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Cheese: A Proteolytic Journey from Curd to Bioactive Peptide

A thesis presented in partial fulfilment of the required for the degree of

Master of Science

in

Chemistry

at Massey University, Manawatu, New Zealand

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2026

Abstract

Cheese is a complex matrix of proteolytic pathways generating numerous peptides, some with bioactive properties such as antihypertensive, antioxidant, and antimicrobial. Manufacturing variables including starter culture, ripening time/temperature, and salt and moisture content affect peptide formation, but comprehensive data linking these process parameters to peptide profiles and quantities across cheese varieties are limited. This study expands that knowledge for cheddar cheese using UHPLC-MS/MS to perform untargeted profiling of peptides and quantify three bioactives: MKPWIQPK, VLNENLLR, and YPFPGPIPN in cheddar manufactured with different starters, ripening temperatures and salt and moisture concentrations. These findings highlight the potential to modulate bioactive peptide yields through manufacturing process control without altering the bioactive peptide profile.

Acknowledgments

I would like to extend my gratitude towards my manager Paul Plieger for enabling me to develop in my career by partaking in this academic endeavour. I also must acknowledge the hard-working Analytical Sciences Team at Fonterra Research and Development Centre who provided me time and space to study and grow.

To my supervisors: Pat Edwards and Gareth Rowlands, you have been endlessly supportive and favourably critical of my work. I have enjoyed learning from you both and I hope this thesis does you proud.

To all the people that have answered my million questions of: Bradley White (mass spectrometer expert), Trevor Loo (peptide expert), Andrew Legg (cheese expert) and Lisa Thomasen (statistics expert). Thank you so much for taking the time to provide me with answers or point me in the direction of them. Special thanks to Bradley White who has helped me develop a better understanding of Mass Spectrometry and the operation of the Exploris 240 (fondly known as Dora).

It cannot go without being said that my journey in this scientific adventure would not be complete without the help and distraction of my loving family Hamish, George and the cat (Stitch).

Abbreviations and Glossary

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ACE-I	Angiotensin Converting Enzyme Inhibition
AGC	Automatic Gain Control
ANOVA	Statistical method for determining variation in datasets
CN	Abbreviation for Casein, one of the major protein groups in dairy.
Codex	A set of standards or rules for the manufacture of a product
DDA	Data Dependent Acquisition
DIA	Data Independent Acquisition
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPP-IV	Dipeptidyl peptidase-4
EPS	Exopolysaccharide
ESI	Electrospray Ionisation
FAO	Food and Agricultural Organisation
FDM	Fat in Dry Matter
GRAVY	Grand Average of Hydropathy
HILIC	Hydrophilic Interaction Liquid Chromatography
IT	Injection time
kDa	Kilodalton
LAB	Lactic Acid Bacteria
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
MALDI	Matrix-Assisted Laser Desorption/Ionization
MIC	Minimal Inhibitory Concentration
MKPWIQPK	Peptide from α_{s2}-CN [205-212] known for bioactive activity
NSLAB	Non-Starter Lactic Acid Bacteria
PDO	Protected Designation of Origin
PGC	Porous Graphitic Carbon
ROS	Reactive Oxygen Species
RP-HPLC	Reverse-Phase High Pressure Liquid Chromatography
S:M	Salt in Moisture ratio
SPE	Solid Phase Extraction
TOF	Time of Flight
UHPLC-MS/MS	Ultra High-Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
VLNENLLR	Peptide released from α_{s1}-CN [30-37] known for bioactive activity
WHO	World Health Organisation
WSE	Water Soluble Extract
WSN	Water Soluble Nitrogen
YPFPGPIPN	Peptide released from β-CN [75-83] known for bioactive activity

Table of Contents

List of Tables and Figures

1 Introduction

Cheese is not just a tasty snack; it is also a complex food matrix steeped in history and tradition. Originating thousands of years ago cheese has evolved alongside humans due to technological innovation, sensory preferences and targeted formulation into the diverse array of cheese varieties seen today.[2] Cheese contains proteins, lipids, carbohydrates, vitamins and minerals. Underlying this is a complement of microorganisms which create the complexity of the cheese matrix through the diverse influences they have on cheese components.

Cheese can arguably be created from any mammalian milk source with the most common being cow, sheep and goat. Grouped by texture, cheese can vary from soft all the way through to hard. Some notable examples are: soft cheese – Brie (France) and ricotta (Italy), semi-soft cheese – Havarti (Denmark) and paneer (India/Afghanistan/Iran), semi-hard cheese – Monterey Jack (USA) and cheddar (England), and lastly hard cheese – Emmental (Switzerland) and Parmigiano Reggiano (Italy).[3] This demonstrates the versatility, extent and global acceptance of cheese as a food source.

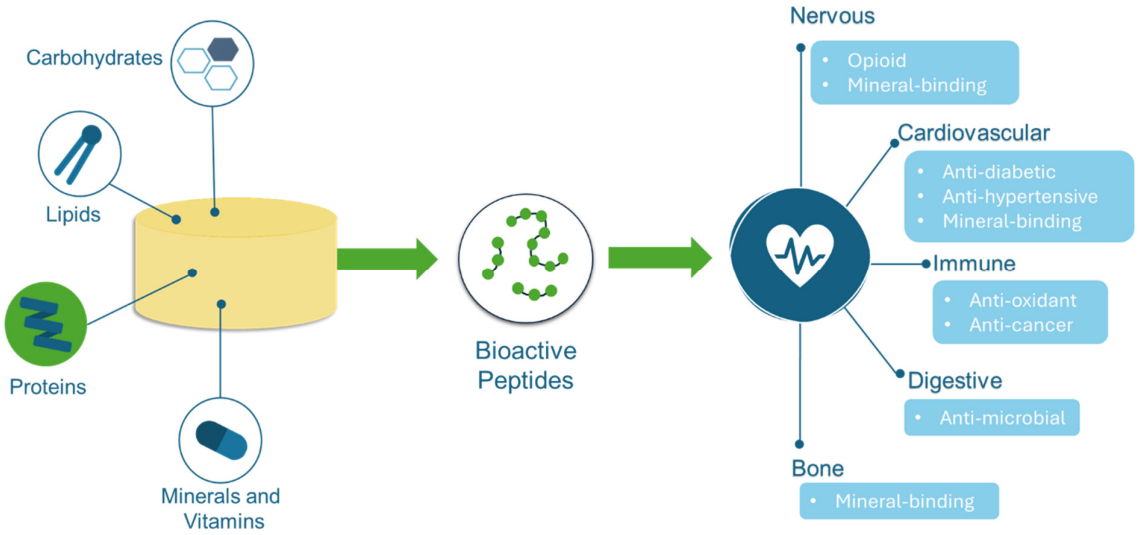

Figure 1. Diagram of cheese matrix and how protein links to bioactivity and health.

As cheese is so widespread, naturally it is the source of many research endeavours exploring how each component works and the impacts they have on human health. Technological advancements, mostly in the mass spectrometry space, have meant that complex systems of biomolecules can be explored with increasing rigor, this means that we can detect new analytes quicker and with more accuracy than before.[4]

Protein omics or 'proteomics' is the large-scale study of protein structure, function and interactions. Proteins can break down into peptides and as the consumption of cheese increases[2] it is natural that the peptide centric subgroup of proteomics – 'peptidomics' has steadily grown in the past 30

years.[5] Peptidomic studies have uncovered that cheese contains a host of peptides many of which are bioactive, meaning they impart a biological effect.

Bioactive peptides have a wide array of beneficial activities that could play a role in promoting human health. Several biological activities have been found that impart potential benefits to various systems such as digestive, cardiovascular, immune, nervous and bone,[6] as displayed in Figure 1. Antihypertensive, antimicrobial and antioxidant are some of the bioactivities currently studied in cheese. Others of note are opioid, mineral-binding, anti-cancer and anti-diabetic.[7]

However, new technology coupled with the breadth of cheese as a source material means that deep understanding of how these bioactive peptides form and function is constantly evolving. Fundamental to this research is the idea that these peptides have bioavailability meaning they can impart an effect after being consumed. While *in vitro* studies indicate bioactivity is retained after digestion, limited studies have investigated whether that activity is imparted in the more complex mammalian system.

Other benefits of deepening our understanding of bioactive peptide profiles are due to the unique fingerprint of cheese brands created by the recipes and techniques used which impart unique peptide profiles. As such peptidomics has been used to fuel Protected Designation of Origin (PDO) claims.[5] Cheese types with PDO status such as Parmigiano Reggiano protects the name from being used for cheese manufactured outside the country of origin. Taking this further, a deeper understanding of how a peptide profile is formed and changes overtime in cheese can guide analysts during critical events such as market complaints or troubleshooting activities during manufacturing.

This review sets out to examine; cheese manufacturing parameters and their impact on the formation and functionality of bioactive peptides, current analytical techniques and their limitations with determination of profiles and quantities contained in cheese, focusing on cheddar.

1.1. Bioactive Peptides in Dairy Products

Bioactive peptides are special sequences of amino acids encrypted within the parent protein that when released, by hydrolysis, activate their bioactivities. It is known that bioactive peptides are found in many dairy products including cheese.[8] They form as part of natural metabolic pathways within these systems, leading to desired organoleptic properties. It is unclear whether these bioactive peptides form out of biological requirements or whether it is coincidental.

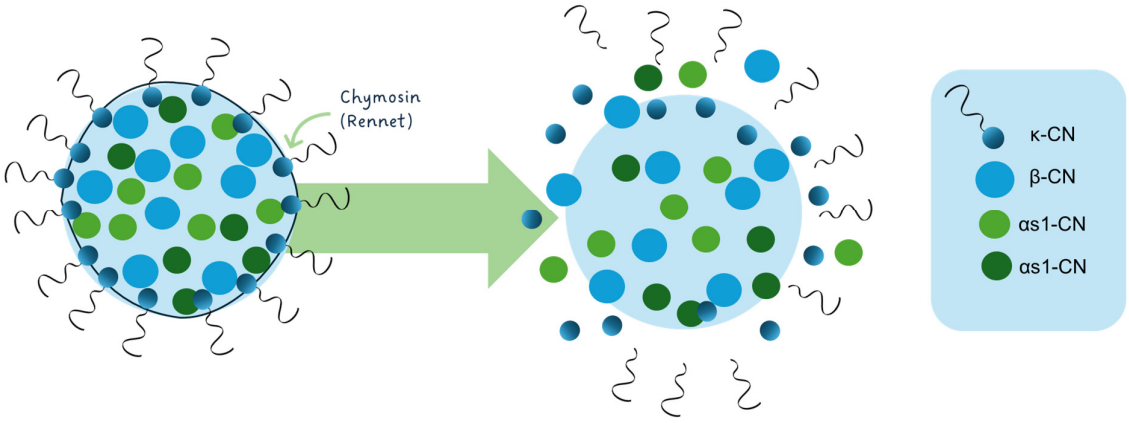

Figure 2. Simple illustration to demonstrate the concept of casein micelle destabilisation through the action of chymosin on the Phe105-Met106 bond of κ-casein.

Bioactive peptides are formed during cheese manufacture and ripening by various enzymatic systems. Milk is transformed into the beginnings of cheese by the action of the enzyme chymosin, also known as rennet, on κ-casein (κ-CN). It acts on the Phe105-Met106 bond hydrolysing κ-casein thus destabilising the casein micelle and freeing the α-caseins (α_{s1}-CN and α_{s2}-CN) and β-casein (β-

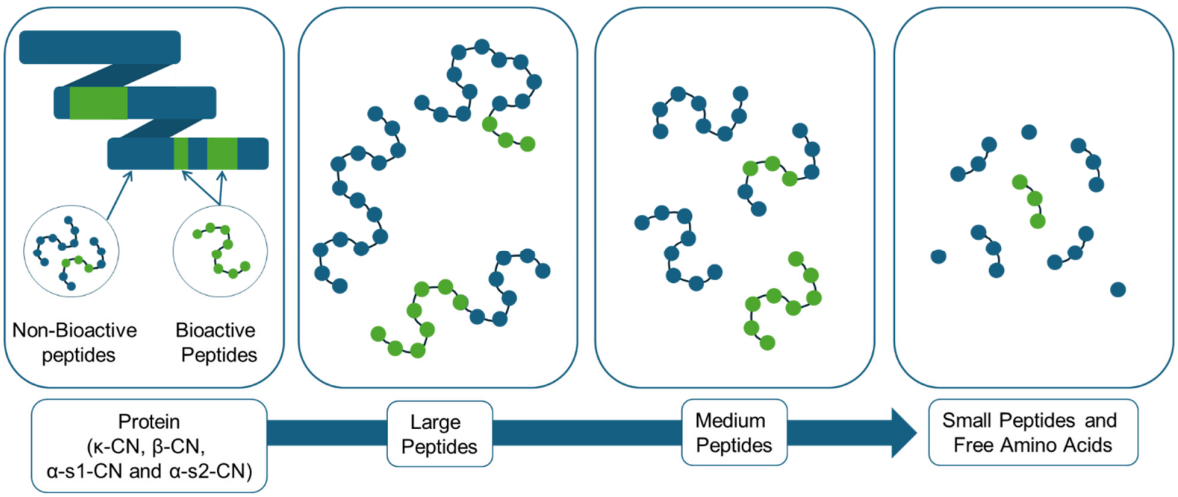

Figure 3. Diagram of casein break-down. Green sections indicate bioactive peptide sequences. Blue sections indicate non-bioactive while blue/green indicates non-bioactive, but a bioactive sequence is still encoded in the larger sequence.

CN) for further enzymatic action, as illustrated in its most basic form in Figure 2. Action of protease enzymes from endogenous and exogenous sources such as Lactic Acid Bacteria (LAB) and Non-Starter Lactic Acid Bacteria (NSLAB) degrades the casein structure further into oligopeptides and, with the help of peptidases, is further degraded into smaller peptides or free amino acids[9] as shown in Figure 3. Along this casein degradation pathway bioactive peptides are released presenting a variety of different bioactive classifications. These include antihypertensive, antimicrobial, antioxidant, opioid, mineral-binding, anti-cancer and anti-diabetic, with antihypertensive, antimicrobial and antioxidant being the most widely studied.

1.2.1. Antihypertensive Bioactivity

Hypertension is one of the main influences on cardiovascular health with investigations into antihypertensive activity being popular. Angiotensin Converting Enzyme inhibition (ACE-I) represents the most studied antihypertensive activity.[7] The ACE metabolic pathway includes the catalysis of Angiotensin I to Angiotensin II which is a vasoconstrictor, or Bradykinin which is a vasodilator to Bradykinin 1-7. Together these act to increase blood pressure which leads to hypertension. Bioactive peptides act by either occupying the active sites inhibiting ACE's ability to act on the metabolic substrates or by chelating the zinc needed for ACE activity. Both of which reduce the enzymes effectiveness leading to reduced hypertension.[7]

1.2.2. Antimicrobial Bioactivity

Pathogenic bacteria such as E. *coli*, B. *cereus* and S. *aureus* are some of the pathogens of interest to the dairy industry due to their prevalence in raw milk.[10] Bioactive peptides with antimicrobial activity can inhibit growth of pathogenic bacteria which may have beneficial applications for packaging conditions and storage time. However, this is a double-edged sword as studies found that desirable bacteria can be inhibited as well.[11]

1.2.3. Antioxidant Bioactivity

Oxidative stress can lead to cell, tissue and DNA damage causing diseases such as cardiovascular, degenerative and cancer.[12] Antioxidant peptides reduce oxidative stress on the body by decreasing or preventing oxidation reactions through mechanisms such as: blocking access to reaction sites thereby inhibiting reactive oxidation species (ROS), binding of metal ions to reduce ROS activity, or scavenging activity which can cause damage to ROS thereby inactivating them.[13]

While bioactive peptides have the potential to positively impact human health, it is important to consider adverse effects when enhancing a product to meet a health claim. While quantifying

bioactive peptides and the cheese consumption required to have an impact; it is wise to be aware of the implications of other compounds that may have an undesirable effect. For example; the peptide β-casomorphin-7 (YPFPGPI) has polarised opinions on its positive or negative impacts on human health.[14, 15] This highlights the importance of *in vivo* studies (as discussed later) and having an understanding of cheese matrices before optimising for certain bio-actives.

1.2. Cheese Manufacturing Parameters

Cheese types vary widely in part due to manufacturing processes as summarised in Figure 4 and can be adjusted to get the desired effect on the end cheese. Decisions made including timing and temperature during the coagulation, cutting, cooking, whey drainage, salt addition, cheddaring, milling, pressing and ripening steps can all be adjusted to meet the final compositional targets. Some of these parameters have known impacts on bioactive peptide production such as salt and temperature of cooking or ripening.[16, 17]

1.2.1. Milk Composition

Dairy is a great source of proteins, fats and minerals and these play an important role in the cheese making process. Milk is standardised prior to curd formation to ensure the right ratio of these components, namely fat and protein. This mostly depends on the cheese variety. Protein is necessary as a structural matrix for cheese, it is the "sponge" that holds together other key components such as fat and moisture and is what ultimately gives cheese its characteristic texture.

Milk quality can vary depending on species used and whether the milk is raw or pasteurised. Cow, sheep, goat, camel and buffalo are similar but vary in protein content, protein sequence and indigenous microbial communities. This coupled with whether it is raw or pasteurised milk being turned into cheese can vastly impact the bioactive peptide profile.[18, 19] This means that the selection of milk is an important factor in bioactive peptide formation.

1.2.2. Cheddar Cheese Process

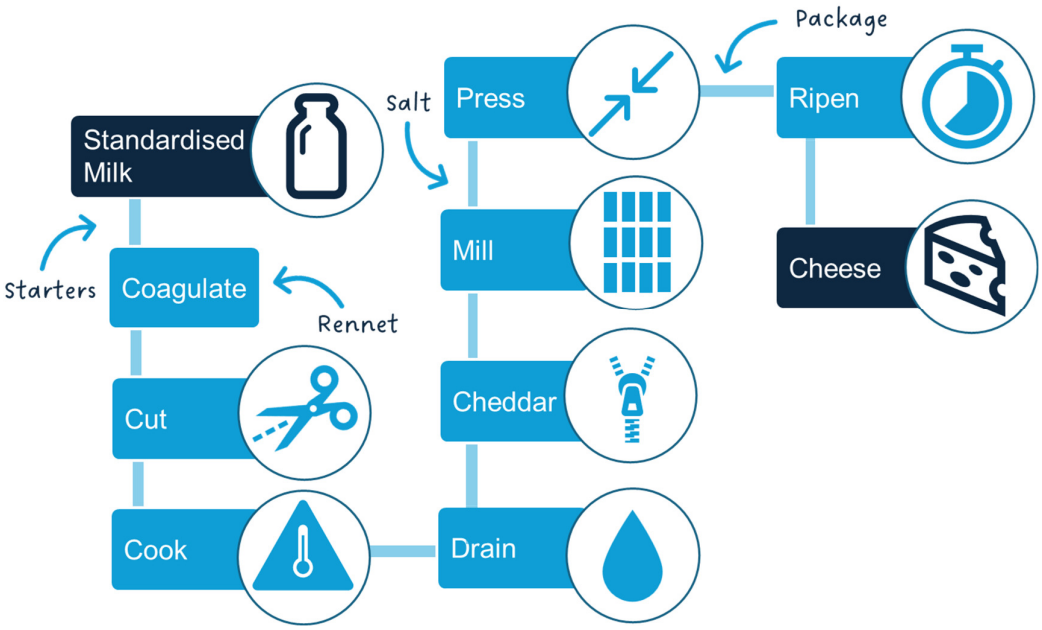

Figure 4. Cheese making process.

Cheddar is a semi-hard matured cheese originating in Cheddar, Somerset, England. Food and Agricultural Organisation standards (Codex) for cheddar requires it to be of white through to orange colouration and can be ripened between 7-15 °C. Other ripening conditions and usage of adjuncts is acceptable provided they do not impair the physical, biochemical and sensory properties. Cheddar should be made from cow or buffalo milk and have a fat in dry matter (FDM) content of 48-60 % however it is permissible to go as low as 22% but must be labelled low-fat.[20]

The process by which cheese is formed is coagulation of standardised milk by acid or enzyme activity, namely rennet, followed by manipulation of the resultant curd into blocks and finally some form of ripening.

The cheddar cheese process, shown in Figure 4, involves standardising milk to correct fat and protein ratios, addition of starter (bacterial cultures), coagulation by means of rennet, cutting of the coagulum to separate curd and whey, cooking to remove moisture from the curd and allow lactic acid development, draining of whey, cheddaring process by which the curd is piled into sections and allowed to knit together becoming smooth and elastic, milling to form even curd "chips", salting, pressing into blocks, packing and ripening at desired temperature.

During this process pH is monitored. Milk typically has a pH of ~6.7 while finished cheese may have a pH of 5.6. This drop in pH is due to the metabolism of lactose present by the active microbial communities. Lactose is broken down to produce galactose and glucose which are then transformed into lactic acid.[21, 22]

1.2.3. Final Cheese Composition

Manufacturing processes are the driving forces of cheese formation, pushing cheese curd towards a singular point – the final cheese composition. Guided by recipes, Codex standards and the need for continuity in commercial application; final cheese composition determines flavour and aroma in the mature cheese which, amongst other compounds, peptides contribute to. Salt, moisture and pH are all critical in determining the mature cheese by creating the environment to benefit and grow or inhibit and slow the bacteria present.

Salt is of interest in studies involving bioactive peptides due to its contribution to health and disease. Salt concentration in cheese is affected by temperature, method of addition, curd size and pH. It is important for bacterial growth, as bacteria can be salt sensitive such as *Lactococcus cremoris* which cannot grow in a 4% salt in moisture ratio (S:M) environment while *Lactococcus lactis* can.[23] Studies tend to agree that there is a difference between low salt and regular salt versions of the same cheese[24, 25] with Ramírez-Rivas *et al.* finding that salt content had different implications for ACE-I and antioxidant peptide formation.[25] It is interesting to note that the type of salt used (sodium or potassium) has little impact on bioactive peptides[26] which is helpful in designing a cheese rich in bio-actives that can avoid the negative impact of traditional salt. Moisture is also necessary for bacterial growth and influences cheese texture, the final moisture value of the cheese is affected by curd size, cooking temperature and time as well as salt. The S:M ratio impacts pH by determining the rate of lactose fermentation and is often used by cheese makers to determine ripening.[23] pH is crucial for controlling enzyme activity, salt absorption and bacterial activity. [27]

1.2.4. Ripening

Cheese ripening is the time when proteolysis, alongside other processes, matures the cheese. Proteins along with fats and sugars are broken down by bacterial action to produce the flavours and textures characteristic of the cheese variety. In cheddar, ripening conditions, namely time and temperature, control flavour development from mild through to aged. These conditions have been thoroughly investigated with many studies finding bioactive peptides are released overtime.[28-30] These bioactive peptides fluctuate in presence and concentration as the sequences of amino acids are broken down by the microbial action of LABs and NSLABs bacteria as well as the residual milk enzymes and rennet.[29]

1.3. Starters, Adjuncts and Probiotics:

Starter cultures, also referred to as primary starters, are a cohort of complementary lactic acid bacteria fundamental to the formation of the cheese matrix through lactic acid production. LABs metabolise lactose present in the milk into galactose and glucose which is then further converted into lactic acid.[31] This causes a decrease in pH which is crucial to curd formation and cheese ripening. The decrease in pH, when carefully controlled, expels moisture and aids rennet in the destabilisation of the casein micelle creating the desired curd texture.

There are two types: mesophilic and thermophilic whose optimum temperatures for growth and performance are ~30 °C and ~45 °C respectively.[32] In cheddar manufacturing mesophilic cultures are often preferred but additional thermophilic cultures have also been used. [33]

Choice of cultures is recipe dependent and can range from a single bacterial species to specialty blends of different species.[21] This choice is dependent on the cheesemaker's preference of sensory outcomes and combating phage - a virus which decreases the potency of starters. In cheddar manufacturing mesophilic cultures are preferred due to the lower cooking temperatures. *L. lactis* and *L. cremoris* are two commonly used LABs used for cheddar production.[12, 21, 34]

Additional bacteria can be added to enhance the cheese in different ways. Adjunct cultures, also referred to as secondary starters, are often added to enhance the flavour, texture and or ripening processes in cheese. Secondary starters could be the moulds added for Camembert or blue type cheese or propionic bacteria added to produce the characteristic holes in Swiss and other cheeses.[35] *Lactobacillus helveticus* is a notably common adjunct for cheddar cheese to add complexity to the flavour profile.[33]

Lastly probiotics, more common in fermented drinks or yoghurt, can be added to cheese and impart specific health benefits depending on the strain added. FAO/WHO describes probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host".[36] In a cheese matrix this has the caveat that the bacteria need to be alive and in adequate amounts which may not always be the case meaning a probiotic claim is not possible even though a probiotic strain has been included in the manufacturing process. However, if the probiotics mechanism of promoting health is through the formation of peptides which are then absorbed by the host, then the conferred benefit doesn't necessarily rely on the probiotic remaining alive as the enzymes responsible for proteolysis may remain active on cell death.[37]

Lactobacillus acidophilus and *Bifidobacterium bifidum* have been proven beneficial for gut health, namely the management of microflora through inhibition of pathogenic bacteria[38, 39] and prevention of cardiovascular disease by reducing cholesterol.[40, 41]

Probiotics are notable for their support in digestive aid, gut health and immune systems. Some recently studied probiotics in the bioactive space are *Lactocaseibacillus casei*, *L. acidophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactiplantibacillus plantarum*, *L. helveticus* and *Lactobacillus rhamnosus* as shown in Table 1.

Cell viability is an important consideration for the application of cheese and desired effects created. Different bacterial strains grow at different rates. Some are fast growers and may be beneficial for imparting acid development crucial to cheese manufacture such as *L. helveticus*.[23] Fast growers and those more proteolytic may also benefit other cultures growing in tandem by providing the amino acid substrates needed for their growth and development.[42] Slow growing LABs such as *L. plantarum*[43] may be beneficial to impart a delayed effect or to outgrow the initial LABs when they die off over the first initial ripening period such as the case with NSLABs.[23] Overall bacterial cell viability is an important factor when considering probiotic claims to ensure that cheese has the correct number of Colony Forming Units (CFUs) at the point of sale.[12]

Ultimately final sensory characteristics are attributed to primary and secondary starter bacteria added to the cheese making process.

1.3.1. Starter Cultures and Their Uses

The importance of starter bacteria and their contribution to the finished product is emphasised by the secrecy surrounding their application. Cheesemakers and commercial starter manufacturers often conceal the specific strains used as intellectual property ensuring the safeguarding of their cheese recipes. Some current applications of starter bacteria as studied in recent years are displayed in Table 1.

Lactococcus lactis and *L. cremoris* are commonly used in tandem for most cheese applications due to their complimentary flavour and acid formation properties. *L. cremoris* is slow growing but creates a nicely flavoured cheese. To enhance this, *L. lactis* is added as it produces lactic acid quickly to help reduce manufacturing times.[21]

Lactobacillus helveticus is known for its proteolytic capability and is an excellent acid producer as such it has been included in cheddar manufacturing to improve flavour and reduce bitterness.[23, 44] When balanced with other cultures it has been described as producing a cheese with "sweet and nutty type flavours".[45]

Lactobacillus delbrueckii subsp. bulgaricus, a thermophilic starter, is commonly utilised in yoghurt manufacture but is useful in cheese manufacturing when high temperatures are needed. Some have utilised this starter as its low proteolytic activity imparts nice aroma and it has shown improvement to pizza cheese properties such as melt and Maillard browning due its production of exopolysaccharide (EPS).[46]

There are no general rules for which starter bacteria should be used for which cheese. However, there are some exceptions to that regarding PDO cheeses where there are controls around the use of starter bacteria, such as Parmigiano Reggiano cheese where addition of starter cultures is prohibited. Instead, this cheese relies on the use of raw milk and mixing partially skimmed milk from the previous day.[47] Codex standards for cheddar indicate the prohibited use of starter strains that produce CO_2 which would lead to eye formation in the cheese.[20]

Table 1. Characterisation of starter bacteria of recent interest in Cheddar and adjacent cheese studies.

Bacteria	Type	Potential Health Claims*	Properties	Cheese application	Bioactivities	Study
L. lactis & *L. cremoris*	mesophilic	-	Medium acid production / pleasant aroma.	Basic starter for most cheese varieties	ACE-I Antioxidant	-
L. rhamnosus	mesophilic	Digestive/Gut Health		Cheddar cheese	ACE-I antioxidant	Hao *et al.* 2023 Hao *et al.* 2021 Liu *et al* 2018
L. plantarum	mesophilic	Cholesterol reducing. Anti-inflammatory	Competitive inhibition / flavour compounds / EPS texture	1. White-brined goat-milk cheese 2. Cheddar cheese (low fat) 3. Cheddar	ACE-I Antioxidant ACE-I Anti-tumor	Kocak *et al.* 2020 Wang *et al.* 2019 Sahingil *et al.* 2024
L. casei	mesophilic	Gut Health / Anti Cancer / Brain Function.		1. White-brined goat-milk cheese	ACE-I Antioxidant	Kocak *et al.* 2020 Hao *et al.* 2023

				2. Cheddar cheese		Hao *et al.* 2021
L. helveticus	thermophilic	Gut Health / Decrease Blood Pressure / Calcium metabolism / Anxiety Reduction	Medium-fast acid production Bitter peptides	1. Cheddar cheese 2. Prato cheese 3. Parmesan 4. Emmental 5. Swiss 6. Mozzarella [23]	ACE-I Antioxidant	Hao *et al.* 2023 Hao *et al.* 2021 Yang *et al.* 2021 Baptista *et al.* 2018 Sahingil *et al.* 2024
L. acidophilus	thermophilic	Gut Health / Reduce Cholesterol	Medium acid production	Cheddar cheese	Antioxidant	Rehman *et al.* 2019
L. bulgaricus	thermophilic	-	Produces peroxide which inhibits pseudomonads [21] Acetaldehyde [23]	1. White-brined goat-milk cheese 2. Cheddar	ACE-I Antioxidant	Kocak *et al.* 2020 Sahingil *et al.* 2024
B. bifidum	thermophilic	-		Cheddar cheese	Antioxidant	Rehman *et al.* 2019

* Health Claims are strain dependent. Specific health claims may be related to a specific strain only.

1.3.2. Bioactive Appeal

While there is a good understanding of the adjuncts, we can use to leverage beneficial flavours and ripening qualities, less is understood about their deeper impact on the cheese matrix. Many studies have been undertaken to uncover the effects that these LABs have on bioactive peptide development. Studies show the inclusion of secondary starters improves the bioactive peptide profile both increasing diversity[48] and boosting bioactive performance.[16]

For a LAB to have acceptable growth and thus have acceptable proteolytic impact the bacteria must have several enzymatic systems to function. A proteinase system to hydrolyse casein into large peptide fragments. They then need a peptidase system to further hydrolyse these into smaller peptides and/or free amino acids. Lastly, they must have a transport and intracellular peptidase system which allows for uptake and hydrolysis of small peptides or free amino acids.[32] This last

system is important as it is the intracellular peptidase that is released upon cell lysis creating a greater impact on proteolysis.[49]

1.3.3. Strain vs Strain: Proteolytic Capability

Bacterial species are complex and unique, and within a species the various strains can differ dramatically in terms of genetic variation. Thus, their proteolytic capability can have a great impact on cheese manufacture in terms of sensory and functional outputs. Additionally, this has impact on the peptides produced by each strain within the same bacterial species due to the variances in enzymes available.

Lactobacillus helveticus is known to have 1-4 different peptidases. Recent studies have included strains 1.0612, ATCC 15009, DPC 4574 and LH-B02 and have shown variations in proteolytic capacity. A recent study by Sahingil *et al* included two strains of *L. helveticus* alongside other bacterial species and investigated bioactive peptide formation and activity. They found differences in how much residual β-CN and α_{s1}-CN was left after 120 days ripening. At 8 °C there appeared to be differences with strain ATCC 15009 having greater proteolytic activity on β-CN and to a lesser extent α_{s1}-CN. However, this difference in proteolytic activity was reduced when the ripening temperature was doubled.[16] Although 16 °C is on the higher end of ripening temperature for cheddar (7-11 °C is more standard).[50] This could indicate that accelerated ripening temperatures could create comparable proteolytic capability regardless of strain. Sahingil *et al* does not differentiate peptides formed at the various temperatures and therefore the different strains could potentially form differing peptides due to the enzymes available to them.

1.3.4. Angiotensin Converting Enzyme-Inhibitory Activity

Antihypertensive properties, specifically the ACE-Inhibitory ability of peptides has been evaluated in various cheese varieties. Starter bacteria have been of interest in the formation of ACE-I peptides as most LABs are known to produce this type of peptide in varying quantities.[9]

Lactobacillus helveticus has been proven to produce water soluble extracts abundant with ACE-I peptides. Several studies have investigated these bacteria, with comparisons of a variety of strains. *Lactobacillus helveticus* strain 1.0612 was utilised in two studies comparing against *L. rhamnosus* and *L. casei*. *In vitro* digestion of cheddar and molecular docking techniques were used to elucidate bioavailability and structural mechanisms of key peptides respectively.[34, 48] These studies showed that *L. helveticus* produces ACE-I peptides and that digestive processes only increased the ACE-I activity with *L. helveticus* having the highest ACE-I activity.

Lactobacillus helveticus' strong performance in the ACE-I space is further backed by Baptista *et al.* whose study investigated *L. helveticus* strain LH-B02 and how it performed in Prato cheese.[51]

Despite the strain difference to that used by Hao *et al.*[34], this adjunct still improved the ACE-I activity during the 120-day ripening time compared to using *L. cremoris* and *L.* lactis. LH-B02 was also investigated by Abd El-Fattah *et al.* in a larger commercial starter study, although this study investigated performance in a fermented milk system it still indicates that *L. helveticus* is a strong ACE-I peptide generator with the highest inhibitory activity at 76 %. This study also showed that a mix of *L. cremoris* and *L. lactis* had the third highest ACE-I activity of 14 different starter strains and/or blends at an inhibitory activity of 51%.[9] This is intriguing as most studies have found that the addition of adjunct cultures have performed better than *L. cremoris* and *L. lactis* alone.

Another two strains of *L. helveticus* (ATCC15009 and DPC4571) were used in a study that compared them to *L. casei*, *L. bulgaricus* and *L. plantarum* in a cheddar-style model cheese. While this study found that the adjunct choice had less significance to bioactivity than ripening time or temperature, it found that both strains performed well in increasing ACE-I activity alongside *L. plantarum* and *L. casei*.[16] This contrasts other studies where *L. helveticus* performed notably better than other adjuncts.[9, 48] Sahingil *et al.* found that at 8 °C both *L. helveticus* strains fluctuated in highest activity with *L. plantarum* over the 90 days. However, at 16 °C *L. casei* and *L. plantarum* had the highest performance. This could be attributed to variations in strains used. Interestingly the study by Sahingil *et al.* may indicate that *L. helveticus* could produce larger ACE-I peptides of proficient activity. Both strains appeared to be higher in ACE-I activity in the 3-10 kDa fraction, although few studies appear to test all their molecular weight fractions, so it is unclear whether this is a unique trait of *L. helveticus*.

1.3.5. Antioxidant Activity

Antioxidant properties are another bioactivity that has been studied recently. With cheddar, cheddar-style model cheese and fermented milk matrices being the subject of investigation. Antioxidant activity is the peptides' ability to reduce oxidative stress. Several methods are employed to establish this activity with some peptides performing better under certain assays.

Sahingil *et al.* found that similar to ACE-I peptides, antioxidant ability was greatest in the <3 kDa size range with the functionality of these peptides being 3-4 times greater than the activity of the other fractions tested.[16] Other studies seem to conclude likewise with Yang *et al.* finding the <3 kDa fractions being highest for antioxidant activity across scavenging assays using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) - both assays where a spectrophotometric change indicates level of antioxidant activity.[12] Sahingil *et al.* found that at 8 °C *L. helveticus* and *L. plantarum* had the highest ABTS scavenging across the 90 days, while at 16 °C *L. bulgaricus* and the control *L. cremoris*/*L. lactis* had the highest.[16] Other studies

have similar findings with Wang *et al.* who found that *L. plantarum* performed better across three different scavenging assays, including ABTS scavenging, than *L. lactis* in a low-fat cheddar cheese.[43] Yang *et al.* also found that *L. helveticus* performed better across two scavenging assays, also including the ABTS assay, compared to *L. cremoris* and *L. lactis* in cheddar cheese.[12] Variances in performance of *L. cremoris*/L. *lactis* compared to adjunct cultures are most likely due to the differences in temperature which Sahingil *et al.* indicates as the most significant decider of bioactive peptide formation.

Abd El-Fattah *et al.* utilised three unique assays to assess various mechanisms of antioxidant activity: DPPH scavenging, reducing power (whereby potassium ferricyanide was reduced and the addition of ferric chloride resulted in a coloured ferric-ferrocyanide complex) and Fe^{2+} chelation. These assays demonstrated that different starters have different prevalences for creating peptides that use different mechanisms to impart antioxidant effects. *Lactocaseibacillus casei* and a mixture containing *L. acidophilus* had the highest DPPH scavenging capacity. *Lactocaseibacillus casei* alongside *L. helveticus* and a mixture containing *L. rhamnosus* were the poorest performers in the reducing power assay while a mixture of *L. lactis* and *L. cremoris* had the best performance. Finally, *L. casei* followed by *L. rhamnosus* (in a mixture) and *L. acidophilus* chelate Fe^{2+} well with *L. helveticus* performing significantly worse.[9]

Liu *et al.* also looked at reducing power in cheddar cheese comparing *L. rhamnosus* to L. *cremoris* and *L. lactis* and contrarily to Abd El-Fattah *et al.* found that *L. rhamnosus* was significantly higher in reducing power pre and post digestion.[52] This could simply be that the fermented milk matrix used by Abd El-Fattah *et al.* isn't comparable to cheese or that the full cohort of milk proteins present are of benefit to *L. cremoris* and *L. lactis*.

This demonstrates that while *L. cremoris* and *L. lactis* may produce antioxidant peptides the inclusion of adjunct cultures in cheese manufacture improves the amount and mechanisms of activity.

1.4. Analysis of Bioactive Peptide Populations

The study of peptides has been growing over the last 30 years with an increasing number of papers being published. Advances in High-Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS) has led to increased exploration of the vast profile of bioactive peptides present in various matrices. There are many challenges in this space such as getting the full peptidomic profile and analysing the formidable amounts of mass spectral data to accurately determine an unambiguous peptide sequence.

1.4.1. Peptide Extraction

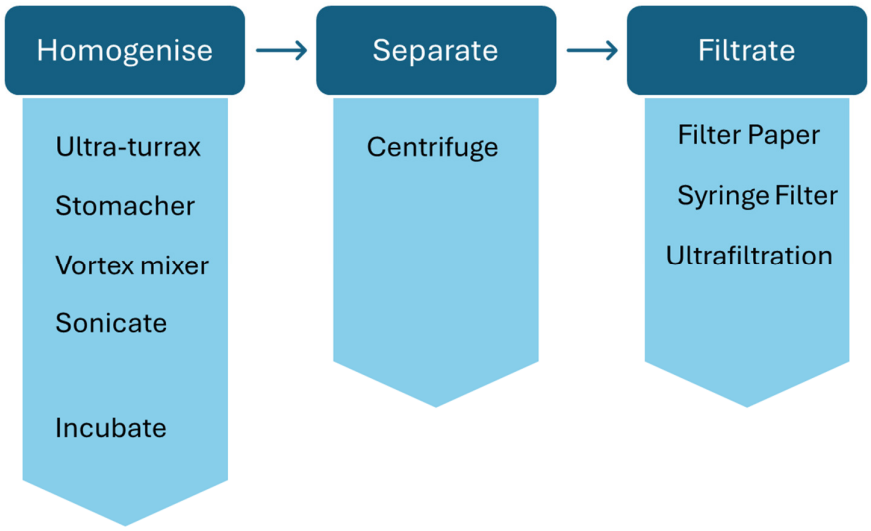

Figure 5. Peptide extraction process flow including commonly used equipment.

Before peptides can be analysed, they must first be extracted from the sample matrix. The most common extraction techniques used for cheese peptide analysis is water soluble extraction which employs homogenising, separating soluble and insoluble material followed by filtration to further remove insoluble material as shown in Figure 5. However, there is no standard methodology for peptide extraction with many modifications on the above process. The amount of cheese used varies from as little as 5 g[53, 54] up to 100 g[30] with the ratio of cheese to diluent varying as well. It is preferable to grate the cheese portion regardless of amount taken due to improvements in sample homogeneity and increased surface area for extraction. Most methods commonly use water as the diluent. However some use a dilute acid to improve the identification of peptides downstream.[53, 54] The homogenisation step has the most variation with a range of instruments used such as dispersal homogenisers (i.e. Ultra-Turrax), sonicator, water baths (with or without shaking functionality) or a combination of these. This step is crucial to extract water soluble peptides into the aqueous layer. Still, it may miss peptide fractions that are not as readily water soluble which can become trapped in the insoluble layers. The length of the homogenisation step also varies with some being completed in under 10 minutes[53, 54] with the use of an Ultra-Turrax while most take 60 minutes and employ incubating in a water bath alongside an initial homogenisation step. Samples are commonly filtered to remove larger particulates, but extra processes have been used to further filter or remove extraneous compounds. Ultrafitration[12, 29, 54] is used to remove any soluble proteins remaining and to fractionate the sample for enrichment of peptides. Use of Folch extraction and solid phase extraction (SPE), while less commonly applied, can be utilised to also remove proteins and concentrate peptides of interest.[5]

1.4.2. High Performance Liquid Chromatography

In proteomics, complex matrices: cells, tissues, biofluids and in this case, cheese typically have their components chromatographically separated before analysis by mass spectrometer. However not all peptides are alike and perform differently under different chromatographic conditions. Reverse

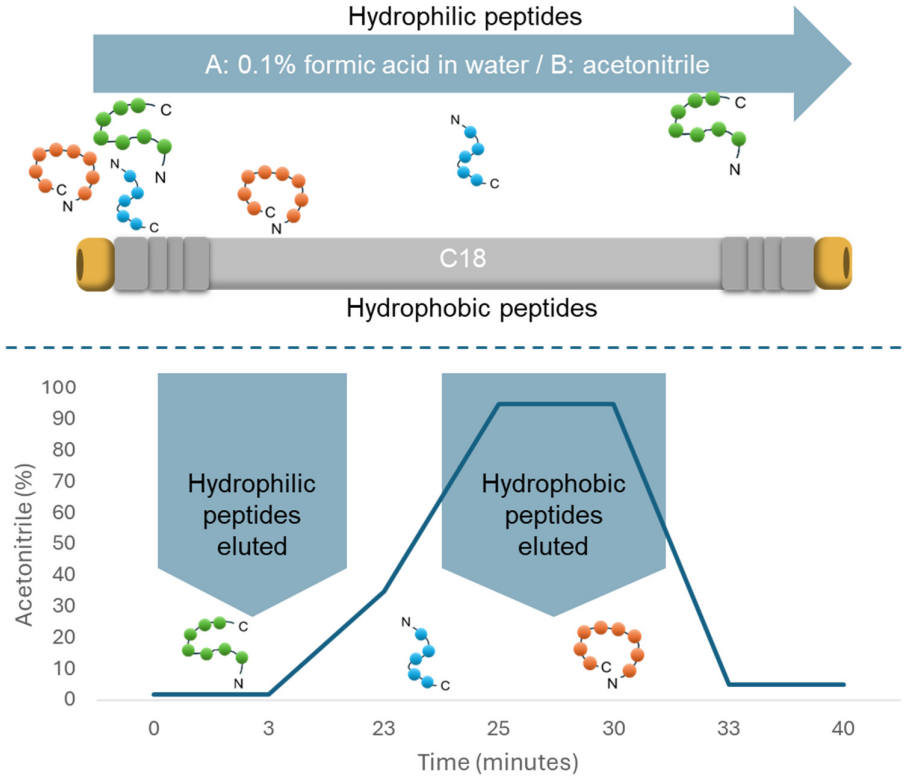

Figure 6. diagram of how RP-HPLC resolves peptides through hydrophobic interactions.

phase HPLC (RP-HPLC) is typically utilised for peptides using C18 stationary phase and gradients of an aqueous mobile phase (0.1% formic acid or trifluoracetic acid in water), and an organic solvent (acetonitrile with or without an additive) used to resolve peptides from one another.[53, 55, 56] This relies on the peptide analytes hydrophobic interactions with the stationary and mobile phases. As shown in Figure 6 hydrophobic peptides, those with more non-polar amino acid residues, interact strongly with the C18 stationary phase while hydrophilic peptides will elute earlier. The increasing acetonitrile over the gradient disrupts the hydrophobic interaction of the peptides retained on the column, allowing them to elute.

This assumes that most peptides resolve and behave as expected under these chromatographic conditions. Some studies have tested the theory that standard RP-HPLC inevitably misses some peptide fractions. Montone *et al.* compared bioactive peptide recoveries of a C18 column and porous graphitic carbon (PGC) column. [57] They used a grand average of hydropathy (GRAVY)

score, which gives an indication to a peptides hydropathy based on amino acid residues present. A peptide can be interpreted as hydrophobic if the GRAVY score is >0 and hydrophilic if <0.[58] This study found an increase of hydrophilic peptides from 12% in C18 to 25% in PGC when the GRAVY score was -1<. PGC also showed a reduction in hydrophobic peptide retention. With respects to bioactive peptides identified; the use of a C18 column resolved 57 peptides while using a PGC column resolved 41, 26 of which were common to each column chemistry.[57] This shows that C18 is good general column for peptidomics but there is a benefit to including different separative chemistries if a more complete peptide profile is desired.

Another approach by Akamatsu *et al.* was utilising Hydrophilic Interaction Liquid Chromatography (HILIC), the inverse of RP-HPLC, where analytes are separated on hydrophilicity with a polar stationary phase and highly organic mobile phase. HILIC proved to be more efficient at peptide recovery compared to C18 at lower concentrations and even at higher concentrations HILIC was still able to identify unique peptides that C18 missed.[59] Again, this highlights the benefit of employing different techniques.

Some studies solely rely on using HPLC to determine peptide profiles and any changes therein. UV determination at 215 nm gives a general comparison of proteolysis between samples and has been used by some to resolve changes in different soluble fractions such as ethanol and pH 4.6.[16] Sahingil *et al.* utilised the chromatogram differences in statistical modelling to better understand changes in peptide profile as effected by bacteria, temperature and ripening time.

Rafiq *et al.* also used HPLC to compare different cheeses over time and found that cheddar cheese made with cow milk cheddar had a greater number of peaks but that buffalo milk cheddar cheese had a higher intensities indicating higher concentration, this was attributed to the higher protein content in buffalo milk.[60]

While HPLC gives good insights into how the different cheeses compare proteolytically to one another it doesn't give specific information on peptide changes or potential bioactivities.

1.4.3. Mass Spectrometry

Mass spectrometry was first developed in the early 1900s but it wasn't until post 1940s that the application of MS became common place.[61]

The process by which a sample is measured in MS, is shown in Figure 7. It starts with the introduction of a sample to the MS either by direct infusion or by coupling a gas chromatography (GC) or liquid chromatography (LC) unit. The sample is then ionised whereby the analytes, peptides in this case, gain a charge. The ions are then filtered by the mass analyser based on selected

ranges. These are then detected and output as a total ion chromatogram (TIC) of the mass to charge ratio (m/z) detected. This data can then be further processed for peptide sequence by software such as Proteome Discoverer[62] or PEAKS[63] or alternatively, manual *de novo* sequencing[64, 65] of data can be done but this is generally considered time consuming and relies on key knowledge of peptide fragmentation rules.

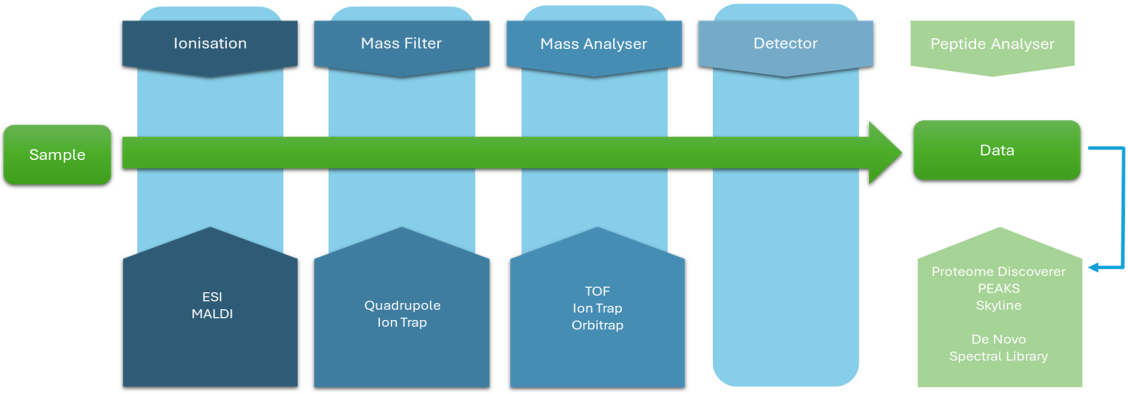

Figure 7. Process flow of peptide analysis through Mass Spectrometry displaying different ionisation techniques, mass Analyser and peptide analysers.

Two common ionisation methods utilised in peptidomics are Matrix Assisted Laser Desorption Ionisation (MALDI) and Electrospray Ionisation (ESI) each has its benefits and disadvantages.

ESI uses an electric field to charge small droplets of sample and is the preferred technique in many peptidomic studies, having the benefit of flexibility with coupled instruments and is found to be highly sensitive especially with nanospray ESI.[66] However, it is susceptible to the presence of salts and/or detergents and therefore sample clean-up is important.[67]

MALDI relies on mixing the sample with a matrix solution (commonly 2,5-dihydroxynenzoic acid, α-cyano-4-hydroxy-trans-cinnamic acid, or sinapinic acid[68]) then a laser is used to generate ions. While MALDI is a technique used for peptidomic research it is less favoured due to its lower sensitivity and incompatibility with other instruments.[67]

Like column chemistries, the ionisation technique influences the peptide sequences detected. Nadler *et al.* studied the impact that ESI and MALDI had on peptide identification and found that ESI accounted for 42 % of unique peptides in a sample while MALDI only found 19 % of unique peptides, the rest being peptides common to both techniques.[66] This shows that ESI has general merit for being able to identify a more complete picture of the peptides present however Nadler *et al.* uses this to demonstrate that the techniques can be complementary to one another. This difference between ESI and MALDI was also highlighted by Caira *et al.* who found that ESI did not provide identical results to MALDI when investigating casein phosphopeptides due to ESI's greater sensitivity coupled with the unexpected ionisation of some non-phosphorylated peptides.[69]

Once ionised, peptides are introduced to the mass analyser which determines the mass to charge ratio (m/z) of the intact peptide this is referred to as MS1. While this information is useful several peptides may have the same mass to charge ratio therefore understanding more about the make-up of the peptide is important to be able to differentiate these. This is where the MS1 peptide is sent for further fragmentation generating MS2 data which contains several fragment ions known as 'b' and 'y' ions. These can be used to elucidate the sequence structure of the intact peptide seen in the MS1 scan. There are many forms of mass analysers that can be implemented such as Time of Flight (TOF) and Orbitrap are two that are most prevalent in peptidomic studies, Ion Trap may also be used as an older model of mass analyser.[67]

The fragmentation of peptides can be completed using either data dependant acquisition (DDA) or data independent acquisition (DIA). DDA is currently most common for peptidomics and is where the instrument sends the most prevalent parent ions (MS1 ions) for fragmentation which tends to overlook the less abundant ions. DIA can overcome this challenge in data acquisition by fragmenting everything. However, accurately deconvoluting the resultant chimeric fragmentation patterns remain a challenge.

A study by Lu *et al.* utilised three different configurations of MS to analyse their peptide molecular weight fractions. Using both nanoESI-MS/MS and ESI-TOF-MS for peptide identification to balance the limitations both instruments had for detection limits. Finally they used a quadrupole ion trap MS/MS for quantification of selected peptide sequences.[29] Other studies tend to stick to a singular MS configuration for analysis however there are limited studies that seek to quantify the peptides in which case the differing requirements for targeted vs untargeted analysis are not of importance.

1.4.4. Quantitative vs Qualitative

There are two steps to investigating a peptidome. The qualitative approach which is simply seeing what bioactive peptides are present – the peptide profile, and the quantitative approach where the absolute concentrations of the peptides present are understood. Both are important to establish bioactivities present and potency for downstream *in vivo* application. Some of the limitations of a qualitative approach have been stated but quantitation has its own challenges.

The most prevalent way in which studies quantify peptides is by creating a calibration curve of the desired peptide. This approach can use isotopic or non-isotopic peptides as standards. Using an isotopic peptide can allow for the standard to be spiked into the sample matrix as an internal standard thereby minimising any matrix effect issues, such as the comparability of signal resolution which could under or overestimate a peptide.[70]

While isotopic peptides are considered a gold standard due to their flexibility in application, they are expensive. To get around this, non-isotopic peptides can work just as well. However, they too can become costly when one considers the range of peptides in a peptidome. Therefore, it is worth considering which peptides are likely to be of most value to quantify. For example peptides VLNENLLR and IKHQLPQE have shown to be very inhibitory against *E. coli* compared to SDIPNPIGSENSEK which has little to no inhibitory capability.[71] Therefore, knowing the peptides of interest for downstream application can save time and money when quantifying. The downside to this method is that it can be influenced by variations in the MS instrument in which case building a robust label-free quantification method is prudent.

1.4.5. Post MS Peptide Analysis

Peptidomic studies generate an overwhelming amount of data, specifically the mass spectral fragmentation patterns for all the peptides. The next challenge is to translate that data into peptide sequences. This can be achieved manually by following the rules of *de novo* sequencing and arduously going through each fragmentation pattern.[64] Today this is sped up by processing software like Proteome Discoverer[62], PEAKS[63] or Skyline.[72] However, they rely on quality data input to ensure quality data output. This relies on the quality of the fragmentation patterns gathered by the MS. The problem is that despite having a perfect MS set up, some peptides produce insufficient fragment ions which leads to ambiguous identities and often null identification. This is more common with short peptides but can still occur with longer peptides.[73]

1.5. Challenges and Knowledge Gaps

The study of bioactive peptides is vast with literature search engines, such as SCOPUS and PubMed, indicating in the past 10 years there have been ~23,000 papers published. Studies on cheese accounts for just ~1 %; considering the breadth of cheese varieties and milk origin, minimising those papers down to ones related to cheddar from bovine origin - the number of studies diminishes to merely 10 papers. This demonstrates there are gaps in knowledge around bioactive peptide formation and more specifically the mechanism of action for those bioactive peptides in cheese, especially over the different varieties of cheese.

Clinical studies are usually regarded as the best model when investigating compounds that are intended to benefit health. While there have been some *in vitro* studies on cheddar cheese[34, 52, 74] to interpret how bioactive peptides might behave in a human model, there have been fewer *in vivo* studies with one study on mice[75] and to the authors knowledge no clinical trials with cheddar cheese have been conducted in the past 10 years. The issue with having limited studies using animal models at the very least or clinical trials is that we cannot establish the impact these bioactive

peptides may have on a human host. The peptides with the strongest bioactivity in theory are ultimately worthless if they cannot impart that benefit due to digestive hydrolysis.

Adjacent to the limited *in vivo* model studies is the limited quantification studies. Some studies display only the relative bioactivities of different fractions – without further peptide identification[30, 43, 52]. Others elucidate the bioactive peptide sequences[12, 28, 34] and some even go further to determine *in silico* structural mechanisms of the peptides and how they may function[48, 76]. However, there are limited studies, especially for cheddar cheese, that determine the quantity of any peptide.[29] These quantification studies are important because without knowing the quantity of bioactive peptides it can be hard to determine impactful dosage rates for the downstream clinical trials.

It is also of value to know how manufacturing variability can impact the bioactive peptides. And whether it is possible to consistently create the quantity of a particular bioactive peptide/group of peptides. Lu *et al.* discovered that comparing two brands of the same style of cheese showed distinct variations in the peptides found and their quantities.[29] It would be worth investigating if this is true of the same brand over different manufacturing makes for continuity.

All these knowledge gaps are restricted by the overarching challenges previously mentioned such as the chromatographic separation, subsequent ionisation and spectral data challenges in acquiring a bioactive peptide profile. There are also the limitations and variability in the automated processing of the mass spectral data from software leading to challenges in comparing studies which have used either different process workflows or different processing software.

1.6. Conclusion

This literature review has given an overview of bioactive peptides and how they operate as released in cheese models - with special attention to the cheddar variety. At its simplest cheese is the proteolytic transformation of milk into a preserved solid state through reduction in moisture. There are many processing conditions from coagulation to pressing curds into a distinguishable cheese shape. Within these processes it was determined that some have more impact than others. Starter bacteria and their different proteolytic systems behave differently to one another and can produce very different levels of bioactivity. This married with the subsequent ripening conditions can give a host of bioactive peptides that fluctuate over time. While these bioactivities can be assessed through bioassays alone, it is the peptide that provides the activity and therefore identification of peptides is important. There are still many unsolved mysteries surrounding how they can be leveraged for human benefit. Studies have come a long way and will continue to do so with the advancements of analytical instrumentation and processing algorithms for determining accurate peptide sequences present. It is important to understand the nuances in bioactive peptide

populations and how they function and fluctuate all because cheese is not just a tasty snack but a potential powerhouse of peptides.

This research aims to address the gaps in understanding about peptides specific to cheddar cheese and how their quantities and bioactivities fluctuate over ripening time. Additionally, manufacturing processes such as ripening temperature and processes leading to varied salt and moisture compositions will be investigated by manufacturing several different cheddar models and subjecting them to untargeted and targeted analysis to understand how manufacturing processes impact peptides.

2 Materials and Methods

2.1. Cheese Manufacture

Cheddar samples were manufactured by the Fonterra Research and Development Centre Pilot Plant. Three different cheddar cheese groups were made:

1 Cheddar with a basic starter culture (**control cheddar**).

2 Cheddar with the basic starter culture and an additional three adjunct bacteria (**adjunct cheddar**).

3 Cheddar with a basic starter culture and a single adjunct was created by varying the processing conditions to achieve a salt and moisture content at high and low varieties: high moisture and high salt (**HMHS**), high moisture and low salt (**HMls**), low moisture and high salt (**lmHS**), low moisture and low salt (**lmls**). Will be referred to as salt and moisture cheddars – not to be confused with salt in moisture ratio.

Samples were ripened over the course of 3 months at 4 °C for the control cheddar, HMHS, HMls, lmHS and lmls cheddars and 12 °C for the adjunct cheddar. In addition to assess the impact of temperature, the control cheddar was portioned and stored at a range of temperatures including –20 °C, 2 °C, 7 °C, 12 °C and 20 °C to follow the effects of temperature on ripening and peptide development.

Time-point zero, referred to as Month 0, was cheese taken the day after the initial cheese manufacture once the cheddar blocks had formed. Consecutive time points, Month 1 and 3, were removed from respective storage conditions for subsequent testing at 30 days and 90 days after manufacture.

Other manufacturing processes (cooking temperature and time, rennet/starter addition concentration, wash steps, etc) are similar between the three cheese groups but details are proprietary information.

2.2. Extraction of Cheese Peptides

Water Soluble Extracts (WSE) were prepared as per Pritchard *et al.* [30] with modifications as displayed in Figure 8. For each sample a 300 g block of cheese was finely grated (GM200 knife mill) and a 6 g portion was freeze-dried (Virtis 25L) in triplicate. Acetic acid (0.1M; 15 mL) was added and subsequently incubated at 40 °C for 60 minutes with intermittent vortex mixing. The mixture was centrifuged at 4000 rpm for 15 minutes at 4 °C (Eppendorf 5810 R). The supernatant was collected and centrifuged again as before. The resulting supernatant was filtered through a 0.22 μm nylon syringe filter. WSE filtrates were stored at –80 °C until further analysis.

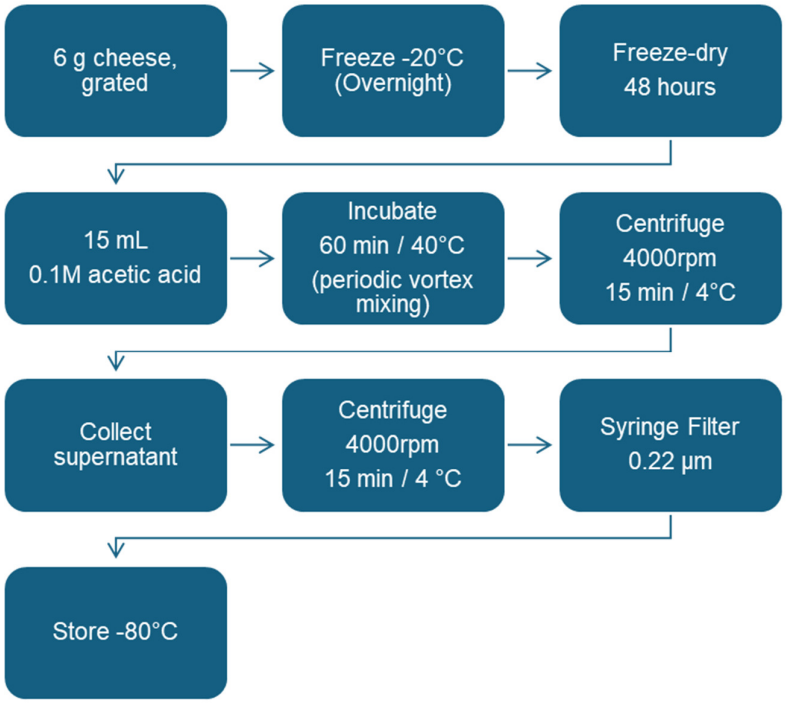

Figure 8. Cheese peptide extraction process

2.3. Macro Nutrient Composition

Protein content was determined by the Kjeldahl nitrogen method,[77] fat content by the Schmid-Bondzynski-Ratzaff fat test,[78] moisture by gravimetric drying at 102 °C for 16 hours, salt by potentiometric titration against silver nitrate,[79] pH was taken using a calibrated pH meter in duplicate. Further to this the extent of proteolysis was measured in duplicate using the Kjeldahl nitrogen method on the WSE to determine the amount of soluble nitrogen.

2.4. Identification of Peptides

The WSE's were analysed using ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). Chromatographic separation was performed using a reverse-phase UHPLC system (Vanquish Flex UHPLC, Thermo Fisher Scientific) equipped with

a C18 column (Hypersil GOLD VANQUISH aQ C18 reversed phase, 2.1 x 100 mm, 1.9 μm particle size). Peptides were separated over a linear gradient of mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile). The 40-minute run time comprised of 0-3 min: 2% B, 3-23 min: ramping to 35% B, 23-25 min: ramping to 95% B, 25-30 min: 95% B, 30-33 min: ramping down to 5% B, 33-40 min: 5% B, with a flowrate of 0.3 mL/minute.

Tandem mass spectrometry was conducted on an Orbitrap Mass Spectrometer (Exploris 240, Thermo Fisher Scientific).

The MS parameters are as follows: Scan range was 125-1500 m/z. with a resolution of 70,000. Positive mode. AGC target $1x10^6$ ions. Maximum IT of 100 ms. MS2 resolution of 17,500. AGC target of $1x10^5$ ions, maximum IT 50 ms. Isolation window of 1.4 m/z, dynamic exclusion of 10s.

Peptide sequencing and identification were performed using Proteome Discoverer software (version 2.5, Thermo Fisher Scientific) against a Bos taurus database (UniProt) using a label-free quantification workflow, see appendix 7.3.

Subsequent identification of bioactive peptides was accomplished using the Milk Bioactive Peptides Database (MBPDB).[80]

Peptide sequence quality was determined if it met a peptide hit rate of 66% - the peptide sequence was found in 6 of the 9 MS injections, Proteome Discoverer XCorr [Sequest] value of >2.0 and a mass accuracy ± 2.5 ppm.

2.5. Quantification of Peptides

Three peptides were purchased from Thermo Fisher covering α_{s1}-CN, α_{s2}-Cn and β-CN with a purity of >95%. MKPWIQPK (α_{s2}-CN [205-212]), VLNENLLR (α_{s1}-CN [30-37]) and YPFPGPIPN (β-CN [75-83]). Peptides were analysed with calibration standards in the range of 0.1-4 μg/mL in 0.1 M acetic acid. Standards were run in brackets at start and end of sample sets with the average being used to create the calibration curve.

2.6. Statistical Analysis

Results were analysed using a two-way ANOVA with Tukey pairwise comparison using Minitab® software. The two-way ANOVA was utilised to discriminate amongst the multiple contributing factors such as time, temperature and cheddar type on the response analysed to determine whether these factors had a significant impact of the response. Tukey pairwise comparison was used to further discriminate which subsets contributed variability to results and determine significantly different groupings. Grouped as A, B or C, where samples with the same letter indicated no significant difference between them.

3 Results and Discussion

3.1. Macro Nutrient Composition of Cheddar

The macro nutrient composition of the cheese samples is shown in Table 2. There was no major difference between the salt content and pH of the control and adjunct cheddar samples. Protein and moisture content were lower in the adjunct while the amount of fat was higher. As expected, the high moisture cheddars were indeed higher in moisture and low salt cheddars had lower salt. Moisture values also varied between the high and low salt cheddars with the higher salt varieties having lower moisture values. As expected, salt content varied depending on whether it was a high or low salt make. Fat and protein differed amongst the varieties with the low moisture cheddars having higher fat and protein content.

Table 2. Moisture, Fat, Protein, Salt (g/100 g) and pH in cheese samples.

	CONTROL	**ADJUNCT**	**HMHS**	**HMls**	**lmHS**	**lmls**
Moisture (± 0.29)	38.69	34.73	34.49	37.96	27.81	32.26
Fat (± 0.23)	31.64	36.35	36.32	34.58	40.36	38.42
Protein (± 0.056)	25.02	24.46	24.17	22.47	26.20	25.36
Salt (± 0.02)	1.92	1.96	2.36	1.45	2.38	1.42
pH (± 0.02)	5.23	5.15	5.32	5.17	5.37	5.36
Salt in Moisture (S:M)	4.96	5.64	6.84	3.82	8.56	4.40

3.2. Proteolysis of Cheddar

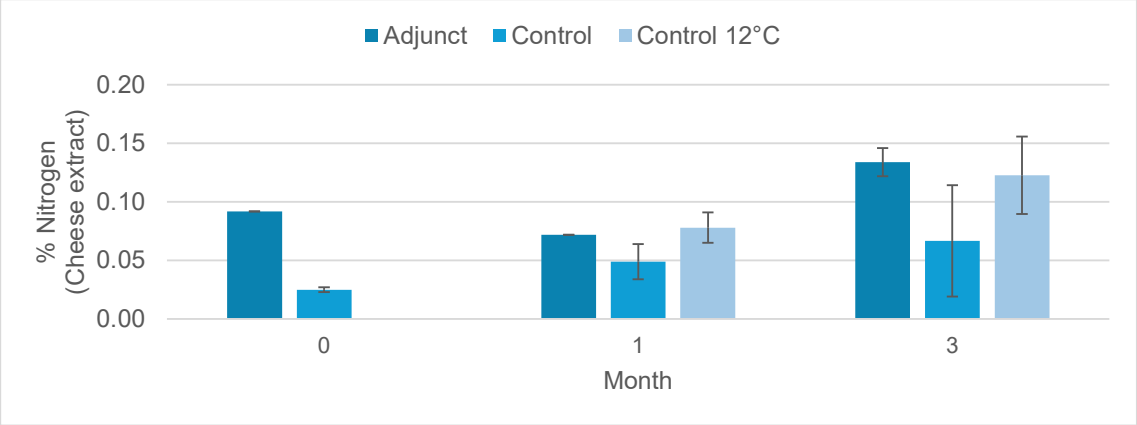

*Figure 9. Percentage of nitrogen extracted in cheddar WSE as effected by starter cultures using adjunct cheddar ripened at 12 °C and control cheddar ripened at 4 °C and 12 °C. (n=2 while * indicates samples where a single value was used). Tukey pairwise comparison grouping: 0 month = B, 1 month = B, 3 month = A. 4°C (control)= B and 12 °C (adjunct and control) = A. adjunct and control cheddar type = A. Those that share a letter are not significantly different.*

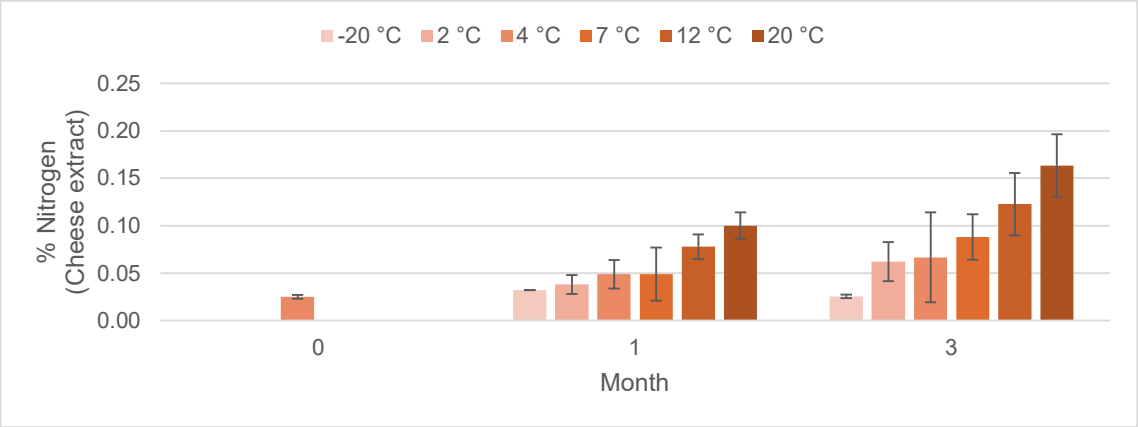

*Figure 10. Percentage of nitrogen extracted in cheddar WSE as effected by ripening temperature in control cheddar. (n=2 while * indicates samples where a single value was used). Tukey pairwise comparison grouping: 0 and 1 month = B, 3 months = A. 20 °C = A, 12 °C = B, 7 and 4 °C = C, 2 °C = C D and -20 °C = D. Those that share a letter are not significantly different.*

Proteolysis of cheddar was determined by the amount of nitrogen extracted in the WSE. There are many measures of the extent of proteolysis, often referred to as degree of hydrolysis, as explored by Rutherfurd.[81] Measuring the percentage of nitrogen extracted does not give information to the source i.e. whether it is peptide, amino acid or other nitrogenous species. Water soluble nitrogen (WSN) is often used to define proteolysis in cheese and it is accepted that an increase in WSN indicates the progression of proteolysis.[82]

In this experiment a Kjeldahl nitrogen test on the WSE was considered the same as WSN, despite the use of the mild acid for the WSE process. This test was used to determine the extent of proteolysis over time due to the different cheddar conditions.

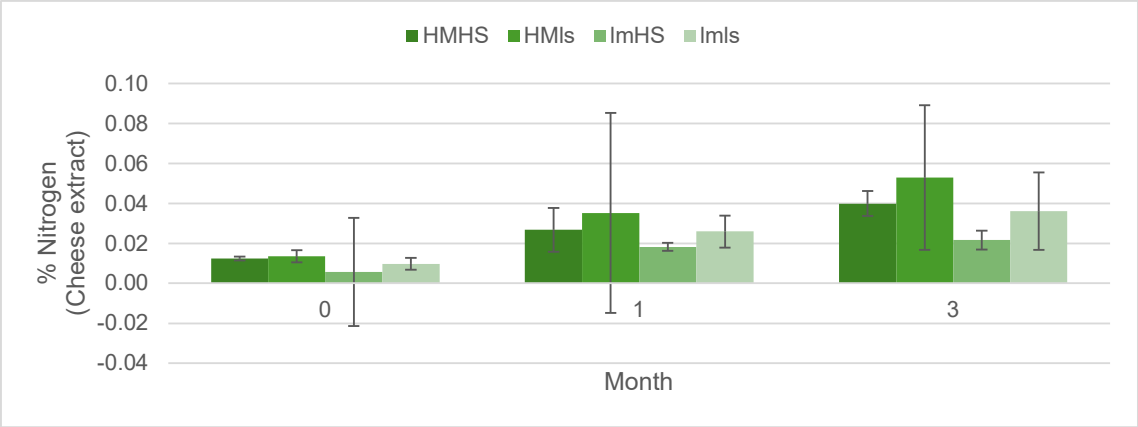

*Figure 11. Percentage of Nitrogen extracted in cheddar WSE effected by processing conditions designed to meet high and low salt and moisture content in a cheddar style cheese. (n=2 while * indicates samples where a single value was used). Tukey pairwise comparison grouping 0 month = C, 1 month = B and 3 months = A. HMls = A, HMHS = A B, lmHS and lmls = B. Those that share a letter are not significantly different.*

It was concluded that temperature, time and the salt and moisture variables impacted the most on the extent of proteolysis. A Tukey pairwise comparison was used to assess which samples were significant from one another by assigning samples to group A, B or C. This is displayed in the figure captions (not on the graph) throughout the results and discussion section see Appendix 7.5 for a full detail of the two-way ANOVA and Tukey pairwise comparison.

The impact of starter cultures was assessed by comparing the adjunct cheddar to the control cheddar. Because both are traditionally ripening at different temperatures 12 °C and 4 °C respectively. The 12 °C ripened control cheddar was also included in the set to balance any effects from temperature. It was determined that starter culture played little role in the extent of proteolysis across the time points as shown in Figure 9. However, it is noted that the adjunct cheddar had a much higher WSN at month 0 compared to the control. This indicates that the adjunct cheddar was more proteolytic during the cheddar manufacture. It would be useful to investigate the composition of the extracted nitrogen to determine free amino acids as this would further determine if the adjunct cheddar had a greater proteolytic capability. Sahingil *et al.* found that model cheeses with additional bacterial cultures proteolyzed the β-CN and α_{s1}-CN more than the control with a basic starter set.[16] This also highlights the limitation of using the WSN as a measure of proteolysis in the comparison of different cheeses.

Temperature was assessed by comparing the control cheddar as ripened between –20 °C and 20 °C over time and as expected was found to have significant impact on the proteolysis. As shown in Figure 10 there are four distinct groups. Cheese at –20 °C and 2 °C had the least amount of proteolysis due to the cold temperatures inhibiting the bacterial activity. There was no significant

difference between 2 °C, 4°C and 7 °C but both 12 °C and 20 °C were significant from one another with the highest amounts of proteolysis.

Salt and moisture showed some interesting trends with the different cheddar varieties leading to differences in proteolysis with the high moisture and low moisture varieties being significantly different from one another, the exception to this was the HMHS cheddar which shared similarities with the low moisture cheddars. This indicates that moisture is more important for proteolysis than salt. This is due to the need for moisture by bacteria for growth and the inhibitory nature of salt. Higher moisture cheddars appear to have more proteolysis and both high salt varieties (HMHS and lmHS) had lower proteolysis. Again, this is attributed to the optimum growth conditions of the starter bacteria. Variability in the results, however, indicate more sample replicates are needed for testing to identify true differences.

Lastly the time ripening shows significant impact on proteolysis. By 3 months ripening each cheddar condition is significantly different than previous months.

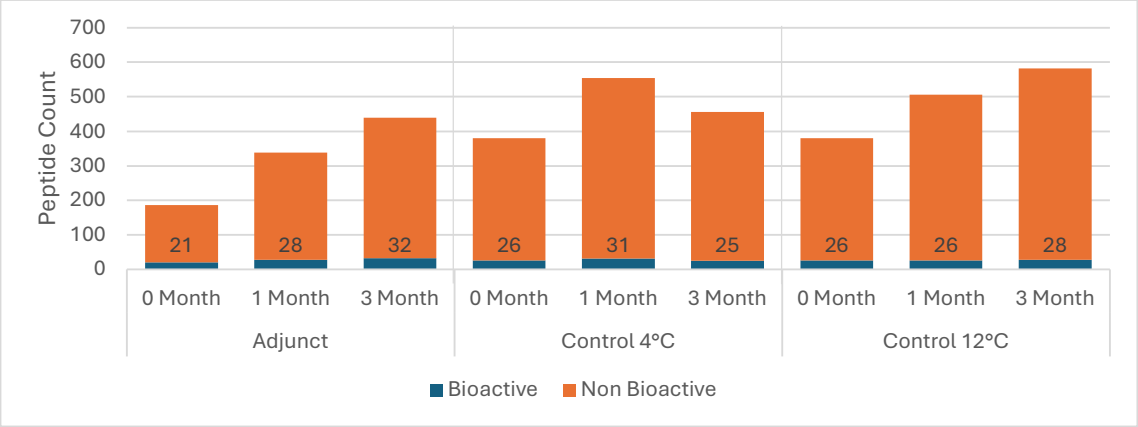

Figure 12. Distribution of peptides between bioactive and not across the ripening period of 3 months. Comparing Adjunct (12 °C) and Control cheddar (4 and 12 °C). Actual number of bioactive peptides displayed on graph.

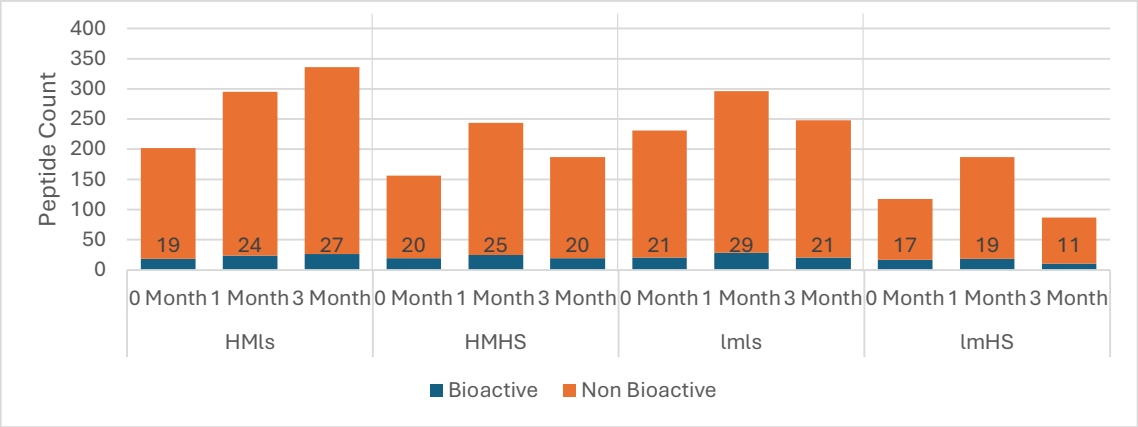

Figure 13. Distribution of peptides between bioactive and not across the ripening period of 3 months. Comparing the different salt and moisture ration cheddars. Actual number of bioactive peptides displayed on graph.

Since the %N of the WSE didn't fully elucidate the extent of peptides being formed; an analysis of the Proteome Discoverer data was used to compare the number of bioactive to non-bioactive peptides that achieved a >66% find rate (peptide was found in a least 6 of the 9 MS data files). This confirmed the WSN findings with similar trends in overall peptide counts see Figure 12, Figure 14 and Figure 13. Interestingly, this demonstrates how few bioactive peptides are present in cheese, accounting for approximately 10% of all peptides. This was also found by Helal *et al.* across different cheese types.[54] The number of non-bioactive peptides appear to increase on average 51% while the bioactive content only increases by 17% over time. This highlights the challenges of identifying bioactive peptides when they are hidden in a metaphorical sea of peptides.

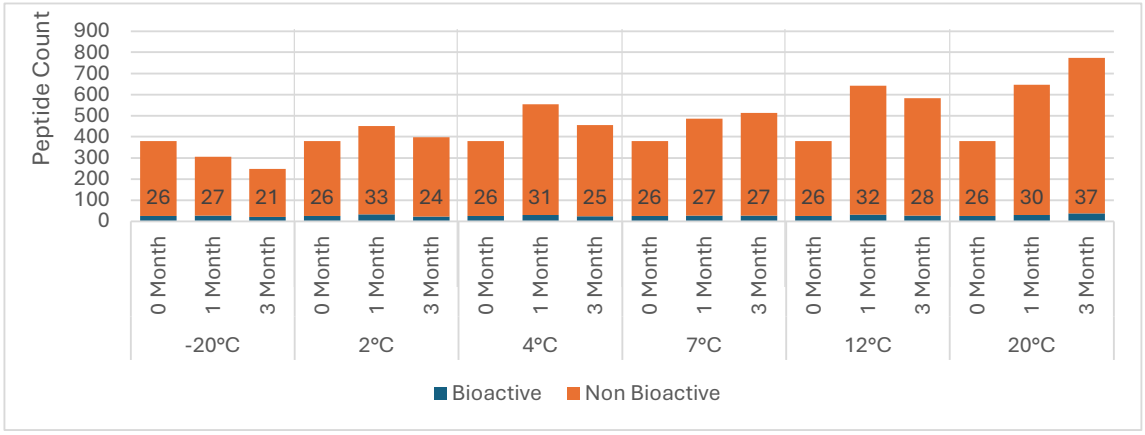

Figure 14. Distribution of peptides between bioactive and not across the ripening period of 3 months. Comparing control cheddar at varying ripening temperatures. Actual number of bioactive peptides displayed on graph.

3.2.1. Extraction Process Recovery of Soluble Nitrogen:

The effectiveness of the extraction was briefly explored by determining the amount of soluble nitrogen that was extracted over two extractions on the same cheese portion: labelled water-soluble extraction 1 and water-soluble extraction 2 in Figure 15. The insoluble cheese pellet was subjected to freeze-drying and re-extracted to determine if any soluble nitrogen remained.

It was determined that a small portion of nitrogen was extracted in comparison to the remaining insoluble portion with ~0.1% extracted nitrogen being soluble over the two extractions. Of this

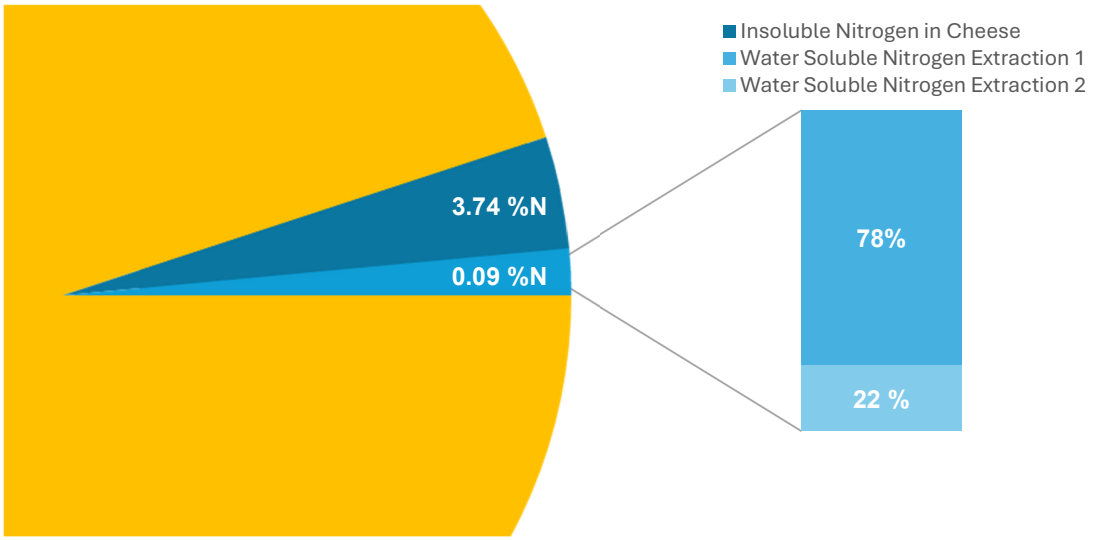

Figure 15. Recovery process of soluble nitrogen through the Extraction process in Figure 5. Left: Pie graph yellow portion represents non nitrogenous composition of cheese. Blue fractions represent nitrogen with 3.74% N being insoluble during the extraction process and 0.09% soluble as extracted over two processes on the same cheese pellet. Right: Bar graph of soluble nitrogen extractions 1 and 2.

78% was extracted in the first round. The 22% attained through further extraction indicated that the first extraction process is not sufficient to extract >80% or that subjecting the cheese pellet to the extraction process again has invoked artificial breakdown of the protein into soluble nitrogen. It would be valuable in future to look at the composition of each extraction to see if that information can be determined.

3.3. Identification of Peptides

Bioactive peptides are a small fraction of peptides present that as a group remain constant in numbers overtime. How these bioactive peptides change in terms of sequences present or bioactivity as effected by the various cheddar conditions were characterised across cheddars by analysing: total bioactive counts, their distribution among caseins, and unique peptides in each cheddar.

3.3.1. Challenges of Identifying Peptides

As mentioned in the introduction, identifying peptide sequences within a sample is not straightforward, with several hurdles to overcome to achieve a quality dataset. For this work the extraction method and processing software for peptide identification were the biggest sources of error.

The chosen extraction process had the benefit of being simple and fast with minimal consumable cost. However, this process meant that the final sample was still messy. This was a problem as large peptides can precipitate out in the gradient conditions of the HPLC leading to gradual blockage of the column and reduced performance over time. This can be overcome by increased cleaning of the column using protein-removing chemicals such as formic acid in acetonitrile mixed with isopropyl alcohol. Additionally, flipping the column to backflush out of any protein material accumulated on the frits can be useful, yet these processes can be time consuming. Alternatively, use of a Folch extraction[5] could be used to precipitate these proteins but how different extraction methods impact the peptides in the resultant sample is yet to be fully assessed.

Further to this, the number of analytes introduced to the MS means reduced scan time per analytes. Limiting the number of times the analytes of interest (bioactive peptides) are fragmented to gain MS2 spectra. This impacts the spectral data as extraneous compounds can interfere with the analytes of interest. While using dynamic exclusion can assist by ignoring analytes that have already been scanned recently, the MS is still focusing on the more abundant analytes, potentially at the expense of those that are bio-actively interesting. Other studies have used sample fractionation to split the sample into the different molecular weights to address these challenges. [12, 29, 54]

Finally, the post MS processing software required to sort through the large dataset provided by the MS and assign fragmentation patterns to peptide sequences can cause challenges. Due to the large amount of data, it was necessary to simplify the process to keep consistency when analysing the various sets of data. While the pros and cons of using software like Proteome Discoverer alone has already been discussed there are also inherent limitations in the way in which the data was analysed. Thresholds were set for this body of work. Peptide hit rate had to be >66 %, indicating it was found in at least 2 of the 3 replicates. The XCorr [Sequest] value had to be >2.0, this is an indicator of goodness of fit for the spectral match as internally quantified by the software. The mass accuracy had to be within a range of 5 ppm as this was an achievable expectation for the Exploris 240 mass spectrometer. Finally, of course the peptide had to be bioactive.

This introduces limitations as a peptide that was found in less than 6 of the 9 injections could still be a legitimate peptide for that sample, especially if when identified met the other criteria. In these cases, it was possible that that the peptide was in lower abundance and was overlooked by the MS. The Xcorr or goodness of fit for the spectral match of greater >2.0 means acceptable and accurate peptides are included but peptides that fall between 1 and 2 could still be real peptides for that sample. The possibility that a peptide doesn't naturally fragment well or didn't receive adequate scan time due to other analytes could drop this score. However, using Xcorr by itself as to

determine goodness of fit is limited since a score of 2.0 indicates different levels of goodness of fit for the spectral match depending on the charge state or the peptide.[83]

3.3.2. How Cheddar Processes Impact Total Bioactive Peptides

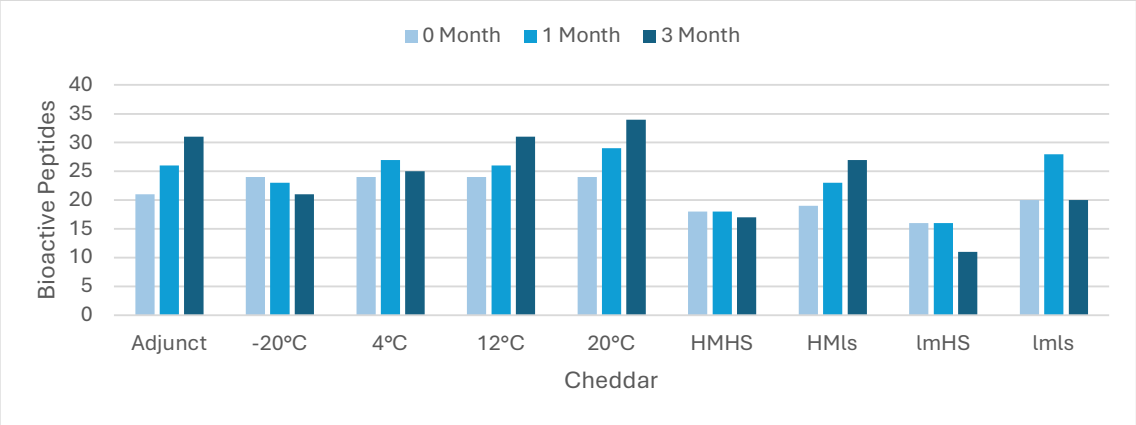

Figure 16. Bioactive peptides over 3 months ripening across cheddars to compare the effects of starter culture, temperature and salt and moisture. Bioactive peptides displayed meet >66% find rate, >2.0 Xcorr [Sequest] value and are within a 5 ppm error range. Tukey Pairwise Comparison: Adjunct = A, Control = A, HMls = A B, lmls = A B, HMHS = A B and lmHS = B. Means that share a letter are not significantly different.

Contrary to initial expectations, there was very little variation in bioactive peptides across the cheddars over time as illustrated in Figure 16. The lmHS cheddar was the only variety to be significantly different from the adjunct and control cheddars. This is due to the cheese environment being inhospitable to bacteria with ~20-30 % less moisture and ~20 % more salt than the adjunct and control cheddar. This means the bacteria have less moisture to survive and more salt inhibiting their activity, both resulting in fewer peptides being formed.

The effect of temperature showed little significant variation which was surprising as by 3 months it is noticeable that there are more bioactive peptides in the 20 °C cheddar vs the –20 °C cheddar. A limitation in how the bioactive peptides are calculated is the most probable cause for the insignificant differences. Although 9 data sets are generated these are compiled into one dataset omitting peptides sequences that are not found in at least 6 of the 9 data sets. This creates one result which drastically reduces the power of the two-way ANOVA. Repeating the measurements to generate more data would increase the discrimination power of the analysis.

Lastly the starter cultures investigated did not impact the total bioactive peptide count. The adjunct cheddar contains three extra cultures alongside the base *L. cremoris* triplet starter culture. Yet when compared to the control has no significant difference. It was expected that more bacteria would increase the number of proteolytic enzymes in the system which would lead to more peptides. However, starter cultures have different proteolytic systems some with very specific functionality

which appeared to be the case here. The adjuncts added all have an enzyme system focusing on debittering, that is breaking down peptides responsible for bitter flavours. This specificity means their focus is only on certain sequences within caseins and casein peptides and not more broadly applicable.

3.3.3. How Cheddar Processes Impact Caseins and Form Peptides

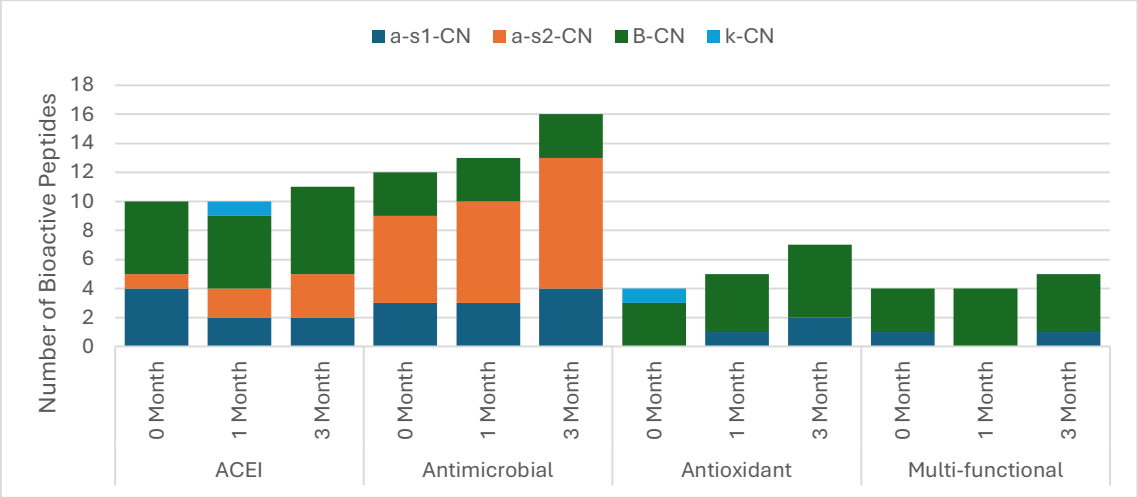

Figure 17. Control cheddar: bioactive peptides over 3 month ripening time (at 12 °C) as expressed per casein group. Bioactive peptides grouped by associated bioactivity. Graph only shows ACE-I, antimicrobial and antioxidant bioactivities as most prominent.

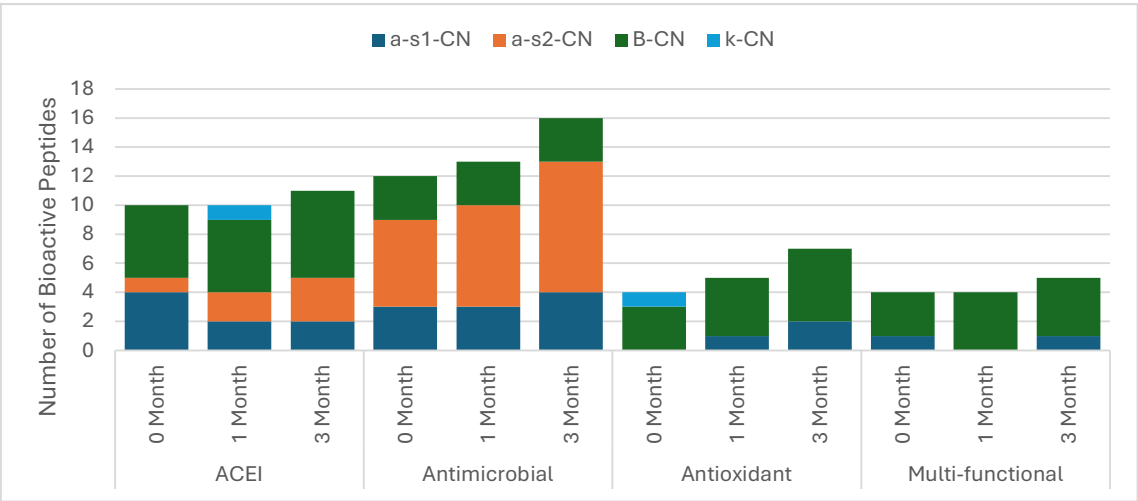

Figure 18. Adjunct cheddar: bioactive peptides over 3 month ripening time (at 12°C) as expressed per casein group. Bioactive peptides grouped by associated bioactivity. Graph only shows ACE-I, antimicrobial and antioxidant bioactivities as most prominent.

Bioactive peptides can be formed from any of the four casein proteins present in cheese: α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN. It was observed that how these proteins contribute to bioactive peptides varied between the adjunct and control cheddars over time (Figure 17 and Figure 18). This was most obvious in the antimicrobial peptides. The control cheddar forms most of the antimicrobial

peptides at 0 months and remains steady over the 3 months with 6-7 peptides. However, the adjunct cheddar at 0 months has minimal antimicrobial peptides with 2 and increases to 9 peptides by 3 months.

This indicates that the bacteria present in the adjunct cheddar are slower to break down α_{s2}-CN into antimicrobial peptides. Utilisation of gel electrophoresis to analyse the intact caseins would provide further information on whether break down of α_{s2}-CN occurs more slowly in the adjunct cheddar. Further time points would also be valuable in determining if this trend continues.

This also shows antimicrobial peptides appear primarily formed from α_{s2}-CN although they can also form from α_{s1}-CN and β-CN. Antimicrobial peptides have been found to be associated with all casein proteins.[84]

It was noted that there were some variations in the κ-CN derived peptides especially for ACE-I activity with peptides found in the control cheddar but not the adjunct. However, they are limited in number and fall into the uncertainty of the Proteome Discoverer workflow as discussed further in appendix 7.4. Alternatively, this variation indicates that the adjunct is more proficient at proteolyzing κ-CN derived peptides into peptides smaller than detectable by Proteome Discoverer or free amino acids.

3.3.4. How Cheddar Processes Impact Peptide Sequence Formation

Where bacteria hydrolyse the protein structure determines the peptide identities. This allows for unique peptides to occur which are specific to the cheddar variety. This is important as not all bioactive peptides are alike; one antimicrobial peptide might be more efficient than another. Comparing sequences between the samples allows for better insight into whether the cheddar conditions have an impact on bioactive peptide formation based on where the protein structure is hydrolysed.

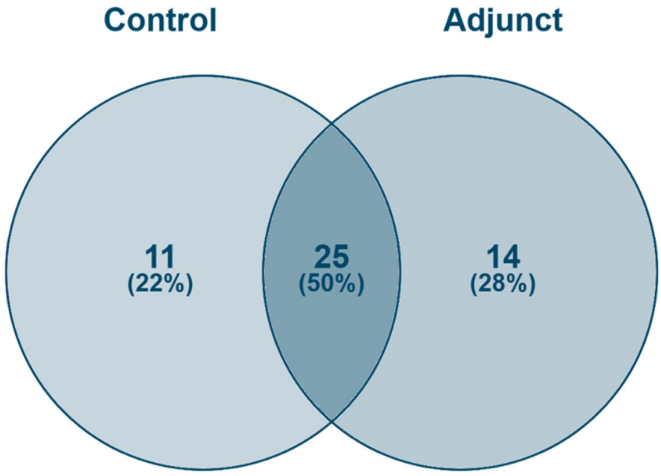

Figure 19. Venn diagram of collated bioactive peptides found in the adjunct and control cheddar (12 °C) over 3 months ripening. Bioactive peptides displayed met >66% find rate, >2.0 Xcorr [Sequest] value and are within a 5 ppm mass accuracy. Made using Venny 2.0.[1]

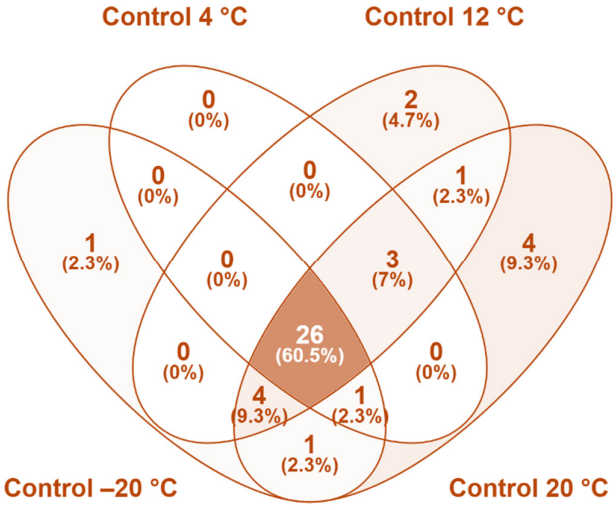

Figure 20. Venn diagram of collated bioactive peptides found in the control cheddar across different ripening temperatures over 3 months ripening. Bioactive peptides displayed meet >66% find rate, >2.0 Xcorr [Sequest] value and are within a 5 ppm mass accuracy. Made using Venny 2.0.[1]

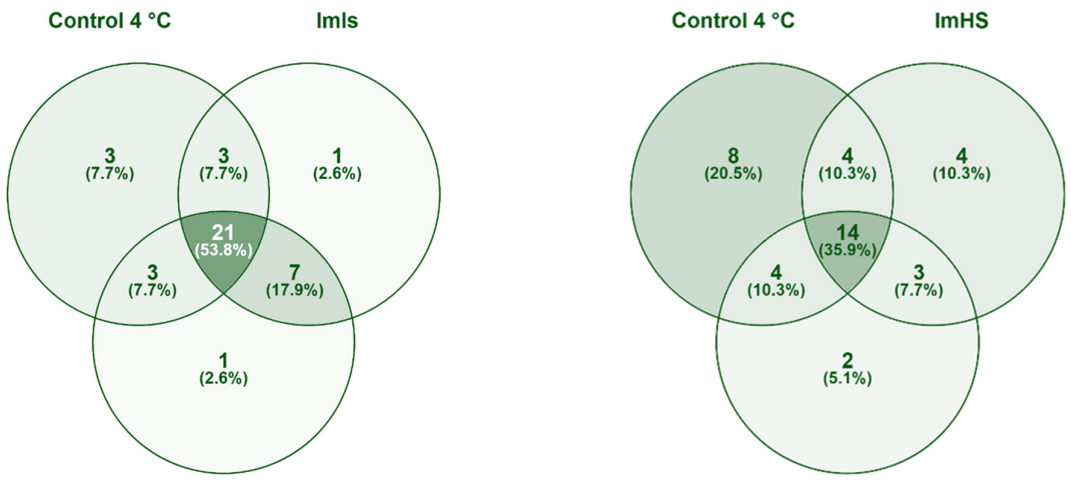

Figure 21. Venn diagram of collated bioactive peptides found in the salt and moisture cheddars over 3 months ripening. Low salt varieties (left) and high salt varieties (right). Bioactive peptides displayed meet >66% find rate, >2.0 Xcorr [Sequest] value and are within a 5 ppm mass accuracy. Made using Venny 2.0.[1]

Starter culture appears to influence the production of unique peptides as shown in Figure 19. Overall, the control and adjunct cheddar share 50% of the peptide sequences and generate a further 22% and 28% respectively that are unique to that cheddar. However, declarations of peptide uniqueness should be treated with caution. Closer analysis of the data shows that some sequences are found in both but that they have been filtered out and discounted. The peptide sequence NLHLPLPLL, a unique peptide to the adjunct cheddar, was also sequenced in the control cheddar but did not meet the hit rate threshold of >66% but was found in all time points and had a good spectral match in the samples where it was found indicating that it is likely this peptide exists in the sample. Also, it was sequenced at 2 °C in the control cheddar which is not included in the Venn diagram. Another example is KVLPVPQK, a peptide unique to the control cheddar, was also sequenced in the adjunct cheddar. However, it did not meet the threshold as found only 4/9 times but was found in each biological replicate and had a high XCorr where identified. This is a good example of the challenges in peptide identification as previously discussed.

Temperature did not influence unique peptide production (Figure 20), which is understandable as temperature was anticipated to affect the rate of peptide formation but not the sequence. Therefore, peptides found with the warmest ripening temperatures should show up in other temperatures at different ripening time points. Warmer temperatures appear to have more unique peptides for this reason. The single unique peptide at –20 °C (SWMHQPHQPLPPT) was again found in other samples but not the higher temperature samples indicating that this peptide degraded more slowly at lower temperatures.

Salt and moisture cheddars showed that salt concentration had an impact on the peptide sequences formed but in a more inhibitory manner as shown in as shown in Figure 21. Low salt varieties had more in common with the control cheddar with >50 % peptide sequences being shared. The high salt varieties still generated unique peptides when compared to the control cheddar. However the reduced overall number of bioactive peptides indicates that conditions for these cheddar varieties would be of reduced efficacy in the respective bioactivities.

3.4. Quantification of Peptides

The peptides MKPWIQPK (α_{s2}-CN [205-212]), VLNENLLR (α_{s1}-CN [30-37]) and YPFPGPIPN (β-CN [75-83]) were chosen based on preliminary screening of ripened cheddar on the basis that they were likely to be present in this study's samples due to a high relative abundance during pre-screening. It was ideal to have a range of peptides accounting for the different casein parent proteins as well as peptides that had different bioactivities. The peptides needed to be longer than six residues to allow for sequencing with Proteome Discoverer but short enough that they were unlikely to interact with themselves which could cause issues in fragmenting– this also had the benefit of minimising cost of synthesis. To the authors knowledge none of these peptides had been quantified in cheddar prior to this study.

MKPWIQPK is a peptide derived from α_{s2}-CN and has been found to have ACE-inhibitory activity.[85] VLNENLLR is a peptide derived from α_{s1}-CN and has been found to have antimicrobial activity against several species including *E. coli* and *L. bulgaricus*.[71, 86, 87] Lastly YPFPGPIPN is a peptide derived from β-CN and has been found to have multiple bioactivities such as ACE-inhibition,[88, 89] antioxidant[88] and DPP-IV inhibition.[90]

Each of these peptides was quantified over 3 months under the different cheddar conditions including starter culture, ripening temperature and manufacturing processes involved in salt and moisture control.

3.4.1. Matrix Effect and its Impact on Peptide Resolution

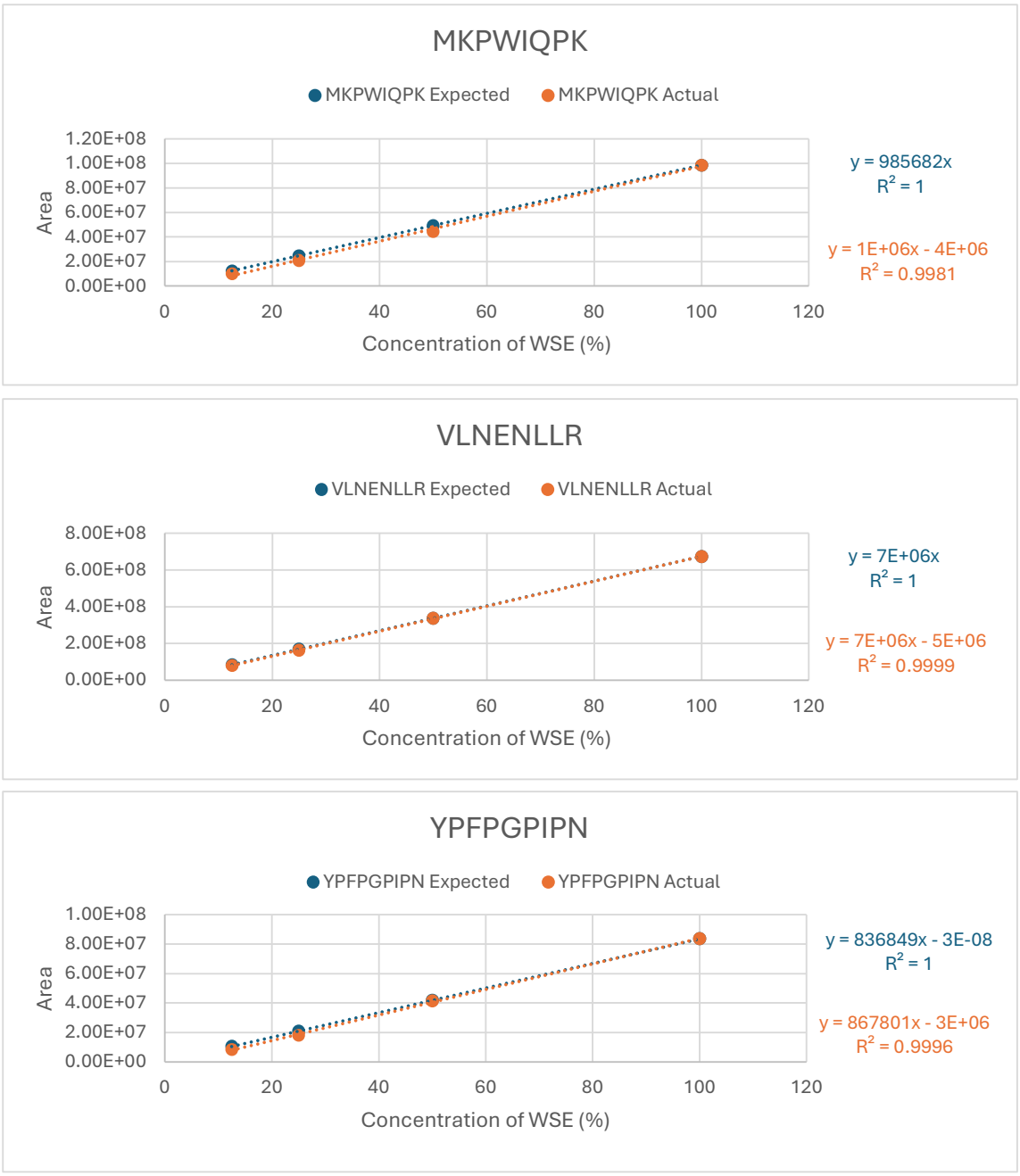

Figure 22. Matrix dilution plot graphs for respective standard. Showing linearity comparability between a theoretical dilution of the standard area vs the actual area of the diluted peptide.

To ensure accurate quantification of peptides it is important to confirm that the peptides present in the standard calibration curve behave like those found in the sample matrix i.e. they produce the same signal intensity and charge states. Therefore, it is important to check the calibration standard matrix is the same as or comparable to that of the WSE. One way to determine the presence of

matrix effect is the Matrix Dilution Plot[91] whereby the sample matrix, in this case the cheese WSE, is diluted over a known range. The area obtained is then plotted against a theoretical curve created sequentially diminishing the attained area at 100% WSE. In this case an area at 100% cheese matrix was halved to obtain a 50%, 25% and 12.5% theoretical area. The theoretical and actual diluted areas are plotted against one another to see whether the dilution of the cheese matrix creates any aberration in the area response. As shown in Figure 22 the reduction in matrix compounds doesn't appear to have a noticeable impact on peptide behaviour. Therefore, it was determined that preparing a calibration curve with 0.1 M acetic acid, the solution used to extract cheese peptides, should give sufficiently accurate results. However, it should be noted that this was based on single cheese matrix material. It is expected that over the ripening times tested the matrix could change which may impact the area response of the samples against the calibration curve.

3.4.2. Effects of Starter Culture on Peptide Quantification

Both cheddar type and time show significant impacts on the quantities of all selected peptides. Most interesting was the near absence of YPFPGPIPN in the adjunct cheddar across all time points and the difference in trends between adjunct and control cheddars for MKPWIQPK and VLNENLLR.

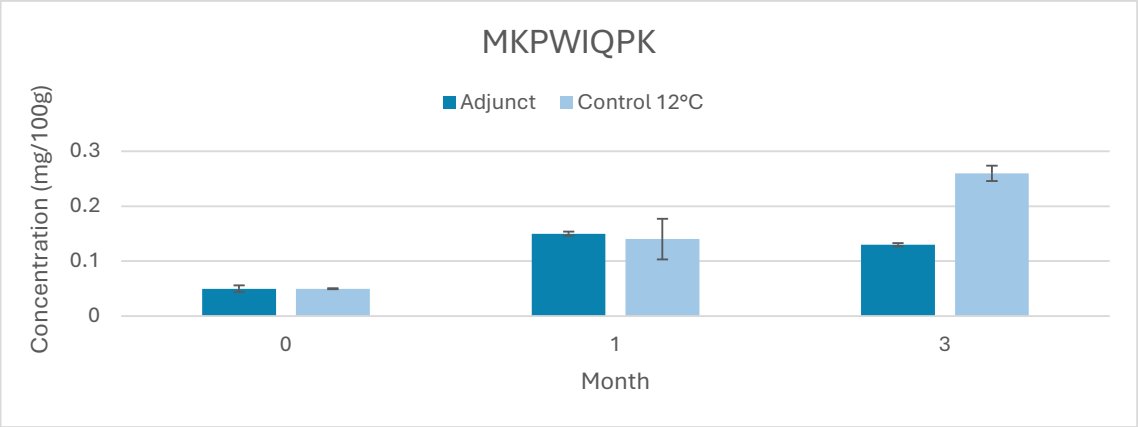

Figure 23. MKPWIQPK quantification across three months ripening comparing effects of starter culture on peptide abundance using adjunct cheddar (ripened at 12 °C) and control cheddar (ripened at 12 °C). n=9. Tukey Pairwise Comparison: Control = A, Adjunct = B, 0 Month = C, 1 Month = B, 3 Month = A. Means that share a letter are not significantly different.

Both cheddar type and time points show significant impact on the quantity of MKPWIQPK with *p*-values <0.001. In the adjunct cheddar an increase over the first 30 days to a maximum of 0.15 mg/100g was observed as shown in Figure 23 and showed little change at 90 days. The control cheddar, however, shows an increased quantity at 90 days with a maximum of 0.26 mg/100g. More time points would be valuable in establishing whether the control cheddar is following an increasing trend over time.

MKPWIQPK was originally discovered by Maeno *et al.* in a casein hydrolysate created by *L. helveticus*. It was found to have an IC_{50} of 300 μM which is the concentration needed to inhibit half ACE-I activity. Its administration to hypertensive rats did not show any significant reduction in systolic blood pressure.[85] Further investigation into this peptide would be useful in confirming these results and determining significant quantities for activity. More active peptides such as KVLPVPQ (β-CN [184-190]) as found in the Maeno *et al.* study may be more worthwhile quantifying in cheddar.

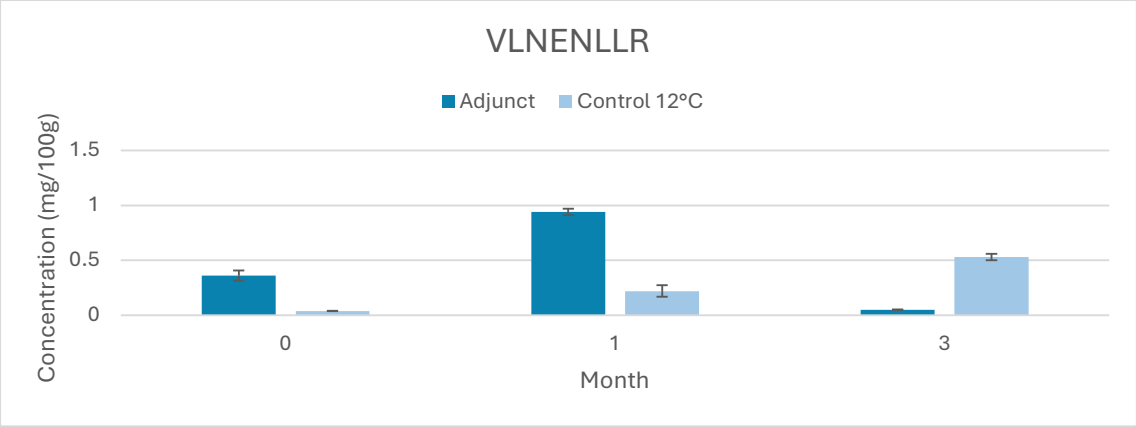

Figure 24. VLNENLLR quantification across three months ripening comparing effects of starter culture on peptide abundance using adjunct cheddar (ripened at 12 °C) and control cheddar (ripened at 12 °C). n=9. Tukey Pairwise Comparison: Control = B, Adjunct = A, 0 Month = B 1 Month = A, 3 Month = B. Means that share a letter are not significantly different.

Both cheddar type and time significantly impacts the quantity of VLNENLLR with p-values ≤0.01 and <0.001 respectively. VLNENLLR shows a differing trend between the control and adjunct cheddar than seen with MKPWIQPK. As shown in Figure 24 the adjunct cheddar rapidly produces VLNENLLR with the concentration increasing to 0.94 mg/100g over the first 30 days. It then promptly drops away to 0.05 mg/100g at 90 days. Meanwhile the control cheddar continuously increases over the 90 days although it only reaches 0.53 mg/100g at 90 days, almost half the maximum concentration in the adjunct. This indicates that the adjunct cultures hydrolyse the proteins/peptides to form VLNELLR early on in ripening before it is subsequently hydrolysed. With more time points it would be interesting to see if VLNENLLR increases in quantity again or whether all this activity happens in the first 3 months. This also suggests that the *L. cremoris* has less preference for hydrolysing this peptide sequence allowing for slow accumulation.

VLNENLLR, also called Caseicin B, has been the subject of many studies investigating its potential as an antimicrobial agent in infant formula to combat *C. sakazakii*. [86, 92] VLNENLLR has been found to have an MIC of 0.22 mM (the minimum concentration of the peptide which shows inhibitory quality) which was comparable to its precursor peptide Isracidin

(RPKHPIKHQGLPQEVLNENLLRF, α_{s1}-CN [16-38]), well known for its antimicrobial activity.[92] This indicates that when Isracidin is hydrolysed its fragments remain active maintaining that antimicrobial activity for longer. It has also proven capable of completely inhibiting *C. sakazakii* over 24 hours at concentrations of 2.91 mg/mL[86] and further studies have also proven that this peptide can reduce *C. sakazakii* in canned infant formula during storage.[93] Based on these minimum concentrations it VLNENLLR does not appear to be in usable quantities naturally in cheddar.

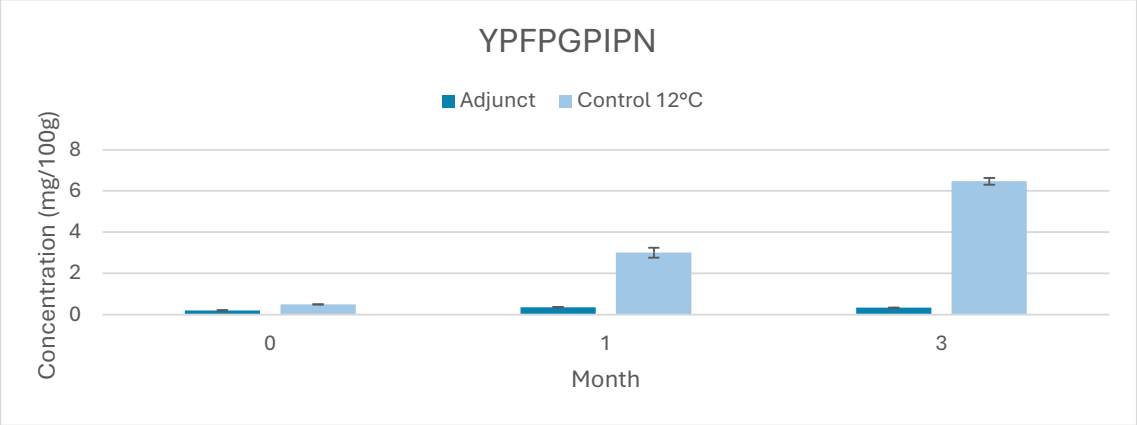

Figure 25. YPFPGPIPN quantification across three months ripening comparing effects of starter culture on peptide abundance using adjunct cheddar (ripened at 12 °C) and control cheddar (ripened at 12 °C). n=9. Tukey Pairwise Comparison: Control = A, Adjunct = B, 0 Month = C, 1 Month = A, 3 Month = B. Means that share a letter are not significantly different.

Again, both cheddar type and time were significant factors in the quantities of YPFPGPIPN with both having *p*-values <0.001. However, this peptide showed a unique trend with the adjunct culture containing consistently minimal amounts of the peptide while the control continued to accumulate as shown in Figure 25. The adjunct cheddar reached a maximum of 0.36 mg/100g at 30 days. The control cheddar however had a maximum of 6.47 mg/100g – the most concentrated of all peptides quantified. This was to be expected as the bacteria in the adjunct cheddar were added for debittering purposes and YPFPGPIPN is a known bitter peptide.[94]

Originally discovered in 1997 by Singh *et al*[95] YPFPGPIPN has since not been the focus of any quantification studies in cheese but was quantified in whey protein hydrolysate and was found to be present at 2.64 g/kg of powder.[94] This indicates that 90 days of ripening is insufficient to generate significant quantities of this peptide. Different ACE-I IC_{50} values have been determined ranging from 14.8 μM[89] to 378.65 μM[88] which shows a major difference in suggested ACE-I activity. This discrepancy is likely due to the difference in methodology implemented. The study by Saito *et al.* [89] further concluded that YPFPGPIPN despite having a desirable IC_{50} value did not show antihypertensive activity in hypertensive rats.

YPFPGPIPN has also been the subject of diabetic investigations and has been found to have a DPP-IV inhibition of 670 μM[90] and antioxidant capability with a value of 1.26 or 1.22 μmol TE2 /μmol of peptide depending on methodology used.[88]

This indicates that YPFPGPIPN would be unsuitable for further investigation given the minor *in vivo* bioactivity coupled with the low amounts present in cheddar. Further investigation to confirm literature values for this peptide would be beneficial given the discrepancies.

3.4.3. Effects of Temperature on Peptide Quantification

Temperature and ripening time both have statistically significant effects on peptide quantities (*p*-values <0.001 for MKPWIQPK, VLNENLLR and YPFPGPIPN). As expected, higher quantities are found in the samples ripened at higher temperatures.

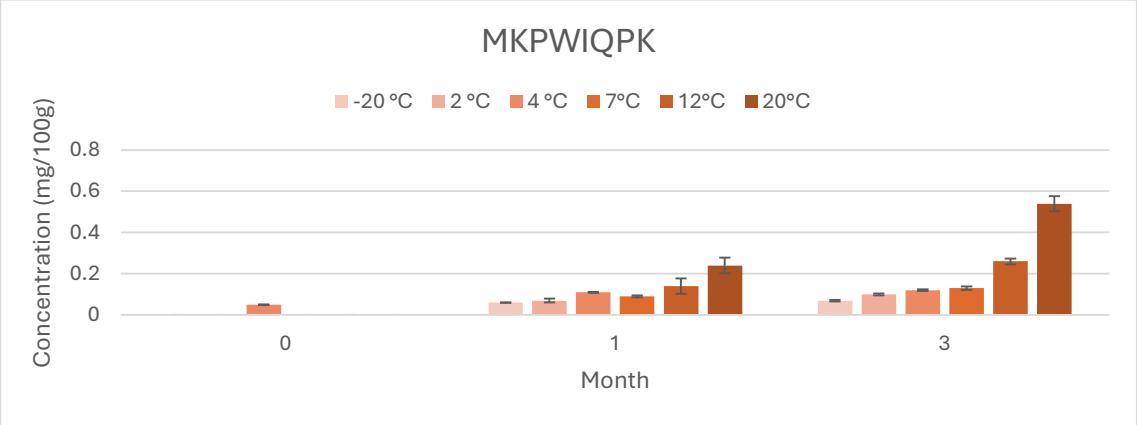

Figure 26. MKPWIQPK quantification across three months ripening comparing effects of temperature on peptide abundance using control cheddar. n=9. Tukey Pairwise Comparison: 20 °C = A, 12 °C = B, 7 °C = C, 4 °C = C, 2 °C = C, –20 °C = C. 0 month = B, 1 Month = B, 3 Month = A. Means that share a letter are not significantly different.

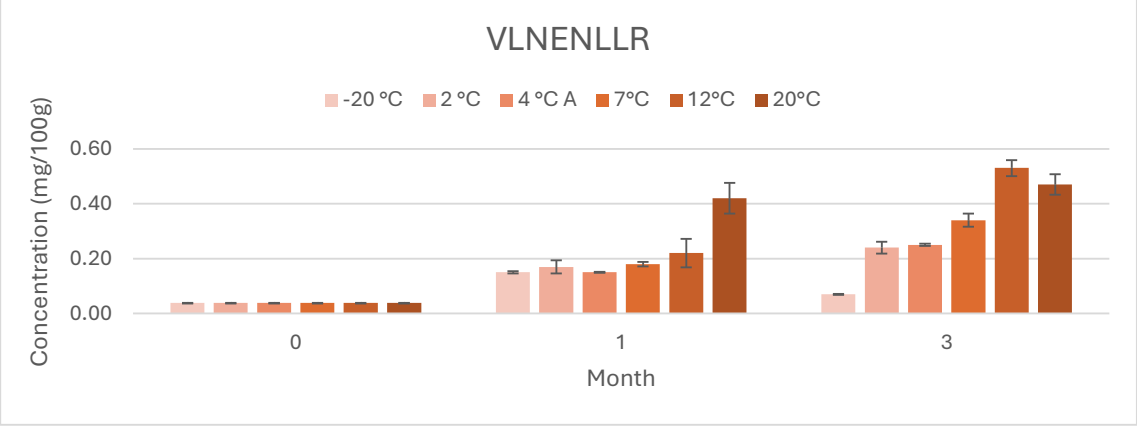

Figure 27. VLNENLLR quantification across three months ripening comparing effects of temperature on peptide abundance using control cheddar. n=9. Tukey Pairwise Comparison: 20 °C = A, 12 °C = B, 7 °C = C, 4 °C = D, 2 °C = C D, –20 °C = E. 0 month = C, 1 Month = B, 3 Month = A. Means that share a letter are not significantly different.

Most of the peptides follow the same trend with 20 °C and 12 °C samples being significantly different to other temperatures. However for VLNENLLR and YPFPGPIPN –20 °C is also significantly different see Figure 26, Figure 27 and Figure 28.

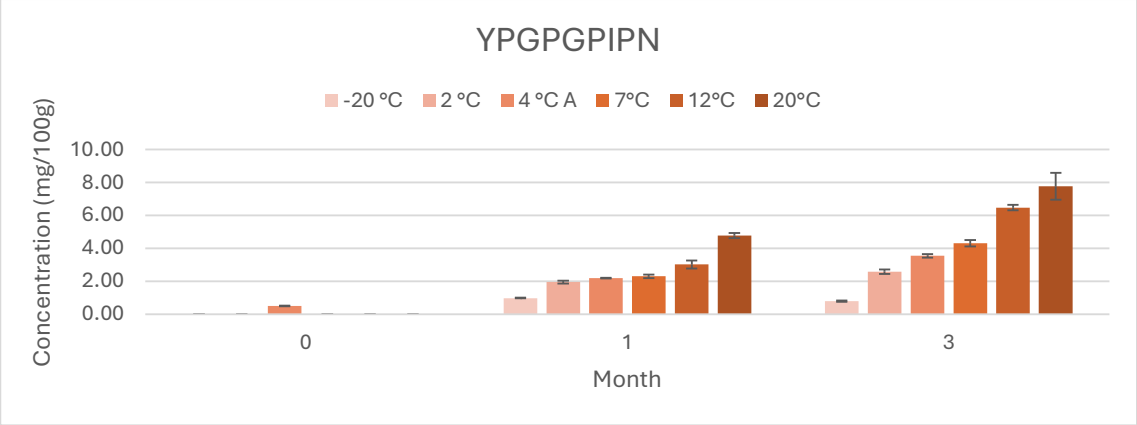

Figure 28. YPFPGPIPN quantification across three months ripening comparing effects of temperature on peptide abundance using control cheddar. n=9. Tukey Pairwise Comparison: 20 °C = A, 12 °C = B, 7 °C = C, 4 °C = C D, 2 °C = D, –20 °C = E. 0 month = C, 1 Month = B, 3 Month = A. Means that share a letter are not significantly different.

Interestingly VLNENLLR shows potential decrease in quantity at higher temperatures at 3 months with 12 °C having a maximum 0.53 mg/100g and 20 °C having 0.47 mg/100g as shown in Figure 27. This makes sense since each peptide will eventually be broken down to form smaller peptides or free amino acids. It would be useful to gather more time points for these peptides to see whether this decrease is real or just part of the measurement uncertainty. This could then indicate that of the three peptides VLNENLLR is more liable to be hydrolysed.

Therefore, temperature could be used to boost bioactive peptides and reduce ripening time, although more investigation would be required to see the impact on other components in cheese.

3.4.4. Effects of Salt and Moisture Processing Levers on Peptide Quantification

Both salt and moisture processing conditions and time are statistically significant factors in influencing the quantity of peptides in cheddar with *p*-values <0.001. The salt and moisture parameters impact the concentration of the selected peptides in different ways, but it is impossible to determine any trends with the current data.

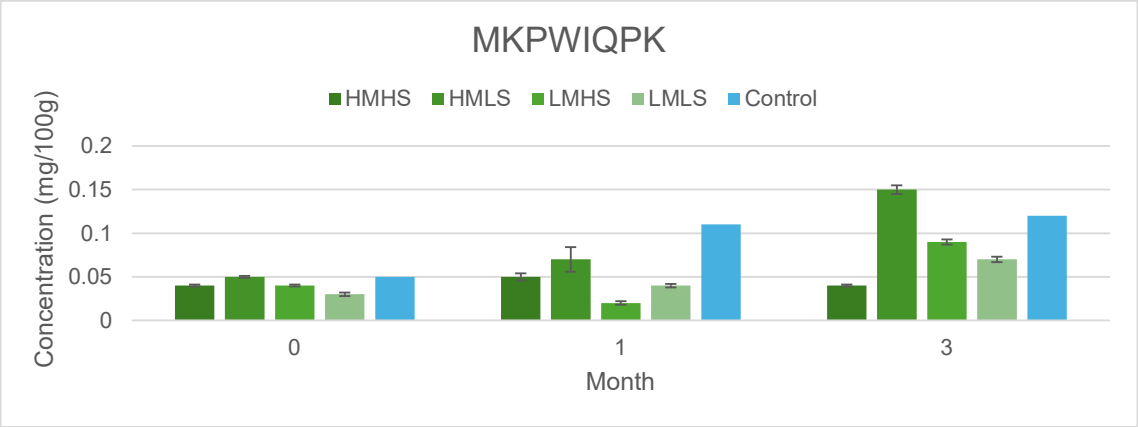

Figure 29. MKPWIQPK quantification across three months ripening comparing effects of salt:moisture composition on peptide abundance using control cheddar. n=9. Tukey Pairwise Comparison: Control = A, HMls = A, lmls = B, HMHS = B C, lmHS = C. 0 month = C, 1 Month = B, 3 Month = A. Means that share a letter are not significantly different.

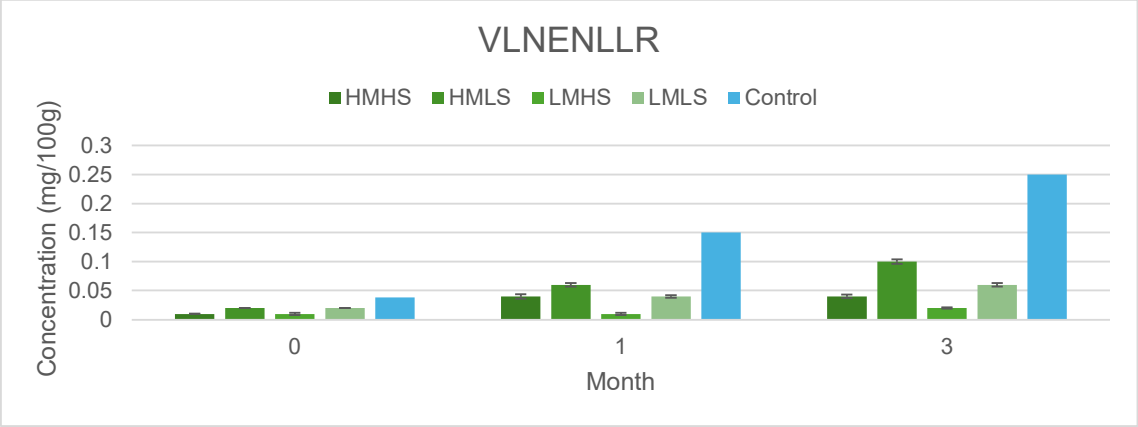

Figure 30. VLNENLLR quantification across three months ripening comparing effects of salt:moisture composition on peptide abundance using control cheddar. n=9. Tukey Pairwise Comparison: control = A, HMls = B, lmls = B C, HMHS = C D, lmHS = D. 0 month = C, 1 Month = B, 3 Month = A. Means that share a letter are not significantly different.

None of the salt and moisture processing conditions were beneficial to boosting VLNENLLR production. The highest amount was reached in the HMls variety at 3 months with 0.1 mg/100g, which is 2.5-fold less than the control cheddar at that time, as shown in Figure 30. It is feasible that this difference is due to the additional bacteria used in the manufacture of the salt and moisture cheddar varieties. Alternatively, this suggests that the hydrolysis of peptide bonds that form

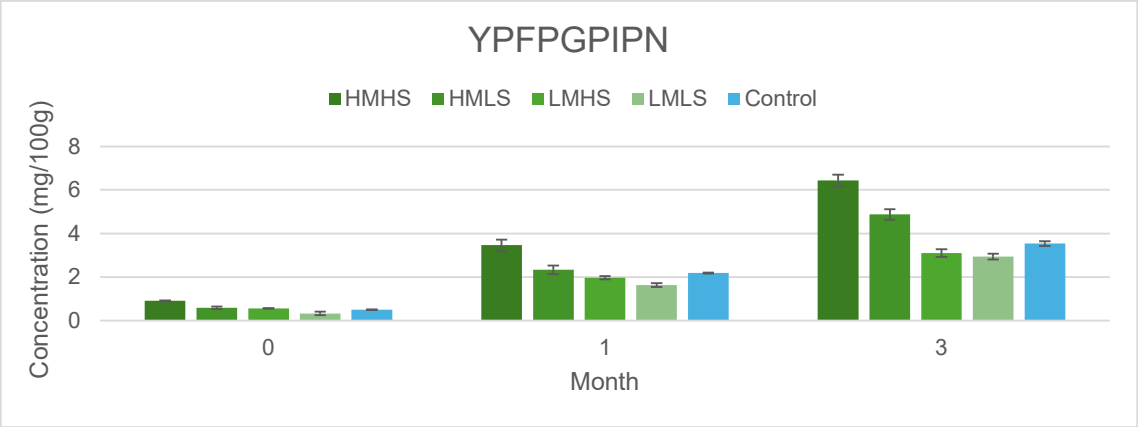

Figure 31. YPFPGPIPN quantification across three months ripening comparing effects of salt and moisture composition on peptide abundance using control cheddar. n=9. Tukey Pairwise Comparison: Control = C, HMls = B, lmls = D, HMHS = A, lmHS = C. 0 month = C, 1 Month = B, 3 Month = A. Means that share a letter are not significantly different.

VLNENLLR are inhibited. Repeating this experiment to control these variables would confirm whether the differences are from the salt and moisture or other manufacturing processes.

HMls cheddar variety produced similar amounts of MKPWIQPK to the control cheddar with 0.15 mg/100g and 0.12 mg/100g respectively at 90 days as shown in Figure 29. Inversely to this lmHS produced similar amounts of YPFPGPIPN to the control cheddar with 3.1 mg/100g and 3.54 mg/100g respectively as shown in Figure 31. While HMls is sensible to be comparable to the control cheddar it is uncertain why a lmHS combination would yield comparable results. It was noted that both high moisture varieties gave increased amounts of YPFPGPIPN than the control with 6.43 and 4.87 mg/100g for the HMHS and HMls varieties respectively. One likelihood is that the generation of this peptide is less impacted by salt concentration with a higher need for a certain moisture value however both cheddars have a lower moisture composition than the control cheddar which indicates that something besides this mechanism is enhancing the generation of this peptide.

Overall, the different parameters show that it is possible to enhance production of certain peptides through use of starter cultures, temperature and salt and moisture.

4 Future Work

Bioactive peptides in cheese offers a variety of opportunities for future work due to the vastness of cheese types and peptides to study.

This study was limited in time points meaning that the full profile of what bioactive peptides do during the ripening course of the cheddars was not fully realised. It is recommended to gather data for at least 12 months bearing in mind cheddar in commercial applications can be ripened up to 24 months or more before sale.

Further to this would be optimisation of processes in the extraction and sequencing of the bioactive peptides. It was established that the extraction process could benefit from clean-up to further remove proteins or large problem peptides or fractionation to improve downstream MS application and sequencing to allow better counting of bioactive peptides. Associated with this would be the potential benefit of investigating whether the extraction process can be optimised by seeing whether different extracting solvents, mechanical processes and column or eluent chemistries have an impact on the bioactive peptide profiles.

Improvements to the quantification process would include establishing limit of detection and limit of quantification to determine significance of those peptides that were in lower concentrations. As well as developing a spectral library of known bioactive peptides to navigate some of the challenges in using sequencing software.

This study did not focus on bioactivity assays or investigation into *in vitro* or *in vivo* impacts. These are critical data points to confirm previous study findings and build on them. Ultimately the goal is to use bioactive peptides in human application for health benefit so deepening the understanding of how these peptides work alone and in tandem with each other to provide an effect is crucial.

5 Conclusion

Cheese is an effective matrix for delivering bioactive compounds due to its wide global acceptance. Yet, cheese is complex, and not all manufacturing conditions have the same result. The small fraction of peptides that are bioactive creates a challenge in their identification and quantification.

This study demonstrated that cheddar manufacturing processes have a quantitative but not qualitative impact on bioactive peptides. The profile of bioactive sequences showed no significant variances between the cheddar samples yet there were significant differences in the quantities of MKPWIQPK, VLNENLLR and especially YPFPGPIPN. These differences were the result of starter culture selection and salt and moisture processing conditions. This proves that enhancement of bioactive peptides is possible during manufacturing. Deeper implications are the processes involved in attaining a sensory suitable cheese has implications for the reduction in certain bioactives due to their other attributes such as bitterness.

This research has deepened the understanding of bioactive peptides in cheese by providing insights into bioactive peptide profiles under different cheddar conditions from starter culture, temperature at ripening and the impacts of salt and moisture concentrations. This study also adds to the quantification studies by quantifying previously unquantified peptides in cheddar allowing a base point in which to proceed if bioactive concentration is required or other manufacturing conditions are assessed.

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7 Appendices

7.1. Result Data Tables

*Table 3. Nitrogen % Mean value (n=2) as performed by Kjeldahl assay on the WSE of the cheese samples. *indicates where a value is generated based on a singular result.*

	Starter Culture		Ripening Temperature (°C)					Salt:Moisture			
	CONTROL	ADJUNCT	-20	2	7	12	20	HMHS	HMls	lmHS	lmls
0	0.025 ±0.002	0.092*	0.025 ±0.002					0.012 ±0.001	0.014 ±0.003	0.006 ±0.027	0.010 ±0.003
1	0.049 ±0.015	0.072 ±0.020	0.032*	0.038 ±0.01	0.049 ±0.028	0.078 ±0.013	0.100*1 ±0.014	0.027 ±0.011	0.035 ±0.050	0.018 ±0.002	0.026 ±0.008
3	0.07 ±0.047	0.134 ±0.012	0.03 ±0.002	0.06 ±0.021	0.09*1 ±0.024	0.12*1 ±0.033	0.16 ±0.033	0.04 ±0.006	0.05 ±0.036	0.02 ±0.005	0.04 ±0.019

Table 4. Quantification of peptides MKPWIQPK, VLNENLLR and YPFPGPIPN in mg/100 g (n=9).

		CONTROL	**ADJUNCT**	**-20 °C**	**2 °C**	**7 °C**	**12 °C**	**20 °C**	**HMHS**	**HMLS**	**LMHS**	**LMLS**
MKPWIQPK	0	0.05 ± 0.001	0.05 ± 0.006	-					0.04 ± 0.001	0.05 ±0.001	0.04 ±0.001	0.03 ±0.002
	1	0.11 ± 0.002	0.15 ± 0.004	0.06 ± 0.001	0.07 ± 0.009	0.09 ± 0.005	0.14 ± 0.037	0.24 ± 0.038	0.05 ±0.004	0.07 ±0.014	0.02 ±0.002	0.04 ±0.002
	3	0.12 ± 0.004	0.13 ± 0.003	0.07 ± 0.004	0.1 ±0.005	0.13 ±0.008	0.26 ±0.014	0.54 ±0.037	0.04 ±0.001	0.15 ±0.005	0.09 ±0.003	0.07 ±0.003
VLNENLLR	0	0.04 ± 0.001	0.36 ± 0.047	-					0.01 ± 0	0.02 ± 0	0.01 ±0.002	0.02 ± 0
	1	0.15 ± 0.003	0.94 ± 0.030	0.15 ± 0.004	0.17 ± 0.024	0.18 ± 0.008	0.22 ± 0.052	0.42 ± 0.056	0.04 ±0.004	0.06 ±0.003	0.01 ±0.002	0.04 ±0.002
	3	0.25 ±0.014	0.05 ± 0.002	0.07 ±0.002	0.24 ±0.022	0.34 ±0.024	0.53 ±0.029	0.47 ±0.037	0.04 ±0.003	0.1 ±0.004	0.02 ±0.001	0.06 ±0.003
YPFPGPIPN	0	0.57 ± 0.056	0.24 ± 0.053	-					0.91 ± 0.022	0.59 ±0.058	0.56 ±0.015	0.33 ±0.079
	1	2.19 ± 0.022	0.36 ± 0.008	0.98 ± 0.023	1.95 ± 0.083	2.30 ± 0.11	3.01 ± 0.243	4.77 ± 0.155	3.46 ±0.265	2.33 ±0.2	1.97 ±0.067	1.63 ±0.089
	3	3.54 ±0.109	0.34 ± 0.009	0.79 ±0.037	2.58 ±0.131	4.31 ±0.19	6.47 ±0.168	7.76 ±0.821	6.43 ±0.269	4.87 ±0.24	3.10 ±0.173	2.94 ±0.129

7.2. List of peptides

Table 5. List of all bioactive peptides found in each cheddar sample. Peptide marked Y if present at any ripening time over the 3 months. - indicates peptide was not identified.

Bioactive Peptide Sequence	Fragment	Activity	Adjunct	Control						HMHS	HMls	lmHS	lmls
				–20 °C	2 °C	4 °C	7 °C	12 °C	20 °C				
AIPPKKNQD	κ-CN [128-136]	ACE-I	-	Y	Y	Y	Y	Y	Y	-	-	-	-
ALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{s2}-CN [190-222]	Antimicrobial	Y	-	-	-	-	-	-	-	-	-	-
AMKPWIQPK	α_{s2}-CN [204-212]	ACE-I	Y	Y	-	-	Y	Y	Y	-	Y	Y	Y
APSFSDIPNPIGSENSE	αs1-CN [191-207]	Antioxidant	-	Y	-	Y	Y	Y	Y	-	Y	-	-
ARHPHPHLSFM	κ-CN [117-127]	Antioxidant	-	-	-	-	-	-	Y	-	-	-	-
AVRSPAQILQWQ	κ-CN [87-98]	Antioxidant	-	-	-	-	-	-	-	-	Y	Y	-
DVENLHLPLPL	β-CN [144-154]	Antimicrobial	-	-	Y	Y	-	-	-	Y	-	Y	Y
EAMAPKHK	β-CN [115-122]	Antimicrobial	-	-	Y	-	-	-	-	-	-	-	-
EPVLGPVRGPFP	β-CN [210-221]	ACE-I	Y	Y	-	-	-	-	Y	Y	-	Y	-
FFVAPFPEVFGK	α_{s1}-CN [38-49]	ACE-I	-	-	-	Y	-	-	-	-	-	Y	-
FPEVFGK	α_{s1}-CN [43-49]	ACE-I	-	-	-	-	-	-	Y	-	-	-	-

Bioactive Peptide Sequence	Fragment	Activity	Adjunct	Control						HMHS	HMls	lmHS	lmls
				–20 °C	2 °C	4 °C	7 °C	12 °C	20 °C				
FPGPIPN	β-CN [77-83]	DPP-IV Inhibitory	Y	Y	Y	Y	Y	-	-	Y	Y	Y	Y
FPKYPVEPF	β-CN [126-134]	Antioxidant	Y	-	-	-	-	-	-	-	-	-	-
FQSEEQQQTEDELQDKIHPF	β-CN [48-67]	Increase Calcium Uptake	Y	-	-	-	-	-	-	-	-	-	-
FVAPFPEVFG	α_{s1}-CN [39-48]	ACE-I				Y							
GVSKVKEAMAPKH	β-CN [109-121]	Antimicrobial	Y	Y	Y	Y	-	Y	Y	-	-	-	-
HIQKEDVPSERYLGYLEQLLRLKKYK	α_{s1}-CN [95-120]	Antimicrobial							Y				
HKEMPFPK	β-CN [121-128]	Antimicrobial	Y	-	-	-	-	-	-	-	-	-	-
HQPHQPLPPT	β-CN [160-169]	ACE-I	Y	-	-	-	-	-	-	-	-	-	-
INNQFLPYPYYAKPA	κ-CN [72-86]	Antioxidant	Y	-	-	Y	Y	-	-	-	-	Y	Y
IQPKTKVIPYVR	α_{s2}-CN [209-220]	Antimicrobial	Y	Y	Y	-	-	Y	Y	Y	Y	Y	Y
IVLNPWDQVK	α_{s2}-CN [119-128]	Antimicrobial	Y	-	-	-	-	-	-	-	-	-	-
KAMKPWIQPKTKVIPYVRYL	α_{s2}-CN [203-222]	Antimicrobial	Y	Y	Y	Y	Y	Y	Y	Y	Y	-	Y
KTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{s2}-CN [196-222]	Antimicrobial	Y	Y	Y	Y	Y	Y	Y	Y	Y	-	Y

Bioactive Peptide Sequence	Fragment	Activity	Adjunct	Control						HMHS	HMls	lmHS	lmls
				–20 °C	2 °C	4 °C	7 °C	12 °C	20 °C				
KVLPVPQK	β-CN [184-191]	Antioxidant/ Immunomodulatory	-	-	Y	Y	-	-	Y	-	-	-	Y
LKKISQRYQKFALPQY	α_{s2}-CN [179-194]	Antimicrobial	Y	Y	Y	Y	Y	Y	Y	-	Y	-	Y
LKTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{s2}-CN [195-222]	Antimicrobial	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
LLYQEPVLGPVRGPFPIIV	β-CN [206-224]	ACE-I	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
LRLKKYKVPQL	α_{s1}-CN [114-124]	Antimicrobial	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
LVYPFPGPIPNSLPQ	β-CN [73-87]	ACE-I	Y	-	Y	-	-	-	-	Y	Y	-	Y
LYQEPVLGPVRGPFPIIV	β-CN [207-224]	Immunomodulatory	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
MKPWIQPK	α_{s2}-CN [205-212]	ACE-I	Y	-	-	-	Y	Y	-	-	-	-	-
NIPPLTQTPV	β-CN [88-97]	ACE-I	-	-	-	Y	-	-	-	-	-	-	-
NLHLPLPLL	β-CN [147-155]	ACE-I	Y	-	Y	-	-	-	-	-	-	-	-
PFPEVFGK	α_{s1}-CN [42-49]	ACE-I	-	Y	Y	Y	-	Y	Y	Y	Y	-	Y
PFPEVFGKE	α_{s1}-CN [42-50]	Antioxidant	-	-	-	-	-	Y	Y	-	-	-	-
QEPVLGPVRGPFPIIV	β-CN [209-224]	ACE-I	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Bioactive Peptide Sequence	Fragment	Activity	Adjunct	Control						HMHS	HMls	lmHS	lmls
				–20 °C	2 °C	4 °C	7 °C	12 °C	20 °C				
QYVLSRYPSYGLN	ϰ-CN [50-62]	Antioxidant	-	-	-	-	-	-	-	-	-	Y	-
RFFVAPFPEVFGKEKVNEL	α_{s1}-CN [37-55]	Osteoanabolic	-	-	-	-	-	-	-	-	Y	-	Y
RPKHPIKHQ	α_{s1}-CN [16-24]	ACE-I	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RPKHPIKHQGLPQEVLNENLLRF	α_{s1}-CN [16-38]	Antimicrobial / Immunomodulatory	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RPKHPIKHQGLPQEVLNENLLRFF	α_{s1}-CN [16-39]	Antimicrobial	Y	Y	Y	Y	Y	Y	Y	-	Y	Y	Y
SQSKVLPVPQKAVPYPQ	β-CN [181-197]	Antioxidant	Y	Y	Y	Y	-	Y	Y	-	Y	-	-
SSSEESITRIN	β-CN [32-42]	Antimicrobial	Y	-	-	-	-	-	-	-	-	-	-
SWMHQPHQPLPPT	β-CN [157-169]	Antioxidant	-	Y	-	-	-	-	-	Y	Y	Y	Y
TKKTKLTEEEKNRL	α_{s2}-CN [163-176]	Antimicrobial	Y	-	-	Y	-	Y	Y	-	-	-	-
TKVIPYVRYL	α_{s2}-CN [213-222]	Antimicrobial	-	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
TPVVVPPFLQP	β-CN [95-105]	ACE-I	Y	Y	Y	-	-	Y	Y	-	Y	Y	Y
TQTPVVVPPFLQPE	β-CN [93-106]	Antioxidant	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Bioactive Peptide Sequence	Fragment	Activity	Adjunct	Control						HMHS	HMls	lmHS	lmls
				–20 °C	2 °C	4 °C	7 °C	12 °C	20 °C				
TVYQHQKAMKPWIQPKTKVIPYVRYL	α_{s2}-CN [197-222]	Antimicrobial	-	Y	Y	Y	Y	Y	Y	-	Y	-	Y
VAPFPE	α_{s1}-CN [40-45]	Cholesterol Regulation	Y	-	-	-	-	-	-	-	-	-	-
VKEAMAPK	β-CN [113-120]	Antimicrobial/Antioxidant	-	Y	Y	Y	Y	Y	Y	-	Y	-	Y
VLGPVRGPFP	β-CN [212-221]	ACE-I	Y	-	-	-	-	-	-	-	-	-	-
VLNENLLR	α_{s1}-CN [30-37]	Antimicrobial	Y	Y	Y	Y	Y	Y	Y	-	Y	-	-
VYPFPGPIPN	β-CN [74-83	ACE-I/Antioxidant	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
VYQHQKAMKPWIQPKTKVIPYVRYL	α_{s2}-CN [198-222]	Antimicrobial	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
YPFPGPI	β-CN [75-81]	ACE-I/Antianxiety/Anticancer/ Antioxidant/ Immunomodulatory/ Increase mucin secretion/Opioid/Satiety	-	-	-	-	-	-	-	Y	-	-	-
YPFPGPIPN	β-CN [75-83]	ACE-I/Antioxidant/DPP-IV Inhibitory	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
YQEPVLGPVR	β-CN [208-217]	ACE-I/Antioxidant/ Antithrombotic/ Immunomodulatory	Y	-	-	-	-	-	-	-	-	-	-

Bioactive Peptide Sequence	Fragment	Activity	Adjunct	Control						HMHS	HMls	lmHS	lmls
				–20 °C	2 °C	4 °C	7 °C	12 °C	20 °C				
YQEPVLGPVRGPFPIIV	β-CN [208-224]	ACE-I/Anticancer/ Antimicrobial/Antithrombotic/ Immunomodulatory	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
YQKFPQYLQY	α_{s2}-CN [104-113]	ACE-I	-	Y	Y	Y	Y	Y	Y	-	Y	Y	Y

Mass Spectrometry Optimisation

7.2.1. Ionisation Parameters

Optimising the ionisation settings is a key step in acquiring the best group of peptides. While it is currently impossible to use optimise the settings to attain all possible peptides, it is valuable to select the settings that gets a good range of those present. Ionisation parameters were tested by adjusting sheath gas flow, spray voltage and mobile phase solvent. A mixed peptide standard at 0.5 ug/mL and a Cheese matrix sample were subjected to the changes and the peak area for YPFPGPIPN, VLNENLLR and MKPWIQPK in the standard and the number of bioactive peptides found in the cheese matrix were used to determine impact.

7.2.1.1. Mobile Phase

There are several mobile phase combinations used for peptide separation prior to ionisation including of 0.1% formic acid (FA) in water with 0.1% FA in acetonitrile [29, 53] or just plain acetonitrile. Some omitted the 0.1% FA altogether [12]. Others prefer the use of TFA instead of formic acid [30] however this is considered a "sticky" substance and often lingers in the MS, because of this it was not included in this experiment. Instead, the effects of inclusion vs exclusion of 0.1 % formic acid in the mobile phase B was tested.

It was found that 0.1% FA in acetonitrile overall gave a better response in terms of peak area and bioactive peptide count regardless of changes to sheath gas or voltage. This difference was most notable with MKPWIQPK with an average difference of +20% peak area. However the differences were more subtle for VLNENLLR and YPFPGPIPN with an average increase in signal of 5% and 3.5% respectively. For this reason, a mobile phase of acetonitrile without the 0.1% FA addition was used. It was also noted that at higher voltages the signal intensity diminished significantly by over 100% for all peptides with a mobile phase of 0.1% FA in acetonitrile which was not seen with just acetonitrile.

7.2.1.2. Sheath Gas

Sheath gas is responsible for the nebulisation of the sample into a fine spray. Aided by the auxiliary and sweep gases; how the sample forms these droplets impacts how each peptide ionises and whether they are allowed passage into the MS. It was determined that sheath gas being the primary mode of nebulisation would have the most impact therefore the effects of 3 sheath gas flow rates was investigated: 40, 50 and 60 arbitrary units.

It was found that a lower flow rate for sheath gas resulted in a higher signal response but that not a higher peptide count. A sheath gas of 50 with the chosen mobile phases yielded the highest

peptide count with a sheath gas of 60 being the lowest. For this reason, a sheath gas flow rate of 50 was chosen.

7.2.1.3. Spray Voltage

Spray voltage is what applies a charge to each peptide allowing them to ionise and be measured by the MS. Too little and the peptides may not ionise while too much and they may fragment before the MS_1 scan convoluting the data. The effects of 6 spray voltages from 3500V to 4000V was assessed.

With the chosen mobile phase very little difference was observed with the signal. Peptide count does appear to be affected with the highest counts being found around 3600-3700V. For this reason, 3700V was chosen to proceed with.

7.3. Proteome Discoverer Processing Workflow

7.3.1. Processing Workflow

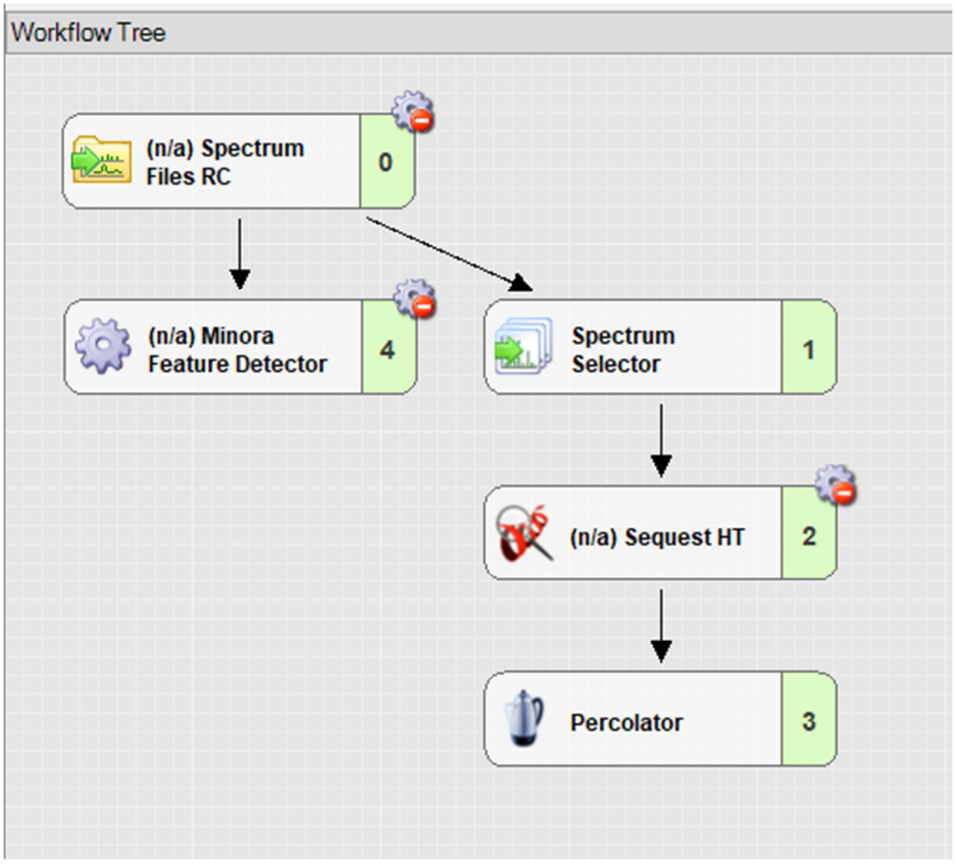

Figure 32. Proteome Discoverer v2.5 Processing Workflow

7.3.2. Consensus Workflow

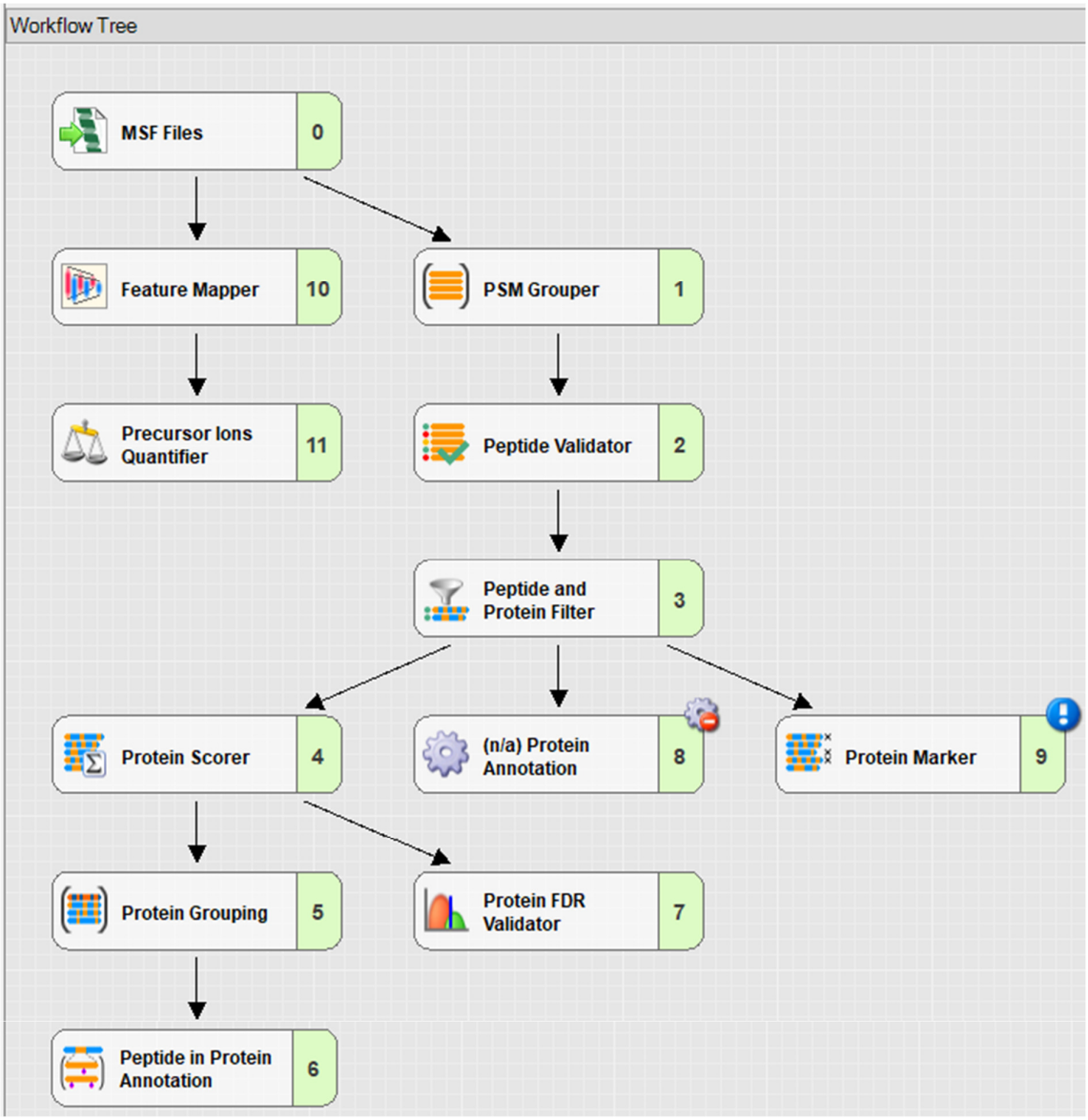

Figure 33. Proteome Discoverer v2.5 Consensus Workflow.

7.4. Proteome Discoverer Software Reproducibility

Due to the "black box" nature of a lot of processing software and the algorithms. It was of interest to determine performance of Proteome Discoverer (PD) and its reproducibility with the same datasets. This was determined by running an individual cheese WSE in triplicate through the MS. The three attained raw MS data files were then digitally copied to determine how PD would handle an exact spectral replica – repeatability in other words. These six files were then run through the PD processing workflow three separate times to determine whether PD handles the same data

consistently – reproducibility if you will. The results of the three separate runs were processed in the same manner as stated previously to attain peptides meeting the quality suitability requirements.

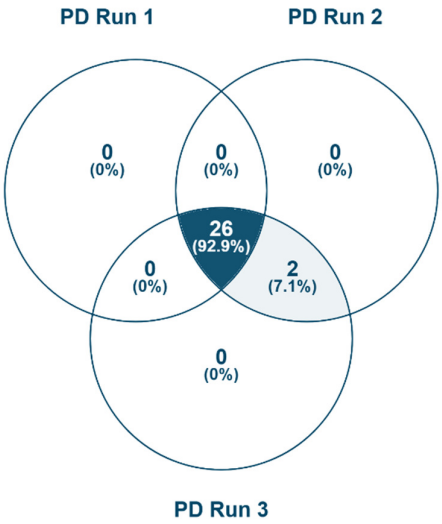

Figure 34. Venn diagram of peptides determined in each PD run.

Figure 34 demonstrates that there is not a 100% correlation in peptides acquired through the PD processing and consensus workflows used. The second and third runs agree that there are an extra two peptides present in the sample tested. These two peptides were discovered by PD however but did not meet the required hit rate across the 3 unique raw MS files or did not meet the Xcorr of >2 for acceptable spectral matching. This indicates that the way peptides were attained in this thesis has an error margin of possibly ± 2 peptides due to the filtering processes for determining quality peptides not necessarily from the PD algorithm. More interestingly however was the relative abundance variation of some peptides between the duplicate raw MS files. It was expected that these should be determined the same as the MS data is identical. Instead, it was found that the algorithm was not consistent with which charge state of the peptide it read. This led to large variation in relative abundances making this less reliable in comparing the wider peptides found between samples.

7.5. ANOVAs

7.5.1. Bioactive peptide count

7.5.1.1. Adjunct vs Control

BIOACTIVE PEPTIDE COUNT
Starter Culture: 2-Way ANOVA [Bioactive Peptide Count]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Time_1	Fixed	3	0, 1, 3
Temperature_1	Fixed	2	4, 12
Cheddar_1	Fixed	2	Adjunct, Control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time_1	2	40.7500	20.3750	2.31	0.247
Temperature_1	1	2.7348	2.7348	0.31	0.616
Cheddar_1	1	0.9167	0.9167	0.10	0.768
Error	3	26.4167	8.8056		
Total	7	79.8750			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.96742	66.93%	22.83%	0.00%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	25.67	1.21	21.19	0.000	
Time_1					
0	-3.17	1.71	-1.85	0.162	1.63
1	0.25	1.48	0.17	0.877	1.50
Temperature_1					
4	-0.79	1.42	-0.56	0.616	1.72
Cheddar_1					
Adjunct	-0.46	1.42	-0.32	0.768	1.72

Regression Equation

Bioactive Peptide_1 = 25.67 - 3.17 Time_1_0 + 0.25 Time_1_1 + 2.92 Time_1_3 - 0.79 Temperature_1_4 + 0.79 Temperature_1_12 - 0.46 Cheddar_1_Adjunct + 0.46 Cheddar_1_Control

BIOACTIVE PEPTIDE COUNT
Starter Culture: Tukey Pairwise Comparison [Bioactive Peptide Count]

Tukey Pairwise Comparisons: Time_1

Grouping Information Using the Tukey Method and 95% Confidence

Time_1	N	Mean	Grouping
3	3	28.5833	A
1	3	25.9167	A
0	2	22.5000	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature_1

Grouping Information Using the Tukey Method and 95% Confidence

Temperature_1	N	Mean	Grouping
12	5	26.4583	A
4	3	24.8750	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Cheddar_1

Grouping Information Using the Tukey Method and 95% Confidence

Cheddar_1	N	Mean	Grouping
Control	5	26.1250	A
Adjunct	3	25.2083	A

Means that do not share a letter are significantly different.

7.5.1.2. Time vs Temperature

BIOACTIVE PEPTIDE COUNT
Temperature: 2-Way ANOVA [Bioactive Peptide Count]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Time	Fixed	3	0, 1, 3
Temperature	Fixed	6	-20, 2, 4, 7, 12, 20

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	2	33.44	16.722	1.85	0.208
Temperature	5	67.78	13.556	1.50	0.274
Error	10	90.56	9.056		
Total	17	191.78			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.00925	52.78%	19.73%	0.00%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	25.889	0.709	36.50	0.000	
Time					
0	-1.89	1.00	-1.88	0.089	1.33
1	1.28	1.00	1.27	0.232	1.33
Temperature					
-20	-3.22	1.59	-2.03	0.070	1.67
2	0.44	1.59	0.28	0.785	1.67
4	-0.56	1.59	-0.35	0.733	1.67
7	-0.89	1.59	-0.56	0.588	1.67
12	1.11	1.59	0.70	0.500	1.67

Regression Equation

Bioactive Peptide = 25.889 - 1.89 Time_0 + 1.28 Time_1 + 0.61 Time_3
- 3.22 Temperature_-20
+ 0.44 Temperature_2 - 0.56 Temperature_4 - 0.89 Temperature_7
+ 1.11 Temperature_12 + 3.11 Temperature_20

BIOACTIVE PEPTIDE COUNT
Temperature: Tukey Pairwise Comparison [Bioactive Peptide Count]

Tukey Pairwise Comparisons: Time

Grouping Information Using the Tukey Method and 95% Confidence

Time	N	Mean	Grouping
1	6	27.1667	A
3	6	26.5000	A
0	6	24.0000	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping
20	3	29.0000	A
12	3	27.0000	A
2	3	26.3333	A
4	3	25.3333	A
7	3	25.0000	A
-20	3	22.6667	A

Means that do not share a letter are significantly different.

7.5.1.3. Salt and moisture

BIOACTIVE PEPTIDE COUNT
Salt and moisture: 2-Way ANOVA [Bioactive Peptide Count]

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Cheddar_2_1	Fixed	5	Control, HMHS, HMls, lmHS, lmls
Time_2_1	Fixed	3	0, 1, 3

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cheddar_2_1	4	240.93	60.233	6.74	0.011
Time_2_1	2	25.20	12.600	1.41	0.299
Error	8	71.47	8.933		
Total	14	337.60			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.98887	78.83%	62.95%	25.58%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	20.600	0.772	26.69	0.000	
Cheddar_2_1					
Control	4.73	1.54	3.07	0.015	1.60
HMHS	-2.93	1.54	-1.90	0.094	1.60
HMls	2.40	1.54	1.55	0.159	1.60
lmHS	-6.27	1.54	-4.06	0.004	1.60
Time_2_1					
0	-1.20	1.09	-1.10	0.304	1.33
1	1.80	1.09	1.65	0.138	1.33

Regression Equation

Bioactive Peptide_2_1 = 20.600 + 4.73 Cheddar_2_1_Control - 2.93 Cheddar_2_1_HMHS + 2.40 Cheddar_2_1_HMls - 6.27 Cheddar_2_1_lmHS + 2.07 Cheddar_2_1_lmls - 1.20 Time_2_1_0 + 1.80 Time_2_1_1 - 0.60 Time_2_1_3

Fits and Diagnostics for Unusual Observations

Obs	Bioactive Peptide_2_1	Fit	Resid	Std Resid	
9	27.00	22.40	4.60	2.11	R

R Large residual

BIOACTIVE PEPTIDE COUNT
Salt and moisture: Tukey Pairwise Comparison [Bioactive Peptide Count]

Tukey Pairwise Comparisons: Cheddar_2_1

Grouping Information Using the Tukey Method and 95% Confidence

Cheddar_2_1	N	Mean	Grouping	
Control	3	25.3333	A	
HMls	3	23.0000	A	
lmls	3	22.6667	A	B
HMHS	3	17.6667	A	B
lmHS	3	14.3333		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Time_2_1

Grouping Information Using the Tukey Method and 95% Confidence

Time_2_1	N	Mean	Grouping
1	5	22.4	A
3	5	20.0	A
0	5	19.4	A

Means that do not share a letter are significantly different.

7.5.2. Proteolysis

7.5.2.1. Adjunct vs Control

PROTEOLYSIS
Starter Culture: 2-Way ANOVA [Proteolysis]

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Time_2	Fixed	3	0, 1, 3
Temperature_2	Fixed	2	4, 12
Cheddar_2	Fixed	2	Adjunct, Control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time_2	2	0.006480	0.003240	23.77	0.000
Temperature_2	1	0.004315	0.004315	31.65	0.000
Cheddar_2	1	0.000076	0.000076	0.56	0.472
Error	10	0.001363	0.000136		
Lack-of-Fit	3	0.001316	0.000439	64.64	0.000
Pure Error	7	0.000048	0.000007		
Total	14	0.018399			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0116764	92.59%	89.63%	83.06%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.07198	0.00358	20.13	0.000	
Time_2					
0	-0.01755	0.00533	-3.29	0.008	1.75
1	-0.01164	0.00430	-2.71	0.022	1.62
Temperature_2					
4	-0.02249	0.00400	-5.63	0.000	1.69
Cheddar_2					
Adjunct	0.00299	0.00400	0.75	0.472	1.56

Regression Equation

WSN_2 = 0.07198 - 0.01755 Time_2_0 - 0.01164 Time_2_1 + 0.02919 Time_2_3
- 0.02249 Temperature_2_4 + 0.02249 Temperature_2_12
+ 0.00299 Cheddar_2_Adjunct
- 0.00299 Cheddar_2_Control

PROTEOLYSIS
Starter Culture: Tukey Pairwise Comparison [Proteolysis]

Tukey Pairwise Comparisons: Time_2

Grouping Information Using the Tukey Method and 95% Confidence

Time_2	N	Mean	Grouping
3	6	0.101167	A
1	6	0.060333	B
0	3	0.054425	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature_2

Grouping Information Using the Tukey Method and 95% Confidence

Temperature_2	N	Mean	Grouping
12	9	0.0944625	A
4	6	0.0494875	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Cheddar_2

Grouping Information Using the Tukey Method and 95% Confidence

Cheddar_2	N	Mean	Grouping
Adjunct	5	0.0749625	A
Control	10	0.0689875	A

Means that do not share a letter are significantly different.

7.5.2.2. Time vs Temperature

PROTEOLYSIS
Temperature: 2-Way ANOVA [Proteolysis]

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Time_3	Fixed	3	0, 1, 3
Temperature_3	Fixed	6	-20, 2, 4, 7, 12, 20

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time_3	2	0.007577	0.003789	25.10	0.000
Temperature_3	5	0.027327	0.005465	36.20	0.000
Error	17	0.002566	0.000151		
Lack-of-Fit	5	0.002489	0.000498	77.59	0.000
Pure Error	12	0.000077	0.000006		
Total	24	0.039266			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0122866	93.46%	90.77%	85.05%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.06017	0.00388	15.51	0.000	
Time_3					
0	-0.02350	0.00709	-3.31	0.004	3.33
1	-0.00441	0.00439	-1.01	0.329	2.93
Temperature_3					
-20	-0.04964	0.00638	-7.78	0.000	1.88
2	-0.02142	0.00564	-3.80	0.001	1.69
4	-0.01367	0.00564	-2.42	0.027	2.07
7	-0.00342	0.00564	-0.61	0.552	1.69
12	0.02833	0.00564	5.02	0.000	1.69

Regression Equation

WSN_3 = 0.06017 - 0.02350 Time_3_0 - 0.00441 Time_3_1 + 0.02791 Time_3_3
- 0.04964 Temperature_3_-20 - 0.02142 Temperature_3_2
- 0.01367 Temperature_3_4
- 0.00342 Temperature_3_7 + 0.02833 Temperature_3_12
+ 0.05983 Temperature_3_20

Fits and Diagnostics for Unusual Observations

Obs	WSN_3	Fit	Resid	Std Resid	
1	0.03200	0.00612	0.02588	2.75	R

R Large residual

PROTEOLYSIS
Temperature: Tukey Pairwise Comparison [Proteolysis]

Tukey Pairwise Comparisons: Time_3

Grouping Information Using the Tukey Method and 95% Confidence

Time_3	N	Mean	Grouping
3	12	0.0880833	A
1	11	0.0557598	B
0	2	0.0366716	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature_3

Grouping Information Using the Tukey Method and 95% Confidence

Temperature_3	N	Mean	Grouping		
20	4	0.120000	A		
12	4	0.088500		B	
7	4	0.056750			C
4	6	0.046500			C
2	4	0.038750			C D
-20	3	0.010529			D

Means that do not share a letter are significantly different.

7.5.2.3. Salt and moisture

PROTEOLYSIS
Salt and moisture: 2-Way ANOVA [Proteolysis]

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Time_1	Fixed	3	0, 1, 3
Cheddar_1	Fixed	4	HMHS, HMls, lmHS, lmls

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time_1	2	0.003009	0.001504	46.38	0.000
Cheddar_1	3	0.000845	0.000282	8.69	0.001
Error	18	0.000584	0.000032		
Lack-of-Fit	6	0.000520	0.000087	16.24	0.000
Pure Error	12	0.000064	0.000005		
Total	23	0.004438			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0056952	86.84%	83.19%	76.61%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.02483	0.00116	21.36	0.000	
Time_1					
0	-0.01448	0.00164	-8.81	0.000	1.33
1	0.00168	0.00164	1.02	0.319	1.33
Cheddar_1					
HMHS	0.00151	0.00201	0.75	0.463	1.50
HMls	0.00907	0.00201	4.50	0.000	1.50
lmHS	-0.00484	0.00201	-2.40	0.027	1.50

Regression Equation

WSN_1 = 0.02483 - 0.01448 Time_1_0 + 0.00168 Time_1_1 + 0.01279 Time_1_3
+ 0.00151 Cheddar_1_HMHS + 0.00907 Cheddar_1_HMls
- 0.00484 Cheddar_1_lmHS
- 0.00574 Cheddar_1_lmls

Fits and Diagnostics for Unusual Observations

Obs	WSN_1	Fit	Resid	Std Resid	
18	0.02003	0.03189	-0.01186	-2.40	R

R Large residual

PROTEOLYSIS
Salt and moisture: Tukey Pairwise Comparison [Proteolysis]

Tukey Pairwise Comparisons: Time_1

Grouping Information Using the Tukey Method and 95% Confidence

Time_1	N	Mean	Grouping		
3	8	0.0376253	A		
1	8	0.0265179		B	
0	8	0.0103558			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Cheddar_1

Grouping Information Using the Tukey Method and 95% Confidence

Cheddar_1	N	Mean	Grouping	
HMls	6	0.0339022	A	
HMHS	6	0.0263415	A	B
lmHS	6	0.0199936		B
lmls	6	0.0190946		B

Means that do not share a letter are significantly different.

7.5.3. MKPWIPQK

7.5.3.1. Adjunct vs Control

MKPWIQPK
Starter Culture: 2-Way ANOVA [mg/100g MKPWIQPK]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Cheddar	Fixed	2	Adjunct, Control
Time	Fixed	3	0, 1, 3
Temperature	Fixed	2	4, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cheddar	1	0.04778	0.047775	42.95	0.000
Time	2	0.08231	0.041154	37.00	0.000
Temperature	1	0.06785	0.067849	60.99	0.000
Error	67	0.07453	0.001112		
Lack-of-Fit	3	0.05195	0.017316	49.08	0.000
Pure Error	64	0.02258	0.000353		
Total	71	0.29635			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0333524	74.85%	73.35%	70.81%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.10190	0.00454	22.45	0.000	
Cheddar					
Adjunct	-0.03488	0.00532	-6.55	0.000	1.72
Time					
0	-0.04866	0.00642	-7.58	0.000	1.62

1	0.00588	0.00556	1.06	0.294	1.50
Temperature					
4	-0.04156	0.00532	-7.81	0.000	1.72

Regression Equation

MKPWIQPK = 0.10190 - 0.03488 Cheddar_Adjunct + 0.03488 Cheddar_Control - 0.04866 Time_0 + 0.00588 Time_1 + 0.04278 Time_3 - 0.04156 Temperature_4 + 0.04156 Temperature_12

Fits and Diagnostics for Unusual Observations

Obs	MKPWIQPK	Fit	Resid	Std Resid	
57	0.08776	0.18422	-0.09646	-3.01	R
58	0.09792	0.18422	-0.08631	-2.69	R
62	0.08538	0.18422	-0.09884	-3.08	R
63	0.10201	0.18422	-0.08221	-2.56	R
64	0.29241	0.22112	0.07129	2.22	R
65	0.29039	0.22112	0.06928	2.16	R

R Large residual

MKPWIQPK
Starter Culture: Tukey Pairwise Comparison [mg/100g MKPWIQPK]

Tukey Pairwise Comparisons: Cheddar

Grouping Information Using the Tukey Method and 95% Confidence

Cheddar	N	Mean	Grouping	
Control	45	0.136774	A	
Adjunct	27	0.067017		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Time

Grouping Information Using the Tukey Method and 95% Confidence

Time	N	Mean	Grouping		
3	27	0.144673	A		
1	27	0.107779		B	
0	18	0.053234			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping	
12	45	0.143460	A	
4	27	0.060331		B

Means that do not share a letter are significantly different.

7.5.3.2. Time vs Temperature

MKPWIQPK
Temperature: 2-Way ANOVA [mg/100g MKPWIQPK]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Time_1	Fixed	3	0, 1, 3
Temperature_1	Fixed	6	-20, 2, 4, 7, 12, 20

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time_1	2	0.21559	0.107797	33.25	0.000
Temperature_1	5	1.34468	0.268936	82.97	0.000
Error	109	0.35333	0.003242		
Lack-of-Fit	5	0.29078	0.058155	96.69	0.000
Pure Error	104	0.06255	0.000601		
Total	116	1.98799			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0569345	82.23%	81.09%	79.68%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.14231	0.00837	17.00	0.000	
Time_1					
0	-0.0404	0.0155	-2.61	0.010	3.38
1	-0.02211	0.00949	-2.33	0.022	3.00
Temperature_1					
-20	-0.0996	0.0123	-8.13	0.000	1.67
2	-0.0736	0.0123	-6.01	0.000	1.67
4	-0.0471	0.0123	-3.84	0.000	2.05
7	-0.0501	0.0123	-4.09	0.000	1.67
12	0.0401	0.0123	3.28	0.001	1.67

Regression Equation

MKPWIQPK_1 = 0.14231 - 0.0404 Time_1_0 - 0.02211 Time_1_1 + 0.06255 Time_1_3
- 0.0996 Temperature_1_-20 - 0.0736 Temperature_1_2
- 0.0471 Temperature_1_4
- 0.0501 Temperature_1_7 + 0.0401 Temperature_1_12
+ 0.2303 Temperature_1_20

Fits and Diagnostics for Unusual Observations

Obs	MKPWIQPK_1	Fit	Resid	Std Resid	
100	0.2138	0.3505	-0.1367	-2.48	R
103	0.2257	0.3505	-0.1248	-2.27	R
106	0.1860	0.3505	-0.1645	-2.99	R
107	0.1754	0.3505	-0.1750	-3.18	R
108	0.2317	0.3505	-0.1187	-2.16	R
109	0.6387	0.4351	0.2036	3.70	R
110	0.5980	0.4351	0.1629	2.96	R
111	0.5481	0.4351	0.1130	2.05	R
112	0.5483	0.4351	0.1132	2.06	R

R Large residual

MKPWIQPK
Temperature: Tukey Pairwise Comparison [mg/100g MKPWIQPK]

Tukey Pairwise Comparisons: Time_1

Grouping Information Using the Tukey Method and 95% Confidence

Time_1	N	Mean	Grouping	
3	54	0.204857	A	
1	54	0.120198		B
0	9	0.101863		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature_1

Grouping Information Using the Tukey Method and 95% Confidence

Temperature_1	N	Mean	Grouping		
20	18	0.372575	A		
12	18	0.182447		B	
4	27	0.095209			C
7	18	0.092207			C
2	18	0.068736			C
-20	18	0.042663			C

Means that do not share a letter are significantly different.

7.5.3.3. Salt and moisture

MKPWIQPK
Salt and moisture: 2-Way ANOVA [mg/100g MKPWIQPK]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Cheese Type_2	Fixed	5	Control, HMHS, HMls, lmHS, lmls
Time_2	Fixed	3	0, 1, 3

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cheese Type_2	4	0.095203	0.023801	63.60	0.000
Time_2	2	0.035161	0.017581	46.98	0.000
Error	128	0.047898	0.000374		
Lack-of-Fit	8	0.044190	0.005524	178.76	0.000
Pure Error	120	0.003708	0.000031		
Total	134	0.178262			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0193444	73.13%	71.87%	70.11%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.06061	0.00166	36.41	0.000	
Cheese Type_2					
Control	0.03460	0.00333	10.39	0.000	1.60
HMHS	-0.01838	0.00333	-5.52	0.000	1.60
HMls	0.02902	0.00333	8.72	0.000	1.60
lmHS	-0.03055	0.00333	-9.17	0.000	1.60
Time_2					
0	-0.01888	0.00235	-8.02	0.000	1.33
1	-0.00167	0.00235	-0.71	0.479	1.33

Regression Equation

MKPWIQPK_2 = 0.06061 + 0.03460 Cheese Type_2_Control
- 0.01838 Cheese Type_2_HMHS
+ 0.02902 Cheese Type_2_HMls - 0.03055 Cheese Type_2_lmHS

- 0.01469 Cheese Type_2_lmls - 0.01888 Time_2_0 - 0.00167 Time_2_1
+ 0.02055 Time_2_3

Fits and Diagnostics for Unusual Observations

Obs	MKPWIQPK_2	Fit	Resid	Std Resid	
38	0.04650	0.08797	-0.04146	-2.20	R
41	0.04799	0.08797	-0.03998	-2.12	R
44	0.04520	0.08797	-0.04276	-2.27	R
46	0.15381	0.11018	0.04362	2.32	R
48	0.15003	0.11018	0.03984	2.12	R
49	0.14988	0.11018	0.03969	2.11	R
51	0.15433	0.11018	0.04415	2.34	R
52	0.15389	0.11018	0.04370	2.32	R

R Large residual

MKPWIQPK
Salt and moisture: Tukey Pairwise Comparison [mg/100g MKPWIQPK]

Tukey Pairwise Comparisons: Cheese Type_2

Grouping Information Using the Tukey Method and 95% Confidence

Cheese Type_2	N	Mean	Grouping		
Control	27	0.0952088	A		
HMls	27	0.0896368	A		
lmls	27	0.0459205		B	
HMHS	27	0.0422361		B	C
lmHS	27	0.0300647			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Time_2

Grouping Information Using the Tukey Method and 95% Confidence

Time_2	N	Mean	Grouping		
3	45	0.0811615	A		
1	45	0.0589423		B	
0	45	0.0417364			C

Means that do not share a letter are significantly different.

7.5.4. VLNENLLR

7.5.4.1. Adjunct vs Control

VLNENLLR
Starter Culture: 2-Way ANOVA [mg/100g VLNENLLR]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Cheese Type	Fixed	2	Adjunct, Control
Time	Fixed	3	0, 1, 3
Temperature	Fixed	2	4, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cheese Type	1	0.16522	0.16522	2.93	0.092
Time	2	0.61500	0.30750	5.44	0.006
Temperature	1	0.31127	0.31127	5.51	0.022
Error	67	3.78377	0.05647		
Lack-of-Fit	3	3.69152	1.23051	853.74	0.000
Pure Error	64	0.09224	0.00144		
Total	71	5.75203			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.237643	34.22%	30.29%	24.36%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.2980	0.0323	9.21	0.000	
Cheese Type					
Adjunct	0.0649	0.0379	1.71	0.092	1.72
Time					
0	-0.1002	0.0457	-2.19	0.032	1.63
1	0.1299	0.0396	3.28	0.002	1.50
Temperature					
4	-0.0890	0.0379	-2.35	0.022	1.72

Regression Equation

VLNENLLR = 0.2980 + 0.0649 Cheese Type_Adjunct - 0.0649 Cheese Type_Control
- 0.1002 Time_0
+ 0.1299 Time_1 - 0.0297 Time_3 - 0.0890 Temperature_4
+ 0.0890 Temperature_12

VLNENLLR
Starter Culture: Tukey Pairwise Comparison [mg/100g VLNENLLR]

Tukey Pairwise Comparisons: Cheese Type

Grouping Information Using the Tukey Method and 95% Confidence

Cheese Type	N	Mean	Grouping
Adjunct	27	0.362824	A
Control	45	0.233102	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Time

Grouping Information Using the Tukey Method and 95% Confidence

Time	N	Mean	Grouping	
1	27	0.427870	A	
3	27	0.268241		B
0	18	0.197778		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping	
12	45	0.386991	A	
4	27	0.208935		B

Means that do not share a letter are significantly different.

7.5.4.2. Time vs Temperature

VLNENLLR
Temperature: 2-Way ANOVA [mg/100g VLNENLLR]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Time_1	Fixed	3	0, 1, 3
Temperature_1	Fixed	6	-20, 2, 4, 7, 12, 20

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time_1	2	0.4231	0.211563	46.36	0.000
Temperature_1	5	1.3238	0.264753	58.01	0.000
Error	107	0.4883	0.004564		
Lack-of-Fit	5	0.3675	0.073495	62.04	0.000
Pure Error	102	0.1208	0.001185		
Total	114	2.5297			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0675544	80.70%	79.43%	77.89%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.21283	0.00997	21.34	0.000	
Time_1					
0	-0.1041	0.0184	-5.66	0.000	3.35
1	0.0009	0.0113	0.08	0.937	2.96
Temperature_1					
-20	-0.1549	0.0146	-10.63	0.000	1.63
2	-0.0619	0.0149	-4.15	0.000	1.66
4	-0.0688	0.0146	-4.72	0.000	2.00
7	0.0007	0.0146	0.05	0.962	1.63
12	0.1074	0.0146	7.37	0.000	1.63

Regression Equation

VLNENLLR_1 = 0.21283 - 0.1041 Time_1_0 + 0.0009 Time_1_1 + 0.1032 Time_1_3
- 0.1549 Temperature_1_-20 - 0.0619 Temperature_1_2
- 0.0688 Temperature_1_4
+ 0.0007 Temperature_1_7 + 0.1074 Temperature_1_12
+ 0.1774 Temperature_1_20

Fits and Diagnostics for Unusual Observations

Obs	VLNENLLR_1	Fit	Resid	Std Resid	
81	0.1900	0.3211	-0.1311	-2.01	R
83	0.1600	0.3211	-0.1611	-2.47	R
84	0.1800	0.3211	-0.1411	-2.16	R
88	0.1400	0.3211	-0.1811	-2.77	R
89	0.1600	0.3211	-0.1611	-2.47	R
90	0.5600	0.4234	0.1366	2.09	R
91	0.5900	0.4234	0.1666	2.55	R

R Large residual

VLNENLLR
Temperature: Tukey Pairwise Comparison [mg/100g VLNENLLR]

Tukey Pairwise Comparisons: Time_1

Grouping Information Using the Tukey Method and 95% Confidence

Time_1	N	Mean	Grouping		
3	53	0.316003	A		
1	53	0.213725		B	
0	9	0.108753			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature_1

Grouping Information Using the Tukey Method and 95% Confidence

Temperature_1	N	Mean	Grouping			
20	17	0.390249	A			
12	18	0.320185		B		
7	18	0.213519			C	
2	17	0.150971			C	D
4	27	0.144074				D
-20	18	0.057963				E

Means that do not share a letter are significantly different.

7.5.4.3. Salt and moisture

VLNENLLR
Salt and moisture: 2-Way ANOVA [mg/100g VLNENLLR]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Cheese	Fixed	5	Control, HMHS, HMls, lmHS, lmls
Type_2			
Time_2	Fixed	3	0, 1, 3

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cheese	4	0.274965	0.068741	77.89	0.000
Type_2					
Time_2	2	0.124073	0.062037	70.29	0.000
Error	128	0.112972	0.000883		
Lack-of-Fit	8	0.109203	0.013650	434.64	0.000
Pure Error	120	0.003769	0.000031		
Total	134	0.512009			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0297084	77.94%	76.90%	75.46%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.05796	0.00256	22.67	0.000	
Cheese					
Type_2					
Control	0.08612	0.00511	16.84	0.000	1.60
HMHS	-0.02738	0.00511	-5.35	0.000	1.60
HMls	-0.00021	0.00511	-0.04	0.968	1.60
lmHS	-0.04162	0.00511	-8.14	0.000	1.60
Time_2					
0	-0.03766	0.00362	-10.42	0.000	1.33
1	0.00109	0.00362	0.30	0.763	1.33

Regression Equation

VLNENLLR_2 = 0.05796 + 0.08612 Cheese Type_2_Control
- 0.02738 Cheese Type_2_HMHS
- 0.00021 Cheese Type_2_HMls - 0.04162 Cheese Type_2_lmHS
- 0.01691 Cheese Type_2_lmls - 0.03766 Time_2_0
+ 0.00109 Time_2_1
+ 0.03657 Time_2_3

Fits and Diagnostics for Unusual Observations

Obs	VLNENLLR_2	Fit	Resid	Std Resid	
109	0.04000	0.10641	-0.06641	-2.30	R
110	0.04000	0.10641	-0.06641	-2.30	R
111	0.04000	0.10641	-0.06641	-2.30	R
112	0.04000	0.10641	-0.06641	-2.30	R
113	0.04000	0.10641	-0.06641	-2.30	R
114	0.04000	0.10641	-0.06641	-2.30	R
115	0.04000	0.10641	-0.06641	-2.30	R
116	0.04000	0.10641	-0.06641	-2.30	R
117	0.04000	0.10641	-0.06641	-2.30	R
128	0.26000	0.18064	0.07936	2.74	R
129	0.28000	0.18064	0.09936	3.43	R
131	0.25000	0.18064	0.06936	2.40	R
132	0.25000	0.18064	0.06936	2.40	R
133	0.24000	0.18064	0.05936	2.05	R
135	0.25000	0.18064	0.06936	2.40	R

R Large residual

VLNENLLR
Salt and moisture: Tukey Pairwise Comparison [mg/100g VLNENLLR]

Tukey Pairwise Comparisons: Cheese Type_2

Grouping Information Using the Tukey Method and 95% Confidence

Cheese Type_2	N	Mean	Grouping		
Control	27	0.144074	A		
HMls	27	0.057750		B	
lmls	27	0.041048		B	C
HMHS	27	0.030577			C D
lmHS	27	0.016339			D

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Time_2

Grouping Information Using the Tukey Method and 95% Confidence

Time_2	N	Mean	Grouping		
3	45	0.0945276	A		
1	45	0.0590518		B	
0	45	0.0202931			C

Means that do not share a letter are significantly different.

7.5.5. YPFPGPIPN

7.5.5.1. Adjunct vs Control

YPFPGPIPN
Starter Culture: 2-Way ANOVA [mg/100g YPFPGPIPN]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Cheese Type	Fixed	2	Adjunct, Control
Time	Fixed	3	0, 1, 3

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cheese Type	1	42.2411	42.2411	109.88	0.000
Time	2	22.7538	11.3769	29.59	0.000
Error	50	19.2217	0.3844		
Lack-of-Fit	2	19.0458	9.5229	2599.13	0.000
Pure Error	48	0.1759	0.0037		
Total	53	84.2166			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.620027	77.18%	75.81%	73.38%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	1.1907	0.0844	14.11	0.000	
Cheese Type					
Adjunct	-0.8844	0.0844	-10.48	0.000	1.00
Time					
0	-0.835	0.119	-7.00	0.000	1.33
1	0.088	0.119	0.73	0.466	1.33

Regression Equation

YPFPGPIPN = 1.1907 - 0.8844 Cheese Type_Adjunct + 0.8844 Cheese Type_Control
- 0.835 Time_0
+ 0.088 Time_1 + 0.748 Time_3

YPFPGPIPN
Starter Culture: Tukey Pairwise Comparison [mg/100g YPFPGPIPN]

Tukey Pairwise Comparisons: Cheese Type

Grouping Information Using the Tukey Method and 95% Confidence

Cheese Type	N	Mean	Grouping
Control	27	2.07519	A
Adjunct	27	0.30630	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Time

Grouping Information Using the Tukey Method and 95% Confidence

Time	N	Mean	Grouping
3	18	1.93833	A
1	18	1.27833	B
0	18	0.35556	C

Means that do not share a letter are significantly different.

7.5.5.2. Time vs Temperature

YPFPGPIPN
Temperature: 2-Way ANOVA [mg/100g YPFPGPIPN]

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Time_1	Fixed	3	0, 1, 3
Temperature_1	Fixed	6	-20, 2, 4, 7, 12, 20

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time_1	2	110.33	55.1669	108.88	0.000
Temperature_1	5	318.45	63.6906	125.70	0.000
Error	106	53.71	0.5067		
Lack-of-Fit	5	42.14	8.4276	73.56	0.000
Pure Error	101	11.57	0.1146		
Total	113	525.36			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.711823	89.78%	89.10%	88.26%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	2.617	0.105	24.85	0.000	
Time_1					
0	-1.576	0.194	-8.14	0.000	3.32
1	-0.068	0.119	-0.57	0.568	2.95
Temperature_1					
-20	-2.524	0.154	-16.43	0.000	1.68
2	-1.077	0.161	-6.68	0.000	1.74
4	-0.542	0.154	-3.53	0.001	2.06
7	-0.101	0.154	-0.65	0.514	1.68
12	1.385	0.157	8.80	0.000	1.71

Regression Equation

YPFPGPIPN_1 = 2.617 - 1.576 Time_1_0 - 0.068 Time_1_1 + 1.645 Time_1_3
- 2.524 Temperature_1_-20 - 1.077 Temperature_1_2
- 0.542 Temperature_1_4
- 0.101 Temperature_1_7 + 1.385 Temperature_1_12
+ 2.860 Temperature_1_20

Fits and Diagnostics for Unusual Observations

Obs	YPFPGPIPN_1	Fit	Resid	Std Resid	
107	8.600	7.122	1.478	2.15	R
114	5.550	7.122	-1.572	-2.28	R

R Large residual

YPFPGPIPN
Temperature: Tukey Pairwise Comparison [mg/100g YPFPGPIPN]

Tukey Pairwise Comparisons: Time_1

Grouping Information Using the Tukey Method and 95% Confidence

Time_1	N	Mean	Grouping
3	52	4.26203	A

1	53	2.54914	B	
0	9	1.04114		C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature_1

Grouping Information Using the Tukey Method and 95% Confidence

Temperature_1	N	Mean	Grouping			
20	18	5.47741	A			
12	17	4.00206		B		
7	18	2.51685			C	
4	27	2.07519			C	D
2	16	1.54016				D
-20	18	0.09296					E

Means that do not share a letter are significantly different.

7.5.5.3. Salt and moisture

YPFPGPIPN
Salt and moisture: 2-Way ANOVA [mg/100g YPFPGPIPN]

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Time_2	Fixed	3	0, 1, 3
Cheese Type_2	Fixed	5	Control, HMHS, HMls, lmHS, lmls

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cheese Type_2	4	48.446	12.111	63.04	0.000
Time_2	2	234.208	117.104	609.49	0.000
Error	128	24.593	0.192		
Lack-of-Fit	8	20.713	2.589	80.07	0.000
Pure Error	120	3.880	0.032		
Total	134	307.247			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.438330	92.00%	91.62%	91.10%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	2.2312	0.0377	59.14	0.000	
Cheese Type_2					
Control	-0.1560	0.0755	-2.07	0.041	1.60
HMHS	0.9392	0.0755	12.45	0.000	1.60
HMls	0.3644	0.0755	4.83	0.000	1.60
lmHS	-0.3539	0.0755	-4.69	0.000	1.60
Time_2					
0	-1.6539	0.0534	-31.00	0.000	1.33
1	0.0848	0.0534	1.59	0.114	1.33

Regression Equation

YPFPGPIPN_2 = 2.2312 - 0.1560 Cheese Type_2_Control
+ 0.9392 Cheese Type_2_HMHS
+ 0.3644 Cheese Type_2_HMls - 0.3539 Cheese Type_2_lmHS
- 0.7936 Cheese Type_2_lmls - 1.6539 Time_2_0

+ 0.0848 Time_2_1
+ 1.5691 Time_2_3

Fits and Diagnostics for Unusual Observations

Obs	YPFPGPIPN_2	Fit	Resid	Std Resid	
49	5.2414	4.1647	1.0767	2.52	R
52	5.4865	4.1647	1.3218	3.10	R

R Large residual

YPFPGPIPN
Salt and moisture: Tukey Pairwise Comparison [mg/100g YPFPGPIPN]

Tukey Pairwise Comparisons: Cheese Type_2

Grouping Information Using the Tukey Method and 95% Confidence

Cheese Type_2	N	Mean	Grouping			
HMHS	27	3.17039	A			
HMls	27	2.59562		B		
Control	27	2.07519			C	
lmHS	27	1.87730			C	
lmls	27	1.43759				D

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Time_2

Grouping Information Using the Tukey Method and 95% Confidence

Time_2	N	Mean	Grouping		
3	45	3.80031	A		
1	45	2.31602		B	
0	45	0.57732			C

Means that do not share a letter are significantly different.