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Detection of residual enzyme activity for assessment of whole milk powder shelf-life stability

A thesis presented in partial fulfilment of the requirements for the degree of Master of Food Technology at Massey University, Manawatū, New Zealand

Zhaohua Ding

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Abstract

Whole milk powder (WMP) is one of the major exported commodities of New Zealand. It is significantly important to maintain quality stability during exportation and storage. The attention to the enzymatic degradation in WMP is limited. This study aims to directly detect enzyme activity in WMP samples and evaluate its relationship with bacterial counts and nutrient content stability, thereby find out the value of direct enzyme activity in assessing WMP shelf-life stability. Sixteen WMP samples were divided into two groups classified by raw milk bacterial loading, ten high-quality samples and six poor-quality samples. Mesophilic and thermophilic bacterial counting were conducted using pouring plate method, incubating at 30 °C and 55 °C for 48 h. Protease activity was detected using azocasein (incubated at 37 ± 1 °C for 20 h) and commercial EnzChek® kit assay (incubated at 22 ± 1 °C for 20 h). Lipase activity was determined using p-nitrophenyl palmitate (p-NPP) assay (incubated at 37 ± 1 °C for 2 h). Fat and protein contents were monitored using FOSS Milkoscan for 6-7 months. Linear mixed-effects models and linear regression models were applied for statistical analysis. Mesophilic bacterial counts in high quality samples were significantly lower than those in poor-quality samples (estimated mean: 2.88 ± 0.04 log cfu/g vs. 3.16 ± 0.05 log cfu/g, $p < 0.001$), which was consistent with the difference of raw milk bacterial counts between these two quality groups. There was no significant difference of thermophilic bacterial counts between the high- and poor-quality groups (estimated mean: high-quality 2.94 ± 0.11 log cfu/g, poor-quality 3.04 ± 0.14 log cfu/g, $p = 0.576$). Protease activity was not detected in all samples using azocasein assay (LOD = 11.53mU/g WMP) and was detected only in one sample that came from high-quality group using EnzChek® kit assay (LOD = 3.69 mU/g WMP, LOQ = 9.86mU/g WMP). The protease activity of that sample was between the LOD and LOQ of the EnzChek® assay. Lipase activity was detected in all samples using p-NPP assay (LOD = 1356 U/g WMP, LOQ = 3002 U/g WMP). Only one sample that came from high-quality group had lipase activity between the LOD and LOQ of the assay, all other samples had quantitative lipase activities. There was no significant difference of lipase activity between high- and poor-quality groups (estimated mean: high-quality 5045 U/g WMP, poor-quality 5213 U/g

WMP, $p = 0.521$). However, the sample that had the highest lipase activity (7894 ± 490 U/g WMP) was from high-quality group, indicating that bacterial counts might not provide reliable enzymatic deterioration in WMP during storage. The fat content maintained stable in high-quality samples over 7-month storage, poor-quality samples over 6-month storage (poor-quality samples were manufactured one month later) ($p = 0.197$). And there was no significant difference between those two groups ($p = 0.114$). Protein content also exhibited stable in high- and poor-quality groups over their respective storage time ($p = 0.352$) and did not significantly vary between the two groups ($p = 0.236$). The FOSS Milkoscan results provided evidence that the fat and protein content conformed regulatory limits over the storage period but failed to provide information about the deteriorative reactions in WMP during storage. The results in the present study suggest that direct enzyme activity may offer complementary information to conventional analysis in a comprehensive evaluation of WMP shelf-life.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Prof. Steve Flint, for his patience, guidance, and support throughout my research project. At the beginning of this project, I was concerned that the scope of the project might be too narrow and wanted to add more experimental components. He advised me to stay focused on the core research objectives, reminding me that it is better to do a few things well than to try to do more. I have carried that advice with me throughout the project, and I believe it genuinely improved the quality of my work. I am also grateful for the additional bacterial cultures he provided during the enzyme activity work, which were very helpful for my experimental progress, and for the time he dedicated to reviewing my thesis and providing detailed feedback.

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1 Introduction

Whole milk powder (WMP) is one of major dairy products in the global trade market due to its extended shelf-life of around 12 months, convenience in storage and transportation, and diverse usages (Phosanam et al., 2020). New Zealand is one of the leading exporters in the international market, supplying markets including East Asia, Southeast Asia, Middle East and Africa (Stats NZ, 2024). However, the long distances and varying storage conditions encountered during export create significant challenges to the WMP stability (Qí et al., 2026). During storage, WMP undergoes a range of deteriorative reactions, including lactose crystallization, Maillard reaction, lipid oxidation and enzymatic degradation. These reactions can compromise the nutritional value, functional properties, and sensory quality of WMP, which can diminish the customer acceptance and commercial value (Thomsen et al., 2005a). Even if the storage conditions such as temperature, relative humidity, packaging and oxygen exposure can be controlled at an ideal level, the deterioration reactions can still occur in WMP, especially with the residual lipase activity presence (Páez et al., 2006; Phosanam et al., 2020). Therefore, it is critical to evaluate the shelf-life stability of WMP at general storage conditions.

Current approaches of WMP shelf-life assessment typically rely on physiochemical indicators such as peroxide value (POV), colour measurement, HMF, volatile compounds, alongside sensory evaluation (An et al., 2018; Jiang et al., 2021; Xing et al., 2021). However, the contribution of residual enzymatic activity, particularly lipase activity, to degradation of WMP during storage has received limited attention, despite evidence that lipolytic degradation can occur even at extremely low water activity conditions. Meanwhile, the reliability of prediction results obtained from those indicators may be limited due to the limited attention to enzymatic influence and the diversity of enzymes present in milk powders (Andrewes, 2022). Therefore, it is necessary to integrate the residual enzyme activity with other conventional physiochemical indicators to comprehensively assess the shelf-life stability of WMP.

This study aimed to directly detect the residual enzyme activity in high- and poor-quality WMP samples (classified by raw milk bacterial loading), conduct plate counts on mesophilic and thermophilic bacteria in these WMP samples, and monthly monitor the fat and protein contents over a 6 – 7 month storage period. Meanwhile, this study also would evaluate the relationships between mesophilic or thermophilic bacterial counts and raw milk counts, residual enzyme activity and raw milk bacterial counts, milk powder bacterial counts, or fat and protein content stability, ultimately aimed to find out the value of direct enzyme activity in assessing WMP shelf-life stability.

This thesis is structured as follows: Chapter 1 is the general introduction of the present study. Chapter 2 provides a review of the literatures on deterioration mechanisms in WMP and current approaches to shelf-life prediction. Chapter 3 describes the experimental materials, methods, method validation, data calculation and statistical analysis. Chapter 4 presents the results of each experiment and discussions of the results. Chapter 5 summarizes the key conclusions and identifies directions of future research.

2 Literature Review

2.1 Introduction

Whole milk powder (WMP) is produced from raw milk by removing approximately 87% water and generally processed by standardization, pasteurization, evaporation, homogenization and spray drying, consists of 26 to 40% milk fats with unsaturated fatty acids approximately 34% (31% monounsaturated and 3% polyunsaturated), approximately 24% protein (20% casein and 4% whey protein), approximately 38% lactose, < 5% moisture, and also minerals and vitamins (Lloyd, Drake, et al., 2009; Park & Drake, 2017; Thomas et al., 2004; Thomsen et al., 2005b). As a dry powder product with water activity about 0.2 (Thomsen et al., 2005b), WMP has a much longer shelf-life than liquid milk for about 12 to 24 months even at room temperature, meanwhile is convenient for storage, transportation, handling, processing, and using as an ingredient in infant formula, yoghurt, ice cream, dairy beverage, confectionary products, or bakery products (Phosanam et al., 2020). These advantages and usages of WMP make it to be one of main milk products in the global trade market (Qí et al., 2026), where New Zealand is the biggest exporter of WMP and occupies 1/3 shares (*NZX, New Zealand's Exchange - Sgx-NZX Dairy Derivatives, Dairy*, n.d.). Approximately 95% of the New Zealand dairy products are exported, contributing 34% of the country's merchandise exports and about 5.6% of national GDP, while about 40% of them is WMP (USDA Foreign Agricultural Service, 2025). The main destinations of WMP of New Zealand are China, Southeast Asia countries, Middle-east countries, and even North Africa countries (Stats NZ, 2024). However, long distribution distance, duration, and relative high temperature and humidity environments of destinations may pose deterioration risks on the WMP (Qí et al., 2026), thus, it is important to maintain the quality, not only for avoiding the rejection by customers but also keeping high-quality reputation and advantages in global market.

The deterioration of WMP generally exhibits off-flavours, changes of colour, and reductions in functional properties (Clarke, Griffin, et al., 2021; Kosasih et al., 2016; Y. H. Li et al., 2019; Lloyd, Drake, et al., 2009; Páez et al., 2006). Off-

flavours such as grassy, painty, cardboard, metallic, tallowy and astringency, which are related with volatile compounds, are developed from lipid oxidation or Maillard reaction, while rancid, soapy, piquant, astringent as well, which are associated to short- and medium-chain free fatty acids (FFAs), may be developed from lipolysis of lipids during storage (Clarke, Griffin, et al., 2021; Lloyd, Drake, et al., 2009; Páez et al., 2006; Park & Drake, 2017). Lloyd et al. (2009) collected WMP samples from 4 US production facilities 5 times over 1 year, with each sample being analysed initially and every 2 months for sensory profile, volatile compounds through 12-month storage. They found that volatile compounds such as hexanal, 2-heptanone, which were the products from lipid oxidation (Y. Li et al., 2012), were related with grassy flavour (R^2= *0.38, P < 0.0001*), while volatiles such as 2-methylbutabal, 3-methylbutanal, which were the products from Strecker reaction, were closely related with painty flavour (R^2= *0.61, P < 0.0001*) (Lloyd, Drake, et al., 2009). Clarke et al. (2021) investigated the relationships between flavours and volatile compounds during WMP storage. They found that painty, solvent-like, metallic aromas, flavours, aftereffects were strong correlated (>0.7), meanwhile, painty flavour and aftereffect were also strong correlated with increasing concentrations of hexanal (>0.7) (Clarke, Griffin, et al., 2021). Páez et al. (2006) studied the changes of FFAs during storage of WMP and found that the rancid flavour positively correlated with $C_{18:1}$ content (*r = 0.42; P = 0.01*) which might be produced by residual lipases in the powder (Páez et al., 2006). In addition, besides the off-flavours directly contributed by FFAs, increasing FFA content produced by the lipolysis of lipids in WMP during storage is susceptible to oxidation. This may result in increased production of other off-flavour volatile compounds such as aldehydes and ketones, which are associated to the metallic and tallowy off-flavours. Former study has demonstrated that lipase activity in WMP is stable during the storage, and the lipolysis of lipid would take place at a constant level even at low water activity values in WMP (Celestino et al., 1997b).

Colour changes could provide information about Maillard degradation (non-enzyme browning) in WMP. CIELAB colour space is usually used to characterize the colour changes. *L** is a measure of lightness or whiteness

(black = 0; white = 100), *b** is a measure of blueness to yellowness value (yellow > 0; blue < 0), *a** is a measure of greenness to redness value (red > 0; green < 0) (Pugliese et al., 2017; Thomsen et al., 2005a). Thomsen et al. (2005) conducted research in the effect of temperature on Maillard reaction during WMP storage. They believed that the combination of measurement of colour with measurements of furosine and 5-hydroxylmethyl-2-furaldehyde (HMF) could provide different stages information of the Maillard reactions. In their study, the *b** values of WMP under different storage temperatures exhibited varied trends, and those of WMP under higher temperatures kept rising over storage time (Thomsen et al., 2005a).

The functional property reductions of WMP mainly include caking and poor rehydration. Thomas et al. (2004) reviewed the reasons and the influencing factors of these two deteriorations as followings. First, lactose crystallization resulted in the formation of solid bridge between powder particles and promoted caking. They also reviewed many studies that associated lactose crystallization and caking as these two phenomena occurred simultaneously. Second, the crystallization of lactose also induced high fat coverage on the powder particle surface and subsequently resulted in the reduction of rehydration. Third, the crystallization of lactose would result in the modification of protein structures, consequently, induce the denaturation and aggregation of proteins, finally reduce the solubility of milk powder. Finally, Maillard reaction and lipid oxidation of WMP during storage also reduced its solubility. Free sulfhydryl groups that were produced during the preheat process and were presented on the surface of protein, would result in the generation of protein polymerization, and cross-linking of protein to Maillard and oxidation reaction products. Cross-linking of protein formed aggregates and subsequently reduced the rehydration of milk powder after storage (Thomas et al., 2004).

Collectively, the deterioration of WMP is primarily driven by these four mechanisms: lactose crystallization, Maillard reaction, lipid oxidation, and enzyme degradation. These reactions also interact synergistically within the WMP matrix. For example, lactose crystallization increases the amount of free fat on the particle surface and releases entrapped water. The subsequent rising in water activity and molecular mobility further accelerates lipid oxidation, the

Maillard reaction, and the lipolysis of lipids (Celestino et al., 1997b; Thomsen et al., 2005a). All the factors that relate to these four mechanisms may pose risks on the shelf-life stability of WMP. These factors that influence the WMP stability range from upstream variables such as raw milk quality (e.g., feeding system, seasonal variations, somatic cell counts, total bacterial counts) to downstream parameters including processing treatments (e.g., homogenization, preheat treatments), compositions (e.g., fat content, water activity, particle surface composition, residual enzymes), storage conditions (e.g., temperature, humidity, light), and packaging specifications (e.g., packaging materials, packaging atmosphere) (Celestino et al., 1997b; Phosanam et al., 2020). Manufacturers generally optimize these factors to maintain the quality of WMP during the production and storage periods. For example, they set requirements of the microbiological quality of raw milk and utilize heat processing to inactivate microorganisms and enzymes to achieve good quality and shelf-life stability of WMP (Muir et al., 1986). On the other hand, manufacturers test some of these factors and the products from those four deterioration mechanisms as parameters to meet the regulation standards and the quality commitments to end-product manufacturers, and predict the shelf-life stability of WMP. For example, they test the POV to assess the WMP quality and predict the shelf-life in an accelerated testing (Jiang et al., 2021). However, these quality control methods may fail to capture some of the latent deterioration risks and the rate that the deteriorations develop during the storage, which may result in early spoilage (Lloyd, Drake, et al., 2009).

This review will comprehensively discuss those four deterioration mechanisms of WMP during storage, meanwhile, overview the shelf-life prediction methods, to provide valuable insights for optimizing the shelf-life management and quality control for WMP products in New Zealand.

2.2 Deterioration Mechanisms

There are four main deterioration mechanisms of WMP during storage, lactose crystallization, Maillard reactions, lipid oxidation and enzymatic hydrolysis. There has been many research and interviews discussing the former three mechanisms, only a few studies focused on the last one - enzymatic hydrolysis.

2.2.1 Lactose Crystallization

Lactose basically presents an amorphous glass state in WMP after the spray-drying and is highly hygroscopic (Jouppila & Roos, 1994a). After the hygroscopic amorphous lactose sorbs water from the environment during the storage, the water content of WMP rises, the mobility of lactose molecules increases, subsequently, the lactose state transitions from amorphous to rubbery, and then the crystallization of lactose takes place (Phosanam et al., 2020). The temperature at which the state of lactose transitions is the glass transition temperature (*Tg*). *Tg* negatively correlates with water content, decreases when the water content rises because of the plasticization effect of water (**Figure 2.1**) (Phosanam et al., 2020). Normally, lactose crystallization will not occur below *Tg* (Meste et al., 2002). *Tg* of WMP with water content of 3.1% (w/w) is estimated at 44 °C (the temperature may be different with different water content), when the storage temperature is higher than *Tg*, the glass state lactose with significantly increased mobility forms different kind of crystalline lactose according to different levels of storage temperature and moisture content, such as anhydrous α-lactose, anhydrous β-lactose or monohydrated α-lactose (Jouppila & Roos, 1994a; Qí et al., 2026). Meanwhile, the difference between the storage temperature and *Tg* (i.e., *T* - *Tg*) impacts the WMP shelf-life, with higher difference resulting in the faster completion of crystallization and shorter shelf-life (Phosanam et al., 2021; Thomsen et al., 2005a). In addition, the crystallization of lactose will release water, which further increases the water content and acts on the crystallization in turn. Therefore, the lactose crystallization will be auto amplified (Thomas et al., 2004). Besides the moisture content and temperature, lactose crystallization is also affected by the formulation of milk powder. In the review study of Thomas et al. (2004), they discussed the effects of milk protein and fat on the lactose crystallization, and found that the milk protein would absorb moisture competing with lactose and delay the lactose crystallization, milk fat might act as a hydrophobic barrier and reduce the rate of lactose crystallization (Thomas et al., 2004).

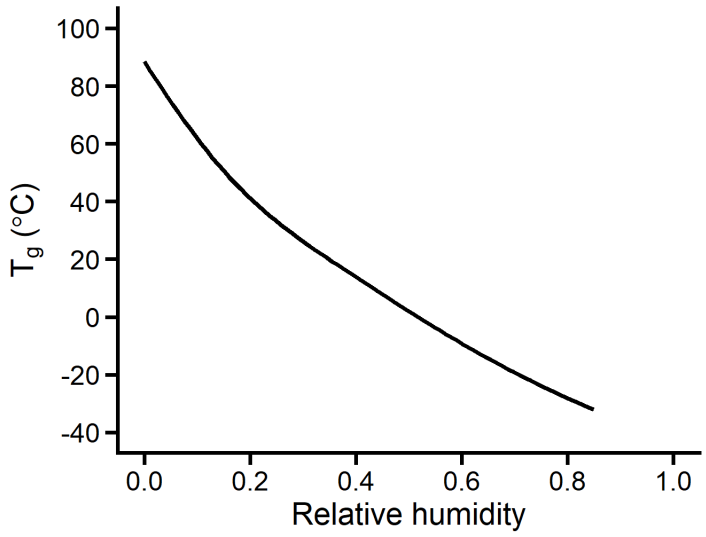


Figure 2. 1
Glass transition temperature (Tg) of whole milk powder as a function of relative humidity at 25°C. (Data adapted from Phosanam et al., 2020 and Jouppila & Roos,1994).

Lactose crystallization alters the surface composition of WMP particles and releases binding water into the matrix, which subsequently leads to physical and chemical deteriorations, including powder caking, loss of flowability and solubility, non-enzyme browning, lipid oxidation, and enzymatic degradation (Thomas et al., 2004; Thomsen et al., 2005b). The water sorbed from environment and condensed on the particle surface, forms liquid bridges between particles, dissolves components on the particle surface, promotes the molecular diffusion and lactose crystallization between particles linked by liquid bridges, finally results in caking (Netto et al., 1998; Teunou & Fitzpatrick, 1999). Caking increases the particle size and forms irregular shapes of powder, which directly reduces the flowability of WMP (Thomas et al., 2004). On the other hand, lactose crystallization increases the fat content on particle surface, which also results in the particle aggregation and subsequently impairs the powder flowability (Thomas et al., 2004). Meanwhile, the increasing fat coverage on particle surface decreases the interaction of powder particle with water due to its hydrophobicity and reduces the solubility of milk powders (Jouppila & Roos, 1994b; Thomas et al., 2004). Besides the changing of fat coverage on particles, lactose crystallization also induces the modifications of proteins, including protein denaturation and aggregation, which also result in the loss of solubility of milk powder (K. Li et al., 2016; Mimouni et al., 2010; Phosanam et al., 2020).

The water released from lactose crystallization further increases the water activity and the molecule mobility, which may lead to the Maillard reaction between lactose and lysine. Former study demonstrated that the Maillard reaction extent significantly increased with 5.4% water content (Guyomarc'h et al., 2000). The study of Thomas et al. (2005) showed that the increasing water activity induced by lactose crystallization clearly resulted in the formation of late-stage Maillard reaction products (Thomsen et al., 2005a). The increasing content of free fat on particle surface leads to not only reduced physical property of WMP, but also lipid oxidation, which is considered as the main deterioration during storage (Phosanam et al., 2021). The lipase in milk powder is stable and active at the lipid-water interfaces (Chen et al., 2003). Thus, the increasing free fat and water activity induced by lactose crystallization will promote the lipolysis by lipase during WMP storage.

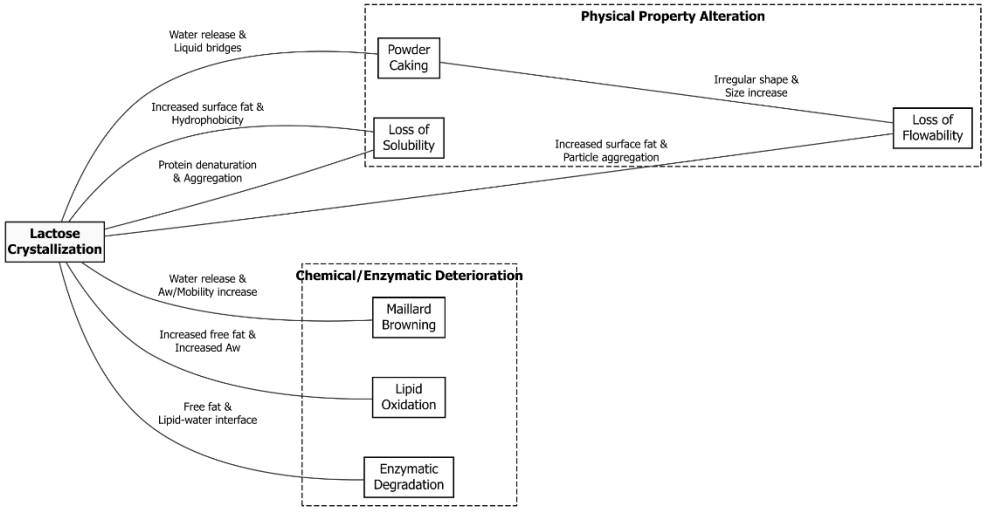


Figure 2. 2
Lactose crystallization initiates a series of physical and chemical changes. Schematic prepared by the author, based on the literature discussed in this section.

Figure 2.2 clearly shows how lactose crystallization induces other deteriorations and indicates the significant importance of controlling it during storage. Besides controlling the relative humidity and temperature of storage, pre-crystallization (induced before spray-drying) and post-crystallization (induced after spray-drying, in fluidized bed) are widely investigated to improve the milk powder storage stability. Yazdanpanah et al. (2013) demonstrated that pre-crystallization surface layer of skim milk powder exhibited more

physiochemically stable than that of controlled sample (Yazdanpanah & Langrish, 2013). The study of Saxena et al. (2020) investigated the stability of infant formula at different levels (from 0.5% to 45%) of pre-crystallization, and their results showed that 18% of pre-crystallization exhibited better stability (Saxena et al., 2020). Qí et al (2026) demonstrated that converting amorphous lactose to anhydrous β-lactose by pre-crystallization at a small crystal size can increase the storage stability of fat-filled milk powder. Therefore, controlling the storage relative humidity and temperature, pre-crystallizing the lactose at some levels at small size, may benefit the shelf-life stability of WMP.

2.2.2 Maillard Reaction

Maillard reaction in milk powder occurs between milk protein and lactose, and is the main cause of non-enzyme browning (Phosanam et al., 2021). There are generally three stages in the Maillard reaction progression. In the first stage, lactose reacts with some amino group of milk protein and forms ϵ -N-deoxylactulosyl-*D*-lysine, a stable Amadori product, also known as lactulosyl-lysine. This progress is called lactosylation. Lactulosyl-lysine can also generate fructoselysine, and in the lab analysis, the later one can generate furoyl-methyl-lysine in the acid condition, also called furosine (Thomas et al., 2004). Furosine is stable, seen as an indicator of the first stage of Maillard reaction, can be directly determined by HPLC, therefore, is widely used to monitor the Maillard reaction in milk powders (Thomsen et al., 2005a). Heat processing and drying can induce Maillard reactions in milk powders. Thus, furosine is also considered as an indicator of heat damage before storage. In the study of Le et al., the furosine content of freshly manufactured WMP ranged from 159 – 163 mg/100 g protein (T. T. Le et al., 2011). During storage, the formation of furosine depends on the temperature and relative humidity. In the research of Thomasen et al. (2005), WMP samples were stored at 37, 45 and 55 °C, the concentration of furosin increased gradually, but the increasing rate rose as the temperature increased (Thomsen et al., 2005a). In the study of Le et al., WMP samples were stored at three relative humidity levels (44%, 66%, 84%) at 30 °C. The content of furosine rose the most quickly at 66% relative humidity, followed by 44% and 84% (T. T. Le et al., 2011). The intensity of preheat treatment also poses risks to the storage stability of WMP by Maillard reaction. In the study of Li et al.

(2021), furosine content increased with 18-month storage time, while the more intense preheat treatment resulted in the higher furosine content (Y. Li et al., 2021). However, when the Maillard reaction goes into the advanced stage, fructoselysine that the source of furosine will be further degraded, and furosine will not be formed (Sørensen et al., 2017).

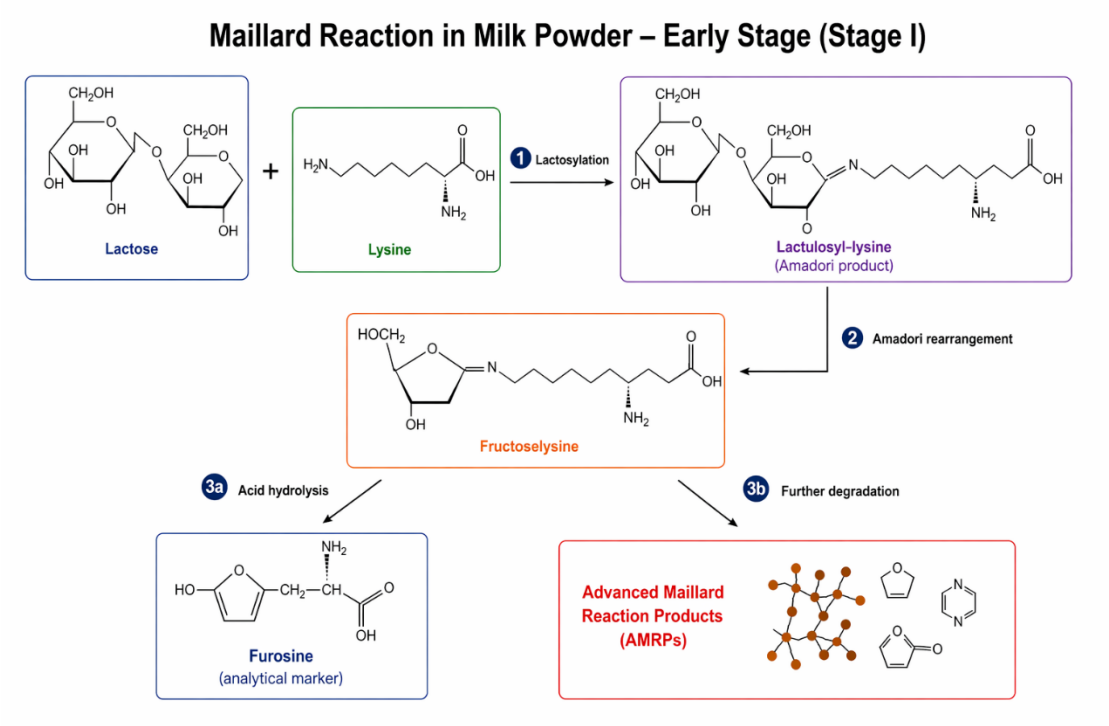


Figure 2. 3
The first stage of Maillard reaction. Schematic prepared by the author, based on the literature discussed in this section.

In the second stage of Maillard reaction, lactulosyl-lysine may decompose into hydroxymethyl furfural (e.g. HMF) by the pathway that is called 1,2 enolization or sometimes 3-deoxyosone route (Aalaei et al., 2019). HMF content is also widely used in many studies to evaluate the degree of Maillard reaction during milk powder storage (H. Yu et al., 2015). Although HMF is considered as the indicator of the second stage of Maillard reaction, it is produced at the beginning of storage. Lactulosyl-lysine is formed in the first stage, and will be degraded through Maillard reaction pathways into HMF or other products (T. T. Le et al., 2011). In the study of Li et al., (2021), they observed that the formation rate in the early storage was higher than that in the late stage of storage. Meanwhile, they also found that the more intense preheat treatment had the higher effect on the content of HMF during WMP storage (Y. Li et al., 2021). The formation

of HMF also depends on the storage temperature and relative humidity, which are demonstrated by some studies.

In the late stage of Maillard reaction, α-dicarbonyl compounds produced during Maillard reaction react with amino acid, then forming Strecker aldehyde by oxidative deamination and decarboxylation (Clarke, McCarthy, et al., 2021; Estévez et al., 2011). Then main Stecker aldehydes in milk powder are 2- and 3-methylbutanal, which are volatile compounds and attributers of the off-flavours such as painty, astringency (Lloyd, Drake, et al., 2009). In addition, in the last stage of Maillard reaction, molecules degraded from Amadori compounds polymerize or copolymerize with protein, and form brown pigments (melanoidins), and this progression is also known as non-enzyme browning (NEB) (Morales & van Boekel, 1998). The monitoring of browning is also common to investigate the content of Maillard reaction, and colour changing is considered as one of the markers of deterioration of milk powders. Tristimulus colorimeter is widely used to determine the content of NEB, which includes three colour parameters as mentioned in the introduction of this review, α* value indicating red/green, *b** indicating yellow/blue, *L** value indicating the lightness (T. T. Le et al., 2011; Thomas et al., 2004). The rate of NEB depends on the storage temperature and relative humidity. The study of Thomasen et al. (2005) showed that the *b** value of WMP stored at 37 °C remained stable, while that of WMP stored at 45 °C and 55 °C increased after 7 and 71 days, meanwhile confirmed browning occurred with the increasing a_w of WMP at low temperature (Thomsen et al., 2005a). The study of Le et al. (2011) showed that *b** value of WMP increased with the storage relative humidity rising (T. T. Le et al., 2011).

Besides the formation of off-flavour volatiles and colour changing, Maillard reaction also results in the loss of solubility. Some intermediate compounds such as methylglyoxal can react with amino acid residuals (e.g. lysine) to generate cross-linked proteins (Singh, 1991), which can result in the loss of solubility (T. T. Le et al., 2011). Finally, Maillard reactions can result in the nutritional reduction in milk powders and even generate products that have healthy risks

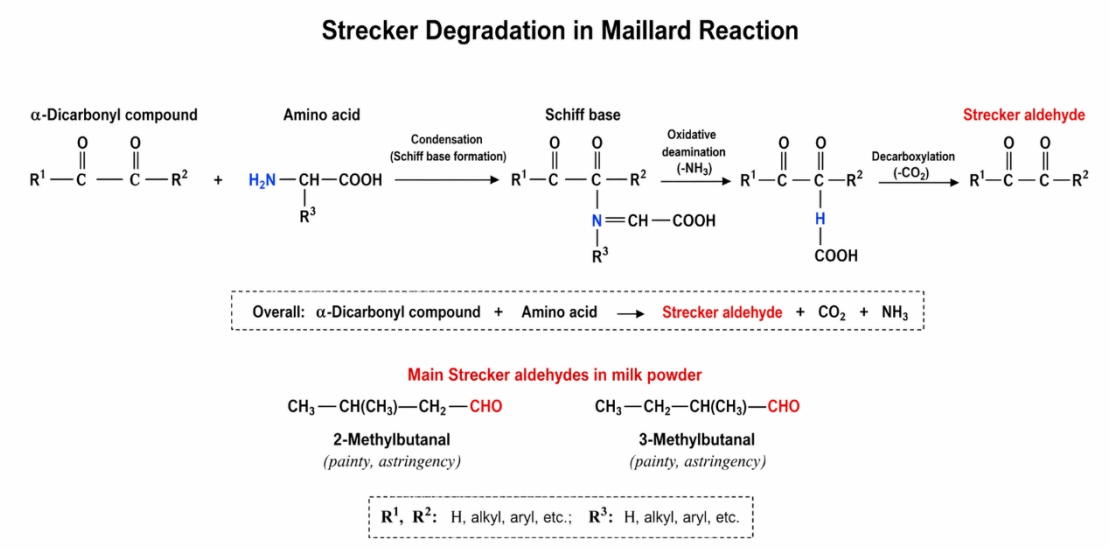


Figure 2. 4
Schematic representation of the Strecker degradation pathway during the advanced stage of the Maillard reaction in milk powder. Schematic prepared by the author, based on the literature discussed in this section.

to human. Essential amino acids (e.g. lysine) react with reduce sugar in Maillard reactions, which directly results in the nutritional loss. The loss of lysine is also used as a marker to estimate the Maillard reactions in milk powders. In the study of Li et., the WMP samples were stored at 25 °C with a relative humidity of 50% for 18 months, and they found 44 - 57% decrease of lysine at the end of storage (Y. Li et al., 2021). Advanced glycation end products (AGEs) are formed in the late stage of Maillard reaction. These products could result in the irreversible loss of lysine, colour and flavour changing, and are reported to be harmful to human health (Van Boekel, 1998).

2.2.3 Lipid Oxidation

Lipid oxidation is widely considered as the main cause of the formation of off-flavour volatile compounds in WMP, and a major cause of deterioration of WMP (Cluskey et al., 1997; Lloyd, Drake, et al., 2009). Lipid oxidation mainly includes three types: auto-oxidation, photo-oxidation and enzymatic oxidation. Auto-oxidation directly occurs between lipid and oxygen and is considered as the most common type in WMP (Fan et al., 2023). The progress of auto-oxidation can be divided into three distinct periods: initiation, propagation and termination. In the initiation period, A hydrogen atom is abstracted from allylic carbon atom neighbouring double bond in an unsaturated fatty acid, forming a free radical.

Subsequently, in the propagation period, the free radical reacts with the ground state oxygen to generate peroxyl radical, which can abstract a hydrogen atom from another unsaturated fatty acid, generating a new free radical and a hydroperoxide. In the last period, with increasing content of radicals, free radicals polymerize with each other and form stable products. Meanwhile, the hydroperoxides that are considered as the primary oxidation products decompose into secondary oxidation products, such as straight-chain aldehydes, ketones, which are believed to be mostly correlated to the off-flavours during WMP storage (Fan et al., 2023; Lloyd, Drake, et al., 2009).

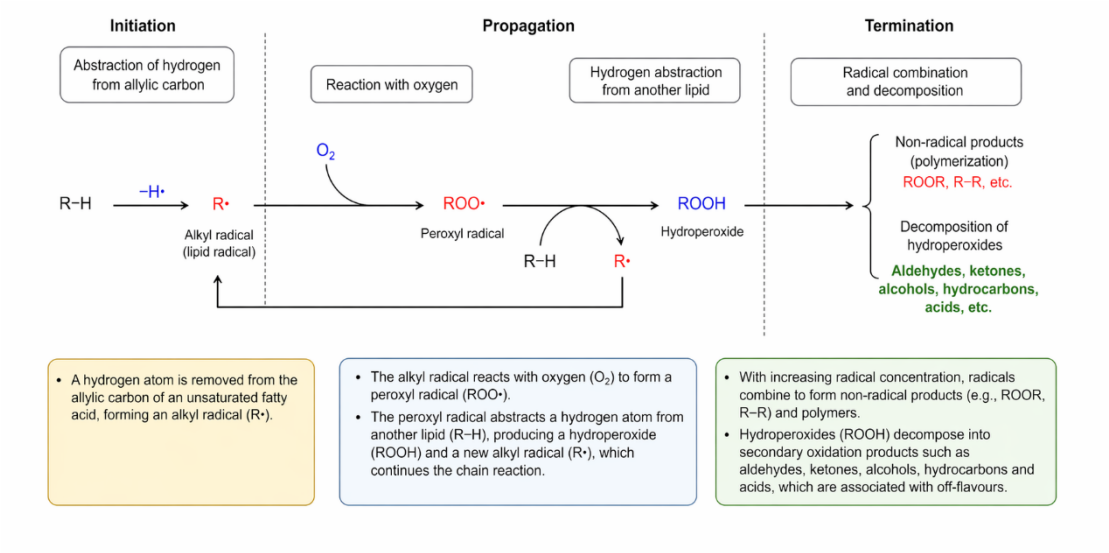


Figure 2. 5
Schematic diagram illustrating the generalised mechanism of free radical lipid autoxidation, divided into initiation, propagation, and termination stages. Schematic prepared by the author, based on the literature discussed in this section.

The peroxide value (POV) that corresponds to the content of hydroperoxides is still widely used to estimate the oxidation extent of dairy products because it is convenient and cheap to use (Clarke, McCarthy, et al., 2021). Former studies also correlated POV with the sensory testing. In the study of Lloyd et al. (2009), they reported a strong correlation between POV and painty flavour (r = 0.86). Meanwhile, their sensory testing panel perceived flavour change before POV reached 1 mEq/kg of fat (Lloyd, Drake, et al., 2009). However, hydroperoxides are unstable, prone to decompose into secondary oxidative compounds. During the storage of WMP, the POV increases at first and then declines. Mahmoodani et al. (2018) studied the POV of WMP samples stored at 20 °C and 40 °C for 12 months. They observed a rapid increase of POV at first and reached a

maximum at the seventh month at both temperatures, and then declined (Mahmoodani et al., 2018). In addition, many factors such as temperature, light exposure, content of unsaturated fatty acids, would affect the POV with non-proportional relationships. POV depends on not only the rate of the formation of hydroperoxides but also how quickly they decompose (Liang, 2000). Thus, POV is not accurate to measure the extent of lipid oxidation but can be used as a qualitative indicator for the initial period of lipid oxidation in milk powders. Meanwhile, because the free radical is formed at the initial step of lipid oxidation, the content of free radical is also used by many studies to estimate the extent of the primary stage oxidation (Y. H. Li et al., 2019; Stapelfeldt et al., 1997; Thomsen et al., 2005a).

TBARS (thiobarbituric acid reactive substances) value is another widely used conventional indicator to evaluate the extent of lipid oxidation, specifically to the secondary lipid oxidation products in milk powders. The major secondary oxidation product is malondialdehyde (MDA), which can react with thiobarbituric acid (TBA) and the products can be detected by absorbance or fluorescence methods (Mahmoodani et al., 2018; Stapelfeldt et al., 1997). Cluskey et al. (1997) found there was an inverse relationship between POV and TBARS of their WMP samples after 6-month storage, with POV decreasing and TBARS rising, indicating lipid oxidation progression from a primary to a secondary stage (Cluskey et al., 1997). However, there are also some disadvantages of this indicator. First, MDA is just one of the aldehydes derived from the secondary oxidation, thus, the TBRAS cannot represent the overall extent of lipid oxidation. Second, TBA does not specifically react with MDA. Sugars also can react with TBA and lead to colour change, which will make the lipid oxidation estimated (Clarke, McCarthy, et al., 2021). Finally, former studies believed this indicator was more sensitive for products with more polyunsaturated fatty acids, not suitable for evaluating the lipid oxidation of products mainly containing oleic acid and linoleic acids, while the polyunsaturated fatty acid content may vary largely between different WMP (Liang, 2000).

Quantitative determination of volatile compounds that are decomposed from the secondary lipid oxidation products is an approach widely used to estimate the lipid oxidation extent. Headspace solid phase-microextraction (HS-SPME)

gas chromatography mass spectrometry (GC-MS) techniques have been widely used to analyse the volatile compounds in milk powders by many studies. In addition, there are still several other techniques that are developed, such as thermal desorption (TD), SAFE, etc. Clarke et al. (2021) comprehensively summarized the techniques that are used to quantify volatile compounds and the advantages, disadvantages of these techniques (Clarke, McCarthy, et al., 2021).

Sensory testing is always combined with the determination methods mentioned above to estimate the lipid oxidation extent in milk powders. Former studies have correlated the sensory characteristics with the volatile compounds and consumer acceptance. Lloyd et al. (2009) found that consumer acceptance was negatively correlated with astringency, painty and grassy flavours, and with volatile compounds from lipid oxidation, such as hexanal, heptanal, nonanal, octanal, 1-octen-3-ol and 3-octen-2-one (Lloyd, Hess, et al., 2009). Another study of Lloyd et al. (2009) demonstrated that hexanal was a compound that both related to grassy and painty flavour, nonanal, 2-heptanone, 1-octen-3-ol were also related to grassy flavour, 3-octen-2-one and octanal were related to painty flavour. Meanwhile, their results showed that the threshold of painty perception for hexanal and nonanal should be 600 and 4 μg/kg (Lloyd, Drake, et al., 2009). Combination of sensory analysis and quantitative determination can provide more information about lipid oxidation extent, threshold of specific volatile compound and customer acceptance. However, although human sensory is much more sensitive than quantitative determination methods, there are still some limitations for sensory testing, such as opinion differences between panellists, low reproducibility (Tunick, 2014).

There are many factors such as feeding system, preheat treatment, other processing conditions, storage conditions (temperature, relative humidity, light, packaging), which will influence lipid oxidation in milk powders. **Table 2.1** shows some examples.

Table 2. 1
Factors influencing lipid oxidation in milk powders.

Factors	How they influence	References
Feeding system	Impacts the fatty acid profile and then influences lipid oxidation.	(Z. Cheng et al., 2022; Clarke, Griffin, et al., 2021)
Diet supplement	Supplement such as Vitamin E, reduces more antioxidative products and reduces oxidation.	(Cluskey et al., 1997)
Microbial quality	Influences content of free fatty acids and then influences lipid oxidation.	(Celestino et al., 1997b)
Heat treatment	Induces more free sulfhydryl groups exposure and reduces lipid oxidation.	(Mahmoodani et al., 2018; Stapelfeldt et al., 1997)
Homogenization	Decreases free fat to reduce lipid oxidation.	(Park & Drake, 2017)
Composition	High content of polyunsaturated fatty acids increases oxidation.	(Romeu-Nadal et al., 2007)
Storage temperature	Accelerates the lipid oxidation.	(Romeu-Nadal et al., 2007; Stapelfeldt et al., 1997)
Water activity	No evident influence when the water activity between 0.11 - 0.24 even 0.33 at room temperature. Promotes lipid oxidation when out of this range. Lipid oxidation occurs before water activity rises at high storage temperatures.	(Chopovda et al., 2018; Y. H. Li et al., 2019; Lloyd, Drake, et al., 2009; Stapelfeldt et al., 1997; Thomsen et al., 2005a; H. Yu et al., 2015)
Packaging	Packaging material influences the water activity through different water vapour permeability during storage. Packaging atmosphere influences the oxygen exposure to milk powders.	(Lloyd, Hess, et al., 2009; H. Yu et al., 2015)

2.2.4 Enzymatic Hydrolysis

There are two groups of enzymes in WMP, indigenous and endogenous enzymes. After experiencing a series of heat treatments during the WMP production, all the enzymes of these two groups left in WMP are heat stable. The main heat stable indigenous enzyme in WMP is plasmin, a kind of native protease derived from blood serum (Chen et al., 2003). Heat stable endogenous enzymes are derived from bacteria that mainly are contaminated in raw milk and bacterial protease and lipase are mainly focused. The raw milk will be contaminated by bacteria from three main sources, the interior of cow's udder, cow's teats, and the equipment for milking and storage (Chen et al., 2003). After milking, the raw milk will be stored or transported at chilled temperature to keep microbiological quality. However, psychrotrophs can keep growing and predominate the total microbiological counts, meanwhile, secrete thermostable proteases and lipases at the chilled temperature (D. Zhang et al., 2020). Zhang et al. (2020) studied bacteria that could produce thermostable enzymes from raw milk in New Zealand. They identified 246 isolates from the fresh and stored raw milk (7 °C for 5 days), found 94 of them were able to produce thermostable proteases and 81 of them could produce thermostable lipases. Meanwhile, they found that there were 9 genera in the stored raw milk, among them, *Pseudomonas* was the predominant one accounting for 93.68%, other 8 genera were *Acinetobacter, Bacillus, Buttiauxella, Carnobacterium, Lactococcus, Hafnia, Rahnella* and *Serratia,* totally accounting for 6.32% (D. Zhang et al., 2020). Although most of these psychrotrophs will be killed by heat treatments during WMP production, however, these thermostable enzymes are likely to be active in WMP and pose risks to the storage stability of WMP, even further deteriorate the end products produced by WMP.

2.2.4.1 Plasmin

There are five elements in plasmin system, plasmin, plasmin inhibitor, plasminogen, plasminogen activator, and inhibitor of plasminogen activator. In milk, plasmin basically exists as its precursor plasminogen, which can be proteolyzed by plasmin activator and converted into plasmin (Chen et al., 2003). The concentration of plasmin and plasminogen vary between different bovine breeds and lactations, become higher from early to late lactation, while

generally plasminogen is 9 times higher than plasmin in milk (Richardson, 1983). Plasmin and plasminogen are mainly associated with casein micelles, plasminogen activators are associated with casein and somatic cells, while inhibitors of plasmin and plasminogen activators are mainly present in milk serum (Chen et al., 2003). Because urokinase-type plasminogen activator is associated with somatic cells, the plasmin level will rise with an increased somatic cell count in milk, due to more plasminogen activators will be dissociated from the somatic cells (Murphy et al., 2016).

Plasmin is a serine protease, has better activity at pH 7.5 – 8.0, similar to trypsin (Reimerdes, 1983). Plasmin can cleave all caseins but prefers to β-, α_{s1}- and α_{s2}-caseins. It exhibits a preferential specificity for bonds on the carboxyl side of lysine and arginine residuals and cleaves lysine bonds at a higher rate than arginine bonds. β -casein has three plasmin sensitive bonds and will be cleaved into γ_1-, γ_2-, γ_3-caseins and proteose peptone-casein. However, plasmin has no activity on β-lactoglobulin and α-lactalbumin. Meanwhile, β-lactoglobulin can be an inhibitor to plasmin due to thiol-disulphide reaction between them (Chen et al., 2003). Plasmin also cleaves plasminogen, and the resultant plasminogen product can be converted to plasmin by plasminogen activators. But the conversion can be slowed by plasminogen activator inhibitor, meanwhile, the proteolysis of caseins by plasmin also can be slowed by plasmin inhibitor (Ismail & Nielsen, 2010).

Plasmin, plasminogen and plasminogen activators are all reported to have high heat stabilities, while inhibitors of plasmin and plasminogen are all reported to be heat labile. There are two parameters used to assess the heat stability of enzymes, half-life (t ½), which means the time that 50% activity reduction needs by heat, and D-value, which means the time that 90% activity reduction needs by heat. Saint Denis et al. (2001) studied the heat stability of plasmin, plasminogen and plasminogen activator in bovine milk. Their results showed that the D value of plasmin, plasminogen and plasminogen activator were 660 s, 660 s and 840 s respectively after 75 °C heat treatment; 71 s , 73 s and 74 s after 90 °C heat treatment; 13 s, 11 s and 16 s after 140 °C heat treatment (Saint Denis et al., 2001). In another earlier study conducted by Lu and Nielsen (1993), the D value of plasminogen activators was 109 min at 70 °C and 32 s

at 140 °C (Lu & Nielsen, 1993). The results from the study conducted by Metwalli et al. (1998) showed that β-lactoglobulin enhanced the inactivation of plasmin, while casein significantly protected plasmin from inactivation, partly compensated the destabilized effect of β-lactoglobulin. Plasmin was unfolded under the high heat, protected by casein, and refolded back to an active form after cooling (Metwalli et al., 1998). These studies indicated that these three elements of plasmin system probably would keep active after the heat treatments during the WMP production. The inhibitors of plasmin and plasminogen activator are reported to be heat labile and plasminogen activator inhibitor is less thermostable than plasmin inhibitor. Prado et al. (2006) studied the heat stabilities of these two inhibitors. Their results showed that 80% activity of plasminogen activator inhibitor and 36% activity of plasmin inhibitor were lost after heat treatment at 74.5 °C for 15 s. Meanwhile, their study also showed that plasmin activity increased after heating at 72 °C, which might be due to the inactivation of plasminogen activator inhibitor and more activation of plasmin, and it significantly increased further after heating at 77 °C and 82 °C, which might be due to the further inactivation of those two inhibitors (Prado et al., 2006).

2.2.4.2 Bacterial Proteases

There are several genera such as *Pseudomonas, Bacillus, Serratia, Chryseobacterium, Citrobacter*, which can produce heat stable protease in raw milk (Morandi et al., 2021; Qin et al., 2023; D. Zhang et al., 2020). According to some studies, *Pseudomonas* species are predominant species that secrete heat stable protease (Machado et al., 2017; Qin et al., 2023; D. Zhang et al., 2020). The study of Zhang et al. (2020) showed that 90 of 94 species that were isolated from raw milk and produced heat stable protease belonged to *Pseudomonas* (D. Zhang et al., 2020). Many studies demonstrated that each of *Pseudomonas* species secretes at least one type of protease (Machado et al., 2017), different species secreted various types (Nicodeme et al., 2005).

The main *Pseudomonas* heat stable protease is AprX, a kind of zinc metalloprotease that is secreted by species such *as P. fluorescens, P. poae, P. fragi, P. tolaasii, P. rhodesiae, P. gessardii, P. proteolytica, P. brenneri, or P.*

chlororaphis (Machado et al., 2017; Morandi et al., 2021). The AprX protein is highly conserved and close within the *Pseudomonas* species, however, is more heterogeneous between species. For example, there is 76 – 99% similarity across *P*. *fluorescens* strains, but 57 – 69% similarity between strains of *P*. *fluorescens* and *P*. *fragi* species (Matéos et al., 2015). AprX has a catalytic domain (Zn^{2+} binding domain) at the N-terminal, and a Ca^{2+} binding domain at the C-terminal, its activity can be stimulated by some divalent cations such as Zn^{2+}, Mn^{2+} and Ca^{2+}, and inhibited by chelators such as EDTA, 1,10-phenanthroline and EGTA, which can chelate Zn^{2+} and Ca^{2+} (C. Zhang et al., 2019). AprX can be active in a large range of pH (4.5 – 10) and temperature (0 – 55 °C) but has optimum activity at pH 6.5 – 9 and 37 – 45 °C, which varies between different species (Martins et al., 2015; Matéos et al., 2015; Volk et al., 2019). AprX can hydrolyse β, κ, α_{s1} and α_{s2} casein, but generally is believed to prefer to hydrolyse β or κ casein, also varying between different species or strains, and is unsensitive to whey proteins (Chen et al., 2003; Stuknytė et al., 2016). The heat stability of AprX is demonstrated by many studies. For example, the study of Volk et al. (2019) showed that the half-life (t ½) of AprX from *P. lundensis* was 42.8 min at 70 °C and 28.9 min at 95 °C, the D value of it was 142 min at 70 °C and 95.9 min at 95 °C. The half-life of AprX from *P. proteolytica* was 28.9 min at 70 °C and 19.3 min at 95 °C, the D value of it was 95.9 min at 70 °C and 64 min at 95 °C (Volk et al., 2019).

Another typical heat stable protease secreted by *Pseudomonas* species is AprA. According to the study of Baur et al. (2015), the full amino acid sequence of AprA from *P*. *panacis* is has 98% similarity to AprX from a *P*. *fluorescens* strain. It has a mass of 49.4 kDa, can be inhibited by EDTA and 1,10-phenanthroline, indicating it probably belongs to metalloprotease, meanwhile, has the highest activity at pH 8 and 40 °C. In addition, their study also showed that the half-life of AprA from *P*. *panacis* was 194 min at 70 °C, 39.8 min at 90 °C, while D value of it was 645 min at 70 °C and 132 min at 90 °C (Baur, Krewinkel, Kutzli, et al., 2015).

Besides AprX and AprA, Stuknytė et al. (2016) found a 25 kDa protease from *Pseudomonas fluorescens* PS19, which was thermostable and was

heterogeneous to any known protein of *Pseudomonas* species (Stuknytė et al., 2016).

Bacillus is another well studied genus. *Bacillus* species are ubiquitous in the environment and can be readily introduced into raw milk or milk products during collection, handling, and processing. In addition, they not only sporulate under heat treatments or sanitation conditions, posing risks at multiple stages, but also produce thermostable enzymes (Chen et al., 2003). They are known to produce diverse extracellular proteases, and some of them have similar characteristics to proteases of *Pseudomonas* species. According to the study of Chen et al. (2004), the proteases from all seven *Bacillus* strains that they isolated from milk powder production line were almost completely inhibited by the combination of two inhibitors, PMSF (inhibitor of serine protease) and 1,10-Phenanthroline (inhibitor of metalloprotease). However, individual strains showed different degrees of inhibition by each inhibitor alone, suggesting the same strain may secrete both serine protease and metalloprotease (Chen et al., 2004). Similar inhibition patterns were obtained by Gonzál et al. (2016). They investigated the inhibition of proteases from two strains of *Bacillus licheniformis* by PMSF and EDTA, and the proteases from each strain were inhibited by both inhibitors. But they proposed that the proteases secreted by each strain were primarily serine protease that required mental cations for activation (mental-activated serine proteases) (J. González et al., 2016). Yang et al. (2021) investigated the proteases from 55 strains of *Bacillus cereus*, which is one of the most prevalent *Bacillus* species in milk. Their results showed that the protease from 25 of the 55 strains were heat stable. A protease from *B. cereus* C58 – one of the 25 strains – was identified as protease Hhoa, had a molecular mass 44 kDa, could hydrolyse β, κ, and α_s casein, could not hydrolyse whey protein, had more than 80% and 40 % activity after being heated at 70 °C for 30 minutes and 100 °C for 10 minutes (Yang et al., 2021). The study conducted by Chen et al. (2004) also showed that the proteases from those strains of *Bacillus* had highest activity at neutral or alkaline pH conditions varying between strains. Meanwhile, they found that the proteases exhibited significant heat stabilities. Most of them still had more than 50% activity after heat treatment at 70 °C for 10 minutes, and proteases from one strain had still

more than 60% activity after heat treatment at 90 °C for 10 minutes (Chen et al., 2004).

Proteases from other genera such as *Serratia, Chryseobacterium*, are also demonstrated heat stable by several studies. Glück et al. (2016) investigated the heat stabilities of proteases secreted by microorganisms isolated from raw milk. At 6 °C, besides *Pseudomonas*, there were other 9 genera such as *Serratia, Acinetobacter, Chryseobacterium*, that could secrete heat stable proteases in their study. The proteases secreted from 7 of 9 genera still had more than 60% activity after heat treatment at 95 °C for 5 minutes. At 30 °C, besides *Pseudomonas*, there were 16 genera including the 9 genera at 6 °C and other 7 genera, of which the proteases had more than 10% activity after being heated 95 °C for 5 minutes. In their study, proteases from mesophiles such as *Enterococcus*, *Staphylococcus* and some species of *Pseudomonas* also exhibited heat stability after heat treatment (Glück et al., 2016).

Proteases can be significantly stabilized against heat treatment and autolysis by calcium ions, which is relatively rich in milk (approx. 0.38mg/ml; 9.5 mM) (Chen et al., 2003; Daniel et al., 1996). Ertan et al. (2015) investigated the role of calcium ions in improving the thermostability of an alkaline metalloprotease from *Pseudomonas* isolated from refrigerated milk. Their results indicted 5 mM calcium ions stabilized the protease stability and activity. They believed that extra Ca2+ ions binding to the C-terminal domain promoted a more compact partially folded protein structure to increase stability, meanwhile, allosterically affected the N-terminal domain to maintain activity (Ertan et al., 2015).

2.2.4.3 Bacterial Lipases

Lipases are generally considered as lipolytic enzymes that can hydrolyse acylglycerols with ≥ 10 carbon-chain fatty acids, while the lipolytic enzymes which can hydrolyse acylglycerols with < 10 carbon-chain acids are called esterase. However, most of lipases can also hydrolyse acylglycerols with < 10 carbon-chain acids and intact fat globules as well. In addition, lipases are generally active at lipid-water interfaces, while esterases are active in aqueous phase (Chen et al., 2003). Bacterial lipases in raw milk are produced by several genera, such as *Pseudomonas, Bacillus, Acinetobacter, Microbacterium,*

Hafnia, Candida, Carnobacterium, Chryseobacterium, Filobasidium, Lactococcus, and Staphylococcus (Baur, Krewinkel, Kranz, et al., 2015; Chen et al., 2004; Vithanage et al., 2016). *Pseudomonas* is generally considered as the predominant genus that produce lipase in raw milk. In the study of Zhang et al. (2020), there were 93 isolates from the raw milk exhibiting lipase activity, and 83 of these isolates belonged to *Pseudomonas* (D. Zhang et al., 2020). Milk microorganisms can secrete various types of lipases that hydrolyse acylglycerols with varying chain length fatty acids, particularly short chain ones, which contribute to off-flavours in dairy products (Chen et al., 2004; Kovacic et al., 2019). According to the study of Kovacic et al. (2019), bacterial lipases are classified into 19 families with the first one being further classified into 8 subfamilies based on conserved sequence motifs and biochemical functions. Lipases from *Pseudomonas* and *Bacillus* were divided into seven and six families and several subfamilies (Kovacic et al., 2019). In the study of Chen et al. (2004), lipases from all seven *Bacillus* strains that isolated from milk powder production line all exhibited lipase activity, with six of them preferable to short chain fatty acid substrates while another one preferable to long chain fatty acid substrates (Chen et al., 2004).

Generally, the bacterial lipases have molecular mass 20 – 75 kDa. Most of them have a conserved α/β-hydrolyse fold and a canonical GXSXG amino acid sequence (most of *Bacillus* species are AXSXG) with nucleophilic serine residual being in it. Most of them have the highest activity between pH 7 – 9 (optimum pH of some lipases from *Bacillus* species are 10 – 11.5, that from *Staphylococcus* species can be lower than 7) (Kovacic et al., 2019). The optimum temperature from them varies between species. The highest lipase activity is at 30 – 45 °C for *Pseudomonas* lipases, while that for most of *Bacillus* is at 60 – 75 °C (Chen et al., 2003). In addition, the presence of calcium ion can influence the lipase activity. Calcium ion is essential for the activity structure of some lipases, such as some *Pseudomonas* and *Serratia* lipases. Meanwhile, some lipases such as some *Bacillus* and *Staphylococcus* lipases are independent of calcium (Kovacic et al., 2019). Bacterial lipases have more specificity to hydrolyse triacylglycerols at sn-1 and sn-3 positions. Because the lipolysis products including 1,2- or 2,3- diacylglycerols and 2-monoacylglycerols

are readily to migrate acyl and convert to 1,3-diacylglycerols and 1-monoacylglycerols, triacylglycerols will be completely lipolyzed by bacterial lipases. In addition, the catalytic efficiency of some of lipases is significantly higher for mono- and diacylglycerols than triacylglycerols (Chen et al., 2003).

Numerous studies have demonstrated that many bacterial lipases are significant heat stable. In a very early study conducted by Andersson et al. (1979), the D value of *Pseudomonas fluorescens* lipases were 23.5 min after heat treatment at 100 °C, 7.3 min at 120 °C and 2 min at 140 °C in skim milk (Andersson et al., 1979). In the study of Vithanage et al. (2016), the lipase activity from *Pseudomonas, Bacillus, Microbacterium* remained 50 – 75% after heat treatment at 142 °C for 4 s (Vithanage et al., 2016). The study of Chen et al. (2004) showed that lipases from *Bacillus* strains were much more stable than protease concomitantly produced by them. Lipases from 5 strains of *Bacillus* remained more than 90 % activity after heat treatment at 70 °C for 10 min, that from 4 strains remained more than 87 % activity after heat treatment at 90 °C for 10 min. Lipases from one strain of their study had half-life 11.5 h being heated at 70 °C for 10 min (Chen et al., 2004). Qin et al. (2023) demonstrated that lipases secreted by *Acinetobacter* retained more than 55% activity after being heated at 140 °C for 5 s (Qin et al., 2023).

Early studies had discussed the possible mechanisms of the thermostability of lipases. First, lipases have abundant hydrophobic residuals, which may contribute to highly compact conformation, thereby increasing the resistance to thermal denaturation and the external environment stress. Second, disulphide and other bonds may provide structural stability for lipases against heat or chemical denaturation (Andersson et al., 1979). Third, the lack of cysteine in the primary sequence of many lipases may protect from disulphide heat aggregation, meanwhile, the low content of heat sensitive amino acids in lipases also may contribute to the less irreversible inactivation (Owusu et al., 1992). Fourth, α_s- and β-casein and calcium ions were demonstrated to be able to protect lipases from inactivation from heat treatment by Kumura et al. (1993) and Shibata et al. (1998) (Kumura et al., 1993; Shibata et al., 1998).

Short chain fatty acids including fatty acids with 4- to 8-carbon chain, such as butyric, caproic and caprylic acid, medium chain fatty acids including 10- and 12-carbon chain fatty acids, such as capric and lauric acid, and long length chain acids including 14- to 18-carbon chain fatty acids, such as myristic, palmitic and stearic acids, will be produced after lipolysis in milk products. Short chain fatty acids always exhibit rancid and astringent flavours, medium chain fatty acids give soapy taste, while long chain fatty acids generally have little flavour (Chen et al., 2003). However, the study of Páez et al. (2006) found a strong correlation between rancidity with an 18-carbon chain fatty acid during WMP storage (Páez et al., 2006). In addition, the unsaturated fatty acids among the lipolysis products are susceptible to be oxidised, forming off-flavour volatile products, such as aldehyde and ketones, further increasing unpleasant flavours and reducing the product quality (Chen et al., 2003; Romeu-Nadal et al., 2007).

2.2.4.4 Protease and Lipase Activity in WMP

Due to the significant thermostability, proteases (plasmin and bacterial proteases) and lipases (mainly bacterial lipases) are unlikely to be inactivated by heat treatments during the WMP production and are expected to be active during the storage. Celestino et al. (1997) detected the protease and lipase activity in WMP and found that their activity was stable throughout the 8-months storage. Meanwhile, they did not find any evidence of the proteolysis in WMP during storage, and believed that degradation of proteins by proteases would not occur due to low water activity (Celestino et al., 1997b). In their another study, they investigated the proteolysis in UHT milk that was produced by WMP. There was more degree of proteolysis in the UHT milk that stored at 25 °C than that stored at 3 °C. They believed that there were more sulphydryl groups that resulted from heat treatment on β-lactoglobulin in UHT milk stored at 3 °C due to less oxidation, the presence of sulphyldryl groups promoted the formation of β-lactoglobulin/κ-casein complex, which diminished the accessibility of proteases to caseins, leading to the reduction of proteolysis degree (Celestino et al., 1997a). During the production of WMP, heat treatment will also result in the formation of β-lactoglobulin/κ-casein complexes (hair-like structures) on casein micelles (McKenna et al., 1999). It is possible for these complexes to inhibit the proteolysis in WMP, thus, the protease activity in WMP may not

reduce the shelf-life stability of WMP during storage. However, Chen (2000) investigated the proteolysis in WMP spiked with proteases from *Bacillus* and the commercial WMP during storage. The researcher believed that the increasing level of insoluble casein particles might be produced by the proteolysis of κ-casein (Chen, 2000). In addition, the protease activity remained in WMP will significantly influence the quality of the end products of WMP, such as UHT milk. In the study of Celestino et al. (1997), the residual proteases kept active in WMP over 8-months storage and significantly reduced the UHT milk quality by proteolysis (Celestino et al., 1997a).

The lipolysis in WMP during storage have been demonstrated by several studies. In the study of Celestino et al. (1997), they found significant increases of FFA due to the lipase activity. They believed that the lipolysis could occur at low water activity values because the milk fat is not water soluble, and the lipolysis does not only occur in water. Meanwhile, they also cited the study by Anderson (1980), in which they found that lipolysis will occur in WMP at constant level at water activity values no more than 0.54 (Celestino et al., 1997b). In the study of Chen (2000), the FFA level in the WMP spiked with *Bacillus* lipases increased rapidly in two weeks at 37 °C during storage and increased gradually in the subsequent storage period. The result of their study is the first evidence that lipolysis can occur in WMP at water activity less than 0.1 (Chen, 2000).

2.3 Shelf-life of WMP

WMP will experience the deterioration through those main mechanisms discussed above during storage, and produce many undesirable products, resulting in sensory change, such as flavour and colour change. It is important to predict the shelf-life of WMP, which is considered as a significantly important index that indicates high quality and safe consuming. The products from those deteriorating mechanisms are usually used as markers in the shelf-life predictions.

An et al. (2018) used peroxide value (POV) as the marker to predict the shelf-life of infant formula powder. In their study, they assumed that the increase of POV over time (t) fitted Huang's three parameter model:

$$POV = POV_0 + k_m \left\{ t + \frac{1}{25} \ln \left(\frac{1 + \exp[-25(t-\lambda)]}{1 + \exp(25\lambda)} \right) \right\} \quad (1)$$

Where *POV* is the peroxide value at time t (meq/kg), *t* is the time of storage (d), POV_0 is initial peroxide value (meq/kg), k_m is maximum oxidation rate constant ($meqkg^{-1}d^{-1}$) and λ is induction period (d).

At a certain temperature and an oxygen concentration, such as 30 °C, 1.5 kPa (N_2 flushed package), they determined POV over storage time. Through the POV increase curve produced by nonlinear regression, values of k_m and λ can be obtained. At a certain oxygen concentration, different values of k_m and λ can be obtained at different temperatures. And then, Arrhenius equation can be applied to find out the relationship between values of k_m and λ, and temperatures.

$$\frac{1}{\lambda} = \frac{1}{\lambda_0} \exp\left(-\frac{E_{a,\lambda}}{RT}\right) \quad (2)$$

$$k_m = k_{m,0} \exp\left(-\frac{E_{a,k}}{RT}\right) \quad (3)$$

Where $E_{a,\lambda}$ and $E_{a,k}$ are activation energies for λ and k_m, λ_0 and $k_{m,0}$ are constants.

Through the different values of k_m and λ, and 1/T, two straight lines can be obtained through the Arrhenius equation, and values of $E_{a,\lambda}$ and $E_{a,k}$ were calculated from the slops of those two lines.

In addition, at a certain temperature, different values of k_m and λ can be obtained at different oxygen concentrations. Another empirical relationship between the values of k_m and λ, and oxygen concentrations.

$$v = \frac{C_1[O_2]}{C_2 + [O_2]} \quad (4)$$

In the equation, *v* is k_m or $1/\lambda$, C_1 and C_2 are constants at the certain temperature, $[O_2]$ is the oxygen concentration.

At the certain temperature, the values of C_1 and C_2 can be obtained from equation (4) based on the different values of k_m and λ at different oxygen concentrations.

Then the values of k_m and λ at any given temperatures and oxygen concentrations can be obtained based on the Arrhenius equation and the calibration by the equation (4). Finally, the shelf-life can be estimated by the equation (5) at any combinations of temperature (20 – 40 °C) and oxygen partial pressure (0.5 – 21 kPa).

$$t = \lambda + \frac{POV_c - POV_0}{k_m} \quad (5)$$

Where POV_c is the peroxide value of the end of the shelf-life, POV_0 is the initial peroxide value. In their study, they set POVc at 15 meq/kg, which was believed to be a quality limit value for fatty foods (An et al., 2018).

The study conducted by Xing et al. (2021) showed a traditional shelf-life prediction method by Arrhenius equation, using Maillard reaction products – HMF and 2-furaldehyde (F) – as the markers. They found the concentration of HMF, and F produced in milk-based formula, UHT milk and pasteurised yoghurt during storage depended on temperature and storage time and followed the zero-order kinetics.

$$C_f = Kt + C_0 \quad (6)$$

Where C_0 and C_f are the initial and the final concentration of HMF or F in the dairy products, t is the storage time, K is the reaction rate.

The relationship between the reaction rate K and temperature is considered to follow Arrhenius equation with the temperature between 25 – 60 °C.

$$lnK = lnK_0 - \frac{E_a}{R}\left(\frac{1}{T}\right) \quad (7)$$

Through the different K values at various temperature (25 – 60 °C), a plot of lnK against 1/T yields a straight line, from whose slope the activation energy (E_a) can be determined. Subsequently, we can have any K value at any temperature. Finally, we can get the shelf-life by the equation (6). For example, the K value can be obtained at 25 °C by Arrhenius equation, then we can get the shelf-life if we know the initial concentration of HMF or F, and the final concentration of HMF or F that can be considered as the shelf-life end (Xing et al., 2021).

There are also other studies using multivariate indicators, such as colour change by Maillard reactions, TBARS and volatile compounds by oxidation, in the shelf-life prediction tests with various models. For example, an early study conducted by Nielsen et al. (1997). They estimated the shelf-life of WMP at very early stage (two days storage) by analysing multivariate indicators screened by PRESS model, using stepwise multiple regression and the sensory outcomes from the accelerated tests (Nielsen et al., 1997). Another study conducted by Jiang et al. (2021) also used multivariate indicators to estimate the shelf-life of milk formula. They chose Vitamins (C, A, E), hydroperoxide, volatile compounds, fatty acids as their prediction indicators, and used principal component analysis (PCA) to find out the variables that changed mostly over time. PC1 scores were found to linearly increase with time at different temperatures. The slops of the linear plots reflected the rate of multivariate kinetic parameters (K values in Arrhenius equation) at respective temperatures. Finally, according to the shelf-life results derived from sensory test in the accelerated test (40 °C), they obtained the shelf-life at the room temperature (Jiang et al., 2021). Marsili et al. (2000) conducted a study using multivariate analysis and electronic-nose instrument (SPME-MS) to build a model for the shelf-life prediction of pasteurised reduced-fat milk and whole-fat chocolate milk over 7 months. They used SPME-MS to analyse the volatiles from the samples that were placed in 19 °C incubator (for accelerated production of bacterial metabolites), and applied MVA analysis based on partial least-squares (PLS) method to predict the shelf-life of the samples. The SPME-MS-MVA-PLS model they built after 7-months period could predict the shelf-life for reduced-fat milk with an accuracy of ± 0.62 day (Marsili, 2000). Nevertheless, there are also some deterioration products and accelerated temperatures are not suitable for shelf-life prediction. Cheng et al. (2017) investigated the shelf-life prediction indicators and the accelerated temperatures for high protein and high lipid infant formulas. Their results showed that AGEs were not suitable for shelf-life prediction and the temperature exceeding 55 °C was not recommended (H. Cheng et al., 2017).

Although these prediction methods, such as MASLT combining Arrhenius modelling, have been widely used for dairy products including milk powders or UHT milk for many years, their reliability may be limited due to the high diversity

enzymes presented in the milk products (Andrewes, 2022). As discussed in previous part of this review, the types, quantities and activities of these enzymes – particularly lipases – often differ substantially in raw milk, which may result in significant difference between the specific samples used in shelf-life trials and the commercial products that ultimately reach the market. Consequently, such predictions may lead to inaccuracies of the actual shelf-life under real-world conditions, too long or too short, because of the enzymatic deterioration. Therefore, it would be necessary to detect the enzyme activities for WMP to make sure the storage stability and avoid risks for its downstream products.

2.4 Conclusion

WMP will experience deterioration during the storage by mechanisms such as lactose crystallization, Maillard reactions, lipid oxidation and enzymatic deterioration. Meanwhile, these mechanisms affected each other in the WMP matrix in complicated patterns. For example, the water activity increase induced by lactose crystallization will promote the Maillard reactions and enzymatic hydrolysis, the increasing free fat on the particle surface due to the lactose crystallization will result in more lipid oxidation, the radicals produced from lipid oxidation will also affect the Maillard reactions, the unsaturated fatty acids produced from lipolysis will promote the oxidation. Finally, the sensory quality of WMP, including colour and flavour, will be reduced because of these deterioration reactions. There are many factors could influence these reactions, referring to various production stages, from initial farming stage to terminal storage period. It is critical to control these factors to assure the quality.

Due to high diversity of the microorganisms in the raw milk, the types, quantities and activities of the enzymes vary significantly in WMP. Many proteases and lipases exhibit heat stability due to their composition, structure or the protections from other elements in the milk matrix. Therefore, these thermostable enzymes are still active during the storage of WMP and may induce hydrolysis, pose risks to the storage stability of WMP and the downstream products. Compared to conventional bacterial enumeration methods, the detection of residual enzyme activity is not only necessary but

also critical for improving the accuracy of shelf-life prediction in WMP. Enzyme activity assays directly reflect the potential impact of these residual enzymes, thereby serving as a valuable and essential complement to multivariate accelerated shelf-life testing models, leading to more reliable commercial shelf-life determinations.

3 Material and methods

3.1 Chemicals

All reagents were of analytical grade unless otherwise stated. The reagents used in this study are listed as follows:

Microbiological Media and Diluent

- Milk Plate Count Agar (Oxoid, Basingstoke, Hampshire, England).
- Peptone Diluent (0.1%; Fort Richard Laboratory, Auckland, New Zealand).

Enzymes, Substrates and Assay Kit

- Standard protease from *Streptomyces* griseus (Type XIV, ≥ 3.5 units/mg solid; Sigma-Aldrich, St. Louis, MO, USA).
- Azocasein (Sigma-Aldrich, St. Louis, MO, USA).
- Standard lipase from *Candida rugosa* (Type VII, ≥ 700 unit/mg solid; Sigma-Aldrich, St. Louis, MO, USA).
- 4-Nitrophenyl palmitate (p-NPP; Sigma-Aldrich, St. Louis, MO, USA).
- EnzChek® Protease Assay Kit (E6638; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) – containing BODIPY FL casein as fluorescence substrate.

Other Organic and Inorganic Reagents

- Sodium dihydrogen orthophosphate anhydrous (NaH_2PO_4; LabServ, Thermo Fisher Scientific, Auckland, New Zealand).
- Di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4; LabServ, Thermo Fisher Scientific, Auckland, New Zealand).
- Potassium chloride (KCl: Scharlau, Sentmenat, Spain).
- Sodium chloride (NaCl: ECP Ltd, Auckland, New Zealand).
- Potassium dihydrogen phosphate (KH_2PO_4; Scharlau, Sentmenat, Spain).
- Hydrochloric acid (HCl; 36%; Ajax Finechem, Thermo Fisher, Auckland, New Zealand).

- Sodium azide (NaN_3; Scharlau, Sentmenat, Spain).
- Sodium hydroxide (NaOH).
- Chloramphenicol (Sigma, St. Louis, MO, USA).
- Tris (hydroxymethyl) aminomethane (Merck KGaA, Darmstadt, Germany).
- Triton X-100.
- Gum Arabic.
- 2-Propanol (anhydrous, 99.5%; Sigma-Aldrich, St. Louis, MO, USA).
- Trichloroacetic acid (TCA, 99.8%; VWR, Leuven, Belgium).
- Ethanol 99.5%.

3.2 Samples

Ten high-quality and six poor-quality (classified by bacterial loads) whole milk powder (WMP) samples manufactured in New Zealand were sent to our lab in July and August of 2025. Sample information including date of manufacture, raw milk aerobic plate count (APC), titratable acidity (TA) are summarised in **Table 3.1**.

Table 3. 1
Description of whole milk powder samples used in this study

Sample ID	DOM	Raw Milk Storage Time (d)	Raw Milk APC (log cfu/mL)	Sifter Tspores (cfu/mL)	TA (%m/v)	NO_2
High-quality samples						
A	14-Jun	-	4.23	< 10	0.126	< 0.21
B1	15-Jun	-	3.93	10	0.111	0.3
B2	15-Jun	-	3.93	10	0.111	< 0.21
C1	20-Jun	-	4.40	900	0.158	0.5
C2	20-Jun	-	4.40	900	0.158	0.3
F1	12-Jun	-	4.34	20	0.129	0.4
F2	12-Jun	-	4.34	20	0.129	0.4
F3	12-Jun	-	4.34	20	0.129	< 0.21
H1	22-Jun	-	4.30	20	0.123	< 0.21
H2	22-Jun	-	4.30	20	0.123	0.7

Poor-quality samples						
A1	-	188	7.60	-	1.77	1.5
A2	-	188	7.60	-	1.77	1.5
A3	-	188	7.60	-	1.77	1.5
K1	15-Jul	188	＞6.00	-	1.77	1.5
K2	15-Jul	188	＞6.00	-	1.77	1.5
K3	15-Jul	188	＞6.00	-	1.77	1.5

3.3 Mesophilic and Thermophilic Plate Counting Assay

3.3.1 Assay Procedure

Milk plate count media was prepared by adding the milk plate count agar (MPCA) powder into distilled water at the ratio of 19.5 g into 1 L, mixing and boiling to dissolve. Subsequently, the media was autoclaved at 121 °C for 15 minutes and stored in 55 °C incubator.

Serial ten-fold dilutions of reconstituted whole milk powder samples were prepared in 0.1% Peptone Diluent (Fort Richard Laboratory, Auckland, New Zealand). For the first set of samples, high-quality samples were diluted from 10^{-1} to 10^{-5}, while poor-quality samples were diluted from 10^{-1} to 10^{-7}, to ensure plates with countable colonies (10 – 300 cfu/plate) (International Dairy Federation, 1991). In the second and third sets of samples, two or three appropriate dilutions were selected for each sample on the expected bacterial load. For example, lower count samples were diluted at 10^{-1} and 10^{-2}, whereas high count samples were diluted at 10^{-2} and 10^{-3}.

For each dilution, 1 mL of the sample was pipetted into a sterile Petri dish. Approximately 15 – 20 mL of MPCA media (cooled to 40 – 45 °C) was then poured into the dish and mixed thoroughly by gently swirling. Six replicate plates were prepared for each dilution, of which three were incubated at 30 °C and the other three at 55 °C for 48 h (Pramularsih et al., 2022).

Plate counts of mesophilic and thermophilic bacteria were carried out on the same sample at multiple time points. High-quality samples were analysed at

three time points (July, August and September of 2025), while poor-quality samples were analysed at two time points (August and September of 2025) due to later receipt of the samples.

3.3.2 Data Calculation

According to the limitation range (10 – 300 cfu/plate), the final plate count outcome of each sample at each time point was calculated from the accountable plates based on the equation as follow (International Dairy Federation, 1991):

$$N = \frac{\sum C}{(n_1 + 0.1n_2)d}$$

Where N is the number of microorganisms per gram WMP sample; ΣC is the total number of colonies on all plates retained; n_1 is the number of plates retained at the first dilution; n_2 is the number of plates retained at the second dilution; d is the dilution factor of the first dilution, for example, d value of 10 fold dilution is 0.1.

3.4 Protease Activity Detection Assay

Two methods were used to detect residual protease activity in whole milk powder (WMP) samples.

3.4.1 Azocasein Assay

The first method was a modified azocasein assay based on Teh et al. (2012) (Teh et al., 2012). A 3% (w/v) azocasein stock solution was prepared in 50 mM phosphate buffer (pH 7.5) (Khadka et al., 2024) containing 1 mg/mL sodium azide and 0.1mg/mL chloramphenicol. This stock solution was diluted by the same buffer to obtain a 0.9% (w/v) working substrate solution.

3.4.1.1 Assay Procedure

WMP samples were reconstituted by dissolving 0.5 g of powder in 4.5 mL Milli-Q water, followed by centrifugation at 15,000 g for 10 min at 4 °C. Then, 0.1 mL of the middle-layer liquid was mixed with 0.9 mL of 0.9% azocasein working solution (reaction group). Positive control groups (protease + substrate) were prepared using standard protease solutions at three concentrations (0.1, 1, 10

μg/mL), and one negative control group (buffer + substrate) was included. All groups were performed in triplicate and incubated in a 37 ± 1 °C water bath for 20 h (T20).

To obtain the initial time point (T0), 0.36 mL of substrate solution was added to 0.04 mL of sample solution and 0.8 mL 20% (w/v) TCA (reaction mixture group of T0). A negative control group of T0 (0.36 mL substrate solution + 0.04 mL buffer + 0.8 mL TCA) was also included. After incubation of T20 groups, the reaction was terminated by adding 0.4 mL of T20 incubation solution (reaction mixture, positive and negative control) to 0.8 mL of 20% TCA. Both T0 and T20 stopped solutions were centrifuged at 20,000 g for 10 min at 4 °C.

Subsequently, 0.2 mL of the resulting supernatant was mixed with 0.2 mL of 1 M NaOH solution (Coêlho et al., 2016; Soboleva et al., 2019). The mixture was briefly centrifuged at 2,000 g for 9 s at 4 °C. Finally, 0.2 mL of the mixture was transferred into a flat-bottom 96-well microplate (Flacon, Corning, NY, USA), and the absorbance was read at 433 nm using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Vantaa, Finland).

The 16 samples were divided evenly into four batches of four samples each. Each batch included four sample reaction groups, three positive control groups (at standard protease concentrations of 0.1, 1, 10 μg/mL) and one negative control group (substrate blank). The four batches were tested on four consecutive days.

3.4.1.2 Method Validation

Several parameters were applied to evaluate the performance of the azocasein assay, including intra-day precision, inter-day precision, recovery rate and the protease activities corresponding to the limit of detection (LOD) and the limit of quantitation (LOQ) by this assay.

Intra-day precision was expressed as the range of relative standard deviation (RSD) values obtained from the 10 μg/mL standard protease across the four batches. The inter-day precision was calculated as the RSD of the four net absorbance values of the 10 μg/mL standard protease across the four batches.

Three WMP samples (B1, F2, K3) were randomly selected for the recovery test to evaluate the reliability of the azocasein assay. A standard protease solution (100 µg/mL) was used for spiking. The spiked group was prepared by mixing 0.1 mL sample solution, 0.9 mL substrate solution and 10 µL standard protease solution (final protease concentration 0.99 µg/mL). A standard protease group (0.9 mL substrate solution + 10 µL standard protease solution + 0.1 mL buffer, no sample matrix), un-spiked sample groups and a substrate blank group were also included. All groups were performed in triplicate and incubated at 37 °C for 20 h.

The LOD/LOQ protease activity that represented the sensitivity of this assay was the highest LOD/LOQ lipase activity across the four batches.

3.4.1.3 Data Calculation

The protease activity in whole milk powder samples was expressed as the units (U) per gram whole milk powder, equivalent to the activity of the standard protease used in our study (Sigma-Aldrich, P5147). One unit (U) of protease activity was defined by the manufacturer as the amount of protease that liberates colour equivalent to 1.0 µmol (181 µg) of tyrosine per minute at pH 7.5 at 37 °C, using casein as the substrate and Folin-Ciocalteu reagent for colour development.

The net absorbance of each sample (A_{nets}) was calculated using the following equation:

$$A_{nets} = M_{T20s} - M_{T0s} - (M_{T20sb} - M_{T0sb})$$

Where M_{T20s} is the mean absorbance reading of triplicate sample absorbance readings at T20 time point; M_{T0s} is the mean absorbance reading of triplicate sample absorbance readings at T0 time point; M_{T20sb} is the mean absorbance reading of triplicate substrate blank absorbance readings at T20 time point; M_{T0sb} is the mean absorbance reading of triplicate substrate blank absorbance readings at T0 time point.

Because the net absorbance was obtained from four independent measurements, the standard deviation (SD) was calculated using error propagation:

$$SD_{nets} = \sqrt{SD_{T20s}^2 + SD_{T0s}^2 + SD_{T20sb}^2 + SD_{T0sb}^2}$$

The net absorbance of standard protease was calculated using the following equation:

$$A_{netsp} = M_{T20sp} - M_{T20sb}$$

Where M_{T20sp} is the mean absorbance reading of triplicate standard protease absorbance readings at T20 time point.

The net absorbance of substrate blank was calculated using the following equation:

$$A_{netsb} = M_{T20sb} - M_{T0sb}$$

The LOD in this azocasein assay was calculated using the following equation:

$$LOD_a = M_{sb} + 3 \times SD_a$$

Where M_{sb} is the net absorbance of substrate blank in each batch; SD_a is the SD value of substrate blank (T20) in each batch.

The LOQ in this azocasein assay was calculated using the following equation:

$$LOQ_a = M_{sb} + 10 \times SD_a$$

Because only the net absorbance of the 10 μg/mL standard protease exceeded the LOQ, the protease activity corresponding to the LOD/LOQ was estimated based on the net absorbance of the 10 μg/mL standard protease with two assumptions: (1) the relationship between protease activity and net absorbance is linear in the region around 10 μg/mL; and (2) the net absorbance at 0 μg/mL standard protease is zero. The protease activity corresponding to the LOD/LOQ was estimated using following equations:

$$P_{LOD,a} = \frac{10 \times 10^{-3}}{A_{netsp}} \times LOD_a \times \frac{3.5}{C_{sample}}$$

$$P_{LOQ,a} = \frac{10 \times 10^{-3}}{A_{netsp}} \times LOQ_a \times \frac{3.5}{C_{sample}}$$

Where $P_{LOD,a}$ is the protease activity (U/g WMP) corresponding to the LOD; A_{netsp} is the mean net absorbance of the 10 μg/mL standard protease calculated from the four net absorbance across the four batches; 10×10^{-3} is the standard protease concentration in mg/mL; 3.5 is the specific activity of the standard protease in U/mg; and C_{sample} is the WMP concentration in the reaction mixture (g/mL).

Recovery was calculated using the following equation:

$$Recovery(\%) = \frac{M_{spiked} - M_{unspiked}}{M_{sp} - M_{sb}} \times 100\%$$

Where M_{spiked} is the mean value of triplicate spiked sample absorbance readings; $M_{unspiked}$ is the mean value of triplicate un-spiked sample absorbance readings; M_{sp} the mean value of triplicate standard protease absorbance readings; M_{sb} is the mean value of triplicate substrate blank absorbance readings.

3.4.2 EnzChek® Assay

The second method for detecting residual protease activity was performed with EnzChek® Protease Assay Kit (E6638).

3.4.2.1 Assay Procedure

WMP samples were reconstituted by dissolving 0.1 g of powder in 0.9 mL of Milli-Q water, followed by centrifugation at 15,000 g for 10 min at 4 °C. The middle-layer liquid was diluted to a final concentration of 0.5 mg/mL and sequentially filtered by 0.45 μm and 0.22 μm filters.

For the reaction, 0.1 mL of sample working solution was mixed with 0.1mL of EnzChek® substrate working solution in a black, flat-bottom 96-well microplate (Nunclon Delta Surface, Thermo Fisher Scientific, Roskilde, Denmark) (reaction group). A positive control group (protease + substrate) was prepared using standard protease at five concentrations (0.5, 1, 2.5, 5, 10 ng/mL). Negative control (substrate + buffer), sample blank (sample working solution + buffer) and buffer blank (only buffer) groups were also included. The negative

group was performed in six replicates, while all other groups were prepared in triplicate.

The microplate was wrapped with aluminium foil and incubated at room temperature (22 ± 1 °C) for 20 h. After incubation, fluorescence intensity was measured using the multimode microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

The 16 samples were divided evenly into four batches of four samples each. Each batch included four sample reaction groups, four sample blank groups, five positive control groups (at standard protease concentrations of 0.5, 1, 2.5, 5, 10 ng/mL), one negative control group, and one buffer blank group. The four batches were tested on four consecutive days.

3.4.2.2 Method Validation

Several parameters were applied to validate the EnzChek® method, including intra-day precision, inter-day precision, recovery rate and the LOD/LOQ protease activity.

The intra-day precision was expressed as the range of RSD values obtained from the1, 2.5, 5, 10 ng/mL standard protease across the four batches. The inter-day precision was expressed as the RSD values of the net fluorescence of 2.5, 5, 10 ng/mL standard protease across four independent batches.

Three WMP samples (A, H1, A3) were randomly selected for the recovery test to evaluate the reliability of the EnzChek® assay. A standard protease solution (250 ng/mL) was used for spiking. To evaluate the influence of the filters used for sample working solution preparation, spiked samples were prepared both before and after filtration for each of the three selected samples. The spiked group was prepared by mixing 0.1 mL spiked sample solution (final protease concentration 2.48 ng/mL) with 0.1 mL substrate solution. The standard protease group (final protease concentration 2.48 ng/mL), the un-spiked sample groups and a substrate blank group were also included. All groups were performed in triplicate and incubated at 22 ± 1°C for 20 h.

The LOD/LOQ protease activity that represented the sensitivity of this assay was the highest LOD/LOQ protease activity across the four batches.

3.4.2.3 Data Calculation

The protease activity in whole milk powder samples was expressed as units (U) per gram whole milk powder (U/g WMP), equivalent to the activity of the standard protease used in our study (Sigma-Aldrich, P5147). One unit (U) of protease activity was defined by the manufacturer as the amount of protease that liberates colour equivalent to 1.0 µmol (181 µg) of tyrosine per minute at pH 7.5 at 37 °C, using casein as the substrate and Folin-Ciocalteu reagent for colour development. Protease activity was determined using the EnzChek assay and expressed as equivalent units (U) of the standard protease.

The net fluorescence of each sample was calculated using the following equation:

$$F_{net} = \bar{F}_r - \bar{F}_{sb} - \bar{F}_{rb} + \bar{F}_{bb}$$

Where F_{net} is the net fluorescence of a sample; $\bar{F}_r$ is the mean value of triplicate fluorescence readings of reaction group; $\bar{F}_{sb}$ is the mean value of triplicate fluorescence readings of sample blank group; $\bar{F}_{rb}$ is the mean value of triplicate fluorescence readings of substrate blank group; $\bar{F}_{bb}$ is the mean value of triplicate fluorescence readings of buffer blank group.

The SD value of the net fluorescence of the samples was calculated using error propagation:

$$SD_{net} = \sqrt{SD_r^2 + SD_{sb}^2 + SD_{rb}^2 + SD_{bb}^2}$$

The net fluorescence of each standard protease was calculated using the following equation:

$$F_{netsp} = \bar{F}_{sp} - \bar{F}_{rb}$$

Where F_{netsp} is the net fluorescence of a standard protease; $\bar{F}_{sp}$ is the mean value of triplicate fluorescence readings of a positive control (standard protease) group; $\bar{F}_{rb}$ is the mean value of triplicate fluorescence readings of substrate blank group.

The SD value of the net fluorescence of each standard protease was calculated using the following equation:

$$SD_{netsp} = \sqrt{SD_{sp}^2 + SD_{rb}^2}$$

The LOD/LOQ values were calculated according to SD values (SD_f) of the substrate blank in each bacth. The LOD of this assay was calculated using the following equation:

$$LOD_f = 3 \times SD_f$$

The LOQ of this assay was calculated using the following equation:

$$LOD_f = 10 \times SD_f$$

The standard curve was established in each batch, and concentrations of standard protease below the LOQ of each batch were excluded from standard curve fitting. The standard curve was expressed using the following equation:

$$y = aX + b$$

The protease activity corresponding to the LOD and LOQ were estimated using the following equations:

$$P_{LOD,f} = \frac{LOD_f - b}{a} \times 10^{-6} \times \frac{3.5}{C_{sample} \times 10^{-3}}$$

$$P_{LOQ,f} = \frac{LOQ_f - b}{a} \times 10^{-6} \times \frac{3.5}{C_{sample} \times 10^{-3}}$$

Where $P_{LOD,f}$ is the protease activity corresponding to the LOD in U/g WMP; $P_{LOQ,f}$ is the protease activity corresponding to the LOQ in U/g WMP; a is the slope of each standard curve in the four batches; b is the intercept of each standard curve in the four batches; C_{sample} is the WMP sample concentration in mg/mL; 3.5 is the specific activity of the standard protease in U/mg; 10^{-6} is the unit conversion factor from ng to mg; 10^{-3} is the unit conversion factor from mg to g.

Recovery was calculated using the following equation:

$$Recovery(\%) = \frac{\bar{F}_{spiked} - \bar{F}_{unspiked}}{\bar{F}_{sp} - \bar{F}_{sb}}$$

Where $\bar{F}_{spiked}$ is the mean value of triplicate spiked sample fluorescence readings; $\bar{F}_{unspiked}$ is the mean value of triplicate un-spiked sample fluorescence readings; $\bar{F}_{sp}$ is the mean value of triplicate standard protease fluorescence readings; $\bar{F}_{sb}$ is the mean value of triplicate substrate blank fluorescence readings.

3.5 Lipase Activity Detection Assay

Residual lipase activity in WMP samples was determined using a modified p-nitrophenyl palmitate (p-NPP) assay based on Teh et al. (2013) with some modifications (Teh et al., 2013).

3.5.1 Assay Procedure

A 0.05% (w/v) p-NPP stock solution was prepared in 50 mM Tris-HCl buffer (pH 8) containing 4 mg/mL Triton X-100, 0.1 mg/mL chloramphenicol, 0.02mg/mL Arabic gum, 1 mg/ml sodium azide and 10% (v/v) 2-propanol. This stock solution was diluted by the same buffer to obtain a 0.01% (w/v) working substrate solution.

WMP samples were reconstituted by dissolving 0.5 g of powder in 4.5 mL of Milli-Q water, followed by centrifugation at 15,000 g for 10 min at 4 °C. Then, 0.1 mL of the middle-layer liquid was mixed with 0.9 mL of the 0.01% p-NPP working substrate solution (reaction group). Positive control groups (lipase +substrate) were prepared using standard lipase at three concentrations (1, 2, 3 mg/mL or 0.5, 1, 2 mg/mL), and a negative control group (buffer + substrate) was also included. All group were performed in triplicates. All three groups were incubated in a 37 ± 1 °C water bath for 2 h (T2).

To obtain the initial time point (T0), a parallel set of all three groups were terminated immediately after mixing. The reaction was stopped by adding 0.6 mL of mixture to 0.6 mL of 99.5% ethanol. Both T0 and T2 groups were centrifuged at 20,000 g for 10 min at 4 °C. Subsequently, 0.2 mL of the supernatant was transferred into a flat-bottom 96-well microplate, and the absorbance was read at 405 and 630 nm (for turbidity correction) using the multimode microplate reader (Rauh et al., 2014; Wesolowska et al., 2018).

The 16 samples were divided evenly into four batches of four samples each. Each batch included four sample reaction groups, three positive control groups (the first batch and the other three batches at standard lipase concentrations of 1, 2, 3 mg/mL and 0.5, 1, 2 mg/mL respectively) and one negative control group. The four batches were tested on four consecutive days.

3.5.2 Method Validation

Several parameters were applied to validate the modified p-NPP lipase assay, including intra-day precision, inter-day precision, recovery rate and the LOD/LOQ lipase activity.

The intra-day precision was expressed as the range of RSD values obtained from all samples and all concentrations of standard lipase across the four batches. The inter-day precision was expressed as the RSD values of the net fluorescence of 0.5, 1, 2 mg/mL standard protease across four independent batches.

Three WMP samples (B1, H2, K1) were randomly selected for the recovery test to evaluate the reliability of the modified p-NPP lipase assay. A standard lipase solution (10 mg/mL) was used for spiking. The spiked group was prepared by mixing 0.01 mL standard lipases solution, 0.09mL sample solution and 0.9 mL substrate solution (final lipase concentration 1 mg/mL). The standard protease group (final protease concentration 1 mg/mL), the un-spiked sample groups and a substrate blank group were also included. All groups were performed in triplicate and incubated at 37 °C for 2 h.

The LOD/LOQ lipase activity that represented the sensitivity of this assay was the highest LOD/LOQ lipase activity across the four batches.

3.5.3 Data Calculation

Lipase activity in whole milk powder samples was expressed as units (U) per gram of whole milk powder (U/g WMP), equivalent to the activity of the commercial standard lipase used in our study (Sigma-Aldrich, L1754). One unit (U) of lipase activity of the standard lipase was defined as the amount of enzyme that liberates 1.0 microequivalent of fatty acid from a triglyceride per hour at pH 7.2 at 37 °C by the manufacturer.

The net absorbance of each sample (A_{nets}) was calculated using the following equation:

$$A_{nets} = M_{T2s} - M_{T0s} - (M_{T2sb} - M_{T0sb})$$

Where M_{T2s} is the mean absorbance of triplicate absorbance readings (corrected by 630 nm) of samples at T2 time point; M_{T0s} is the mean absorbance of triplicate absorbance readings (corrected by 630 nm) of samples at T0 time point; M_{T2sb} is the mean absorbance of triplicate absorbance readings (corrected by 630 nm) of substrate blank at T2 time point; M_{T0sb} is the mean absorbance of triplicate absorbance readings (corrected by 630 nm) of substrate blank at T0 time point.

The SD value of the net absorbance of each sample was calculated using error propagation:

$$SD_{nets} = \sqrt{SD_{T2s}^2 + SD_{T0s}^2 + SD_{T2sb}^2 + SD_{T0sb}^2}$$

The net absorbance of standard lipase (A_{netsl}) was calculated using the following equation:

$$A_{netsl} = M_{T2sl} - M_{T2sb}$$

Where M_{T2sl} is the mean absorbance of triplicate absorbance readings (corrected by 630 nm) of standard lipase at T2 time point.

The SD value of the net absorbance of standard lipase was calculated using error propagation:

$$SD_{netsl} = \sqrt{SD_{T2sl}^2 + SD_{T2sb}^2}$$

The net absorbance of substrate blank (A_{netsb}) was calculated using the following equation:

$$A_{netsb} = M_{T2sb} - M_{T0sb}$$

The LOD in this p-NPP lipase assay was calculated using the following equation:

$$LOD_p = M_{sb} + 3 \times SD_p$$

Where M_{sb} is the net absorbance of substrate blank in each batch; SD_p is the SD value of substrate blank (T2) in each batch.

The LOQ in this p-NPP lipase assay was calculated using the following equation:

$$LOQ_p = M_{sb} + 10 \times SD_p$$

The standard curve was established in each batch, and concentrations of standard protease below the LOQ of each batch were excluded from standard curve fitting. The standard curve was expressed using the following equation:

$$y = aX + b$$

The lipase activity of the WMP sample was calculated using the following equation:

$$Ls = \frac{A_{nets} - b}{a} \times \frac{700}{C_{sample}}$$

Where Ls is the lipase activity of WMP sample in U/g WMP; A_{nets} is the net absorbance of WMP sample; a is the slope of each standard curve in the four batches; b is the intercept of each standard curve in the four batches; C_{sample} is the WMP sample concentration in g/mL; 700 is the specific activity of the standard lipase in U/mg.

The SD value of lipase activity (SD_{la}) was obtained by further propagating the SD value of the net absorbance (SD_{nets}) through the standard curve (using the slope of the curve):

$$SD_{la} = \frac{SD_{nets}}{a} \times \frac{700}{C_{sample}}$$

The lipase activity corresponding to the LOD/LOQ (L_{LOD}/L_{LOQ}) was calculated using the following equations:

$$L_{LOD} = \frac{LOD_p - b}{a} \times \frac{700}{C_{sample}}$$

$$L_{LOQ} = \frac{LOQ_p - b}{a} \times \frac{700}{C_{sample}}$$

Recovery was calculated using the following equation:

$$Recovery(\%) = \frac{M_{spiked} - M_{unspiked}}{M_{sl} - M_{sb}} \times 100\%$$

Where M_{spiked} is the mean value of triplicate spiked sample absorbance readings; $M_{unspiked}$ is the mean value of triplicate un-spiked sample absorbance readings; M_{sl} the mean value of triplicate standard lipase absorbance readings; M_{sb} is the mean value of triplicate substrate blank absorbance readings.

3.6 Fat and Protein Content Monitoring Using FOSS Milkoscan FT1

The fat and protein contents of WMP samples were determined monthly over a storage period of 6 – 7 months (high-quality 7 months, poo-quality 6 months) using a FOSS Milkoscan FT1 (FOSS Analytical A/S, Hilleroed, Denmark).

Briefly, 5 g of WMP was reconstituted in 45 mL distilled water and mixed thoroughly. Although pre-heating at 40 °C for 30 min is recommended for Milkoscan to ensure complete melting of fat, all samples were measured at room temperature (22 ± 1 °C) for convenience as the main objective was to monitor the trend of fat and protein contents over storage rather than obtaining absolute values.

The fat and protein contents were converted to a dry matter basis using the following equation:

$$X_{db} = \frac{X_{wb}}{TS} \times 100\%$$

Where X_{db} is the dry basis content of fat or protein; X_{wb} is the wet basis content of fat or protein measured by FOSS Milkoscan; TS is the total solid content measured by FOSS Milkoscan.

The SD of dry basis content (SD_{db}) was calculated using the following equation:

$$SD_{db} = \frac{SD_{wb}}{TS} \times 100\%$$

Where SD_{wb} is the SD of wet basis content of fat or protein measured by FOSS Milkoscan.

3.7 Statistical Analysis

All statistical analyses were performed in R (version 4.5.3; R core Team, 2026). All figures were generated using the ggplot2 package (Wickham, 2016). Statistical significance was set at $p < 0.05$ (two sided).

Because repeated measurements of bacteria counts were taken within the same sample over time, resulting in non-independence observations, meanwhile, missing data being present, linear mixed-effects models (LMMs) were fitted using restricted maximum likelihood estimation (REML) through the lmer function from lme4 package (Bates et al., 2015; Z. Yu et al., 2022). The mesophilic and thermophilic data were log-transformed to meet the assumptions of normality and homoscedasticity. The model included quality group as a fixed effect and sample ID as a random effect. Model assumptions including linearity, normality and homoscedasticity were assessed using diagnostic plots and Shapiro-Wilk test (**Appendix Figure S1, S2, Table S1**). Emmeans package was applied to obtain estimated marginal means and pairwise comparison (Lenth & Piaskowski, 2026).

Lipase activity data were analysed using a multiple linear regression model (LM), with assay batch and turbidity change included as covariates. Model assumptions (linearity, normality, homoscedasticity of residuals) were verified using residual diagnostics (**Appendix Figure S3, Table S3**). A sensitivity analysis was conducted by comparing model results with and without sample B1 to assess its influence on the quality effect estimate. Type III analysis of variance (Type III ANOVA) was performed using car package (John Fox & Sanford Weisberg, 2019). Emmeans package was applied to obtain estimated marginal means and pairwise comparison.

Fat and protein content change trends in high- and poor-quality samples were analysed using LMMs, with sample quality, storage time and their interactions as fixed effects, sample ID as random effects. The significance of each effect was assessed using Type III ANOVA. The slopes of fat or protein content over time were estimated using the emtrends function from emmeans package. Marginal R^2 values, which represent the proportion of variance explained by fix effects, were calculated using r.squaredGLMM function in the MuMIn package

(Bartoń, 2026). Model assumptions including linearity, normality and homoscedasticity were verified using diagnostic plots and Shapiro-Wilk test (**Appendix Figure S4, S5, Table S6**).

4 Results and Discussion

4.1 Mesophilic and thermophilic plate counts

In order to assess the correlations between aerobic plate counts (APC) in raw milk and bacteria counts in milk powder, and between bacteria counts in milk powder and enzyme activity in milk powder, mesophilic and thermophilic bacteria counts were enumerated in WMP samples.

The assumptions of LMMs for mesophilic plate counts were generally met (**Appendix S1, S2, Table S1**). The normality assumption of random effects in the thermophilic model was marginally violated (Shapiro-Wilk: W = 0.08, p = 0.043), driven by sample C2, which exhibited much higher plate count results than other samples. A sensitivity analysis excluding sample C2 produced consistent results of quality effect on thermophilic plate counts. The assumptions of LMMs excluding sample C2 were generally met. These results confirmed that the inclusion of sample C2 would not substantially affect the conclusion. Therefore, all the analysis below included sample C2.

The mesophilic and thermophilic bacteria count of high-quality samples were from 2.60 to 3.28 and from 2.10 to 3.98 log cfu/g, while those of poor-quality samples were from 3.05 to 3.32 and from 2.71 to 3.47 log cfu/g (**Table 4.1**). The LMMs revealed that high-quality samples exhibited significantly lower mesophilic counts (estimated mean: 2.88 ± 0.04 log cfu/g, 95% *CI* [2.80 – 2.97]) compared to poor-quality samples (estimated mean: 3.16 ± 0.05 log cfu/g, 95% *CI* [3.05 – 3.27]), with a mean difference of 0.28 log cfu/g (95% *CI* [0.14 – 0.42], t (14.8) = 4.22, p < 0.001) (**Figure 4.1**). This is consistent with the difference of APC between high- and poor-quality samples, which indicates that the mesophilic bacteria might mainly come from raw milk contamination. The study conducted by Paludetti et al. (2019) showed a similar correlation between APC of raw milk and the mesophilic bacteria counts in skim milk powders (Paludetti et al., 2019).

Table 4. 1
Mesophilic and thermophilic bacteria plate count results from July to September of 2025. Poor-quality samples (A1, A2, A3, K1, K2, K3) were received one month later, thus, there were no plate count data of them in July.

Sample ID	Mesophilic bacteria (log cfu/g)			Thermophilic bacteria (log cfu/g)		
	July	August	September	July	August	September
A	2.81	2.92	2.95	3.45	2.10	2.76
B1	2.91	3.21	3.10	2.67	2.41	2.92
B2	2.60	2.74	2.68	NA	3.06	2.55
C1	2.83	3.28	3.08	3.11	3.69	2.87
C2	2.85	3.22	2.90	3.43	3.94	3.98
F1	2.65	NA	2.81	NA	NA	2.47
F2	2.71	NA	2.77	2.44	NA	2.58
F3	2.66	NA	2.92	2.56	2.90	2.63
H1	NA	3.06	2.75	3.01	3.08	2.92
H2	2.89	NA	2.94	2.91	3.13	3.58
A1	-	3.14	3.10	-	2.94	2.94
A2	-	3.11	3.22	-	3.08	3.39
A3	-	3.17	3.20	-	2.71	2.98
K1	-	3.19	3.32	-	2.88	3.03
K2	-	3.13	3.16	-	2.90	3.11
K3	-	3.13	3.05	-	3.07	3.47

Note: NA means the plate count outcome of each replicate at each dilution exceeded 10 – 300 cfu/plate, which was considered as the countable limitation range.

In contrast, the thermophilic bacteria counts did not differ significantly between high- and poor-quality samples (estimate = 0.10, *95% CI* [-0.28, 0.49], *t* (14.6) = 0.57, *p* = 0.576). On average, poor-quality samples (estimated mean: 3.04 ± 0.14 log cfu/g, 95% *CI* [2.74 – 3.35]) had 0.10 log cfu/g higher thermophilic bacteria counts than high-quality samples (estimated mean: 2.94 ± 0.11 log cfu/g, 95% *CI* [2.71 – 3.17]) (**Figure 4.2**). The thermophilic bacteria in these two quality samples might be the result of contamination from manufacturing facilities rather than that from raw milk (Dettling et al., 2020; Scott et al., 2007), therefore, the thermophilic bacteria counts did not exhibit the similar difference between high- and poor-quality samples as that of APC and mesophilic bacteria counts.

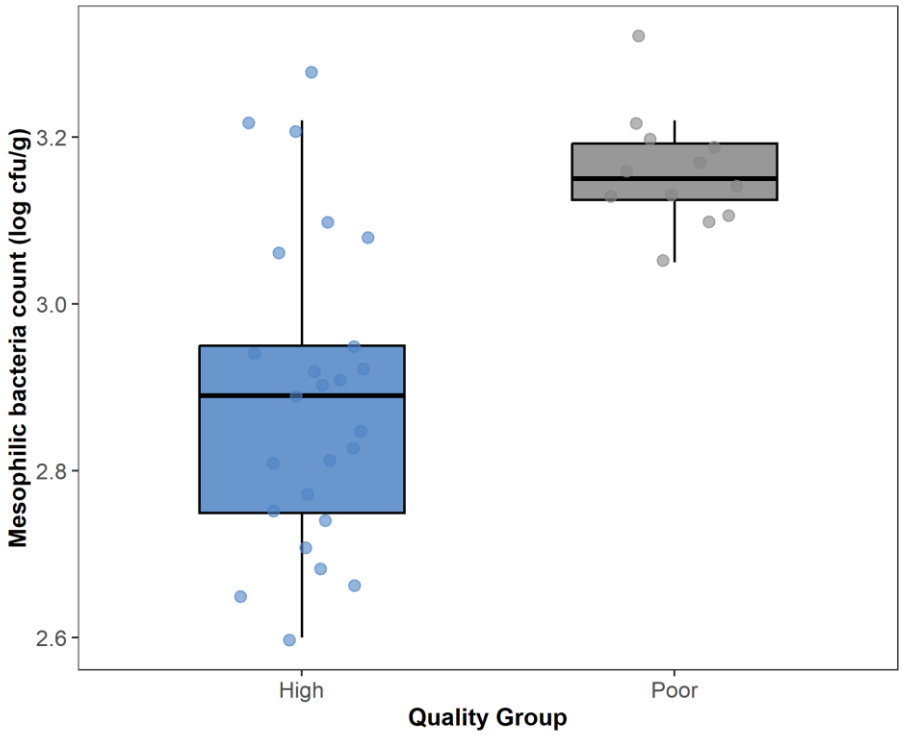


Figure 4. 1
Comparison of the mesophilic bacteria counts between high- and poor-quality samples.

The mesophilic and thermophilic bacteria count results of both qualities in our study are higher than those from Pramularsih et al. (2022), of which mesophilic and thermophilic count results were 2.22 and 1.82 log cfu/g respectively (Pramularsih et al., 2022). The mesophilic count result in our study is similar to Paludetti et al. (2019), while the thermophilic count result is lower than theirs. Those in their study were 2.96 and 3.61 log cfu/g in skim milk powder samples respectively (Paludetti et al., 2019). The study of Pramularsih et al. (2022) also showed that the dominant specie at 30 °C was *Bacillus licheniformis*, and that at 55 °C was *Geobacillus stearothermophilus* in WMP samples (Pramularsih et al., 2022). In addition, Scott et al. (2007) found that the dominant thermophilic spores in WMP samples were from *Anoxybacillus* and *Geobacillus* species (Scott et al., 2007). However, all of these species have been demonstrated that they could produce heat stable proteases and lipases (J. González et al., 2016; Medjahdi et al., 2025; Sadiq et al., 2016), which may maintain activity in milk powder during storage and pose shelf-life stability risks to milk powder and downstream products.

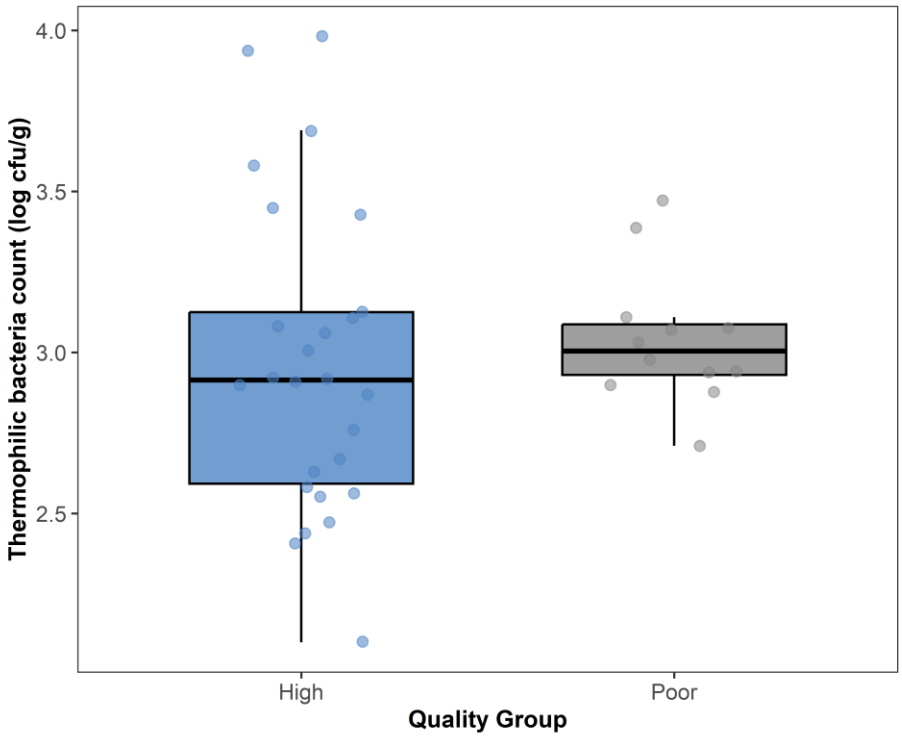


Figure 4. 2
Comparison of thermophilic bacteria counts between high- and poor-quality samples.

4.2 Protease Activity

4.2.1 Azocasein Assay Validation

The stability and reproducibility of the modified azocasein assay was evaluated by intra-day, inter-day precisions, and recovery test (**Table 4.2**). The validation results indicate that the precision of this modified azocasein assay was good and the recovery was acceptable. In addition, the LOD/LOQ protease activities of this modified azocasein assay in our WMP samples was 11.53 and 26.20 mU/g WMP. The sensitivity of azocasein assay in the present study was higher than the value of 29.6 mU/g WMP estimated by converting the LOD reported by Bendicho et al. (2002) for whole milk (3.26 mU/mL) to a dry matter basis using a total solids content of 11% (Bendicho et al., 2002). The difference may be due to the substantially longer incubation time in present study (20 h vs. 15 min).

Table 4. 2
Validation parameters of the modified azocasein assay for residual protease activity in WMP.

Parameters	Values	Unit	Remarks/Calculation basis
Intra-day precision (RSD)	1.4 - 5.8	%	The range of RSD from the 10 µg/mL standard protease across the four batches
Inter-day precision (RSD)	3.5	%	RSD of the four net absorbance values of the 10 µg/mL standard protease across the four batches
Recovery (mean ± SD)	124.9 ± 4.5	%	Spiking at 0.99 µg/mL of final standard protease in three randomly selected WMP samples (B1, F2, K3); n = 3.
LOD (absorbance - based)	0.0088	-	M + 3 * SD; see the section 3.4.1.3.
LOQ (absorbance-based)	0.0200	-	M + 10 * SD; see the section 3.4.1.3.
Estimated LOD protease activity	11.53	mU/g WMP	Estimated based on the net absorbance of the 10 µg/mL standard protease.
Estimated LOQ protease activity	26.20	mU/g WMP	Estimated based on the net absorbance of the 10 µg/mL standard protease.

4.2.2 Protease Activity by Azocasein Assay

Due to the sensitivity limit of azocasein assay, the protease activity was not detected in both high- and poor-quality WMP samples (**Table 4.3**). Based on the protease activity of LOD of this azocasein assay in present study, the protease activities of all 16 WMP samples might be below 11.53 mU/g WMP. The study of Chen (2000) demonstrated the presence of residual protease activity by SDS-PAGE and RP-HLPC methods in WMP samples. Meanwhile, their study indicated that milk protein in the reaction mixture might significantly compete with the azocasein on the residual protease, remarkably reducing the sensitivity of the assay (Chen, 2000). Button et al. (2011) spent 336 h (14 days) in detecting the extreme low level protease activity in UHT milk using casein as the substrate (Button et al., 2011). Therefore, the detection of residual protease activity using azocasein assay may need a much longer incubation period.

Table 4. 3
Protease Activity in WMP samples determined by the modified azocasein assay.

Sample	Net Absorbance Readings	Protease Activity (mU/g WMP)
A	0.0000 ± 0.0024	＜LOD
B1	-0.0005 ± 0.0023	＜LOD
B2	0.0008 ± 0.0023	＜LOD
C1	0.0009 ± 0.0024	＜LOD
C2	0.0000 ± 0.0018	＜LOD
F1	-0.0001 ± 0.0018	＜LOD
F2	0.0001 ± 0.0019	＜LOD
F3	0.0009 ± 0.0018	＜LOD
H1	0.0016 ± 0.0016	＜LOD
H2	0.0017 ± 0.0019	＜LOD
A1	0.0026 ± 0.0018	＜LOD
A2	0.0020 ± 0.0015	＜LOD
A3	0.0018 ± 0.0014	＜LOD
K1	0.0011 ± 0.0011	＜LOD
K2	0.0023 ± 0.0019	＜LOD
K3	0.0038 ± 0.0022	＜LOD

Note: All WMP samples showed net absorbance values below the LOD (LOD = 0.0088; estimated LOD protease activity = 11.53 mU/g WMP). Therefore, protease activity was reported as below the LOD (< LOD).

4.2.3 EnzChek Assay Validation

Intra-day precision (RSD) was 0.5 – 2.3 % based on the RSD values of the 1, 2.5, 5 and 10 ng/mL standard protease across the four batches. Inter-day precision was showed in **Table 4.4**. The net fluorescence values of all concentrations of standard protease in the first batch were much higher than the later three batches (might be because the substrate solution was freshly prepared in the first batch, the substrate solution used in the later three batches had been stored in 4 °C room for 1-3 days), resulting in higher inter-day RSD values. However, the inter-day RSD values were significantly lower in the later three batches. These inter-day RSD values indicate that the inter-day precision of EnzChek assay was acceptable.

Table 4. 4
Inter-day precision (RSD) of the EnzChek assay using standard protease.

Standard protease (ng/mL)	Net fluorescence of Batch 1	Net fluorescence of Batch 2	Net fluorescence of Batch 3	Net fluorescence of Batch 4	RSD[a] （%）	RSD[b] （%）
2.5	2.8093 ± 0.1929	1.9017 ± 0.0736	1.9890 ± 0.1594	1.8255 ± 0.0573	21.4	4.3

5	5.2873 ± 0.1894	3.9097 ± 0.0893	4.0333 ± 0.2035	3.8845 ± 0.1528	15.8	2.0
10	10.5073 ± 0.3602	8.1483 ± 0.2647	8.7440 ± 0.2887	8.3625 ± 0.1441	12.0	3.6

Note: [a]The value is the RSD value of the net fluorescence across four batches; [b]the value is the RSD value of the net fluorescence across the later three batches.

The recovery results were showed in **Table 4.5**. The recovery rates of samples after filtration were not lower than those of samples before filtration, which indicates that filters used in this assay did not reduce the concentration of protease in the samples. The recovery rates also indicate that the protease activity in spiked WMP sample solution was significantly higher than that in buffer. This might be attributed to the increasing plasmin activity in sample solution with the presence of the standard protease, a kind of bacterial protease. The study of Fajardo-Lira et al. (2000) showed that plasmin were substantially released from casein micelle with the presence of protease from *Pseudomonas fluorescens* (Fajardo-Lira et al., 2000). Frohbieter et al. (2005) reported that the activity and catalytic efficiency of plasmin activator in bovine milk increased by 2.5- and 4-fold with the presence of protease from *Pseudomonas fluorescens* (Frohbieter et al., 2005). On the other hand, elements such as calcium ions in milk matrix might promote protease stability and activity, resulting higher activity in milk matrix than in buffer (Ertan et al., 2015). Meanwhile, fluorescence assay is considered much more sensitive than azocasein assay (Button et al., 2011; Chen et al., 2003), which also was confirmed in the present study. This high sensitivity might further increase the recovery rate of fluorescence assay than azocasein assay.

Table 4. 5
Recovery test results of EnzCheck assay.

Sample ID	Recovery of sample before filtration (%)	Recovery of sample after filtration (%)
A	198.6 ± 12.0	224.7 ± 15.5
H1	193.1 ± 13.3	193.1 ± 14.6
A3	179.0 ± 6.2	182.9 ± 4.5

The parameters of standard curves in each batch and the sensitivity of EnzChek assay are showed in **Table 4.6**. The LOD/LOQ fluorescence of this

assay were 0.3711 and 1.2370, and the LOD/LOQ protease activities by EnzChek assay in our WMP samples were 3.69 and 9.86 mU/g WMP.

Table 4. 6
Standard curve parameters and the sensitivity of EnzChek assay.

Batch	Slope (RFU· mL/ng)	y - intercept	R^2	Linear range (ng/mL)	Numbers of point	Blank SD (n=6)	LOD (mU/g WMP)	LOQ (mU/g WMP)
1	1.0304	0.2012	0.9985	1 - 10	4	0.0751	0.15	3.33
2	0.8145	-0.061	0.9994	1-10	4	0.0636	1.93	5.35
3	0.8773	-0.1465	0.9981	2.5- 10	3	0.1237	3.69	9.86
4	0.8387	-0.1382	0.9982	1 - 10	4	0.0416	1.96	4.13

Note: The value of LOD/LOQ is the protease activity corresponding to the LOD/LOQ fluorescence in each batch; the concentration points used in the standard curve are those that have higher net fluorescence than the LOQ fluorescence.

4.2.4 Protease Activity by EnzChek Assay

Protease activity determined by EnzChek assay was detected exclusively in sample A. According to the conservative LOD/LOQ values in the present study, the protease level in sample A could be detected (> 3.69 mU/g WMP) but was not high enough to be quantified (< 9.86 mU/g WMP), while that in other samples might be below 3.69 mU/g WMP (**Table 4.7**). Considering the competition of milk protein with the artificial substrate in the assay, the real protease activity in WMP samples might be higher than these results.

Table 4. 7
Protease activity in WMP samples determined by EnzChek assay.

Sample	Net Fluorescence	Protease Activity (mU/g WMP)
A	0.8131 ± 0.0829	> LOD, < LOQ
B1	-0.1112 ± 0.0913	＜LOD
B2	-0.3886 ± 0.0856	＜LOD
C1	-0.1265 ± 0.0920	＜LOD
C2	-0.1733 ± 0.0679	＜LOD
F1	-0.2247 ± 0.1045	＜LOD
F2	-0.2653 ± 0.0712	＜LOD
F3	-0.0567 ± 0.0793	＜LOD
H1	-0.1158 ± 0.1466	＜LOD
H2	-0.0630 ± 0.1291	＜LOD
A1	-0.1213 ± 0.1295	＜LOD
A2	-0.0616 ± 0.1558	＜LOD
A3	-0.1437 ± 0.0537	＜LOD
K1	-0.0505 ± 0.1101	＜LOD
K2	0.0205 ± 0.2316	＜LOD

K3	-0.0068 ± 0.0460	＜LOD

Note: LOD = 3.69 mU/g WMP and LOQ = 9.86 mu/g WMP were the most conservative values determined from the four batches and were applied uniformly to all samples for reporting. Sample A showed net fluorescence above the LOD fluorescence (0.3711) but below the LOQ fluorescence (1.2370) and was therefore reported as > LOD, < LOQ.

The residual protease activity in WMP is very stable and may still pose risks to product quality during storage. The study of Celestino et al. (1997) showed storage period had no significant influence on the protease activity in WMP samples (Celestino et al., 1997a). In the study of Chen (2000), a new N-terminal sequence was detected by RP-HPLC method, and the proteolysis in WMP samples during storage was believed to be the only cause of this new N-terminal sequence (Chen, 2000). In addition, the residual protease activity in WMP will also be significantly detrimental to the downstream products such as UHT milk. Celestino et al. (1997) found that residual protease activity resulted in gelation in UHT milk processed from freshly manufactured WMP in the third month (Celestino et al., 1997a). Therefore, it is critical to investigate the residual protease activity in WMP to ensure the shelf-life stability.

Sample A was classified as high-quality grade based on the bacterial load of the raw milk. However, even within the high-quality group, neither the raw milk bacterial load nor the mesophilic and thermophilic bacterial counts in the corresponding milk powder were the highest. Despite this, sample A exhibited relatively high residual protease activity. These findings indicate that bacterial counts in raw milk or milk powder may not reliably predict the level of residual protease activity in the final product. Therefore, direct measurement of protease activity in milk powder may be a necessarily complementary approach to assess product stability prior to market release.

4.3 Lipase Activity

4.3.1 p-NPP Lipase Assay Validation

Intra-day precision (RSD) was 0.81 – 5.16% based on the RSD values of all samples and all three concentrations of standard lipase across the four batches. Inter-day precision (RSD) was showed in Table **4.8**.

Table 4. 8
Inter-day precision (RSD) of p-NPP assay expressed by standard lipase.

Standard lipase concentration	Batch 1[a]	Batch 2[a]	Batch 3[a]	Batch 4[a]	RSD (%)
0.5		0.0413 ± 0.0047	0.0440 ± 0.0037	0.0418 ± 0.0038	3.39
1	0.0810 ± 0.0056	0.0896 ± 0.0091	0.0853 ± 0.0100	0.0897 ± 0.0039	4.82
2	0.1520 ± 0.0128	0.1617 ± 0.0152	0.1482 ± 0.0033	0.1516 ± 0.0031	3.79
3	0.1931 ± 0.0040				

Note: [a]The values are net absorbance of standard lipase in each batch.

Due to the high variability (RSD = 12.1%) observed in the spiked replicates for sample B1 during the first recovery experiment of the p-NPP lipase assay, a repeat recovery experiment was performed using the same WMP samples. The results of both recovery experiments are presented in **Table 4.9**. The standard lipase activity showed relative consistently high recovery rates in all three samples in the first experiment, and consistently low recovery rates in all three samples in the second experiment. This indicates a complex interference by the milk matrix to the p-NPP assay. Milk fats might significantly compete with the substrate (p-NPP) used in the assay, and free fatty acids could considerably inhibit the lipolysis of the p-NPP (Blake et al., 1996), which would underestimate the real lipase activity in WMP samples, meanwhile, reduce the recovery rates of the standard lipase. On the other hand, calcium ions in milk are able to increase the lipase activity (Martins et al., 2015), which may make the recovery rates of standard lipase rising in milk matrix. In addition, our study confirmed that the turbidity of the milk matrix would considerably influence the absorbance readings of the samples. Even though 630 nm absorbance readings were applied, the turbidity influence could not be completely diminished because it might produce different absorbance readings at 450 and 630 nm. Moreover, the turbidity varied between batches, which also might be one reason that resulted in the different recovery rates between the two recovery experiments. **Table S1** (see the **Appendix**) showed the turbidity (630 nm absorbance readings) change of the same three samples between unspiked and spiked incubation from the two recovery experiments. This variance might be due to the different extent of hydrolysis in the matrix of each batch, which further destroyed the emulsion system of the matrix at different level and then resulted in different

turbidity in the supernatant after centrifugation (Bourlieu et al., 2012). Our recovery results indicate that the direction and extent of the matrix interference (as indicated by recovery rates) appeared consistent across samples proceeded within the same experimental batch.

Table 4. 9
Recovery test results of the p-NPP assay.

Sample	Recovery (%)	
	First-time	Second-time
B1	94.9 ± 4.6	69.0 ± 0.8
K1	147.1 ± 3.4	78.5 ± 1.0
H2	126.7 ± 2.5	74.9 ± 0.9

The parameters of standard curves in each batch and the sensitivity of the p-NPP assay are showed in **Table 4.10**. The LOD/LOQ absorbance of this modified p-NPP lipase assay was 0.0194/0.0404, and the LOD/LOQ lipase activity by this assay in our WMP sample was 1356/3002 U/g WMP.

Table 4. 10
Standard curve parameters and the sensitivity of the p-NPP assay

Batch	Slope (RFU·mL/ng)	y - intercept	R^2	Linear range (ng/mL)	Blank SD (n=3)	LOD (U/g WMP)	LOQ (U/g WMP)
1	0.065	0.009	0.9806	1 - 3	0.0023	744	2320
2	0.0812	0.0021	0.9963	0.5-2	0.003	1356	3002
3	0.0735	0.005	0.9924	0.5-2	0.0013	597	1385
4	0.0759	0.0043	0.9899	0.5-2	0.0009	553	1082

Note: The value of LOD/LOQ is the lipase activity corresponding to the LOD/LOQ absorbance in each batch; the concentration points used in the standard curve are those that have higher net fluorescence than the LOQ absorbance.

4.3.2 Lipase Activity by p-NPP Assay

The lipase activity of each WMP sample were showed in **Table 4.11**. The lipase activity value of sample B1 was calculated using the standard curve of batch 1 for the statistical analysis. Sensitivity analysis showed that inclusion or exclusion of sample B1 did not change the conclusions regarding the milk quality effect, therefore, all the statistical analysis below included sample B1. Residual diagnostics indicate that model assumptions were generally met (**Appendix Figure S3, Table S2**).

Table 4. 11
Lipase activity in WMP samples determined by the p-NPP assay.

Sample	Net absorbance (630nm Corrected)	Lipase activity (U/g WMP)
A	0.0658 ± 0.0062	5264 ± 537
B1	0.0339 ± 0.0087	> LOD, < LOQ[b]
B2	0.0668 ± 0.0096	5354 ± 831
C1	0.0405 ± 0.0034	3070 ± 294
C2	0.0747 ± 0.0219	5903 ± 1836[a]
F1	0.0432 ± 0.0126	3261 ± 1056[a]
F2	0.0607 ± 0.0115	4732 ± 964
F3	0.0444 ± 0.0157	3362 ± 1316[a]
H1	0.052 ± 0.0099	4210 ± 969
H2	0.0896 ± 0.0050	7894 ± 490
A1	0.0727 ± 0.0110	6236 ± 1077
A2	0.0492 ± 0.0083	3936 ± 813
A3	0.0962 ± 0.0108	7372 ± 846
K1	0.0986 ± 0.0108	7565 ± 846
K2	0.0763 ± 0.0051	5812 ± 400
K3	0.0777 ± 0.0088	5927 ± 690

Note: [a]Samples showing high variability (RSD > 25%). These results were calculated using full error propagation but exhibited large standard deviations, possibly due to matrix effects. [b]LOD = 1356 mU/g WMP and LOQ = 3002 mu/g WMP were the most conservative values determined from the four batches and were applied uniformly to all samples for reporting. Sample B1 showed net fluorescence above the LOD fluorescence (0.0194) but below the LOQ fluorescence (0.0404) and was therefore reported as > LOD, < LOQ.

Linear regression model (LM) showed that the quality of our WMP samples did not significantly affect the lipase activity after controlling batch and turbidity change (F (1, 10) = 0.443, p = 0.521). The estimated marginal mean lipase activity was 5213 U/g WMP (95% *CI*:4961 – 5465) in high-quality samples and 5045 U/g WMP (95% *CI*: 4667 – 5423) in poor-quality samples (**Figure 4.3**). The difference between these two groups was not significant (estimate = 168, *SE* = 252, t (10) = 0.665, p =0.521). The LM model explained 98.5% of the total variance (adjusted R^2 = 0.978; F (5, 10) = 133, p < 0.001).

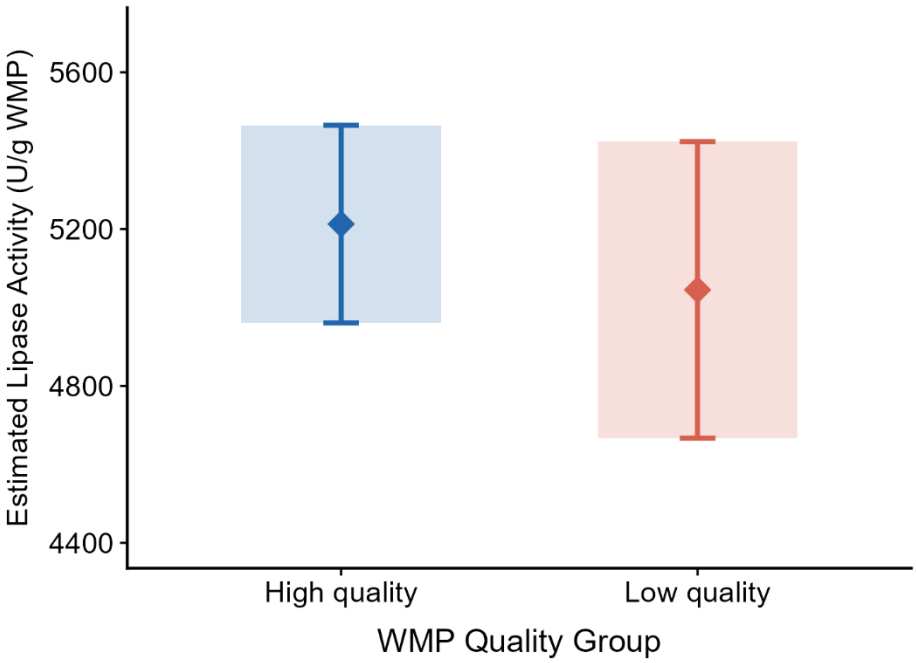


Figure 4. 3
Estimated marginal means of lipase activity in high- and poor-quality WMP sample groups. Diamond symbols represent the estimated marginal means; error bars and shaded regions indicate 95% confidence intervals.

It should be noted that turbidity change had a significant positive effect on lipase activity values (F (1, 10) = 365.733, $p < 0.001$). This indicates the lipase activity results obtained from p-NPP assay were substantially influenced by matrix effects (turbidity). In addition, the batch effect also had a statistically significant influence on the lipase activity values (F (3, 10) = 11.049, $p = 0.002$). Moreover, the experimental design was imbalanced, with most batches containing only one quality samples (all high-quality or all poor-quality). This imbalanced design resulted in partial confounding between sample quality and batch, which limited the ability of statistical model to identify the effect of sample quality on lipase activity. Therefore, the absolute values of lipase activity in our samples, comparisons of relative lipase activity among samples from different batches, and the non-significant difference between high- and poor-quality samples should be interpreted with caution. Future studies using p-NPP assay in WMP samples are recommended to balance the allocation of quality groups across batches, increase the overall sample size, and considering processing all samples within few batches where possible, to improve the reliability of the results.

However, samples within the same batch were processed and analysed under standardised conditions, and the model included turbidity change as a covariate to statistically adjust for matrix effects. Consequently, comparisons of relative lipase activity among samples within the same batch are considered reliable. In the first batch, which included both high-quality samples (H1 and H2) and poor-quality samples (A1 and A2), the lipase activity of sample H2 was notably higher than those of A1 and A2 (**Table 4.11**). This example indicates that the bacterial counts of raw milk or milk powder may not reliably predict the residual enzyme activity in WMP. Although the bacterial counts did not exceed the regulatory limit, enzymatic deterioration in WMP would still occur during storage (Food Standards Australia New Zealand, 2025). These findings support the value of direct enzyme activity measurements as a complementary approach to evaluate the shelf-life stability of the final dairy products. Former studies also reported that the determination of bacterial number would not provide reliable predictions of potential spoilage in dairy products (Chen, 2000).

The study of Celestino et al. (1997) showed that lipase activity could be stable in WMP sample over 8 months. During the storage, they found significant increase of free fat acid (FFA) from the second month at 25 ± 1 °C (Celestino et al., 1997b). Chen (2000) also investigated the storage stability of WMP samples and found that the lipase activity in WMP samples was affected by storage temperature. In her study, the FFA level in WMP samples started to increase from 9 months at 22 °C, 105 days at 37 °C and 15 days at 55 °C, and short-chain fatty acids were the main products from the lipolysis, which were believed to be correlative to rancid flavour. Meanwhile, her study also showed that lipolysis in WMP samples could occur at extreme low water activity (a_w < 0.1) (Chen, 2000). Páez et al. (2006) investigated the FFA production in WMP during storage at different temperatures and the correlation between the FFAs and rancid flavour. Their study showed that short-chain FFAs increased from the twelfth month at 21 °C and the second month at 40 °C. In addition, they found that rancid flavour could be detected when short-chain FFA concentration reached 90 – 125 mg/kg of milk powder, a concentration four times lower than the theoretical threshold reported in the study of Chen (2000). They also found a positive correlation between rancid flavour and the concentration of a long-

chain FFA ($C_{18:1}$) (Páez et al., 2006). Another study conducted by Celestino et al. (1997) showed that the residual lipase would cause increases of FFAs in the UHT milk manufactured from whole milk powder even at 3 ± 1 °C (Celestino et al., 1997a). Moreover, it is known that FFAs are the substrates of lipoxygenase in milk powder or the precursors of oxidation products, are more readily to be oxidized during milk powder storage than the corresponding glycerides. The oxidation of the FFAs will further intense the off flavour with the formation of compounds such as aldehydes, ketones and alcohols (Celestino et al., 1997b; Ning et al., 2025; Páez et al., 2006). Therefore, the detection of lipase activity is critically necessary to comprehensively evaluate the shelf-life stability of whole milk powders during storage.

While previous studies of lipase activity in milk powders mainly focused on monitoring FFA accumulation over time as an indirect indicator of lipase activity rather than the lipolytic potential in milk powders at any given point, or lipase activity that could hydrolyse short to medium-chain substrates, direct quantification of lipase activity capable of hydrolysing long-chain lipids in WMP has received less attention. In the present study, lipase activity was detected directly using p-nitrophenyl palmitate (p-NPP) as the substrate, which contains a long-chain fatty acid moiety ($C_{16:0}$, palmitate). The results of the present study showed direct evidence of lipase activity that can hydrolyse long-chain lipids in WMP, confirming that enzymatic lipolytic potential persists in WMP during storage. Our results suggest that direct lipase activity measurement may offer complementary information to conventional analysis in WMP shelf-life assessment.

4.4 Fat and Protein Content Monthly Monitoring

The fat and protein content data of high- and poor-quality WMP samples over 7- and 6-month storage are presented in **Appendix Table S3** and **S4**. The diagnostic plots (**Appendix Figure S4**) and the results of Shapiro-Wilk test (**Appendix Table S5**) indicate that the assumptions of LMMs were generally met.

LMMs showed that fat content was stable throughout the storage time in both high- and poor-quality WMP samples (F (1, 87.3) = 1.687, p = 0.197).

Meanwhile, there was no significant difference of fat content between the two quality groups (F (1, 64.9) = 2.568, p = 0.114). The interaction between quality group and storage time was marginally significant (F (1, 87.3) = 4.352, p = 0.040), indicating that the fat content in these two quality groups exhibited different trends over time. High-quality samples showed a negligible positive trend (0.007%/month, t (88) = 0.754, p = 0.453), while poor-quality samples showed a slight decreasing trend (-0.031%/month, t (88) = -1.984, p = 0.050). However, the total content changes of both groups over the storage period were not significant (< 0.2%). The marginal R^2 of the model was 0.033, indicating that fixed effects including sample quality, storage time and their interaction explained only 3.3% of the total change of fat content, confirming that fat content of both sample groups were stable over the storage period.

Protein content also showed no significant difference between high- and poor-quality samples (F (1, 72.9) = 1.428, p = 0.236), and no significant change over storage period (F (1, 88.5) = 0.877, p = 0.352). The interaction between sample quality and storage time was marginally significant (F (1, 88.5) = 3.836, p = 0.053), suggesting different trends of protein content change between high- and poor-quality samples. Specific slope estimates showed that protein content of high-quality samples decreased significantly over time (-0.025%/month, t (88) = -2.772, p = 0.007), while poor-quality samples kept stable (0.009%/month, t (88) = 0.599, p = 0.551). However, the total protein content change of both groups over time were not significant (< 0.2%). The model marginal R^2 was 0.323, which indicates that sample quality, storage time and their interaction accounted for 32.3% of total protein content variance.

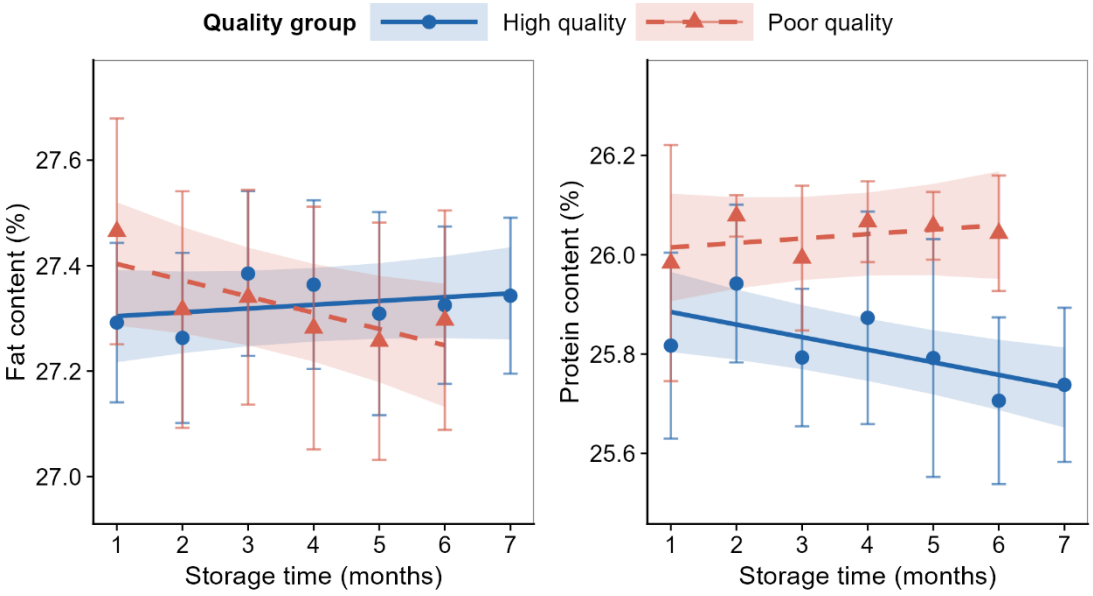


Figure 4. 4
Fat and protein content of high- and poor-quality WMP samples monthly trend during storage.

Points and error bars represent group means ± SD (High-quality n = 10, Poor-quality n = 6) at each month; Lines represent model estimated marginal means from LMMs with shaded areas represent 95% CI; High- and poor-quality samples were monitored for 7 and 6 months respectively.

FOSS Milkoscan used in our study applies Fourier Transform Infrared (FTIR) spectroscopic technique (using wavelengths between 2,500 – 25,000 nanometres) to quantify the fat and protein content in milk by utilizing the fundamental vibrations of -CH groups within the fatty acid chains, carbonyl groups in easter bonds of fat, and peptide binds between amino acids (Mohamed et al., 2021; Richard Mills, 2018). Therefore, this instrument may not be able to capture the chemical deteriorations (such as enzymatic hydrolysis, lipid oxidation or protein denaturation) during storage period of the WMP samples. The practical value of the slight changes of fat or protein content during our study period may be limited. These changes might be due to sampling errors rather than true decreasing. Therefore, the correlation between the FOSS Milkoscan monitoring results and the residual enzyme activity determined in present study cannot be established. However, although the fat and protein content remained stable and conform to the regulatory composition standard during our study period, the deteriorative reactions might still occur (Food Standards Australia New Zealand, 2017). The detection of deteriorative reactions requires specific methods such as enzyme activity testing, POV

determination for primary fat oxidation, HMF determination for Maillard reactions, HS-SPME-GCMS for volatile compounds, gas chromatography for free fatty acids and sensory evaluation (Clarke, Griffin, et al., 2021; Y. H. Li et al., 2019). Consequently, it is critical to combine nutrient monitoring with other chemical indicators to comprehensively evaluate the shelf-life stability of whole milk powders.

5 Conclusion

This study demonstrates that the raw milk microbial quality is a significant determinant of mesophilic bacterial counts in WMP, confirming that raw milk microbial quality control at the production stage is critical to reduce microbial contamination in the final product. The insignificant difference of thermophilic bacterial counts between the high- and poor-quality samples supports the conclusion that thermophilic contamination primarily originated from the milk powder production plant environment or facilities rather than raw milk, highlighting the importance of facility hygiene management as an independent risk factor.

The detection of protease activity in only a single high-quality sample, the omnipresence of lipase activity across all samples regardless of quality group, and the inconsistent trends between lipase activities and bacterial counts together suggest that conventional microbiological quality indicators cannot reliably predict the enzymatic hydrolysis potential in WMP.

Furthermore, the stability of fat and protein content throughout the storage period confirms that FTIR-based compositional monitoring is insufficient to capture deteriorative changes that may compromise WMP quality during storage.

Collectively, the present study suggests that bacterial counts in raw milk or corresponding milk powder and compositional monitoring using FTIR techniques might not be able to predict the deteriorative risks during WMP storage. Direct enzyme activity measurement may provide complementary information to other conventional deterioration indicators in a comprehensive assessment of WMP shelf-life.

In future studies, a more sensitive method, such as reversed-phase HPLC (T. X. Le et al., 2006), should be employed to investigate whether significant proteolysis occurs in WMP during storage. In addition, a validated and standardised method for the absolute quantification of lipase activity in WMP should be established, and the correlation between initial lipase activity and FFA accumulation during storage should be investigated to evaluate whether

lipase activity at the point of production can be an early predictive indicator of lipolytic deterioration before the off-flavour thresholds are reached.

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Appendix

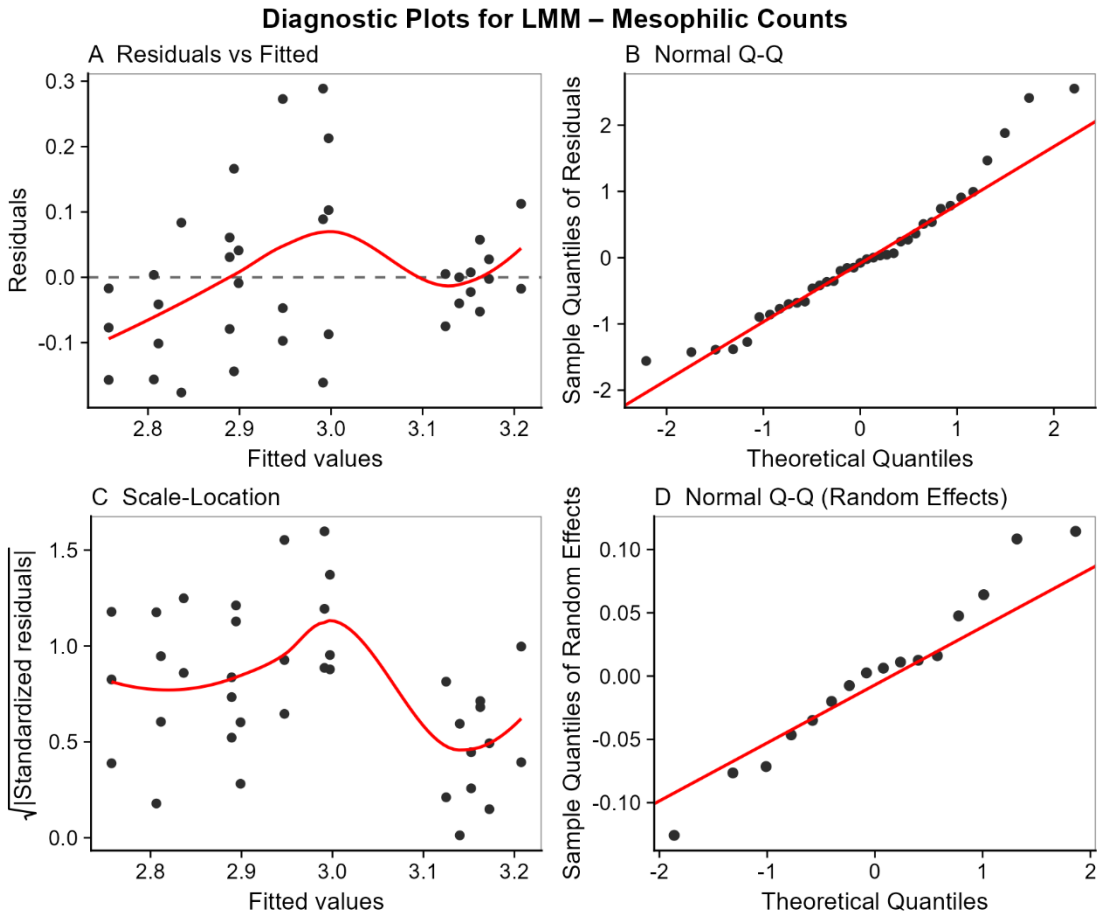


Figure S1

Diagnostic plots assessing the assumptions of LMMs for mesophilic bacteria counts. (A) Residuals versus fitted values plot; (B) Normal Q-Q plot of residuals; (C) Normal Q-Q plot of random effects; (D) Normal Q-Q plot of random effects.

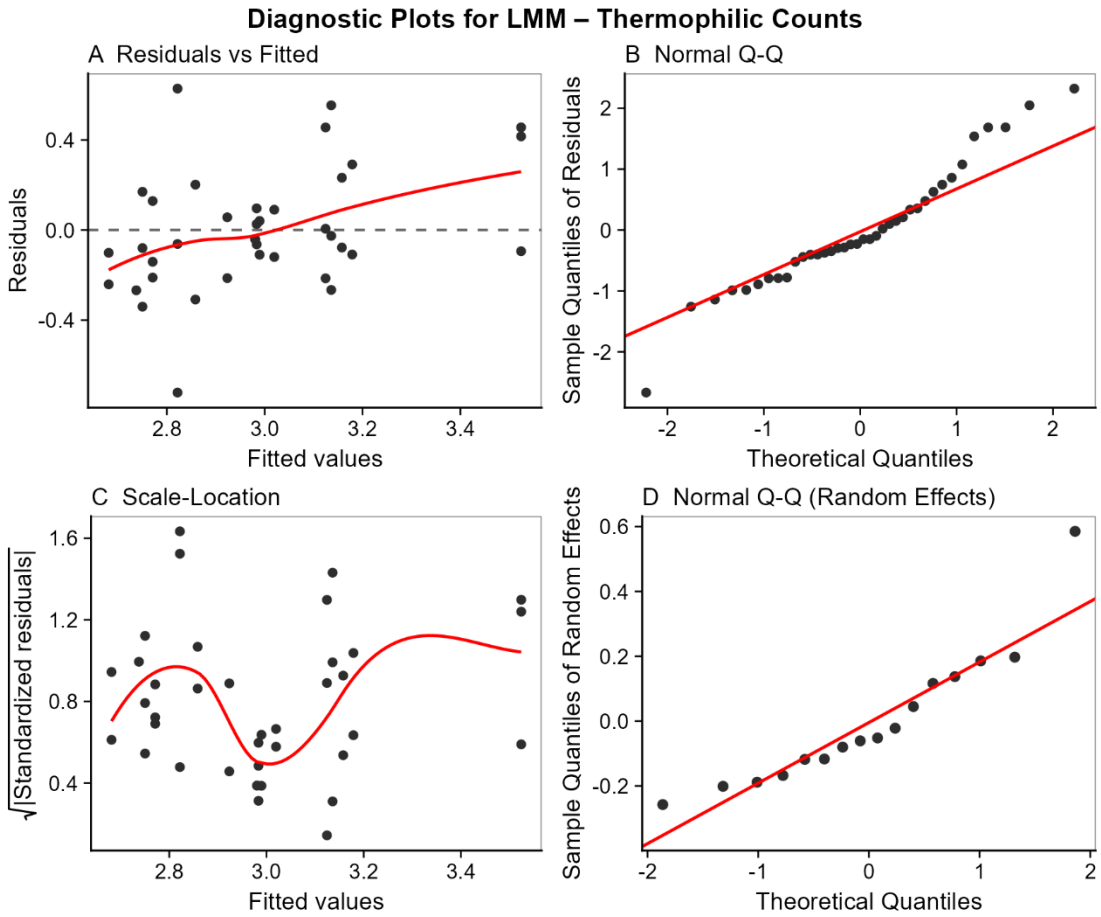


Figure S2

Diagnostic plots assessing the assumptions of LMMs for thermophilic bacteria plate counts. (A) Residuals versus fitted values plot; (B) Normal Q-Q plot of residuals; (C) Normal Q-Q plot of random effects; (D) Normal Q-Q plot of random effects.

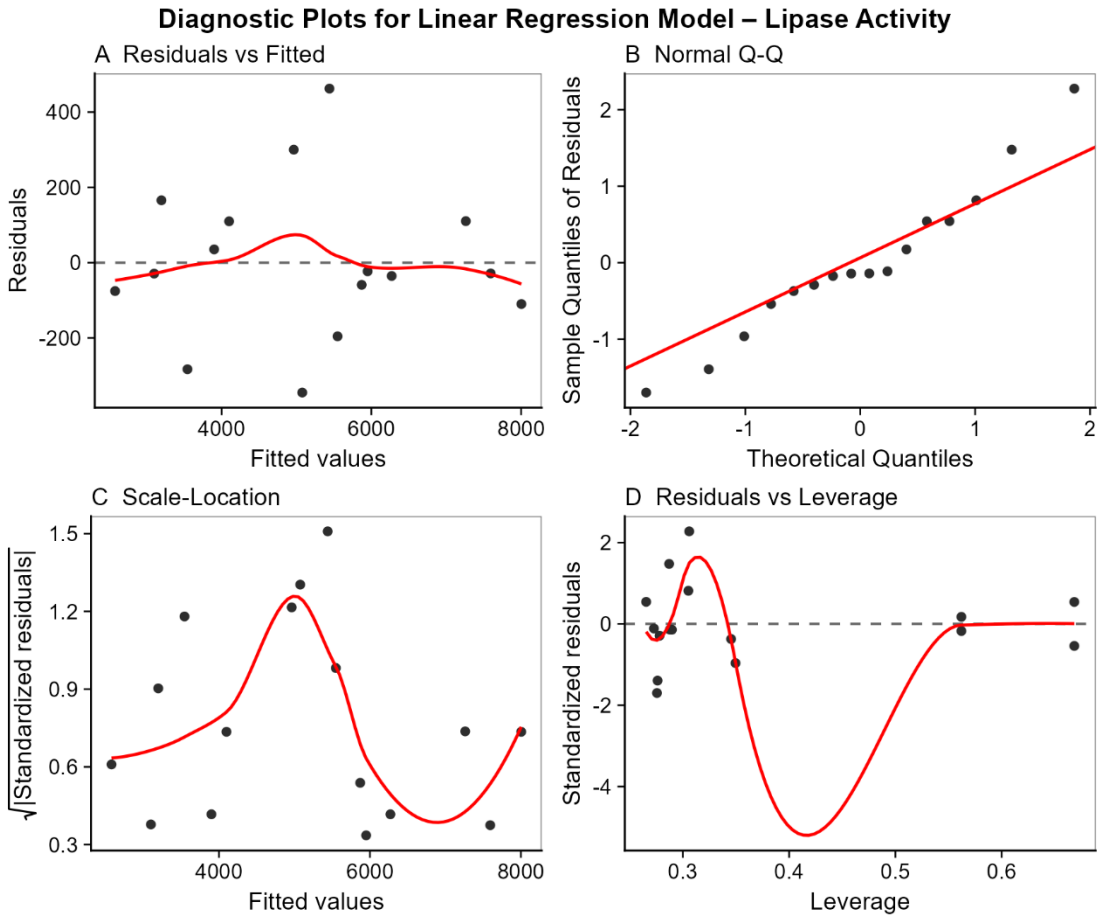


Figure S3

Diagnostic plots assessing the assumptions for lipase activity linear regression model. (A) Residual versus fitted values; (B) Normal Q-Q plot; (C) Scale-Location plot; (D) Residuals versus leverage. Red lines represent locally weighted smoothing trends. Dash lines indicate reference values (zero residual or Cook's distance contours).

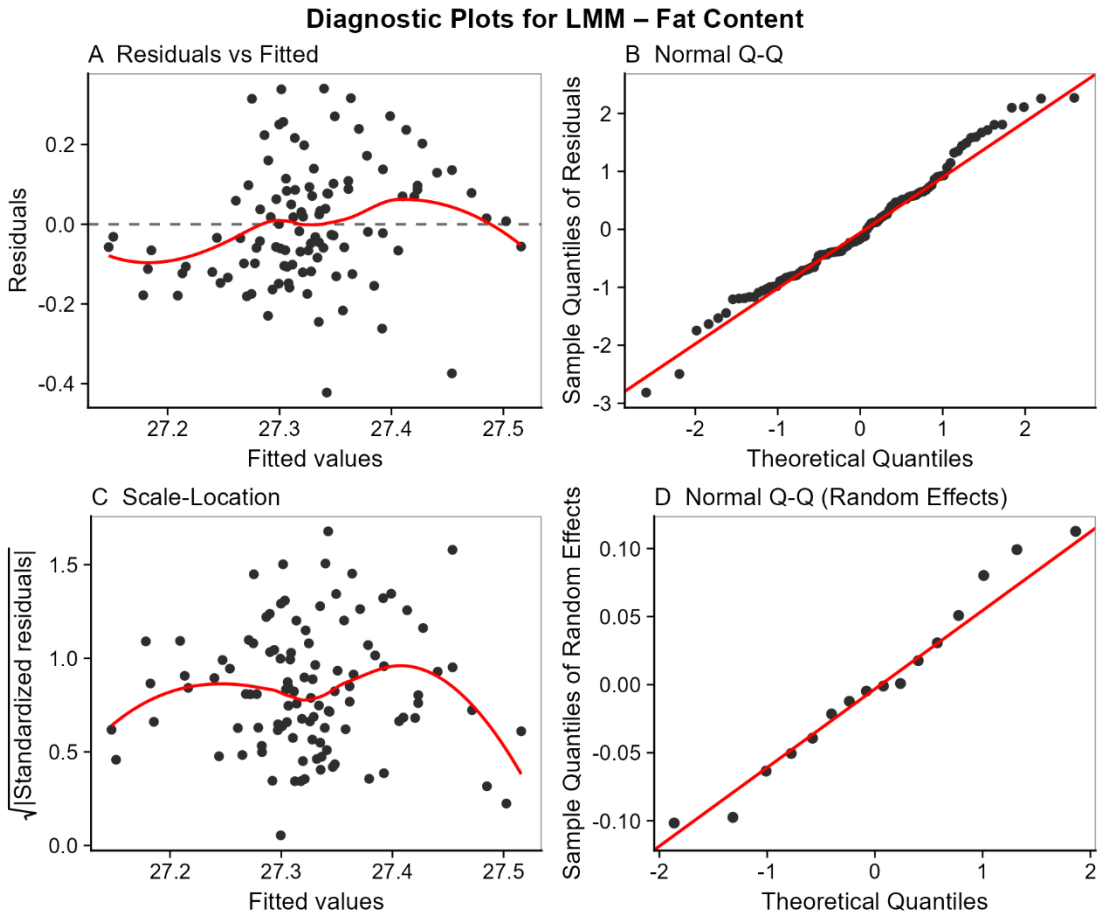


Figure S4

Diagnostic plots assessing the assumptions for fat content linear regression model. (A) Residual versus fitted values; (B) Normal Q-Q plot; (C) Scale-Location plot; (D) Normal Q-Q plot (random effects).

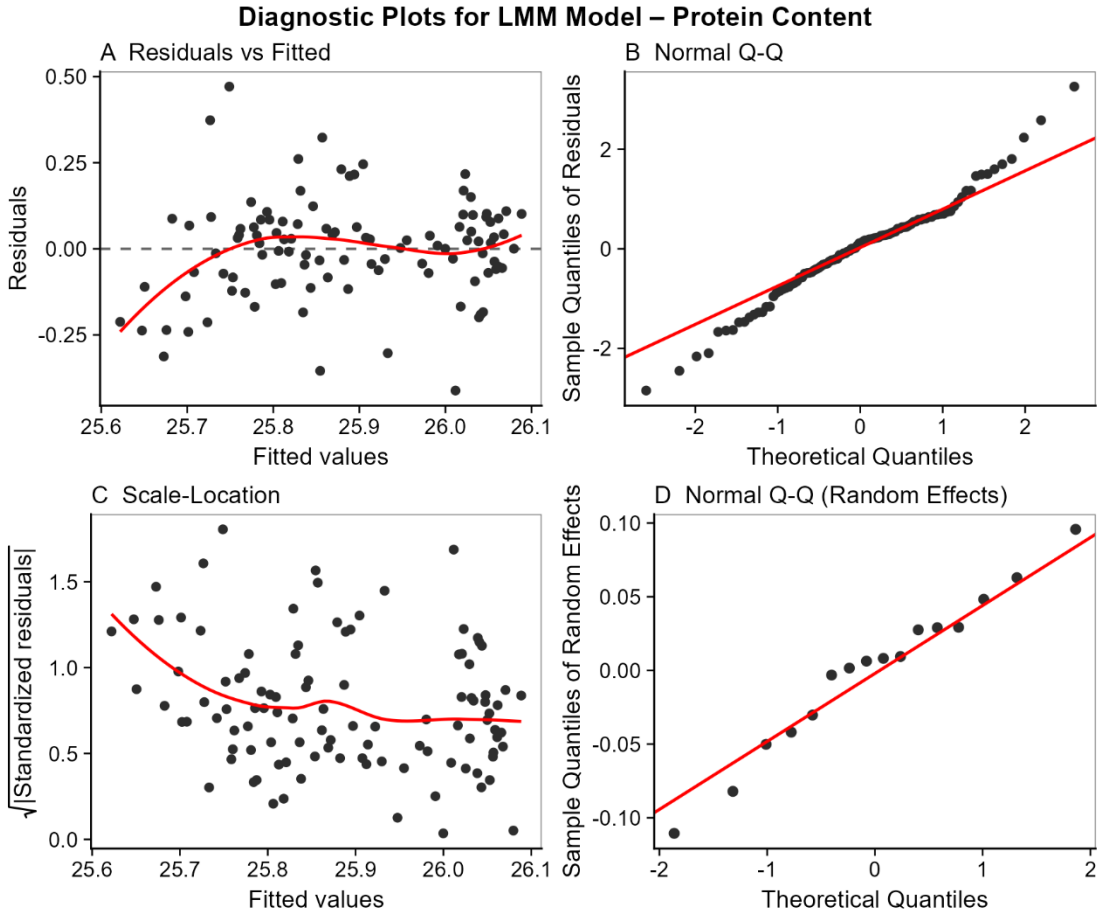


Figure S5

Diagnostic plots assessing the assumptions for protein content linear regression model. (A) Residual versus fitted values; (B) Normal Q-Q plot; (C) Scale-Location plot; (D) Normal Q-Q plot (random effects).

Table S1

Shapiro-Wilk test results for bacterial count LMMs.

Model	Test Target	W	p-value	Outcome
Mesophilic	Residuals	0.95	0.080	Satisfied
Mesophilic	Random Effects	0.97	0.894	Satisfied
Themophilic	Residuals	0.96	0.132	Satisfied
Thermophilic	Random Effects	0.88	0.043	Unsatisfied

Table S2

Turbidity (630 nm absorbance readings) of each incubation matrix in the two recovery experiments.

Sample	Recovery experiment 1		Recovery experiment 2	
	Unspiked[a]	Spiked[a]	Unspiked[a]	Spiked[a]
B1	0.1145 ± 0.0047	0.1281 ± 0.0034	0.1229 ± 0.0022	0.1187 ± 0.0016
K1	0.1284 ± 0.0046	0.1297 ± 0.0066	0.1225 ± 0.0019	0.1151 ± 0.0040
H2	0.1161 ± 0.0044	0.1211 ± 0.0031	0.1343 ± 0.0033	0.1249 ± 0.0008

Note: [a]The mean absorbance reading of each incubation matrix at 630 nm (mean ± SD).

Table S3

Assumption diagnostics for lipase activity linear regression model.

Test	Statistic	df	p-value	Outcome
Shapiro-Wilk (normality)	w = 0.960	-	0.668	Satisfied
Breusch-Pagan (Homoscedasticity)	BP = 10.732	5	0.057	Satisfied

Table S4

Fat content (%, mean ± SD) of high- and poor-quality WMP sample during storage.

Sample	Fat						
	1	2	3	4	5	6	7
A	27.32 ± 0.08	27.45 ± 0.01	27.36 ± 0.04	27.20 ± 0.02	27.21 ± 0.11	27.25 ± 0.04	27.26 ± 0.05
B1	27.23 ± 0.02	27.13 ± 0.10	27.67 ± 0.04	27.34 ± 0.06	27.65 ± 0.07	27.49 ± 0.04	27.63 ± 0.05
B2	27.09 ± 0.05	26.92 ± 0.09	27.62 ± 0.08	27.14 ± 0.01	27.68 ± 0.11	27.61 ± 0.10	27.55 ± 0.03
C1	27.56 ± 0.06	27.36 ± 0.04	27.30 ± 0.10	27.15 ± 0.05	27.30 ± 0.08	27.28 ± 0.00	27.32 ± 0.08
C2	27.52 ± 0.07	27.40 ± 0.04	27.37 ± 0.02	27.42 ± 0.03	27.22 ± 0.06	27.30 ± 0.11	27.24 ± 0.06
F1	27.24 ± 0.03	27.33 ± 0.04	27.35 ± 0.07	27.42 ± 0.07	27.25 ± 0.00	27.38 ± 0.01	27.32 ± 0.05
F2	27.30 ± 0.01	27.20 ± 0.08	27.40 ± 0.06	27.34 ± 0.03	27.21 ± 0.05	27.36 ± 0.04	27.42 ± 0.03
F3	27.31 ± 0.03	27.15 ± 0.09	27.39 ± 0.07	27.53 ± 0.04	27.20 ± 0.09	27.28 ± 0.06	27.29 ± 0.09
H1	27.23 ± 0.06	27.37 ± 0.03	27.22 ± 0.03	27.51 ± 0.04	27.13 ± 0.04	27.24 ± 0.05	27.16 ± 0.04
H2	27.12 ± 0.05	27.32 ± 0.06	27.17 ± 0.02	27.59 ± 0.09	27.24 ± 0.10	27.06 ± 0.05	27.24 ± 0.08
A1	27.46 ± 0.08	27.50 ± 0.03	27.59 ± 0.12	27.52 ± 0.07	27.53 ± 0.07	27.45 ± 0.04	
A2	27.51 ± 0.03	27.55 ± 0.05	27.57 ± 0.03	27.48 ± 0.02	27.36 ± 0.07	27.45 ± 0.07	
A3	27.08 ± 0.02	27.51 ± 0.04	27.37 ± 0.07	27.47 ± 0.07	27.47 ± 0.13	27.55 ± 0.10	
K1	27.68 ± 0.06	27.15 ± 0.10	27.18 ± 0.11	27.10 ± 0.03	27.11 ± 0.08	27.12 ± 0.09	
K2	27.64 ± 0.05	27.09 ± 0.03	27.12 ± 0.07	27.03 ± 0.05	27.00 ± 0.08	27.09 ± 0.09	
K3	27.42 ± 0.08	27.10 ± 0.11	27.21 ± 0.07	27.09 ± 0.09	27.07 ± 0.03	27.12 ± 0.04	

Note: Values are expressed as mean ± SD from three replicate measurements per sample per month. High-quality samples were stored and monitored for 7 months. Poor-quality samples were produced one month later and were therefore monitored for 6 months. Fat content was determined using the FOSS Milkoscan FT1 and converted to dry basis. All data are presented on a dry matter basis.

Table S5

Protein content (%, mean ± SD) of high- and poor-quality WMP sample during storage.

Sample	Protein						
	1	2	3	4	5	6	7
A	25.91 ± 0.13	26.22 ± 0.01	25.51 ± 0.13	25.56 ± 0.16	25.36 ± 0.01	25.41 ± 0.03	25.41 ± 0.07
B1	26.11 ± 0.08	25.91 ± 0.05	25.73 ± 0.04	25.81 ± 0.14	25.90 ± 0.09	25.64 ± 0.04	25.67 ± 0.05
B2	25.87 ± 0.08	26.10 ± 0.12	25.78 ± 0.13	25.82 ± 0.09	25.84 ± 0.05	25.77 ± 0.12	25.82 ± 0.03
C1	25.95 ± 0.06	25.86 ± 0.03	25.96 ± 0.09	25.92 ± 0.08	25.97 ± 0.03	25.85 ± 0.06	25.88 ± 0.14
C2	25.94 ± 0.08	25.77 ± 0.06	25.92 ± 0.13	25.79 ± 0.08	25.89 ± 0.12	25.87 ± 0.15	25.80 ± 0.10
F1	25.65 ± 0.08	25.71 ± 0.14	25.80 ± 0.06	25.79 ± 0.02	25.72 ± 0.06	25.64 ± 0.15	25.77 ± 0.05
F2	25.50 ± 0.14	26.09 ± 0.08	25.85 ± 0.10	25.61 ± 0.14	25.67 ± 0.08	25.82 ± 0.13	25.77 ± 0.08
F3	25.70 ± 0.09	25.84 ± 0.07	25.63 ± 0.01	26.10 ± 0.07	25.46 ± 0.16	25.44 ± 0.06	25.54 ± 0.02
H1	25.63 ± 0.10	25.94 ± 0.08	25.85 ± 0.13	26.18 ± 0.14	26.00 ± 0.06	25.80 ± 0.02	25.82 ± 0.05
H2	25.91 ± 0.05	25.98 ± 0.10	25.90 ± 0.01	26.15 ± 0.03	26.11 ± 0.13	25.82 ± 0.12	25.90 ± 0.05
A1	26.24 ± 0.08	26.13 ± 0.12	25.85 ± 0.08	25.98 ± 0.07	26.00 ± 0.05	26.11 ± 0.07	
A2	26.19 ± 0.09	26.08 ± 0.07	25.84 ± 0.09	26.15 ± 0.09	26.02 ± 0.02	26.01 ± 0.02	
A3	26.08 ± 0.10	26.05 ± 0.07	25.94 ± 0.10	26.03 ± 0.03	26.13 ± 0.03	26.01 ± 0.09	
K1	25.86 ± 0.05	26.07 ± 0.14	26.15 ± 0.12	26.18 ± 0.12	26.08 ± 0.08	26.19 ± 0.13	
K2	25.93 ± 0.07	26.02 ± 0.11	26.00 ± 0.05	26.00 ± 0.05	25.98 ± 0.15	25.85 ± 0.09	
K3	25.60 ± 0.09	26.12 ± 0.04	26.18 ± 0.02	26.06 ± 0.12	26.14 ± 0.13	26.09 ± 0.02	

Note: Values are expressed as mean ± SD from three replicate measurements per sample per month. High-quality samples were stored and monitored for 7 months. Poor-quality samples were produced one month later and were therefore monitored for 6 months. Fat content was determined using the FOSS Milkoscan FT1 and converted to dry basis. All data are presented on a dry matter basis.

Table S6

Shapiro-Wilk normality test results for nutrient content LMMs.

Model	Test Target	W	p-value	Outcome
Fat Content	Residuals	0.984	0.213	Satisfied
Fat Content	Random Effects	0.968	0.813	Satisfied
Protein Content	Residuals	0.978	0.081	Satisfied
Protein Content	Random Effects	0.973	0.885	Satisfied