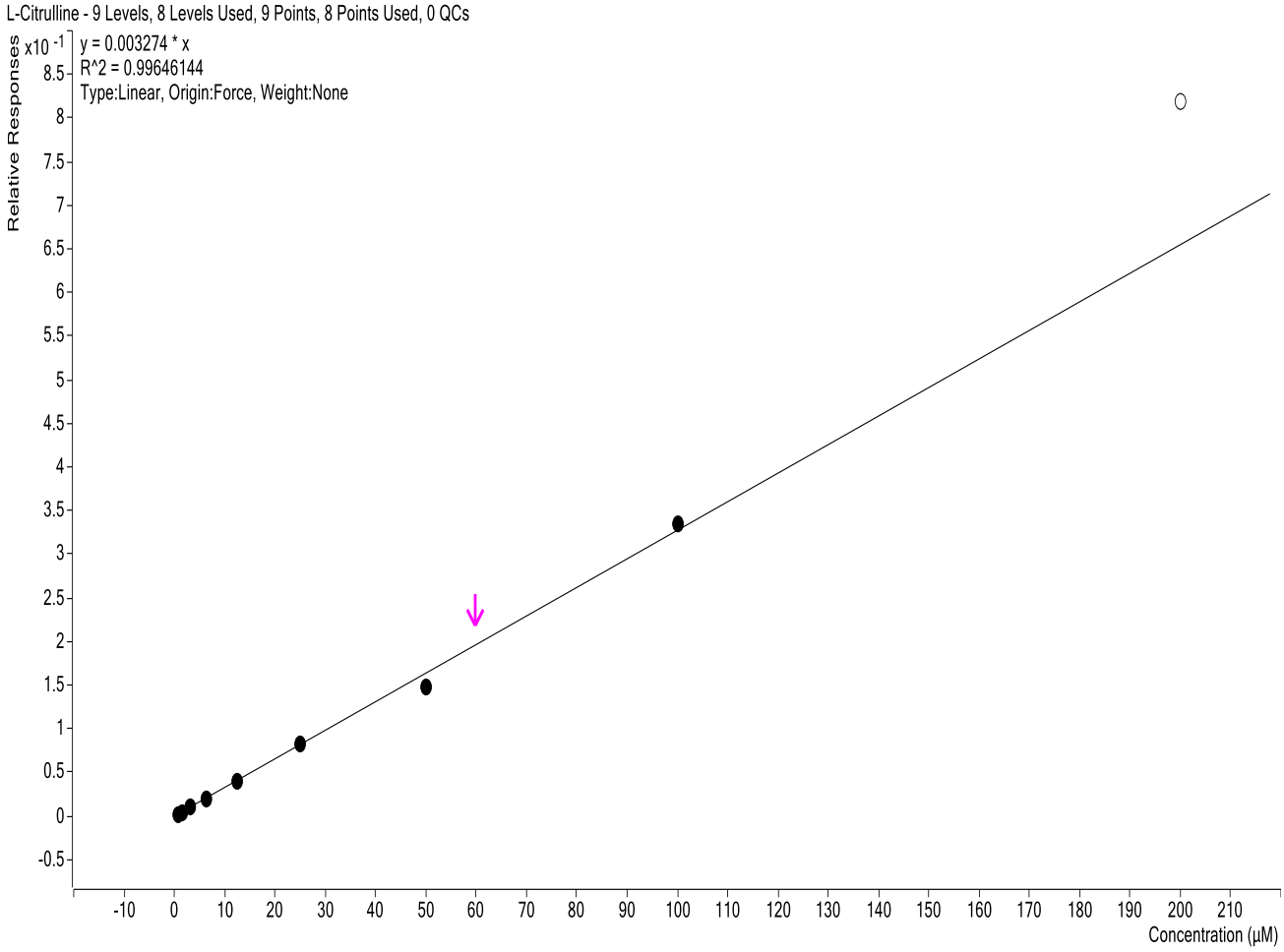
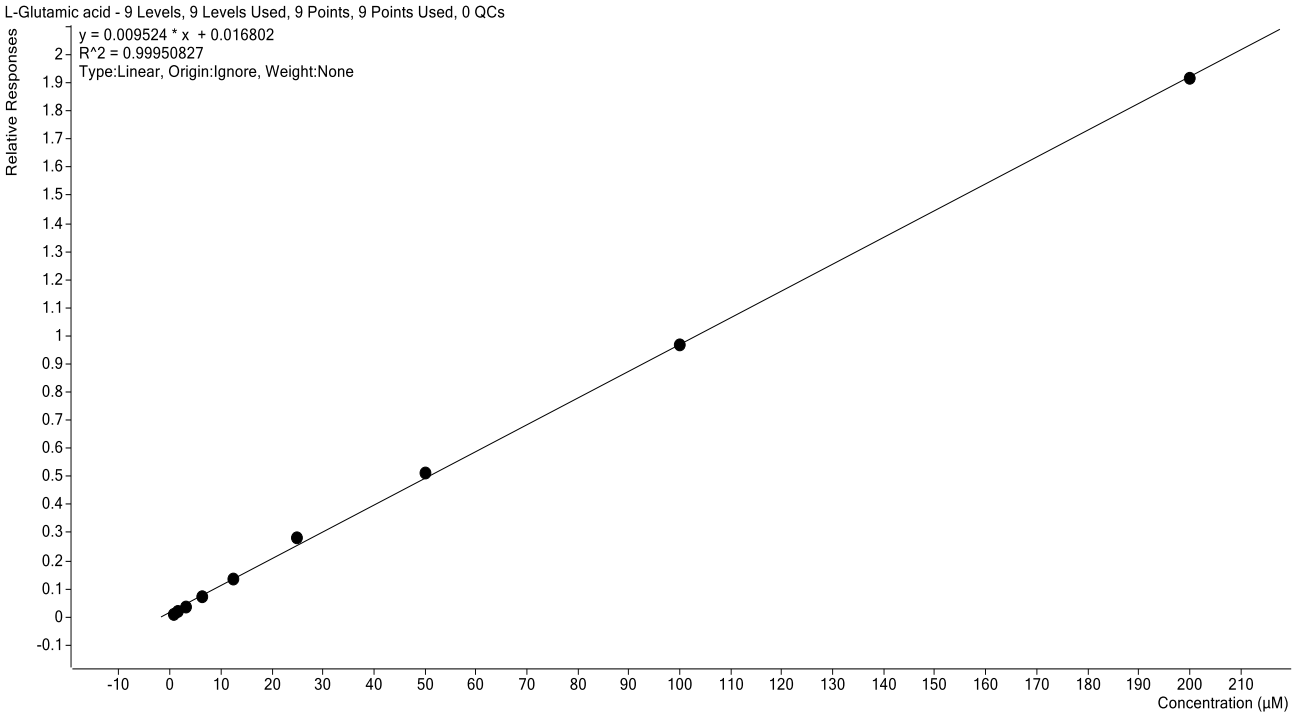
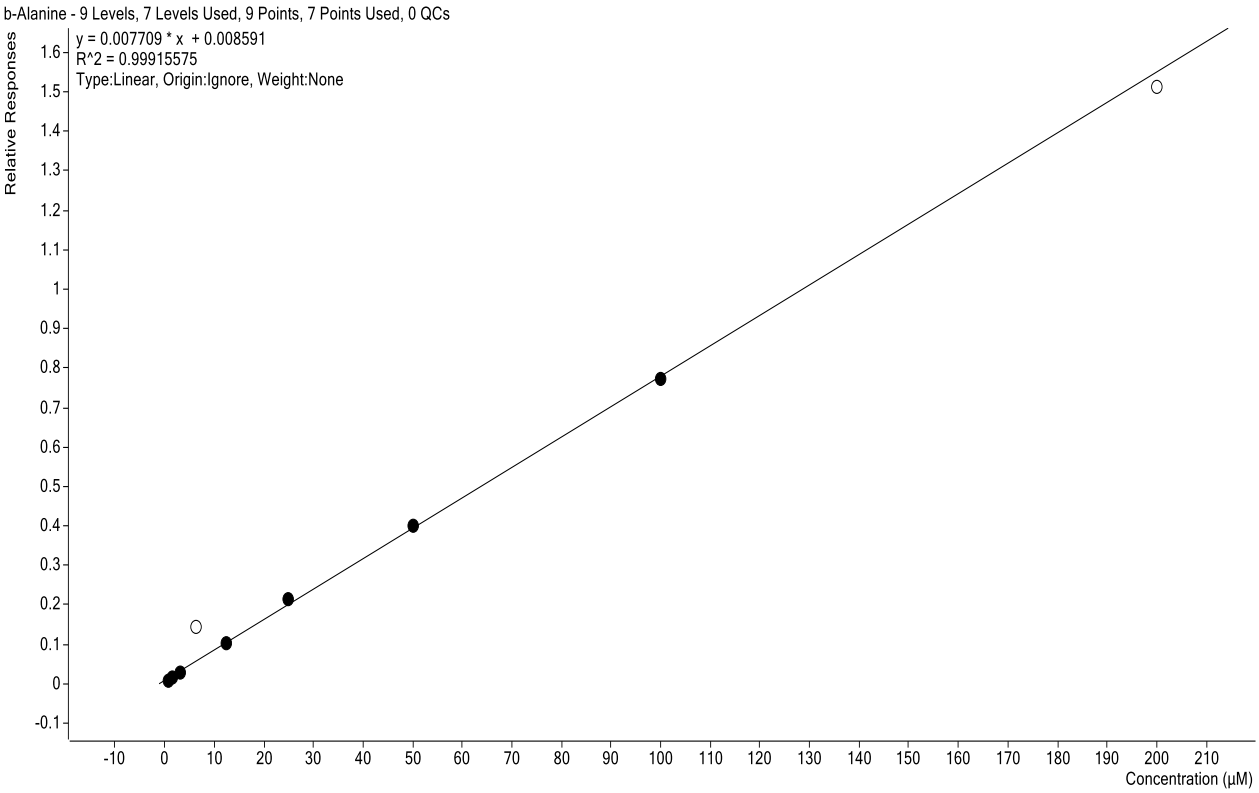
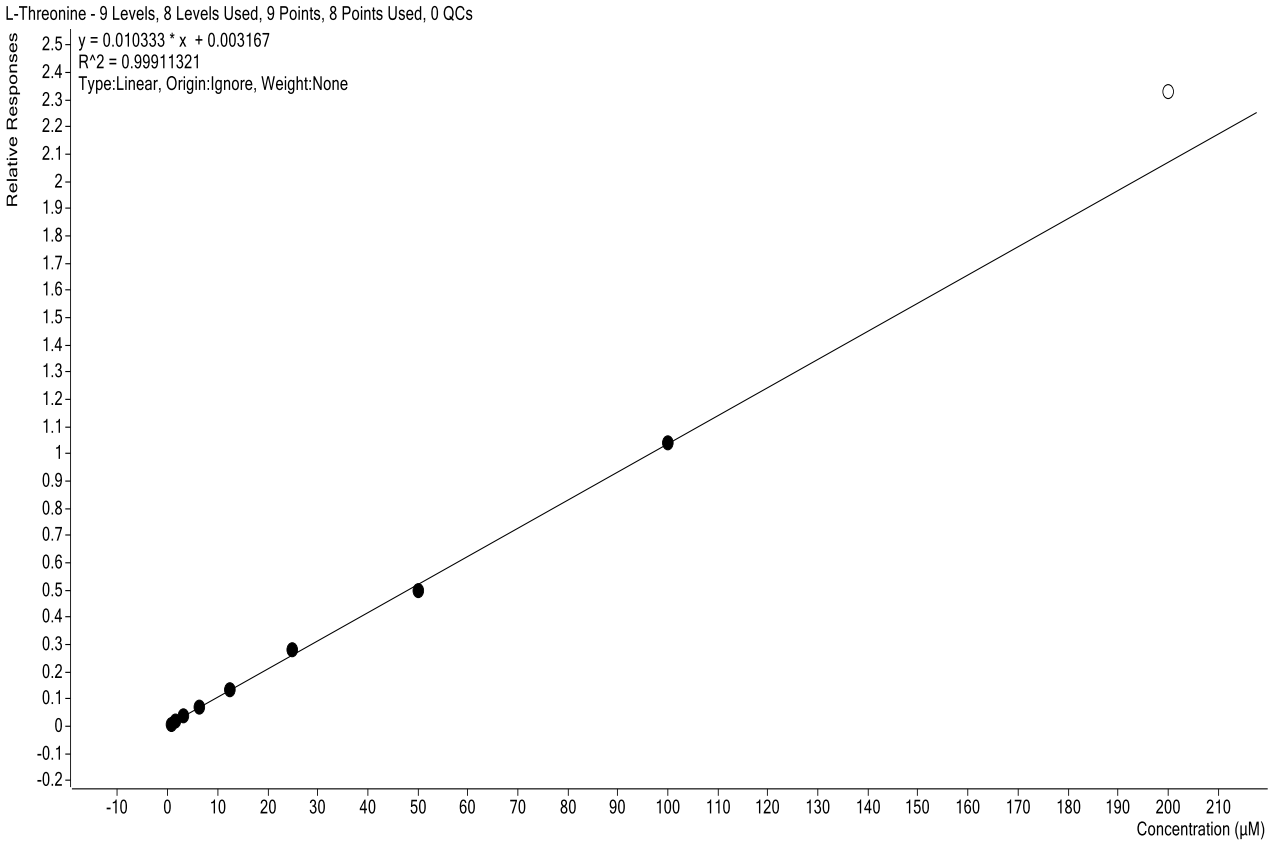
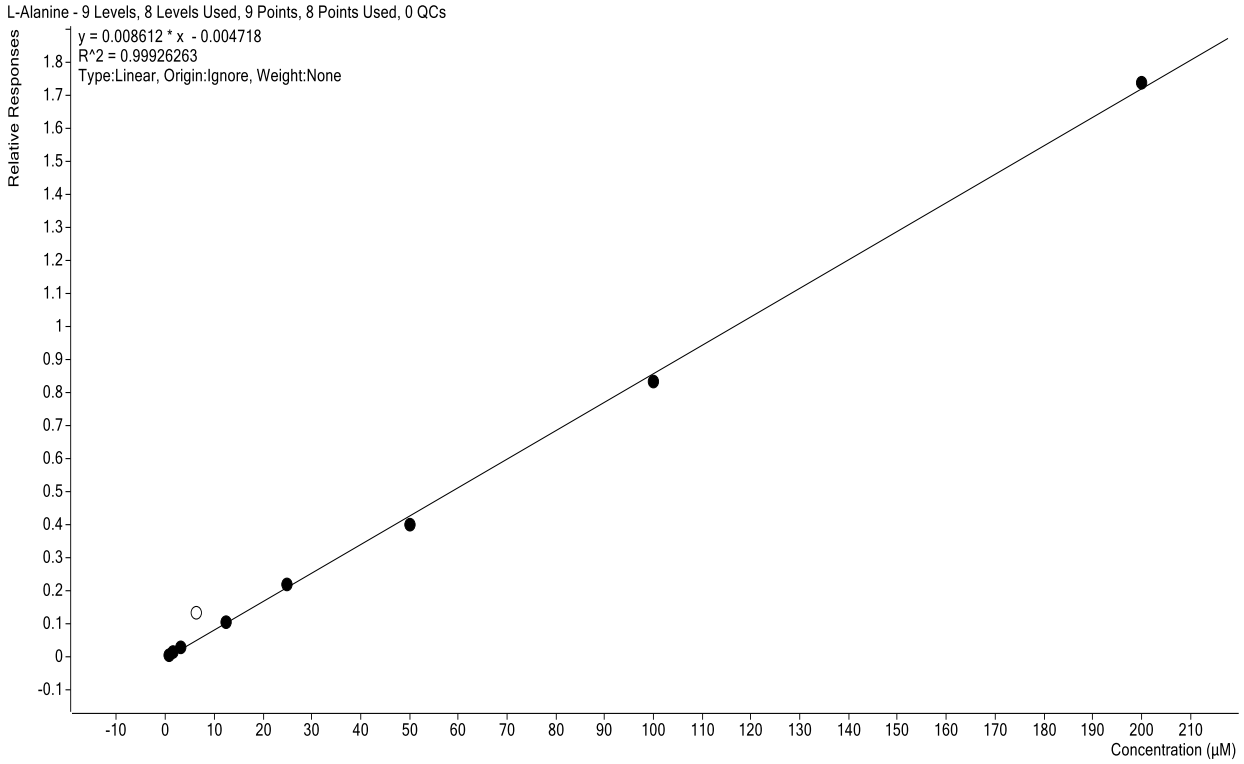
Relative Responses
Concentration (µM)


L-Citrulline - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.003274 * x
R^2 = 0.99646144
Type:Linear, Origin:Force, Weight:None
Relative Responses
x10 -1
Concentration (µM)

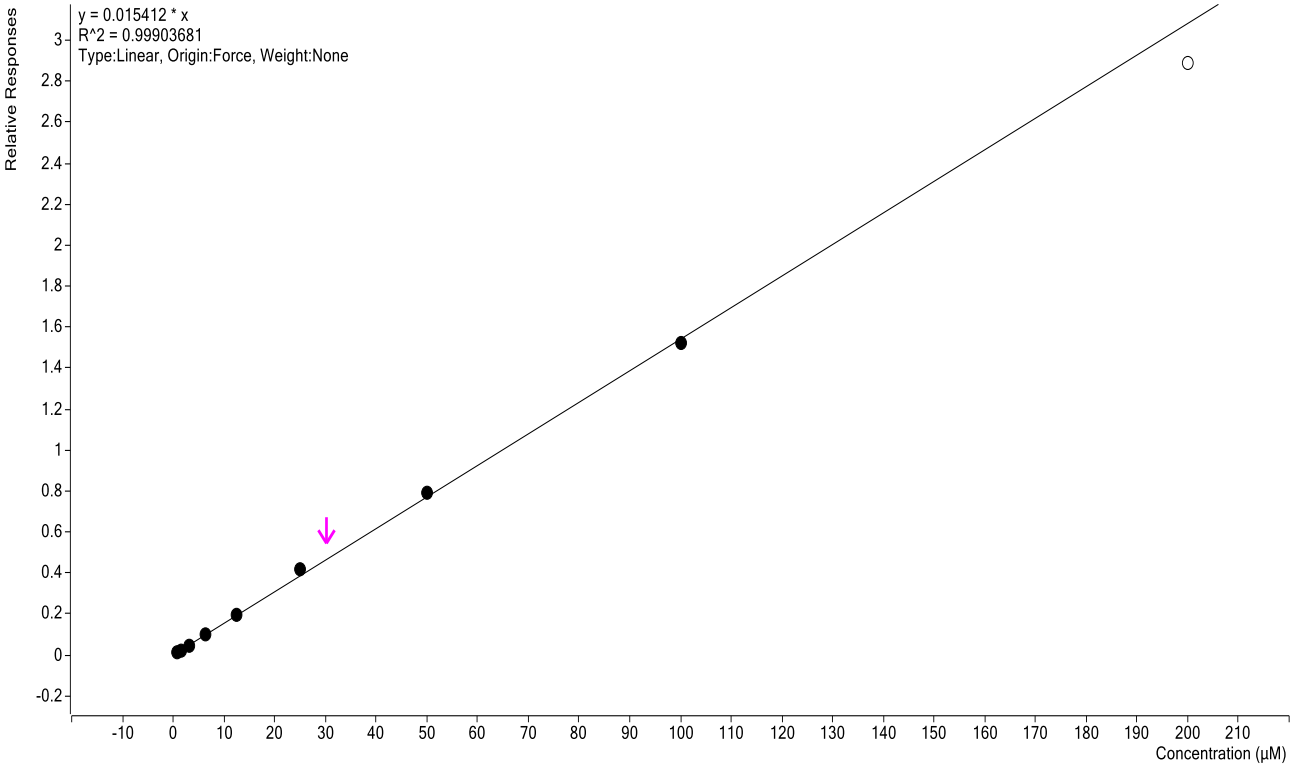

L-Glutamic acid - 9 Levels, 9 Levels Used, 9 Points, 9 Points Used, 0 QCs
y = 0.009524 * x + 0.016802
R^2 = 0.99950827
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)


b-Alanine - 9 Levels, 7 Levels Used, 9 Points, 7 Points Used, 0 QCs
y = 0.007709 * x + 0.008591
R^2 = 0.99915575
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

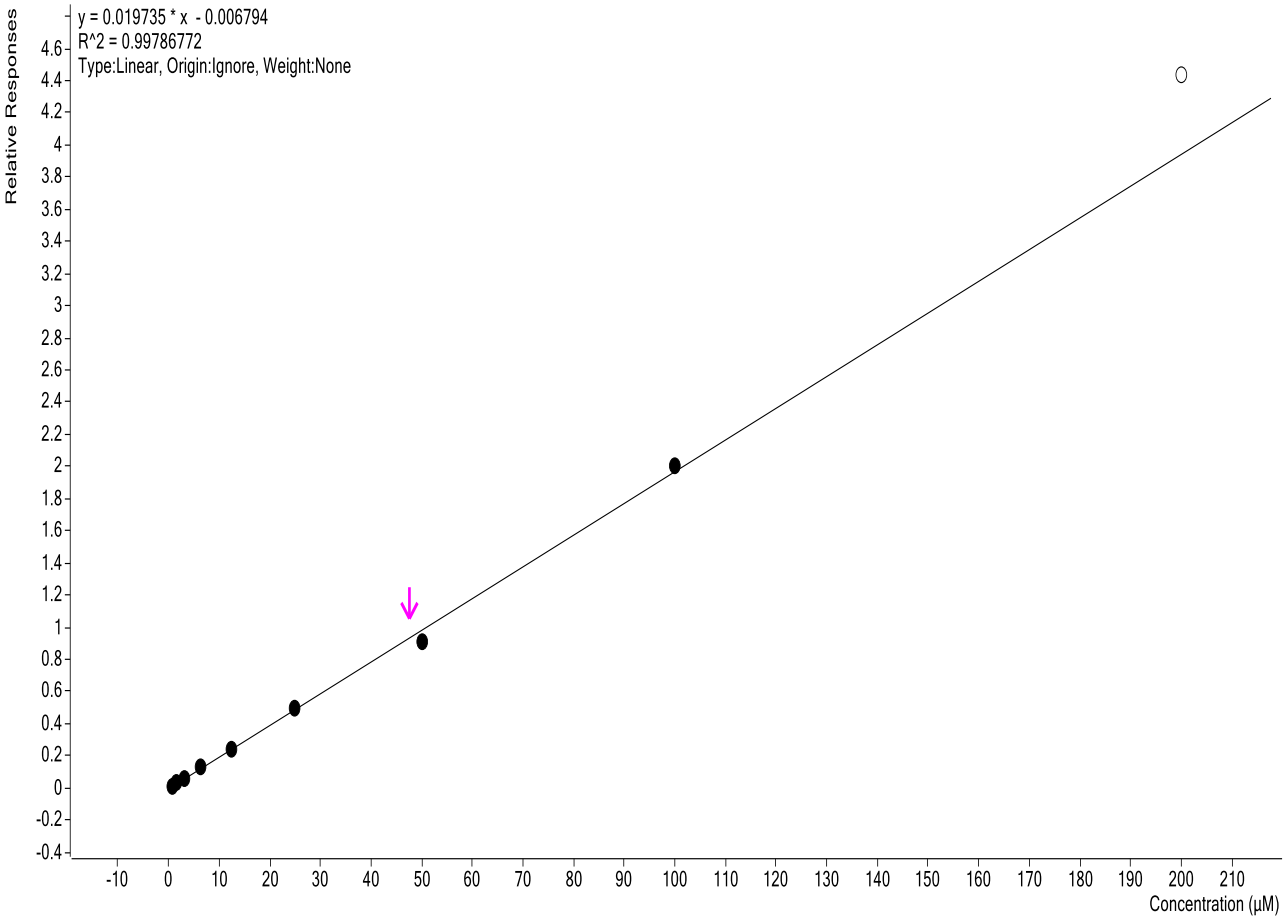

L-Threonine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.010333 * x + 0.003167
R^2 = 0.99911321
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

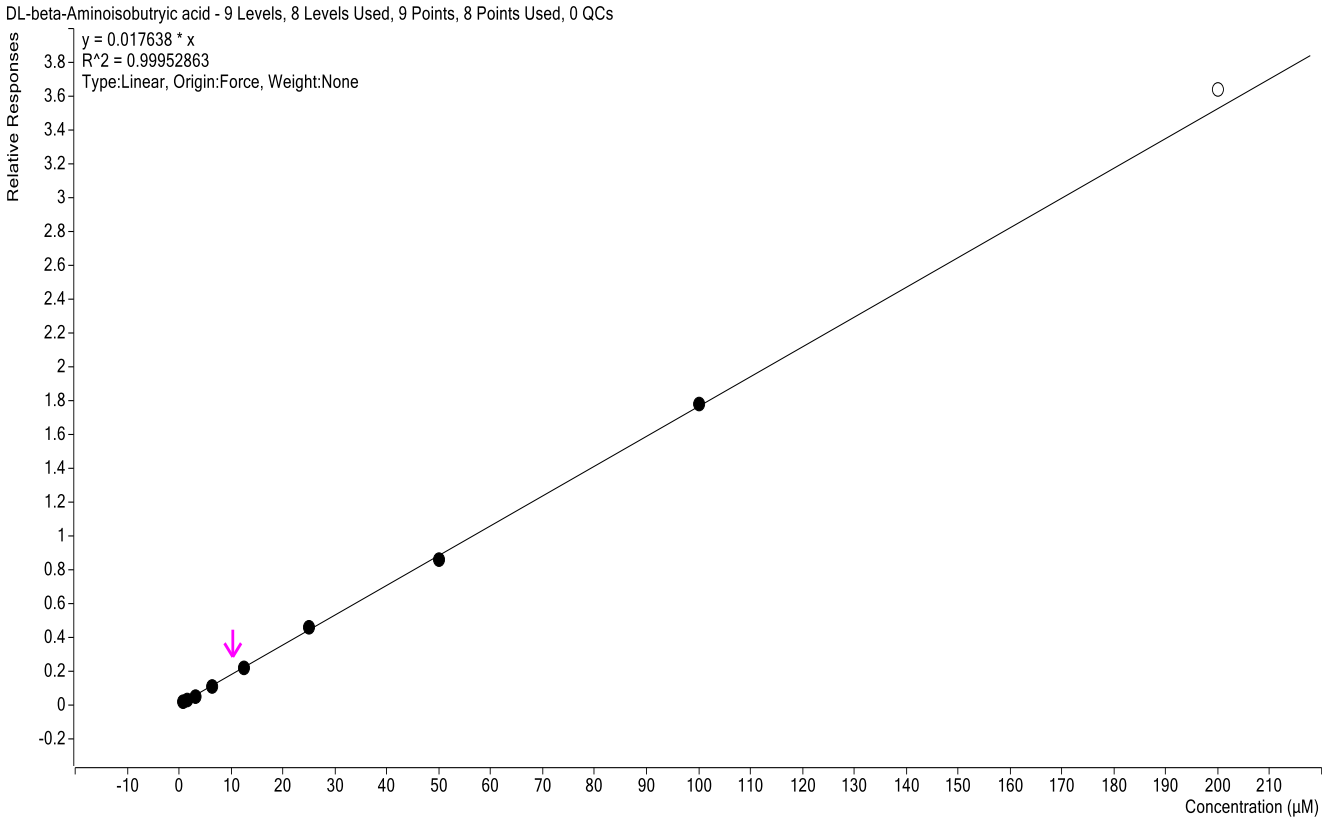

L-Alanine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.008612 * x - 0.004718
R^2 = 0.99926263
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

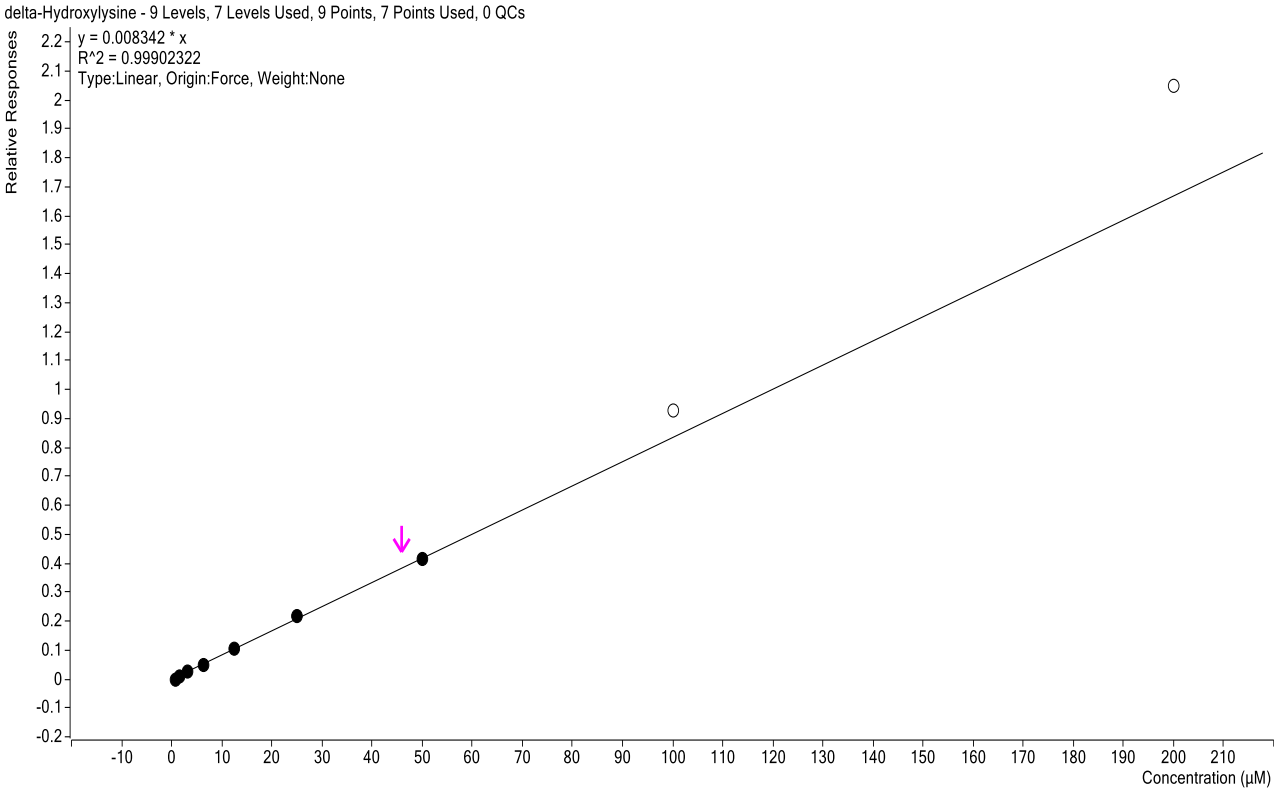
gamma-Amino-n-butyric acid - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs



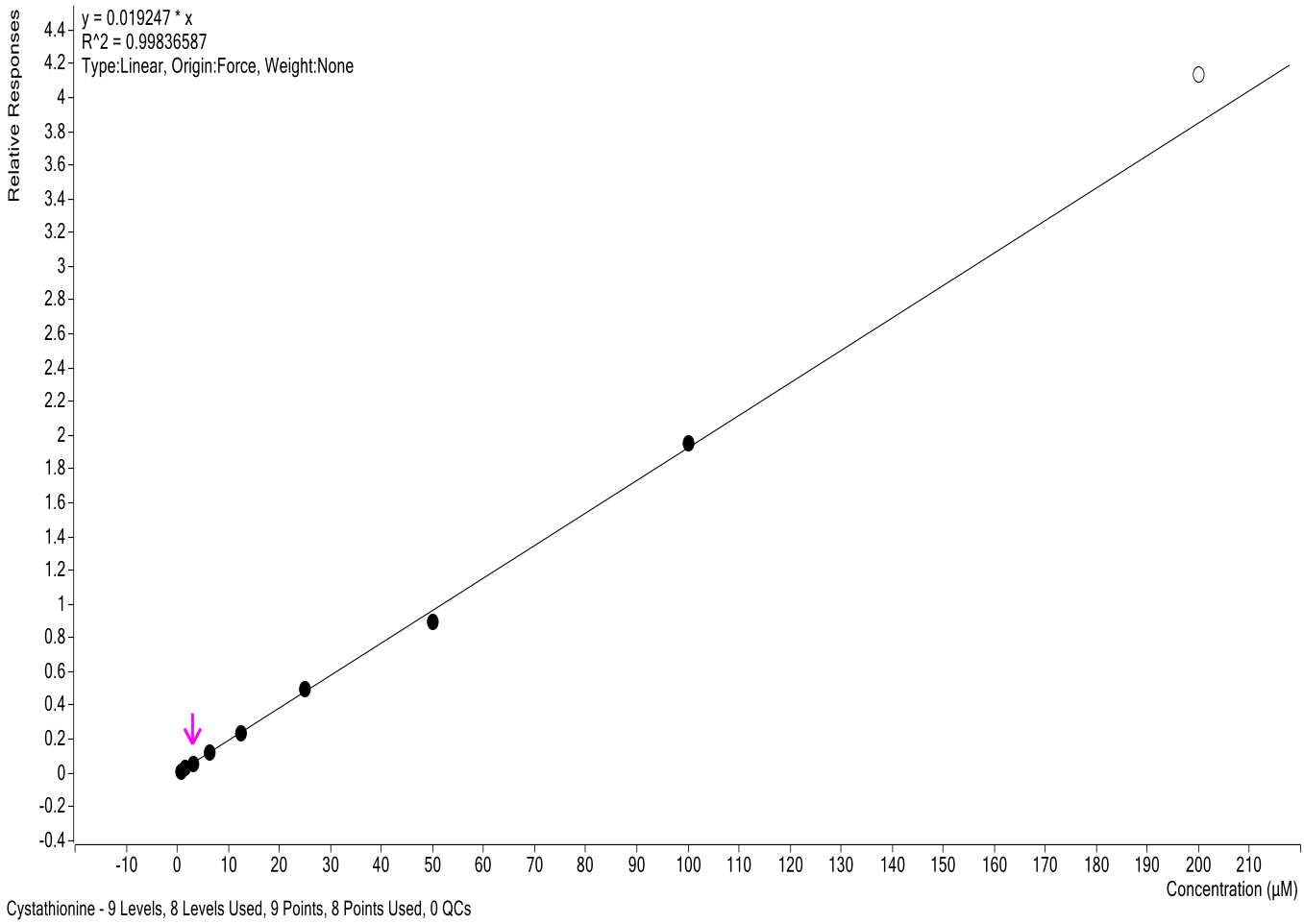
L-Proline - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs



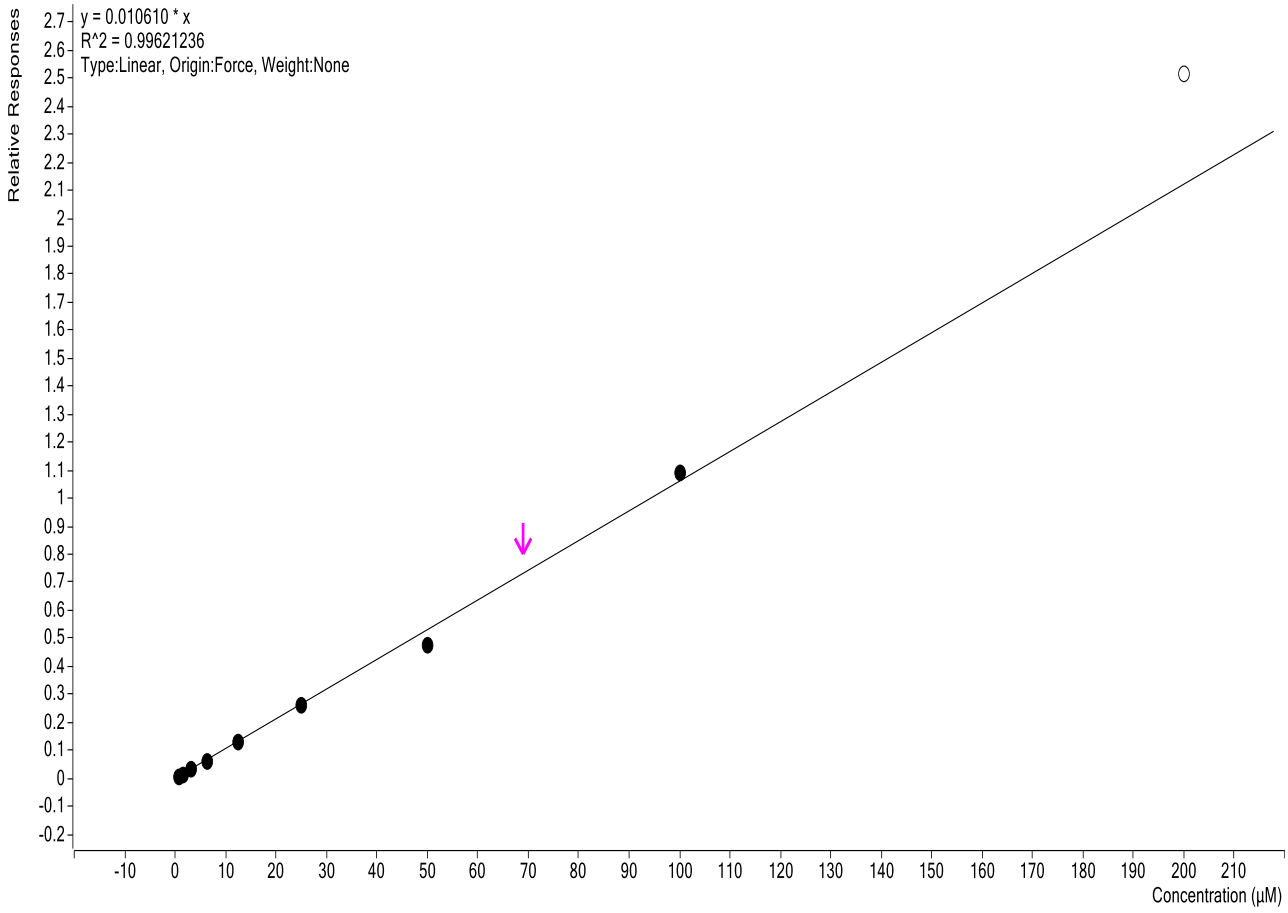

DL-beta-Aminoisobutryic acid - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017638 * x
R^2 = 0.99952863
Type:Linear, Origin:Force, Weight:None
Relative Responses
Concentration (µM)

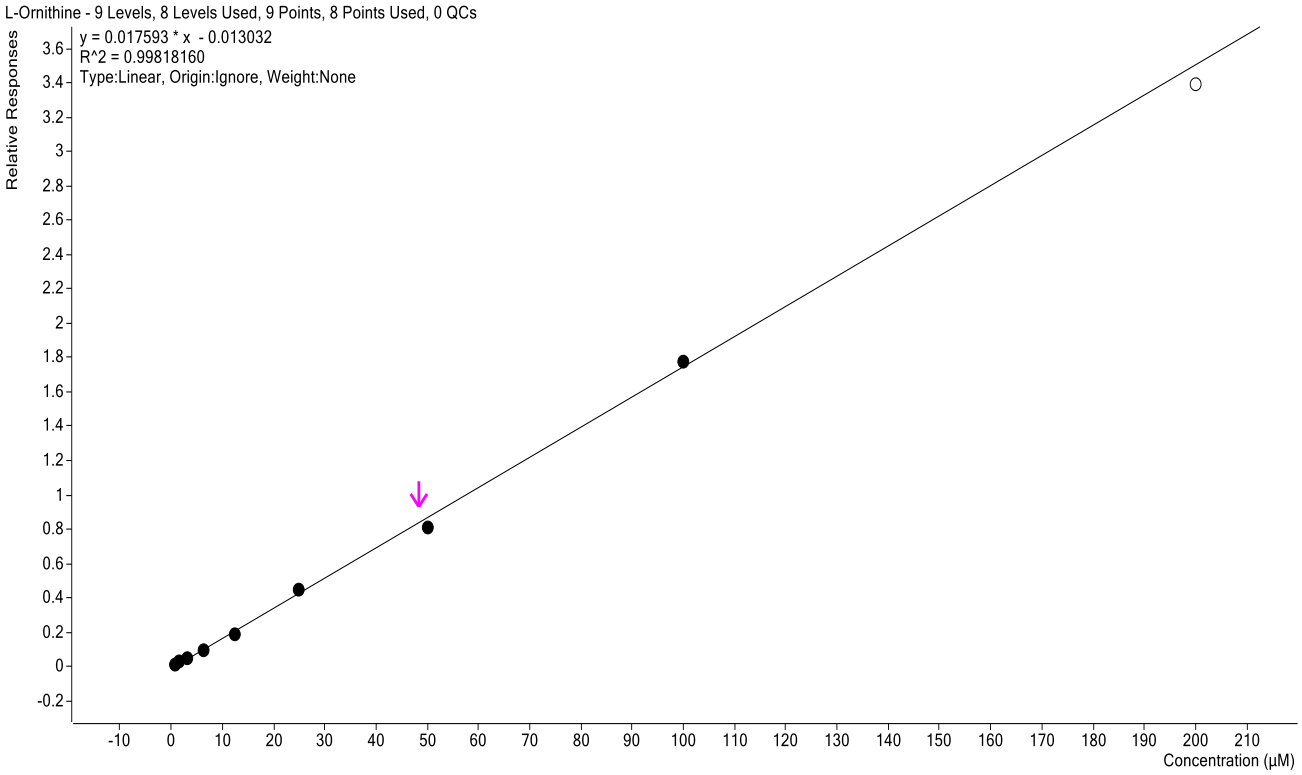

delta-Hydroxylysine - 9 Levels, 7 Levels Used, 9 Points, 7 Points Used, 0 QCs
y = 0.008342 * x
R^2 = 0.99902322
Type:Linear, Origin:Force, Weight:None
Relative Responses
Concentration (µM)

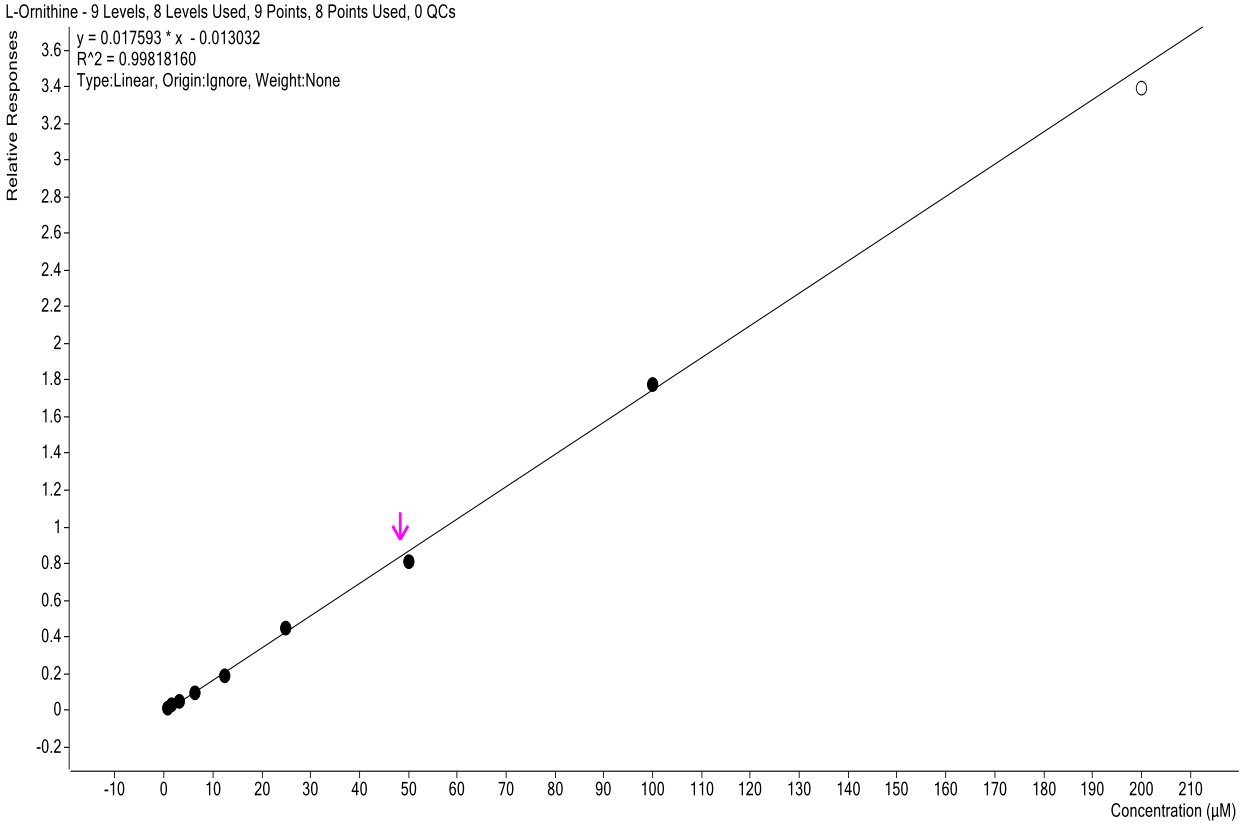
L-alpha-Amino-n-butyric acid - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs

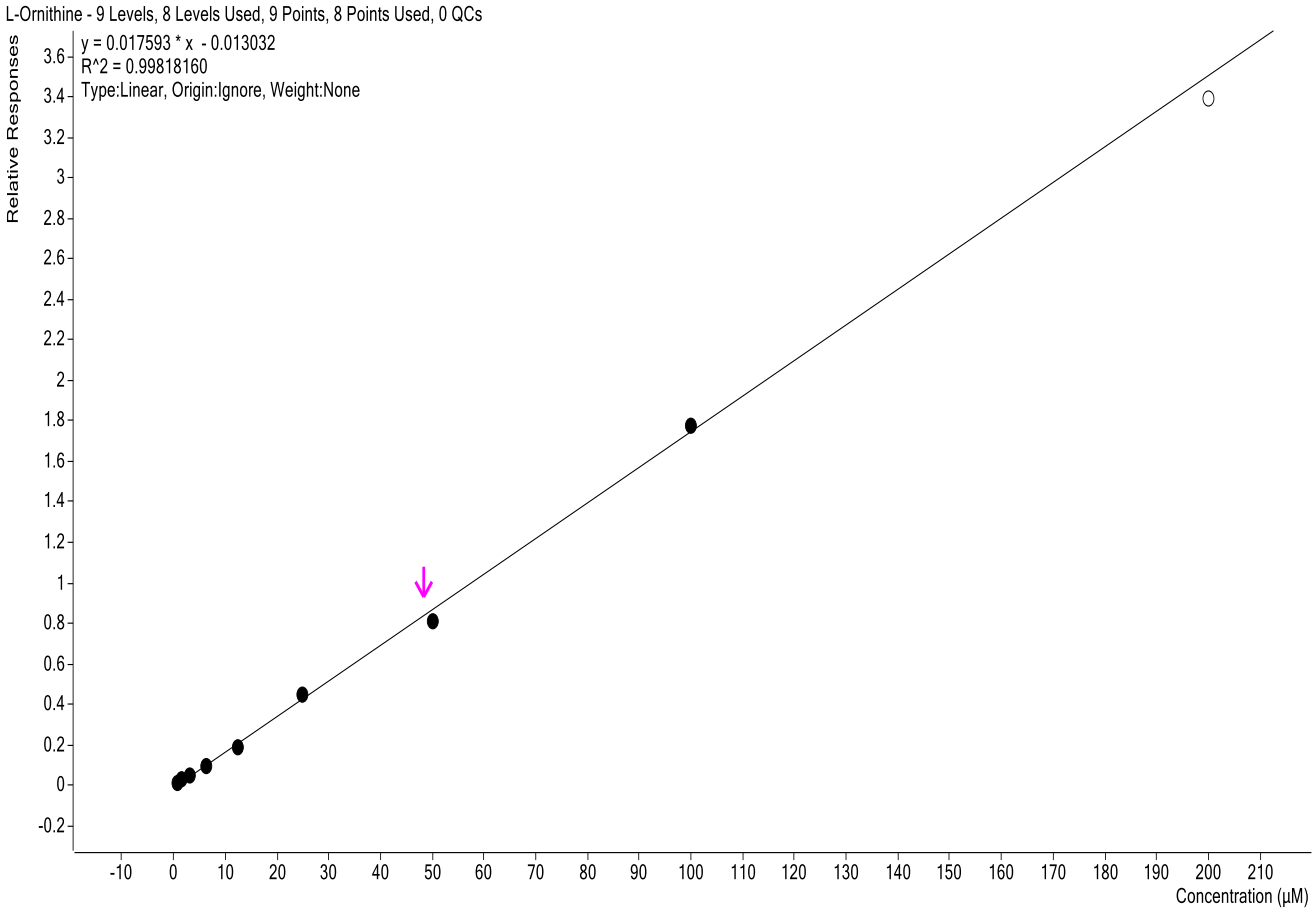


Cystathionine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs




L-Ornithine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017593 * x - 0.013032
R^2 = 0.99818160
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)


L-Ornithine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017593 * x - 0.013032
R^2 = 0.99818160
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)


L-Ornithine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017593 * x - 0.013032
R^2 = 0.99818160
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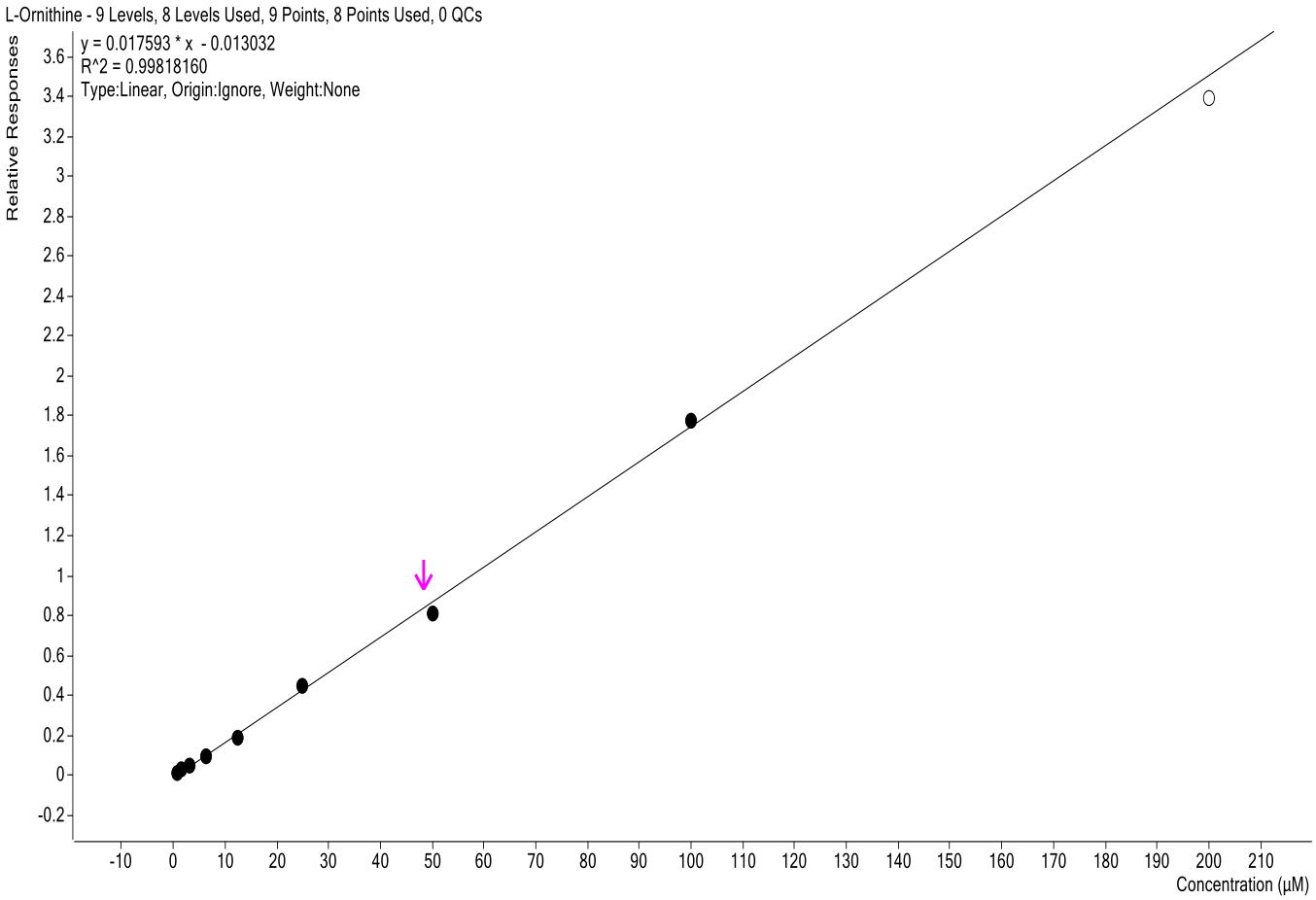
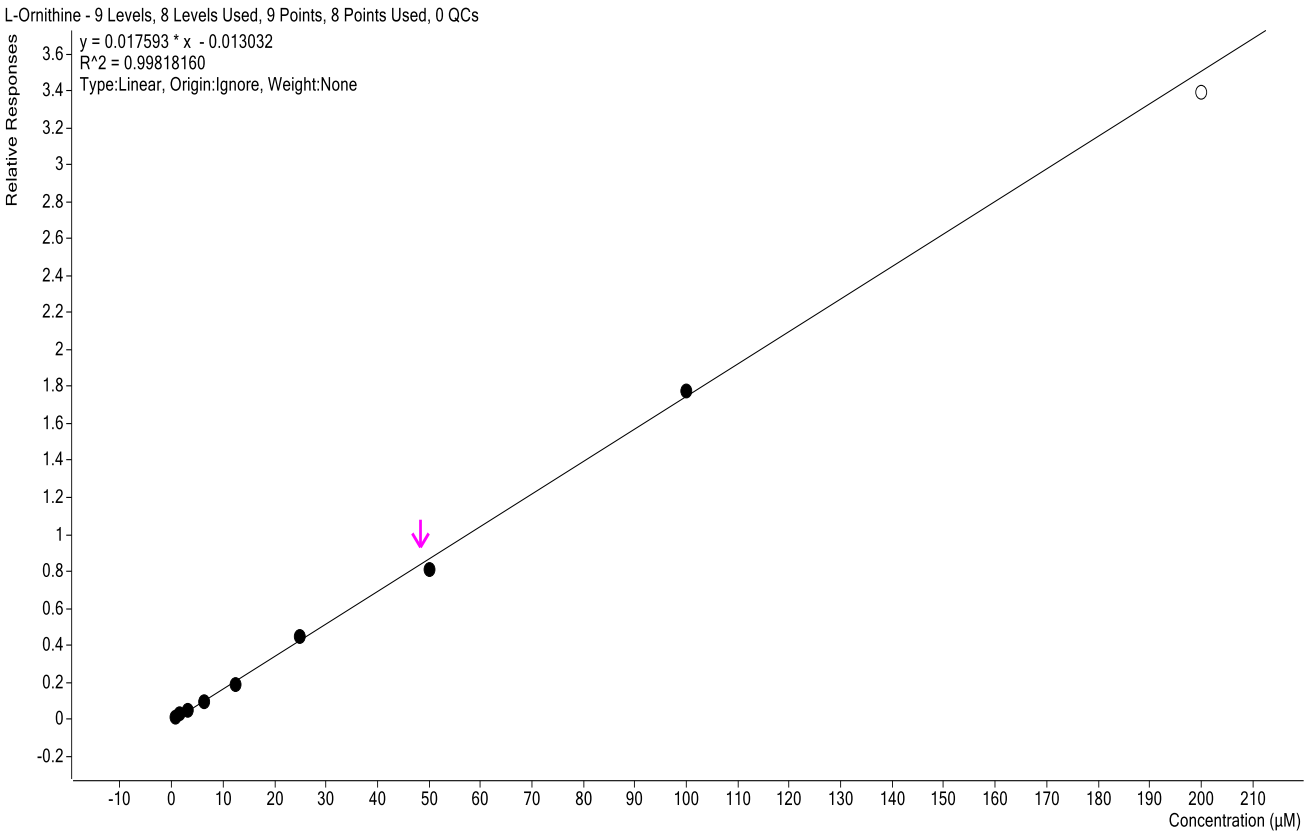
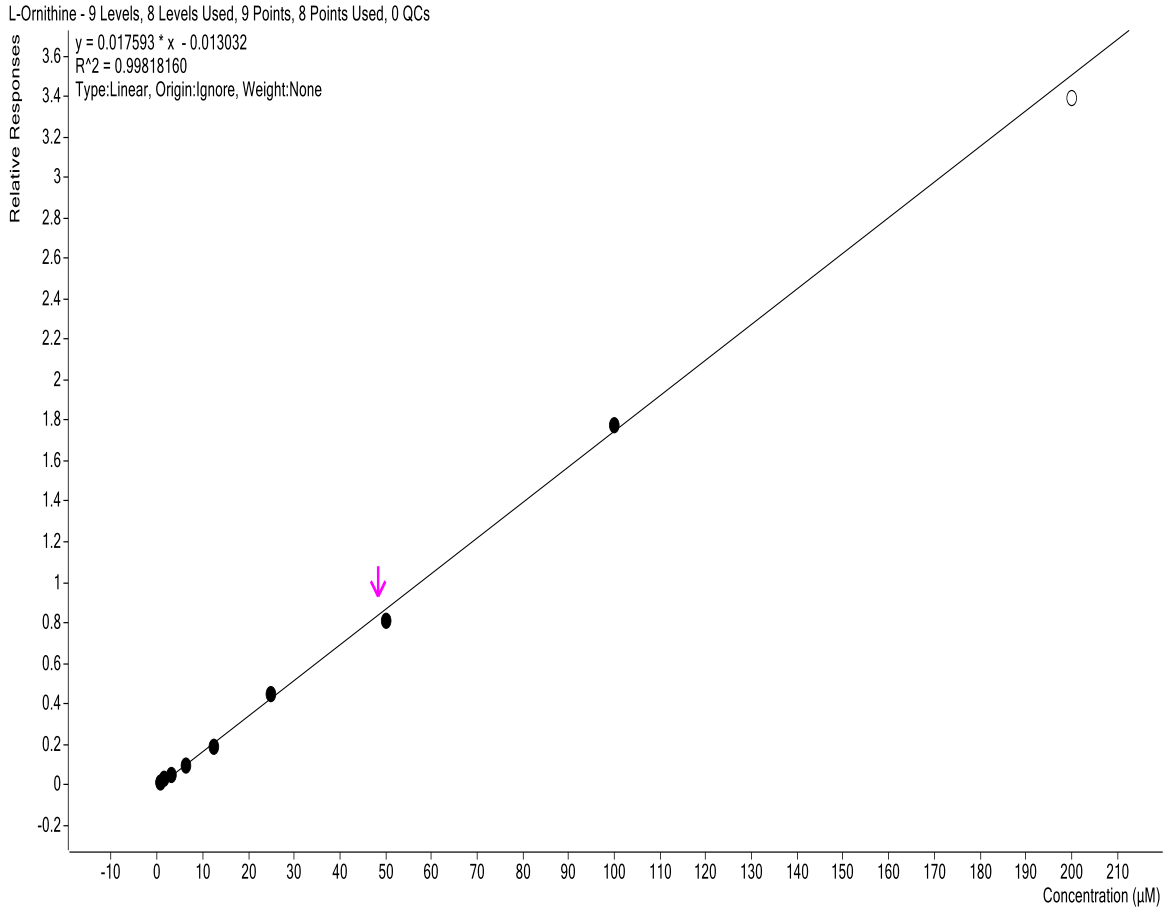
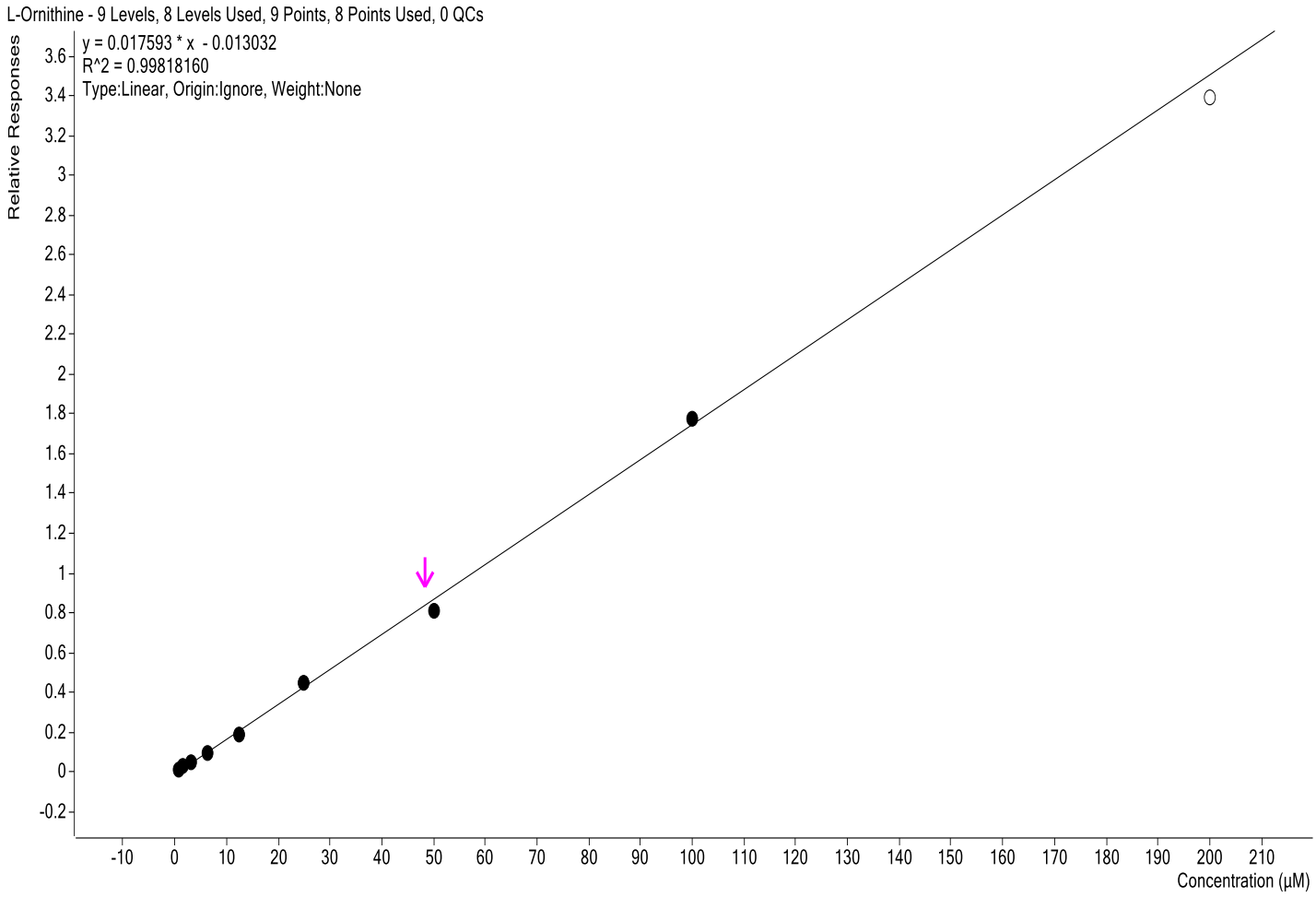
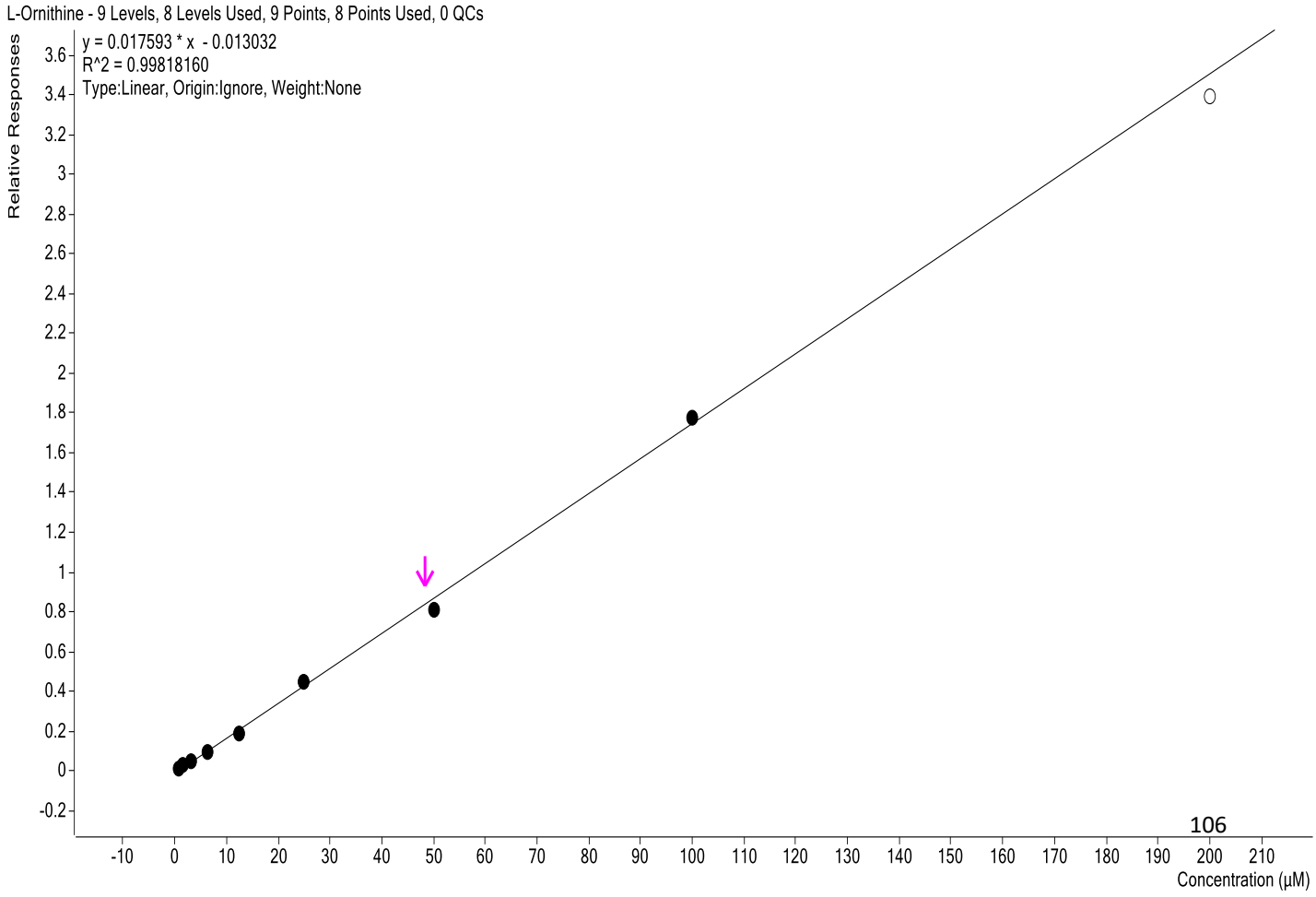
Relative Responses
Concentration (µM)

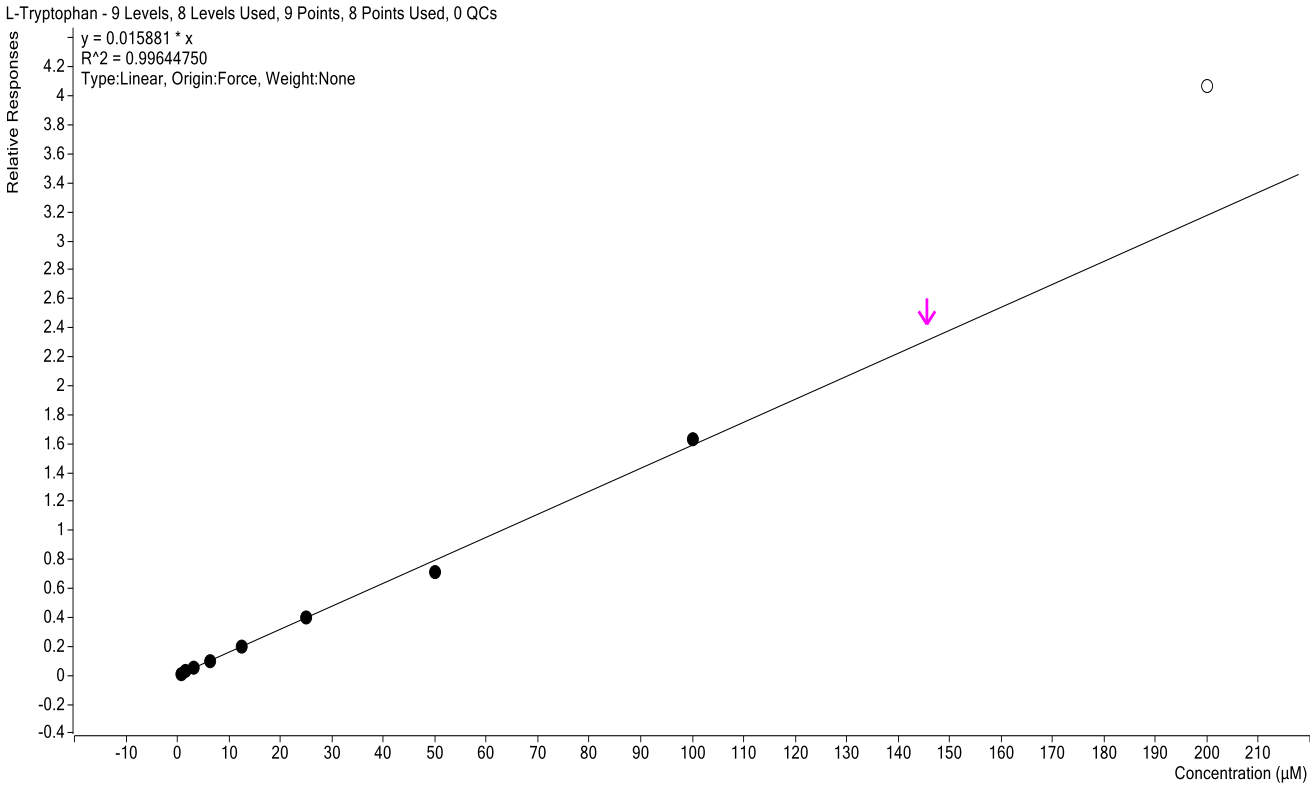

L-Ornithine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017593 * x - 0.013032
R^2 = 0.99818160
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

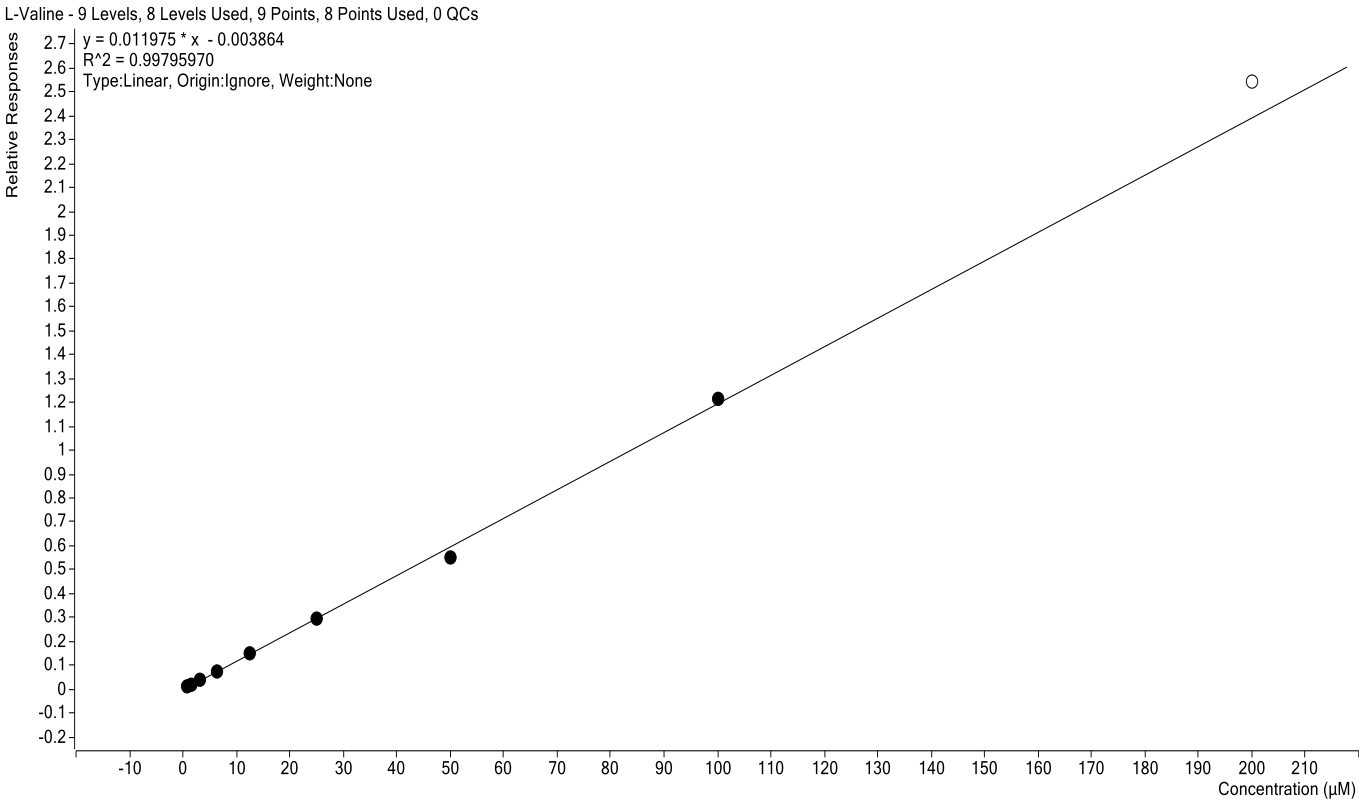

L-Ornithine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017593 * x - 0.013032
R^2 = 0.99818160
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

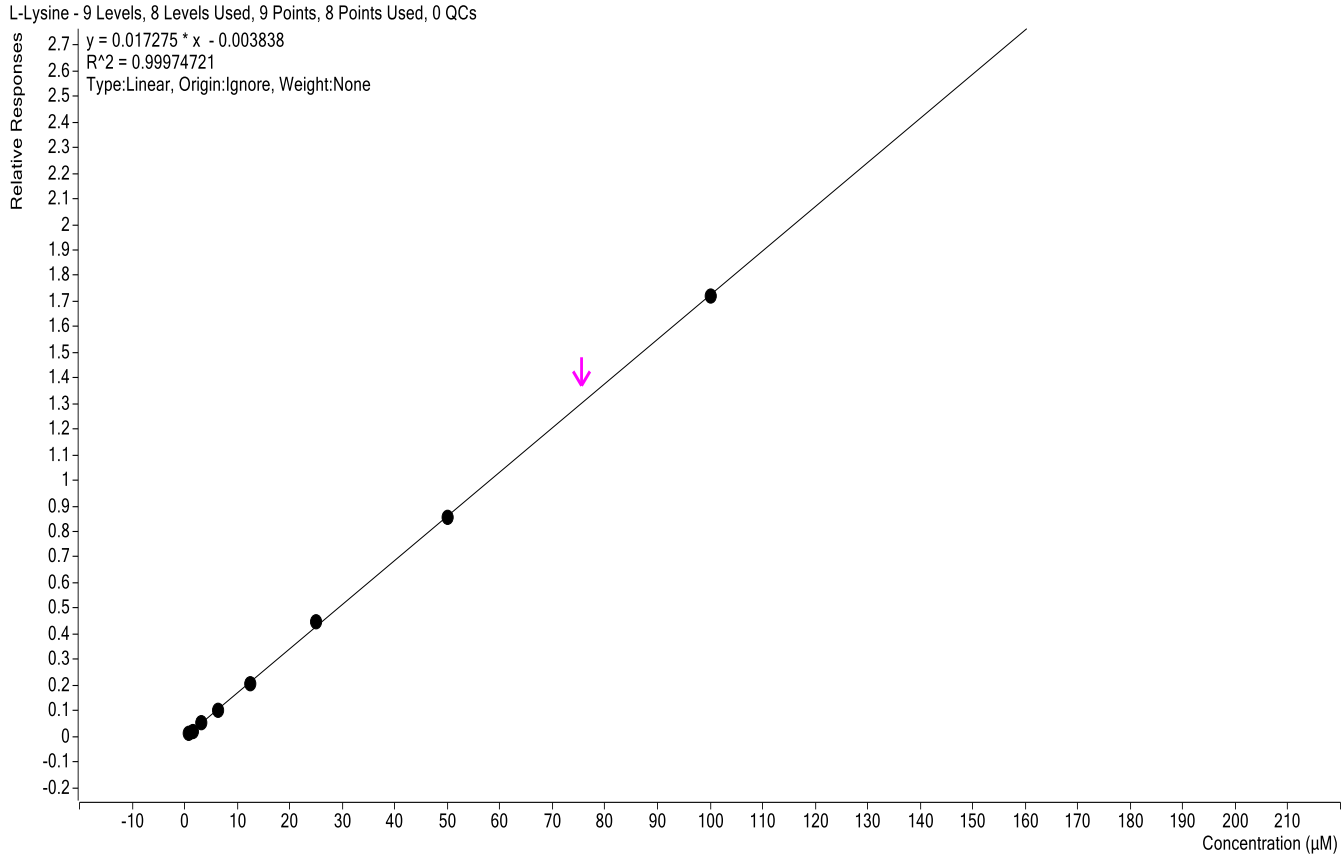

L-Ornithine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017593 * x - 0.013032
R^2 = 0.99818160
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

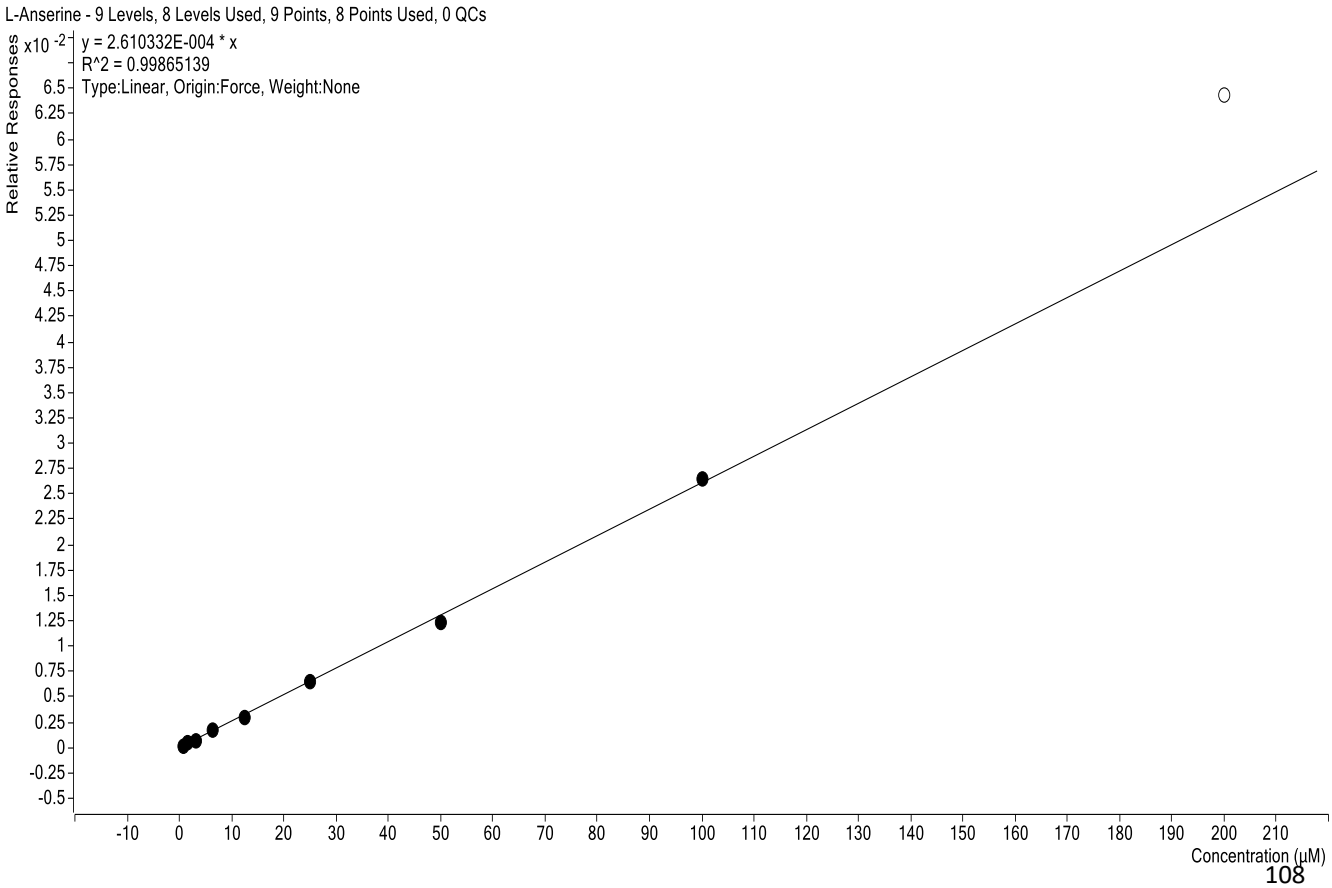

L-Ornithine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017593 * x - 0.013032
R^2 = 0.99818160
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

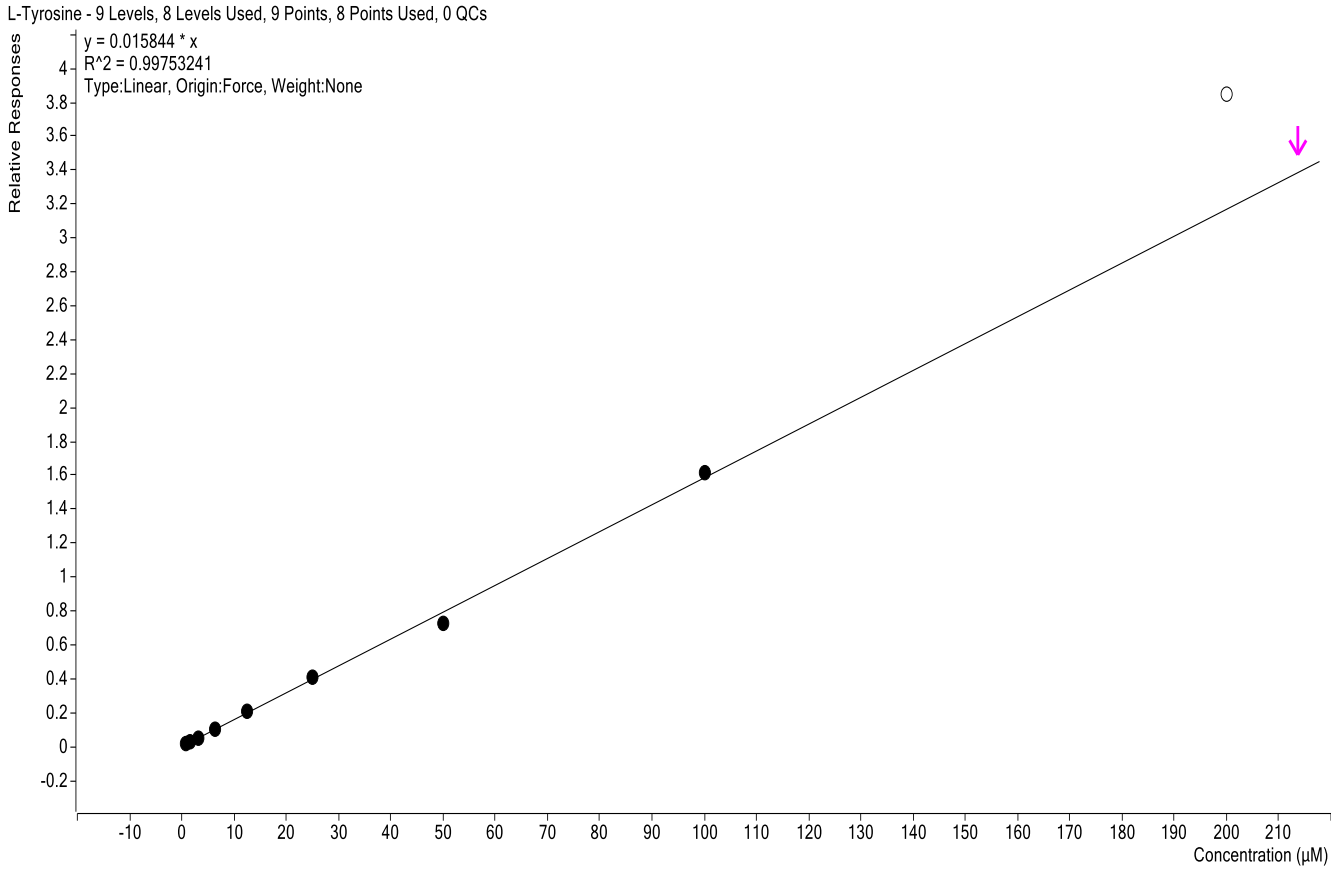

L-Ornithine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017593 * x - 0.013032
R^2 = 0.99818160
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

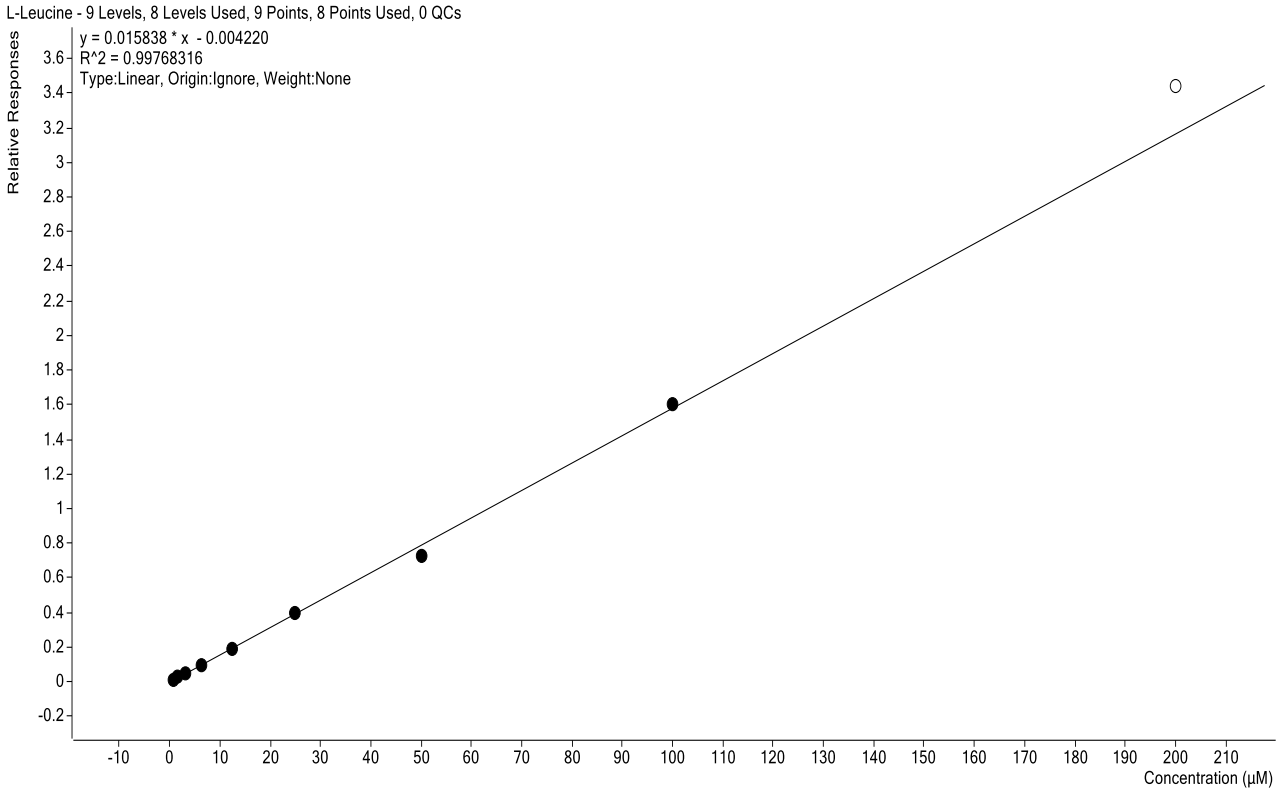

L-Tryptophan - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.015881 * x
R^2 = 0.99644750
Type:Linear, Origin:Force, Weight:None
Relative Responses
Concentration (µM)

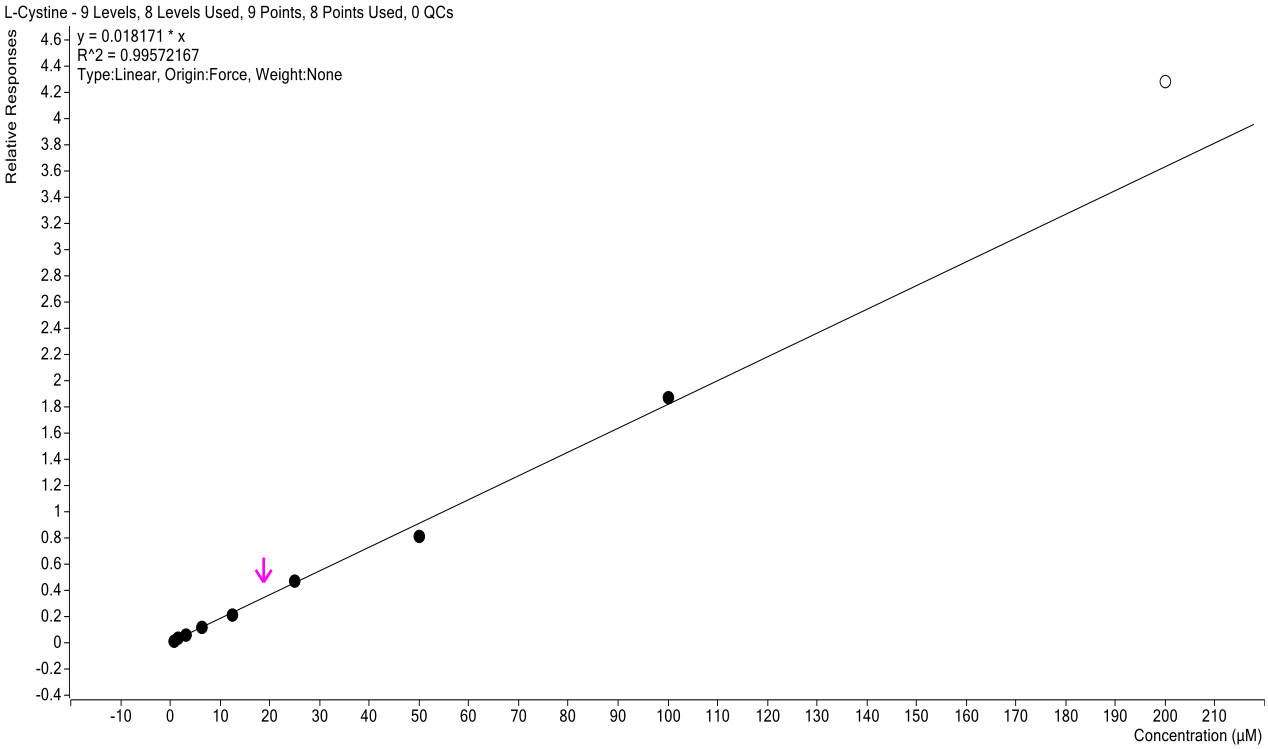

L-Valine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.011975 * x - 0.003864
R^2 = 0.99795970
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

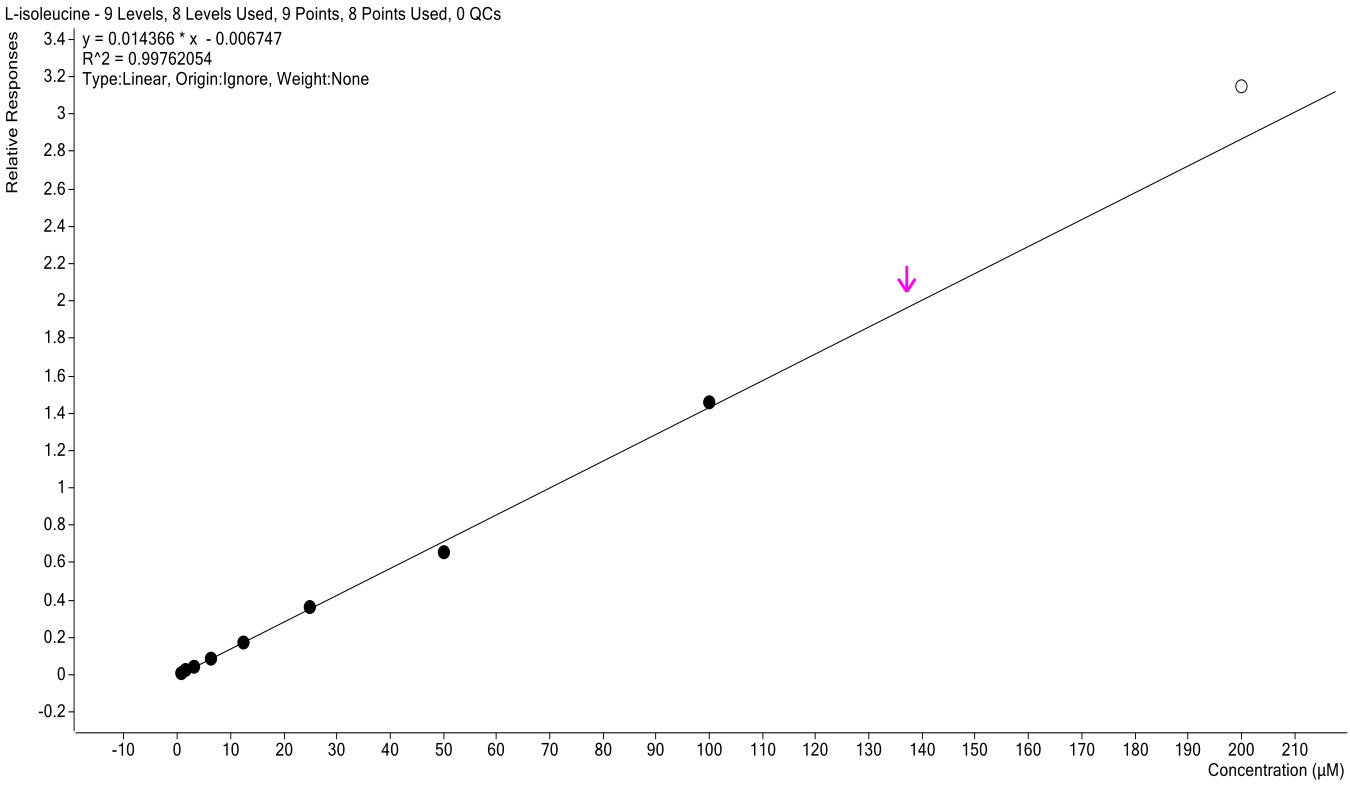

L-Lysine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.017275 * x - 0.003838
R^2 = 0.99974721
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)

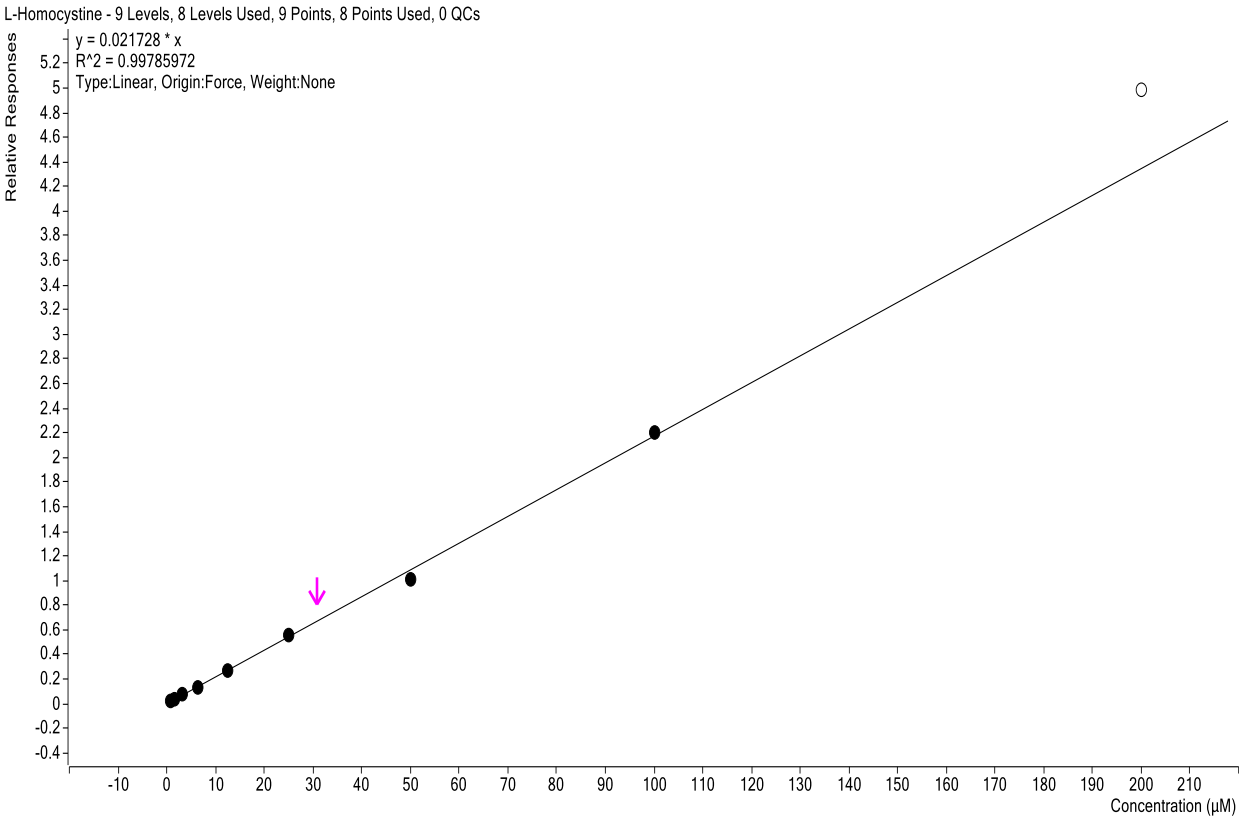

L-Anserine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 2.610332E-004 * x
R^2 = 0.99865139
Type:Linear, Origin:Force, Weight:None
Relative Responses
x10 -2
Concentration (µM)

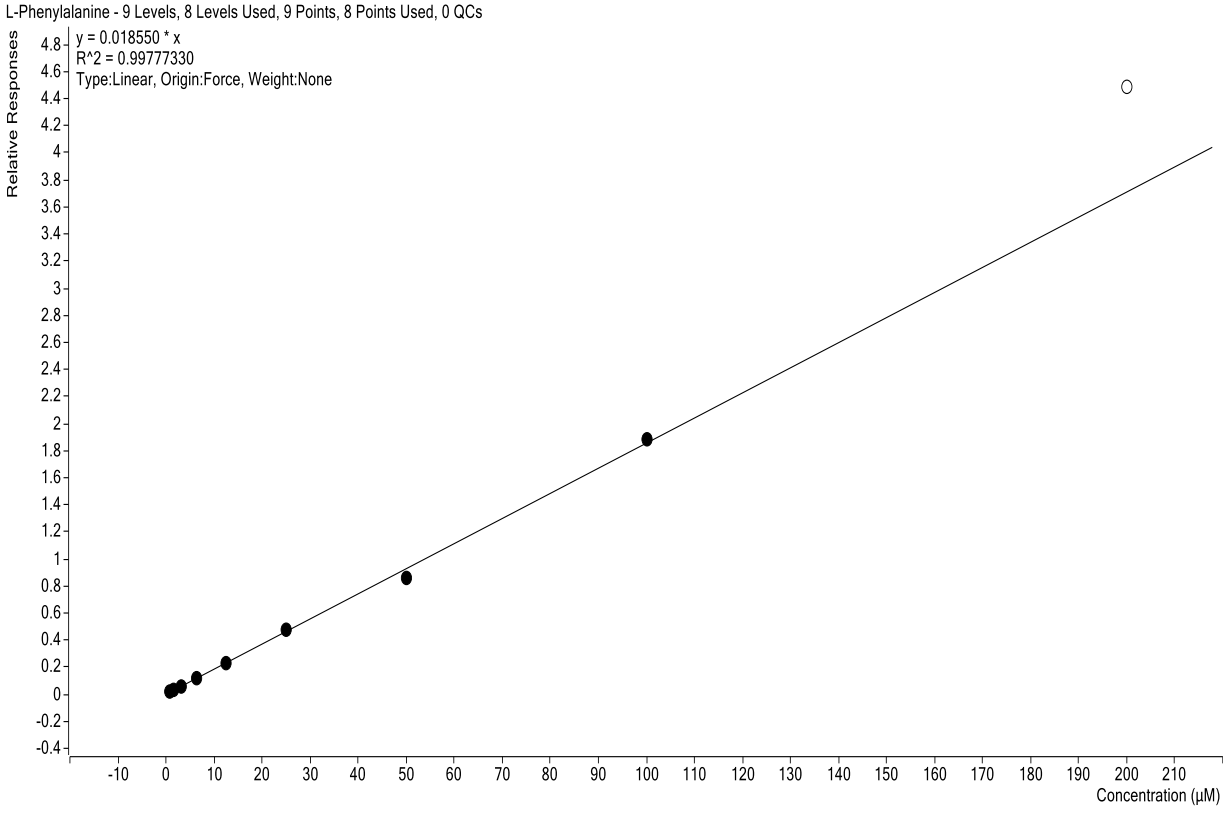

L-Tyrosine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.015844 * x
R^2 = 0.99753241
Type:Linear, Origin:Force, Weight:None
Relative Responses
Concentration (µM)


L-Leucine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.015838 * x - 0.004220
R^2 = 0.99768316
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)


L-Cystine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.018171 * x
R^2 = 0.99572167
Type:Linear, Origin:Force, Weight:None
Relative Responses
Concentration (µM)


L-isoleucine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.014366 * x - 0.006747
R^2 = 0.99762054
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Concentration (µM)


L-Homocystine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.021728 * x
R^2 = 0.99785972
Type:Linear, Origin:Force, Weight:None
Relative Responses
Concentration (µM)


L-Phenylalanine - 9 Levels, 8 Levels Used, 9 Points, 8 Points Used, 0 QCs
y = 0.018550 * x
R^2 = 0.99777330
Type:Linear, Origin:Force, Weight:None
Relative Responses
Concentration (µM)