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# Exploring Milk Management Strategies for Improved Food Safety and Health Outcomes in the Neonate

Thesis presented by

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for the degree of

**Doctor of Philosophy**

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## Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

### Contributions

Chapter 3: Dr. Kevin Linehan organized farmer recruitment. Bioinformatic analysis of 16S rRNA sequencing data was performed in collaboration with Dr. Dhrati Patangia.

Chapter 4: Mass spectrometry was performed by Dr. Paula O'Connor. Annotation and part of the genome analysis of whole genome sequences was performed by Dr Dhrati Patangia.

Chapter 5: Dr. Therese Uniacke-Lowe and Ms. Carol-Anne O'Shea assisted with sample collection from Cork University Maternity Hospital. The parallel nutritional analysis was performed by Dr. Fanyu Meng as part of a collaboration with the School of Food and Nutritional Sciences under the supervision of Prof. Alan Kelly. Dr. Fanyu Meng and Mr Jim McNamara participated in the running of processing treatments outlined in this study.

Chapter 6: Nutritional analysis was performed by Dr. Fanyu Meng in collaboration with the School of Food and Nutritional Sciences. Dr. Fanyu Meng also participated in the running of treatment trials outlined in this study.

Signature of the candidate: 

Date: 22.06.2025

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## Abstract

Milk fulfils the nutritional and energy requirements of the neonate to ensure adequate development and immunological protection during the initial critical period, with long-lasting effects across the lifespan. Mammals milk produced in the first 24 hours post-partum is known as colostrum. In bovines, colostrum consumption in calves is associated with improved health and reduced mortality. In humans, fresh mothers' own milk is regarded as the optimum nutrition for the newborn infant. When mother's own milk is unavailable the recommended alternative is donor human milk. Receipt of donor human milk is imperative for pre-term infants, with a very low birth weight (<1,500 g) or a serious illness, due to their increased risk of negative medical outcomes. The nutritional, bioactive and microbial properties of milk can be influenced by an array of extrinsic and intrinsic factors. This thesis investigates the influence of two common management practices of milk, in both the bovine and human context, and their subsequent influence on microbial composition. Firstly, the influence of farm management strategies, mainly antibiotic administration during dry cow therapy, on bovine colostrum. Secondly, the impact of processing techniques utilized in the milk bank setting on donor human milk. The results demonstrate factors which may affect the quality of milk and detail potential strategies to optimize milks benefit for the neonate. Additionally, the exploration of bovine colostrum as a source of microbes with health-promoting properties is undertaken.

Chapter 1 outlines the value of donor human milk to vulnerable pre-term infants in the absence of mother's own milk. Milk bank processing can influence the value and safety of donor human milk. This chapter consolidates the effects of holder pasteurization and alternative thermal and non-thermal methods, evaluating their ability to preserve milk's nutritional and bioactive properties whilst ensuring microbial inactivation.

Chapter 2 provides an overview of the literature on carotenoid-fortified dairy products and infant formula. The origin and role of carotenoids in bovine and human milk, influential factors on the carotenoid content of milk and potential

mechanisms to manipulate the carotenoid profile of bovine milk are discussed. In addition, challenges commonly associated with functional foods including bioacessibility and stability are presented herein.

Chapter 3 details the microbial profile of Irish bovine colostrum and the impact of antibiotic treatment. The influence of parity and inter-farm level variation were also assessed. DNA extraction and 16S rRNA sequencing of colostrum samples from 90 dairy cows revealed the most abundant phyla (Firmicutes, Actinobacteriota, Bacteroidota and Proteobacteria) and genera (*Acinetobacter, Corynebacterium, Facklamia, Jeotgalicoccus, Lactococcus, Leuconostoc, Psychrobacter and Staphylococcus*) in colostrum. Antibiotic treatment resulted in a significant difference in the microbial composition and diversity of colostrum when compared with the colostrum of cows following natural dry cow therapy. Furthermore, a significant difference in microbial composition based on the choice of antibiotic (Cephagaurd and UbroRed) was demonstrated. Beta diversity revealed significant differences between all groups, with distinct microbial clustering in the non-antibiotic group. These results substantiate the presence of a diverse microbial population in bovine colostrum, which can be influenced by farm management practices. Our findings support the use of non-antibiotic alternatives for drying off in cows.

Chapter 4 presents lactic acid bacteria strains isolated from pooled bovine colostrum. *In vitro* and *in silico* analysis revealed potential probiotic attributes of colostrum isolates, including exopolysaccharide activity, bile salt hydrolase activity and GABA production. In particular, four bacterial isolates were identified as putative bacteriocin producers following a series of well diffusion assays. Whole genome sequencing and predictive analysis confirmed the presence of five bacteriocin gene clusters. Our results demonstrate that bovine colostrum is a source of microbes with positive attributes that could be further investigated for potential use by the food or health sectors.

Chapter 5 substantiates the efficacy of holder pasteurization and assesses the ability of high-pressure processing in reducing the microbial growth in human milk. In addition, we determine if the microbial safety of donor milk can be

sustained for an extended storage period of 7 days. Bacterial screening on non-selective media demonstrated that milk treated with holder pasteurization or high-pressure processing at 600 MPa resulted in milk within acceptable growth limits (<10 CFU $mL^{-1}$). In addition, the microbial safety of milk was maintained when stored at 4 °C for 7 days. HPP at 400 MPa achieved a 3.45-log reduction in microbial counts, with undetectable growth at subsequent time-points. The results of this study suggest the microbial safety of holder pasteurised milk is maintained when stored at 4 °C for 7 days. In addition, we propose high pressure processing as a potential non-thermal alternative for donor milk treatment.

Chapter 6 presents findings on previously unexplored methods for the processing of donor human milk including, the application of the bacteriocin, nisin A, microfiltration and the direct inclusion of the enzyme, lactose oxidase. A > 6-log reduction in microbial growth on non-selective media and media targeting *Pseudomonas* and coliforms growth was achieved following microfiltration or a hybrid treatment of nisin A and holder pasteurisation. This study provides novel insights into the potential incorporation of non-conventional processing techniques in human milk banking, resulting in milk within acceptable microbial safety standards.

Overall, this thesis details beneficial properties of milk from a microbial perspective with results that can help optimize the management and handling of milk for optimal value, providing improved health benefits to the neonate. Furthermore, milk as a source of health-promoting agents (carotenoids and microbes) with potential industrial applications was explored.

## Publications

**Chapter 1 Conboy-Stephenson, R.**, Ross, R. P., Kelly, A. L., & Stanton, C. (2024). Donor human milk: the influence of processing technologies on its nutritional and microbial composition [Review]. *Frontiers in Nutrition*, *11*. https://doi.org/10.3389/fnut.2024.1468886

**Chapter 2 Conboy Stephenson, R.**, Ross, R. P., & Stanton, C. (2021). Carotenoids in Milk and the Potential for Dairy Based Functional Foods. *Foods*, *10*(6), 1263. https://www.mdpi.com/2304-8158/10/6/1263

### Co-authored publications

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## Abbreviations

α: Alpha

β: Beta

δ: Delta

γ: Gamma

ABTS: 2,2’-azino-di-3-ethylbenzthiazoline-6-sulphonic acid

ADI: Acceptable daily intake

ALP: Alkaline phosphatase

AMRG: Antimicrobial resistance gene

ANI: Average nucleotide identity

BLAST: Basic local alignment search tool

BMI: Body mass index

bp: Base pair

BSH: Bile-salt hydrolase

BSSL: Bile salt-stimulated lipase

CaCl2: Calcium chloride

CAMP: Collection of Anti-Microbial Peptides

CARD: Comprehensive antibiotic resistance database

CAZyme: Carbohydrate-active enzyme

CD36: Cluster determinant 36

CDS: Coding sequence

CFU: Colony forming unit

CFS: Cell-free supernatant

CRP: C-reactive protein

DCT: Dry cow therapy

DHM: Donor human milk

DM: Dry matter

DNA: Deoxyribose nucleic acid

EFSA: the European Food Safety Authority

EGF: Epidermal growth factor

EPO: Erythropoietin

EPS: Exopolysaccharide

EU: European Union

FAO: Food and Agriculture Organization of the United Nations

FDA: Food and Drug Administration

GABA: Gamma-aminobutyric acid

GF: Growth factor

GH: Glycoside hydrolases

GI: Gastrointestinal

GLP: Glucagon-like peptide

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GRAS: Generally recognized as safe

GT: Glycosyltransferase

H2O2: Hydrogen peroxide

HGF: Hepatocyte growth factor

HIPEF: High-intensity pulsed electric fields

HMO: Human milk oligosaccharide

HoP: Holder pasteurization

HPP: High-pressure processing

HTST: High-temperature short time

IFN: Interferon

Ig: Immunoglobulin

IGF: Insulin-like growth factor

IL: Interleukin

IPA: Isopropyl alcohol

IR: Infrared

LAB: Lactic acid bacteria

LBS: Lactobacillus selection

LC/MS: Liquid chromatography-mass spectrometry

LDL: Low-density lipoprotein

LPL: Lipoprotein lipase

LPO: Lactoperoxidase

LTLT: Low-temperature long time

MIP: Macrophage inflammatory protein

MRD: Maximum recovery diluent

MRS: de Man, Rogosa, Sharpe

MUFA: Monounsaturated fatty acids

NCBI: National Centre for Biotechnology Information

NEC: Necrotizing enterocolitis

NICE: the National Institute for Health and Care Excellence

NPC1L1: Niemann-Pick C1 like

ns: Nanosecond

orf: open reading frame

OTU: Operational taxonomic unit

PcOA: Principle Co-Ordinate Analysis

PCR: Polymerase chain reaction

PEF: Pulsed electric field

PERMANOVA: Permutational Analysis of Variance

Pln: Plantaricin

PUFA: Polyunsaturated fatty acids

QPS: Qualified presumption of safety

RAPD: Random amplified polymorphic DNA

RCA: Reinforced clostridial agar

RGI: Resistance gene identifier

RNA: Ribose nucleic acid

SD: Standard deviation

SFA: Saturated fatty acids

SCC: Somatic cell count

SIBO: Small intestinal bacterial overgrowth

SR-BI: Scavenger receptor class B

TAC: Total antioxidant capacity

TCC: Total coliform count

TDCA: Taurodeoxycholic acid

TFA: Trifluoroacetic acid

TGF: Transforming growth factor

TMR: Total mixed ration

TNF: Tumour necrosis factor

TPC: Total plate count

TUS: Thermo-ultrasonication

UHT: Ultra-high temperature

UV: Ultraviolet

VEGF: Vascular endothelial growth factor

VLDL: Very low-density lipoproteins

vs.: Versus

WDA: Well diffusion assay

WGS: Whole genome sequencing

WHO: World health organization

**Units**

°C: degree Celsius

€: Euro

µL: microlitre

µm: micrometre

µs: microsecond

µg: microgram

cm: centimetre

Da: Dalton

*g*: g-force

g: gram

h: hour

Hz: hertz

J: joule

kDa: kilodalton

kg: kilogram

kGy: kilogray

Kcal dL-1: kilocalories per decilitre

kV: kilovolt

L: litre

mbar: millibar

mg: milligram

min: minute

mL: millilitre

mm: millimetre

mM: millimolar

MPa: mega pascal

nm: nanometre

nmol: nanomol

psi: pounds per square inch

s: sec

U: unit

V: volt

W: power

w/v: weight/volume

v/v: volume/volume

# Chapter 1

## Literature Review

### Donor Human Milk: The Influence of Processing Technologies on its Nutritional and Microbial Composition

Ruth Conboy-Stephenson, R. Paul Ross, Alan L. Kelly, Catherine Stanton

Conboy-Stephenson, R., Ross, R. P., Kelly, A. L., & Stanton, C. (2024). Donor human milk: the influence of processing technologies on its nutritional and microbial composition [Review]. *Frontiers in Nutrition*, *11*. https://doi.org/10.3389/fnut.2024.1468886

## Abstract

Human milk is regarded as the gold standard nutrition for newborn infants, providing all nutrients required for adequate growth and development from birth to 6 months. In addition, human milk is host to an array of bioactive factors that confer immune protection to the newborn infant. For this reason, the supply of human milk is crucial for premature, seriously ill, or low birth weight infants (<1,500 g). When a mother's own milk is unavailable, donor human milk is the recommended alternative by the World Health Organization. Prior to consumption, donor human milk undergoes pasteurization to ensure the eradication of bacterial agents and prevent the transfer of potentially pathogenic organisms. Currently, Holder Pasteurization, a heat-based treatment, is the widely adopted pasteurization technique used by milk banks. Holder pasteurization has demonstrated degradative effects on some of milk's biologically active factors, thus depleting critical bioactive agents with known functional, protective, and beneficial properties, ultimately reducing the immunoprotective value of donor human milk. As a result, alternative strategies for the processing of donor human milk have garnered much interest. These include thermal and non thermal techniques. In the current review, we describe the effects of Holder pasteurization and alternative milk processing technologies on the nutritional and bioactive properties of milk. In addition, the capacity of each technique to ensure microbial inactivation of milk is summarized. These include the most extensively studied, high temperature short-time and high-pressure processing, the emerging yet promising techniques, microwave heating and UV-C irradiation, and the lesser studied technologies, thermoultrasonication, retort processing, pulsed electric field, and gamma irradiation. Herein, we collate the findings of studies, to date, to allow for greater insight into the existing gaps in scientific knowledge. It is apparent that the lack of a cohesive standardized approach to human milk processing has resulted in contrasting findings, preventing a direct comparative analysis of the research. We conclude that donor human milk is a unique and valuable resource to the health sector, and although substantial research has been completed, persistent data

disparities must be overcome to ensure optimal nutrition for the vulnerable newborn preterm infant group, in particular.

# 1 Introduction

Milk is a unique species-specific fluid produced by mammals post-partum. Human milk is the established optimum nutrition for newborn infants, contributing to the newborn infant's health and development with long-lasting effects across the lifespan. Human milk has been linked with reduced incidence of infections in infants, including necrotizing enterocolitis (NEC), diarrhoea, and respiratory diseases, and is associated with reduced rates of infant morbidity and mortality (1–3). Human milk is a rich source of macro- and micronutrients, an array of bioactive components and microbes (4). The mammary gland confers the unique ability to adjust milk composition during lactation to the requirements of the growing infant. As a result, human milk composition varies based on a number of maternal and environmental factors, including gestational age, stage of lactation, stage of milk (fore vs. hind), age, diet, geographical location, and diurnal variations (5).

Although fresh mothers' own milk is regarded as the optimal food for newborn infants (6), donor human milk (DHM) is recommended by the World Health Organization (WHO) when mothers' own milk is not available (7, 8). DHM is particularly beneficial to infants born prematurely, with a very low birth weight (<1,500 g) or a serious illness (9, 10). Preterm and low birth weight infants are at increased risk of serious health complications, including NEC, due to an underdeveloped immune system (11, 12). For these infants, DHM contributes to the optimal recovery, growth, and health of the newborn and reduces the risk of negative medical outcomes (13–16). However, increasing evidence suggests that there is lower availability of favourable immunoprotective compounds in DHM than in fresh milk as a result of the collection, storage, and processing of DHM (17).

## 1.1 Milk banks—current handling practices and techniques

Human milk banks provide an essential service to infants who are unable to access their mothers' own milk. Milk banks are responsible for the collection, processing, storage, and distribution of DHM. Currently, there are 282 human milk banks

supplying DHM in Europe, with a further 18 planned (18). Similar to Europe, there has been an upsurge in the number of milk banks in the USA, with 11 banks in 2012 which increased to 32 as of 2023 (19). Furthermore, in the USA, a number of for-profit milk banks have also been established. However, for the purpose of this review, we will only focus on non-profit milk banks. As the number of milk banks increases, so too does the number of hospitals and patients using their services. An analysis of surveys conducted by the Centers for Disease Control and Prevention on Maternity Practices in Infant Nutrition between 2007 and 2011 demonstrated an increase in the use of DHM by intensive care units from 25.1 % in 2007 to 45.2 % in 2011 (20). Indeed, a 2015 survey notes a 74 % increase in neonatal facilities using DHM compared with 2011 (9).

The women who become donors donate on a volunteer basis if they have excess milk supply. Donors undergo health evaluation including behavioural and serological screening to prevent the transfer of infectious diseases (21). Although no international legislation exists for donor screening and milk processing, a number of guidelines have been developed. The European Milk Banking Association has published recommendations on milk banking procedures; these recommendations align closely with those of the Human Milk Bank Association of North America, the National Institute for Health and Care Excellence (NICE), and The Perron Rotary Express Bank Australia (19, 22–25).

Following the donation, milk undergoes a series of processing steps including pooling, pasteurization, and bacteriological screening (see Figure 1) (26). For pooling, milk from multiple donors is thawed and pooled to create a uniform batch of donor milk with a relatively standard level of macronutrients. Although no official criteria exist regarding the number of donors per batch, Colaizy et al. (27) suggest that three to five donors per pool is sufficient to reduce variability and optimize macronutrient content. Once pooled, milk undergoes pasteurization and bacterial screening. The microbiological quality and safety of DHM are a primary concern for human milk banks. In addition to the naturally occurring microorganisms of breast milk, DHM is at risk of exogenous contamination during the handling and processing steps (28, 29).

Holder pasteurization (HoP), also known as low-temperature long-time pasteurization, is performed to ensure the inactivation of potentially harmful bacterial agents and viruses. HoP is the standard preservation technique adopted by milk banks worldwide (22), subjecting milk samples to a temperature of 62.5 °C for 30 min. Following treatment, milk is rapidly cooled and frozen awaiting use. Current guidelines regarding bacterial screening by milk banks vary. NICE recommends that samples should be screened prior to pasteurization and discarded if total microbial counts exceed $10^5$ CFU $mL^{-1}$ (24). Furthermore, counts of $10^4$ CFU $mL^{-1}$ or higher of *Enterobacteriaceae* or *Staphylococcus aureus are* unacceptable. Post-pasteurization, no level of bacterial growth in milk is permissible.

Preterm infants are the primary recipients of DHM, highlighting the pivotal role of milk banks in healthcare settings (30). Current research endeavours to better understand the impact of handling, processing, and storage procedures on DHM composition and address any subsequent nutrient gap. In particular, optimizing the pasteurization method used to ensure minimal loss of bioactive factors of milk while preserving its microbial safety is desirable.

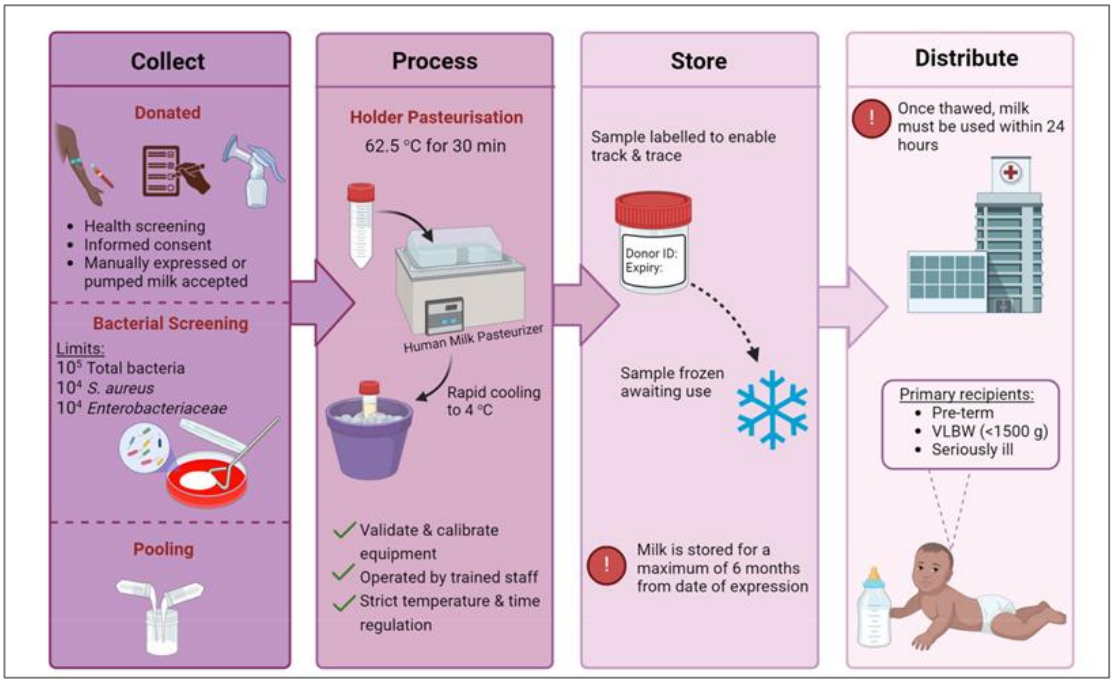



Figure 1: The handling and processing steps of human milk in a milk bank setting

# 2 The effect of holder pasteurization on the composition of donor human milk

## 2.1 Macronutrient composition of human milk

Human milk is a tailor-made liquid food that provides complete nutrition to the developing infant (31). The nutritional constituents of milk originate from three key sources, the lactocytes, dietary origins, and maternal stores (32). The macronutrients in human milk constitute lipid, carbohydrate, and protein. Human milk composition consists of approximately 87% of water, 3.8% of fat, 7% of lactose, and 1% of protein (33, 34).

### *2.1.1 Lipids*

In milk, 98–99 % of the lipid content is comprised of triglycerides in the form of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) (16). Triglycerides are composed of a glycerol molecule with three fatty acids attached. These fatty acids can be combined in over 45 different positions and combinations (31). The remaining lipid portion of milk includes cholesterol, phospholipids, gangliosides, and sphingolipids, located within the milk fat globular membrane (16). The lipid content of human milk accounts for approximately 50 % of milk's energy supply and is linked with improved neurodevelopment, immune function, and metabolism in the infant (33, 35).

To date, several studies have investigated the effect of HoP on the lipid content of DHM. The majority of findings suggest the retention of DHM lipid content following HoP (36–42). Pitino et al. noted that the fatty acid profile of milk is preserved following HoP (43). Indeed, no modifications to the fatty acid composition of DHM were observed post-HoP (36, 38, 44–48). However, an increase in the proportion of medium-chain fatty acids alongside a reduction in oleic acid was reported (49). In contrast, some studies have reported reductions in the total lipid content of DHM of 3.5–8.9 % (50–53) with a subsequent loss in total energy of 2.8–2.9 % (50, 51). Similarly, an even greater decrease in fat and energy of 25 and 16 %, respectively, was documented in DHM from a South Indian milk bank (54). In addition, Capriati et al. (55) noted a reduction in the triglyceride content of DHM of 21 %. A reduction in

the total lipid content of DHM is unexpected, and the reason for these reported reductions remains unclear.

### *2.1.2 Carbohydrates*

Human milk consists of many complex carbohydrates, of which the major and most studied are lactose and human milk oligosaccharides (HMOs). Lactose is a disaccharide sugar and is the main carbohydrate in milk (56), accounting for 40% of the energy supply (57) and 60–70 % of the total osmolarity of human milk (16). In addition, lactose facilitates the absorption of minerals and is associated with the innate immune response (58, 59). Unlike other macronutrients, lactose levels remain relatively constant over 12 months of lactation, with a reported mean concentration of 61.4 g/L (60). HMOs are a group of structurally diverse unconjugated glycans unique to human milk (61). After lipids and lactose, they are the third most abundant solid component of breast milk, constituting 10–15 g/L of mature milk (62). Over 200 structures of HMOs have been identified in human milk to date (63). HMOs have an important role in the immune system due to their anti-adhesive properties against pathogenic microbes, their promotion of a Bifidobacterium-rich gut microbiome, and their modulation of intestinal cell responses (62, 64).

There is consensus regarding the effects of HoP on DHM carbohydrate levels. Adhisivam et al. observed no significant reduction in carbohydrate levels following HoP (54). Indeed, despite one report of a slight reduction (0.92 %) in lactose levels (53), the stability of carbohydrate levels post-HoP has been further demonstrated elsewhere (37, 39–42, 50, 52, 65–67). Additionally, other milk carbohydrates were preserved following HoP, including myo-inositol (65, 66), glucose (51, 65, 66), glycosaminoglycans (68), and oligosaccharides (67, 69).

### *2.1.3 Proteins*

Accounting for just 1%, the protein content of human milk is low but has high bioavailability (70). Human milk proteins can be categorized into three main classes, whey protein, caseins, and mucin proteins (71). Whey protein is the predominant protein in milk, with a whey protein-to-casein ratio of 90:10 in colostrum and 60:40 in mature milk (72). Milk proteins contribute to a wide range of

functions, including providing nitrogen and amino acids for tissue growth and maintenance, antimicrobial activity, immunomodulatory effects, and aiding digestion (34, 73). Overall, findings indicate that HoP does not reduce the total protein content of DHM. Indeed, a number of studies reported the retention of total protein levels post-HoP (37, 39–42, 50, 51, 74–76). However, similar to the lipid content, contrasting data on the effect of HoP on the total protein content of DHM have been reported. For instance, a reduction in protein levels following HoP of 12.5–13 % was demonstrated (77, 54), with a less but still significant reduction of 2.5–3.9 % also being reported (52, 53). Importantly, there have been reported changes to the protein profile of HoP-treated DHM in comparison with raw milk (76, 78). In particular, Silvestre et al. recorded a significant reduction in lysine levels of DHM following HoP. Lysine is an essential amino acid found in human milk and can be an indicator of the protein quality of milk. HoP caused a significant loss in the average available lysine of 29.3 % when compared with untreated milk (75). These findings suggest that although HoP may preserve the total protein content, the protein quality of milk may be modified or even diminished. For instance, decreased lysine levels are often used as an indicator of early-stage Maillard reaction (79). Furthermore, a separate study noted increased production of three key Maillard reaction products, furosine, carboxymethyllysine, and N-epsilon-carboxyethyllysine, following HoP (80). Taken together, these findings suggest that HoP-treated DHM may have increased levels of Maillard reaction products, potentially affecting the nutritional value of donor milk. Future studies should investigate the effect of HoP on the protein quality, the potential formation of Maillard reaction products, and any subsequent health effects on the newborn infant.

Overall, despite the observed heterogeneity within the available data, studies to date, indicate the ability of HoP processing to preserve the total lipid, carbohydrate, and protein content of DHM. However, the potential modification of protein quality warrants further investigation. Future studies should endeavour to further elicit not only the effect on macronutrient concentration but also any subsequent consequences for the activity, quality, and bioavailability of nutritional

constituents. The variability in findings may be due to the absence of a systematic approach to study design, sample characterization (sample volume, number of donors, and number of freeze–thaw cycles), and the analysis method. The sample storage history, mainly freezing, can result in alterations to certain fractions of milk, including the milk fat globule membrane, leaving components more vulnerable to thermal processing (27). Additionally, a high starting concentration of solid components may impact the efficacy of the analysis method. The use of older instruments or varying pasteurizer designs can increase the lag times for heating and cooling, altering the duration of thermal exposure for milk components. In particular, the choice of the analytical method appears to be a key influential factor, with studies choosing from a range of laboratory reference methods or commercially available infrared (IR) analyzers.

Milk banks primarily use IR analyzers to assess the nutritional composition of DHM. IR analyzers use near-, mid-, or Fourier transform IR spectroscopy. Recently, a mid-IR analyzer (Miris Human Milk Analyzer) was approved by the FDA and is available commercially (81). IR analyzers have the advantage of performing rapid analysis on low-volume milk samples. Perrin et al. confirmed that IR analyzers have a high degree of accuracy for crude protein and fat analysis (82). In contrast, carbohydrate determination was less precise, with Fourier- and mid-IR analysis reporting lower concentrations, while filtered- and near-IR led to increased levels (20–50 %) when compared with reference values. These findings suggest that IR analysis is accurate at determining the protein and fat content but may not be the optimum choice for carbohydrate quantification. Furthermore, the correct calibration, validation, and operation of the instrument are critical to ensure consistent accuracy.

In addition to IR analyzers, laboratory-based analysis methods are still widely used and considered favourably. A review of laboratory techniques for macronutrient quantification has recently been performed (83), in addition to a review of methodologies for lactose analysis (57). These include the Kjeldahl or Dumas method for protein determination, a combination of solvent extraction and gravimetry for lipid analysis, and either high-performance lipid chromatography or high-performance anion exchange for carbohydrate quantification. Both study

design and method of analysis have the potential to impact results, while a standardized choice of analytical method and predetermined selection criteria for the milk sample may reduce the inconsistencies noted in findings to date.

Overall, despite some contrasting findings, HoP appears to adequately preserve the macronutrient portion of DHM and provide high nutritive value to the infant.

### 2.2 Micronutrients

Micronutrients in milk refer to essential vitamins and minerals that aid human growth, development, and cell function (84). Vitamin and mineral intakes are linked with a variety of physiological functions, including ocular development, hormone synthesis, enzymatic and metabolic functioning, and neurodevelopment (16, 85).

Only a small number of studies have examined the effect of HoP on the mineral content of DHM. HoP did not cause a significant reduction in calcium (37, 55). Additionally, phosphorus, iron, copper, and zinc levels remained stable following HoP (37). However, a change in the distribution pattern of zinc following processing was observed, which could impact zinc bioavailability (37). Usually, zinc concentration is at its highest in the whey portion of milk with lower levels in the fat and casein fractions. Goes et al. demonstrated that although this was the case pre-processing, following pasteurization, the levels of zinc in the whey portion decreased and an increase occurred in the fat fraction of milk.

Findings indicate the potential stability of mineral concentrations post-processing. However, only a small number of minerals have been quantified. Future studies should expand to include a wider range of minerals, such as potassium, magnesium, and chloride. Furthermore, the consequences of any potential modifications to the distribution pattern of minerals and other nutrients as a result of pasteurization should be further examined.

Studies to date indicate a vulnerability of some water-soluble vitamins to thermal processing conditions of HoP. In particular, the degradation of vitamin C as a result of HoP leads to losses of up to 36% (44, 46, 86). Similarly, folacin and vitamin B6 underwent significant losses of 31 and 15%, respectively (86). In contrast, other studies reported the stability of both (74, 87). The variability in results is likely due

to differing analytical techniques. The retention of riboflavin (B2), biotin (B7), niacin (B3), thiamine (B1), vitamin B2, vitamin B12, and pantothenic acid following HoP has been observed (74, 86, 87). It has been reported by some studies that fat-soluble vitamins, including vitamin A (37, 86), vitamin D (86), and vitamin E (44, 86) are robust to the processing conditions of HoP. However, a study by Ribeiro et al. noted a 34 % reduction in vitamin A with high-performance liquid chromatography following pasteurization (88). A similar reduction of 32.5 % in vitamin A was also reported (89). Vitamin E levels following HoP underwent a reduction of 17–25.5, 13–47.3, and 33 % in α-, γ- and δ-tocopherols, respectively (45, 46).

Potential changes to the vitamin concentration and bioavailability of DHM following processing are concerning as low birth weight infants, the primary recipients of donor milk, have increased vitamin requirements. Interestingly, vitamin A level analysis in both HoP-treated and untreated DHM suggests that neither supply adequate levels, providing only 43.6 and 66 %, respectively, of the recommended nutrient requirement (88, 89). The clinical implications of reduced vitamin bioavailability in DHM require further consideration. Evidently, HoP has varying effects on the vitamin profile of DHM, which are dependent on the vitamins' ability to withstand thermal exposure. Any future milk processing technique should endeavour to optimize micronutrient preservation.

## 2.3 Bioactive milk components

Human milk is a valuable source of bioactive compounds with a range of anti-inflammatory and antimicrobial properties that contribute to immune development and gut colonization. These include hormones, enzymes, cell signalling molecules, and bioactive agents such as immunoglobulins (Igs), lactoferrin, and lysozyme. A number of studies have indicated the negative influence of HoP on these bioactive compounds, mainly due to their proteinaceous structure.

### *2.3.1 Immunoglobulins*

There are five classes of Igs in human milk IgA, IgG, IgM, IgD, and IgE (90). The most abundant is IgA followed by IgG and IgM, with mean concentrations of 2.785, 0.059, and 0.036 g/L, respectively, across gestational age ranges (77). Indeed, secretory IgA (sIgA) accounts for 80–90 % of the Ig profile of human milk (91). The Ig content

of milk has a critical role in the protective capacity of human milk against infection (92).

The Ig profile of DHM has been widely studied, and findings suggest significant reductions in Ig levels as a result of HoP. Adhisivam and investigators reported a reduction in IgA of 30 % (54). Similar reductions in IgA of >20 % have been noted in a number of studies (77, 93–112) and even higher reductions of 50–60 % have also been reported (65, 101, 102). Furthermore, numerous studies demonstrated a reduction in sIgA levels (74, 103–105). In addition, a decrease in IgG levels following HoP has been widely reported (96) although the extent of loss varied, from 23–34 % (98, 106) to 60–79 % (54, 77). All studies to date describe significant reductions in IgM concentration following HoP (65, 77, 96–98, 105). Overall, it is apparent that the processing conditions of HoP have severe degradative consequences on the Ig profile of DHM.

### *2.3.2 Lactoferrin*

Lactoferrin is a multifunctional glycoprotein of the transferrin family with enzymatic activity and antimicrobial, anti-inflammatory, and anti-infective properties (107, 108). Lactoferrin is best known for its role as an iron-binding protein. Indeed, lactoferrin has been shown to bind 20–45 % of iron in human milk (108). Lactoferrin is the second most abundant protein in human milk (107). Concentrations of lactoferrin in milk vary according to the stage of lactation, with the highest levels in colostrum (7 g/L) and declining in mature milk (2–4 g/L) (109). The findings reported thus far indicate a degradative effect of HoP on the lactoferrin content of DHM. Mayayo et al. demonstrated that 80 % of lactoferrin undergoes denaturation as a result of thermal exposure during HoP (110). Furthermore, the group speculate that lactoferrin degradation occurs as a result of protein aggregation caused by disulfide bonds. Separate investigations also noted a reduction in lactoferrin following HoP of 57–81 % (93, 97, 101, 102, 105, 106). Goulding et al. (111) suggest that the heat stability of lactoferrin is influenced by environmental conditions including the degree of iron saturation, pH levels, and protein composition. Evidently, lactoferrin is poorly preserved during HoP, undergoing thermally induced degradation likely as a result of changes in the protein structure.

### *2.3.3 Lysozyme*

Lysozyme is a major enzyme in human milk with anti-infective properties (34), contributing to the bacteriostatic capacity of human milk. Findings to date demonstrate a reduction in the concentration of lysozyme following HoP (74, 101, 102). Koenig et al. noted reductions of 65–85 % in colostrum (77). A similar decrease of 60.6 % was reported in mature milk (93). Moreover, the effect on lysozyme activity was measured in a series of *Micrococcus lysodeikticus* turbidimetric and lyso-plate assays and determined reductions of 36–44 % (98, 100, 112). Sousa et al. hypothesized that the loss of both lysozyme concentration and activity following HoP was due to the neutral pH environment of milk (7–7.4) (98). Indeed, lysozyme is heat stable at acidic pH (3–4) but heat labile at pH >7 (113). It is apparent that the thermal processing conditions of HoP, likely in combination with the pH environment of milk, result in lysozyme inactivation.

### *2.3.4 Human milk enzymes*

Human milk lipase activity consists of two enzymes, bile salt-stimulated lipase (BSSL) and lipoprotein lipase (LPL). BSSL facilitates the digestion and absorption of fat (114, 115). LPL participates in the production of milk lipids in the mammary gland (116), and although it has no known function in milk, it is thought to have a protective role during cold storage (117).

The evidence thus far indicates the near complete destruction of BSSL following HoP (47, 118, 119). In a study by Hamprecht et al. (74) <1 % of lipase activity was retained. Similarly, Henderson et al. (38) noted a 100 % reduction in LPL and BSSL activity. Comparable findings were reported by Kontopodi et al. in two separate studies (101, 102); the group noted low retention of BSSL levels (2–3 %) and activity (4–7 %) following HoP. The inactivation of BSSL as a result of thermal processing is not surprising. For instance, Pan et al. indicated that BSSL remained stable when exposed to 45 °C for 30 min (120). However, following an increase in temperature, the stability of BSSL was significantly reduced at 50 °C and completely destroyed at 60 °C (120). It can be concluded that HoP treatment results in the denaturation of BSSL. BSSL is particularly important for newborn infants, due to their limited secretion of pancreatic lipase and bile salts. Indeed, pasteurization of DHM results

in a reduction of lipid absorption of 30 % in preterm infants (113). Discernibly, a loss of BSSL interferes with the lipid metabolism of the neonate and is a serious limitation of HoP.

Fewer studies have assessed the impact of HoP on other enzymes, including amylase and alkaline phosphatase (ALP); however, reports indicate their reduction. Amylase contributes to the digestion of starch; its presence in human milk negates the limited salivary and pancreatic amylase activity in the first few months of life (121). Similar to milk lipases, amylase is relatively heat-labile (113). However, amylase appears more resistant to HoP processing with a reduction of 6–15 % (38, 122). ALP, due to its thermal resistance, is often used as a marker of adequate pasteurization (123, 124). Indeed, two studies noted the complete inactivation of ALP post-HoP (74, 102). Evidently, thermal exposure during pasteurization induces structural modifications, which can result in a loss of enzyme activity, functionality, and concentration. The degree of denaturation is dependent on the optimum conditions of the enzyme.

### *2.3.5 Cell signalling molecules*

Cell signalling molecules in DHM are important regulators of the inflammatory and immune response. Cell signalling molecules include cytokines, chemokines, and growth factors (GF). The influence of HoP on a variety of cytokines has been assessed with inconsistent results, see Table 1. The majority of findings to date suggest interleukin (IL)-2, IL-4, IL-5, IL-12, and IL-13 are not influenced by HoP (45, 49, 65); however, contrasting results have been noted (99, 125). It appears that HoP has no effect on IL-17 (45, 65). Both macrophage inflammatory protein (MIP)-1β and erythropoietin (EPO) were reduced by HoP (65, 126). Similarly, a reduction in IL-1β IL-6, IL-10, and tumor necrosis factor (TNF)-α was observed (45, 49, 99, 125, 126), although Espinosa-Martos et al. noted no effect of pasteurization on these cytokines (65). Similarly, a reduction post-HoP of interferon (IFN)-γ was reported (49, 125) but no effect was noted elsewhere (45). Interestingly, an increase in the concentration of IL-7, IL-8, and monocyte chemotactic protein (MCP)-1 following pasteurization has been recorded (45, 49, 65, 99). However, no increase in IL-8 and

MCP-1 was reported (65, 125), although high levels of IL-8 retention occurred (89 %) (125).

Similarly, HoP has variable effects on milk GFs (see Table 2). No effect of HoP on the following GFs has been reported, Transforming GF (TGF)-β1, TGF-β2, and epidermal GF (EGF) (65, 99, 126, 127). In contrast, reductions in hepatocyte GF (HGF), insulin-like GF (IGF)-1, IGF-2, IGFBP-2, and IGFBP-3 were recorded (49, 127), while increased levels of granulocyte–macrophage colony-stimulating factor (GM-CSF) following pasteurization were reported (65). It appears as though the effect of HoP on both cytokines and GFs is molecule-dependent. The mechanism by which cytokines and GFs are impacted by HoP is unknown but may be dependent on their protein structure. Espinosa-Martos et al. (65) attributed changes in concentration to their release from cellular or fat compartments into the aqueous fraction following heat exposure.

Overall, findings indicate a vulnerability of some cell signalling molecules to thermal pasteurization conditions; however, due to the limited and contrasting data a total effect cannot be determined. Due to the essential role of these molecules in the inflammatory response, minor alterations may influence the immunoprotective value of DHM. Further evaluation of changes in the balance of pro- and anti-inflammatory cells following HoP is necessary, as a shift could significantly alter the clinical value of DHM.

Table 1 Influence of Holder Pasteurization on the cytokine content of donor human milk.

| Cytokine | Effect | Volume pasteurized (mL) | Pooled (no. of donors) | Reference |
|---|---|---|---|---|
| IL-2 | – | 119 | Yes (4) | (49) |
| | – | <10 | No | (65) |
| | ↓ (47%) | <50 | No | (125) |
| IL-4 | – | 119 | Yes (4) | (49) |
| | – | <10 | No | (65) |
| | ↓ (>70%) | <50 | No | (125) |
| IL-5 | – | 119 | Yes (4) | (49) |
| | – | <10 | No | (65) |
| | ↓ (>45%) | <50 | No | (125) |
| IL-12 | – | 20 | Yes (6) | (45) |
| | – | 119 | Yes (4) | (49) |
| | – | <10 | No | (65) |
| | ↓ (42%) | <50 | No | (125) |
| IL-13 | – | 119 | Yes (4) | (49) |
| | – | <10 | No | (65) |
| | ↓ (>90%) | Not specified | No | (99) |
| IL-17 | – | 20 | Yes (6) | (45) |
| | – | <10 | No | (65) |
| MIP-1β | ↓ | <10 | No | (65) |
| EPO | ↓ | Not specified | No | (126) |
| IL-1β | ↓ | 119 | Yes (4) | (49) |
| | ↓ (>70%) | <50 | No | (125) |
| | – | <10 | No | (65) |
| IL-6 | ↓ (75%) | 20 | Yes (6) | (45) |
| | ↓ | Not specified | No | (99) |
| | ↓ (>50%) | <50 | No | (125) |
| | – | <10 | No | (65) |
| IL-10 | ↓ (24%) | 20 | Yes (6) | (45) |
| | ↓ | 119 | Yes (4) | (49) |
| | ↓ (>75%) | <50 | No | (125) |
| | ↓ | Not specified | No | (126) |
| | – | <10 | No | (65) |
| | – | Not specified | No | (99) |
| TNF-α | ↓ (95%) | 20 | Yes (6) | (45) |
| | ↓ | 119 | Yes (4) | (49) |
| | ↓ | Not specified | No | (99) |
| | ↓ (>80%) | <50 | No | (125) |
| | – | <10 | No | (65) |
| IFN- γ | ↓ | 119 | Yes (4) | (49) |
| | ↓ (>60%) | <50 | No | (125) |
| | – | 20 | Yes (6) | (45) |
| IL-7 | ↑ | <10 | No | (65) |

*(continued)*

| Cytokine | Effect | Volume pasteurized (mL) | Pooled (no. of donors) | Reference |
|---|---|---|---|---|
| IL-8 | ↑ (41%) | 20 | Yes (6) | (45) |
| | ↑ | 119 | Yes (4) | (49) |
| | ↑ | Not specified | No | (99) |
| | – | <10 | No | (65) |
| | – | <50 | No | (125) |
| MCP-1 | ↑ (62%) | 20 | Yes (6) | (45) |
| | – | <10 | No | (65) |

(↑) A significant increase in cytokine level occurred, (↓) A significant reduction in cytokine level occurred, (–) No significant effect on cytokine concentration was observed, (%) The percentage reduction or increase is noted in brackets.

### *2.3.6 MicroRNA*

Human milk is a rich source of microRNAs (miRNAs), defined as short (19–24 nucleotides), non-coding segments of RNA, that act as post-transcriptional regulators of gene expression (128). miRNAs are located within extracellular vesicles called milk exosomes. Milk exosomes encapsulate miRNAs, protecting them from degradation by RNases, digestion, or low pH, facilitating their transport to target cells and tissues via the bloodstream (115, 129). Although the extent of miRNA activity is still under investigation, evidence suggests a role in cell functionality and modulation of genes involved in physiological processes, such as metabolism, neurocognitive development, and immune function (90, 130).

There have been relatively few studies assessing the impact of HoP on miRNAs. A recent study noted that miRNA, within whole milk material and milk exosomes, undergoes significant degradation as a of HoP (131). Smyczynska et al., demonstrated an 82-fold decrease in whole material miRNA reads and a 302-fold decrease in miRNA exosome reads following HoP. This significant reduction prevented the group from further analyzing and characterizing the effects of HoP on the miRNA composition and function in DHM. However, in a subsequent study, Lamberti et al. observed a significant modification in the diversity of miRNA content of HoP-treated milk exosomes, noting 33 differential miRNAs (132). The differential miRNAs were implicated in five key pathways associated with immune and cellular

function, highlighting the potential for alterations to the immunomodulatory activity of DHM as a result of processing. As it stands, the impact of HoP on the miRNA composition of DHM remains relatively unexplored. Further investigations are required to establish the full effect of HoP, however, based on the findings to date a shift in the abundance and diversity of miRNA as a result of HoP has been observed.

Table 2 Influence of Holder pasteurization on the growth factor composition of human milk.

| Growth factor | Effect | Reference |
|---|---|---|
| TGF- β1 | – | (99, 126) |
| TGF- β2 | – | (65, 99) |
| EGF | – | (99, 126, 127) |
| HGF | ↓ | (49) |
| IGF-1 | ↓ (39%) | (127) |
| IGF-2 | ↓ (10%) | (127) |
| IGFBP-2 | ↓ (19%) | (127) |
| IGFBP-3 | ↓ (7%) | (127) |
| GM-CSF | ↑ | (65) |

(↓) A significant reduction in growth factor level occurred, (–) No significant effect on growth factor concentration was observed, (↑) A significant increase in growth factor occurred.

### *2.3.7 Milk hormones*

Milk hormones are non-nutritive bioactive compounds (69). More recently, links between hormones and infant health and development, particularly in metabolic health, have been reported (133, 134).

A small number of studies have assessed the response of milk hormones to HoP treatment. Marousez et al. demonstrated a significant effect of HoP on a variety of metabolic hormones. They reported a decrease of 63, 41, 11, 100, 41, and 83 % in insulin, nesfatin-1, cortisol, leptin, apelin, and GLP-1, respectively (69), whereas adiponectin levels remained unchanged following treatment. Ley et al. (51) also noted a reduction in insulin of 46% and, unlike the above study, a decline in adiponectin of 33 %. Furthermore, melatonin, a key hormone in the regulation of the sleep/wake cycle, was unaffected by HoP (135). The potential for alterations in the

levels of milk hormones as a result of processing raises concern due to their roles in energy regulation and the metabolic development of infants. The potential for long-term effects on the development and physiological function should be assessed.

Although HoP-treated milk retains a significant portion of the nutritive value of DHM, HoP also depletes a range of biologically active milk components. The loss of irreplaceable bioactive agents, including Igs, lactoferrin, and BSSL is a substantial limitation to the use of HoP. The affected components have known functional and beneficial properties for the newborn. It is apparent the thermal conditions of HoP negatively disrupt the structure of bioactive milk proteins. This shortcoming calls for the development and implementation of alternative techniques with less degradative outcomes.

### 2.4 Holder pasteurization and microbial inactivation

Although bacterial levels in milk vary, starting bacterial counts of 2.7–4.1 $\log_{10}$ CFU $mL^{-1}$ and 2.6–5.2 $\log_{10}$ CFU $mL^{-1}$ in DHM have been reported (65, 66). Furthermore, the predominant bacteria were of the *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Bacillus* genera. The primary objective of HoP is to remove any pathogenic microbes that could potentially infect an infant upon ingestion. A number of studies have demonstrated the efficacy of HoP at eliminating potentially harmful bacteria in DHM, both naturally occurring and through artificial inoculation.

The efficacy of HoP against naturally occurring microbes in DHM has been determined (65, 136). Lima et al. (104) noted no aerobic or coliform growth post-HoP. Additionally, in a study by Landers et al. (137), pre-HoP culturing demonstrated that of 303 milk pools, 87 % were colonized with coagulase-negative *Staphylococcus*, 16 % with *Enterococcus*, 8 % with *α-Streptococcus*, 4 % with *S. aureus*, and 61 % of samples were colonized with at least one Gram-negative rod. Following HoP, 93 % of the samples showed no bacterial growth. The persistent bacterial growth was mainly *Bacillus* growth (5 %) and also coagulase-negative *Staphylococcus* (1 %). This study emphasizes the need for mandatory pasteurization due to the prevalence and variability of bacterial growth in DHM. Moreover, the results to date indicate the efficacy of HoP in eliminating the non-

spore-forming bacterial community of DHM. Indeed, Czank et al. assessed the capacity of HoP to deactivate inoculated bacterial strains (93). HoP was carried out on milk samples with a concentration of $1\times10^5$ CFU $mL^{-1}$ of five bacterial strains (*S. aureus*, *Enterobacter cloacae*, *Bacillus cereus*, *Staphylococcus epidermis*, and *Escherichia coli*). The tested concentration of bacteria is the maximum advisable level of growth in DHM, according to NICE guidelines (24). The study demonstrated that HoP reduced the number of bacterial species by at least 99.9 %. Furthermore, the group determined the most to least heat-resistant strains were as follows: *S. aureus*, *B. cereus*, *E. cloacae*, *E. coli*, and *S. epidermis*. Similarly, findings by Raptopoulou-Gigi et al. (138) noted the ability of HoP to eliminate bacterial growth in samples inoculated with $2\times10^7$ of *S. aureus* and *E. coli*.

The ability of HoP to eliminate a high concentration of non-spore-forming bacteria has been established, but an ongoing concern for milk banks is the presence of bacterial spores. A number of studies demonstrated the inefficiency of HoP in eliminating bacterial spores, particularly *B. cereus* (65, 66). In a study of 21 DHM samples, 14 % of samples had *B. cereus* growth post-pasteurization (66). Indeed, the enhanced growth of *Bacillus* following exposure to thermal pasteurization conditions has been observed. Landers et al. reported *B. cereus* growth in 17 of 303 pooled milk samples post-HoP (137). Of the 17 samples, 10 were positive for *B. cereus* growth pre-pasteurization; however, the remaining 7 were only positive subsequent to HoP processing. Similarly, Lima et al. (104) reported *B. cereus* growth in 3 of 12 samples post-HoP. Again, pre-HoP culturing noted only one sample positive for *B. cereus* prior to pasteurization. These findings suggest that HoP is not only inefficient at eliminating *B. cereus* growth but also poses a risk of increasing *B. cereus* levels by inducing sporulation during thermal processing. Clearly, post-processing, DHM is at increased risk of *B. cereus* growth.

The starting levels of bacterial growth in DHM and the potential for bacterial spore contamination emphasize the need for routine pre-HoP bacterial screening. However, any post-pasteurization screening must be carried out with caution to reduce the risk of further contamination (24). It is evident that although HoP is effective in eliminating the majority of bacterial growth, this does not extend to

robust organisms such as spores, in this case, *B. cereus*. This is a key challenge facing the milk banking sector, which is largely reliant on strict post-processing handling and storage protocols to minimize the risk of bacterial sporulation. The inactivation of microbial spores and other thermo-tolerant organisms should be considered when future studies evaluate the efficacy of HoP or undertake the development of alternative processing techniques.

# 3 Alternative thermal techniques

As a result of the degradative effects of HoP on bioactive agents of milk and the inability to fully eradicate bacterial spores, a variety of other thermal processing techniques are under investigation. These methods alter the processing conditions applied (temperature and duration of exposure) or the mode of thermal application.

## 3.1 High-temperature short-time pasteurization

High-temperature short-time (HTST) pasteurization is an established, effective, and efficient method of pasteurization in the commercial dairy industry (139, 140). This method requires the rapid heating of milk to 72–75 °C for a short period of time (5–15 s), usually using continuous-flow plate heat exchangers. In recent years, HTST has emerged as an alternative to HoP, due to its ability to eradicate the microbes of DHM. However, it remains to be established if a reduction in the length of thermal exposure better preserves the nutritional and bioactive factors of DHM.

### *3.1.1 HTST and the nutritional composition of DHM*

HTST conditions (72.5 °C/15 s) applied to DHM, with either a laboratory-scale continuous-flow device or water bath immersion, did not significantly affect the macronutrient content of the DHM (39, 122). In addition, no significant changes in the fatty acid profile or levels of malondialdehyde, an indicator of lipid oxidation, were observed (122). Further studies noted the retention of the total protein content of DHM (74, 75, 102) and a better-retained protein profile than HoP-treated milk following HTST (118). Moreover, Silvestre et al. reported a superior retention of the mean lysine concentration following HTST than HoP, 85.11 and 70.69 %, respectively (75), suggesting improved protein quality with HTST-treated DHM. Again, these findings highlight the possibility that milk processing techniques may

modify the nutritional quality of DHM to varying degrees despite retaining the overall nutrient levels.

Few studies have examined the effect of HTST on the vitamin micronutrient composition of DHM. The retention of folic acid and some vitamins, including vitamins B1, B2, B6, B12, and C, was reported following HTST (72 °C /5–15 s) (74, 141). In contrast, Martysiak-Żurowska et al. (122) noted a reduction in vitamin C of 50.2 %. However, despite the observed reduction in vitamin C, the total antioxidant capacity (TAC) of milk was maintained (122, 142).

In conclusion, HTST processing maintains the macronutrient composition of DHM. Moreover, findings suggest that HTST may be superior at retaining the protein profile and quality of DHM. The full effect of HTST on the micronutrients in DHM is yet to be established due to the limited number of studies. However, findings propose some B vitamins and the TAC are retained with HTST treatment. Further studies examining a wider range of vitamins and indeed minerals should be carried out to allow for comparison with HoP-treated DHM.

### *3.1.2 HTST and the bioactive factors in DHM*

Reports on the Ig content of DHM following HTST are limited, with the majority of data only reporting on the IgA and sIgA content of DHM. A number of studies noted high levels of retention. Indeed, following HTST (72 °C/5–15 s) retention of IgA of 74–84 % and sIgA of 79–100 % was reported (74, 102, 141, 143), although a depletion in IgA of 36–57 % was also documented (97, 144). In addition, a reduction in IgG of 42 % and IgM of 100 % was noted following HTST at 72 °C for 15 s (97). The evidence suggests that the Ig content of DHM may incur losses following HTST; however, the extent of IgA reduction when compared with HoP suggests that HTST is superior for IgA retention. Clearly, further investigation on the IgG and IgM content following HTST is warranted.

Contrary to the findings of Goldblum et al. (141), a considerable decrease in lactoferrin levels, of 42–73 %, following HTST has been widely reported (102, 122, 143). The observed reductions in lactoferrin are likely influenced by the form of lactoferrin and therefore the degree of iron saturation (122, 145). Indeed, the iron-

depleted (apo) form of lactoferrin denatures at temperatures higher than 70 °C at a pH of ~7.0 (145). Taken together, the evidence suggests lactoferrin is not retained following HTST.

Several studies demonstrate the maintenance of lysozyme concentration and activity following HTST (102, 122, 146), and two studies reported increased lysozyme activity (141, 144). However, a reduction in lysozyme levels of 80 % was reported elsewhere (74). The contradictory findings may be caused by different methods of biochemical analysis. For instance, studies noting lysozyme retention used turbidimetric *M. lysodeikticus* assays (122, 141, 144) or liquid chromatography–mass spectrometry (LC/MS) (102), whereas Hamprecht et al. employed radial immunodiffusion to evaluate lysozyme levels (74). Overall, the evidence supports the stability of lysozyme during HTST. As discussed previously, lysozyme is heat labile at the pH of milk (113), suggesting the shorter exposure time of HTST processing prevents the destruction of lysozyme.

Similar to HoP, a significant depletion in human milk lipase, particularly BSSL, occurs following HTST (74, 141, 146). Kontopodi et al. noted low retention of BSSL levels and activity of 9 and 19 %, respectively (102). Nonetheless, the observed reductions in HTST-DHM are significantly less than those of HoP-treated DHM (102, 146). In addition, the digestive enzyme α-amylase underwent a reduction in activity of 7 % following HTST (122). The complete destruction of ALP following HTST has been repeatedly demonstrated, confirming the efficacy of HTST pasteurization conditions (74, 102, 143).

Studies on the influence of HTST on cell signalling molecules are sparse. However, a reduction in IGFBP-2 and the retention of IGF-1, IGF-2, IGFBP-3, and EGF have been demonstrated (127). These findings are in contrast to HoP, where in the same study the authors noted a significant reduction following HoP in all of the GFs analyzed, except EGF (127).

The contents of lactoferrin, lysozyme, and Ig of DHM are likely contributors to the intrinsic bacteriostatic ability of milk. Indeed, a negative correlation between a reduction in these three proteins and the growth rate of bacteria was identified

(102). Thus far, findings are in agreement that HTST impacts the bacteriostatic capacity of milk, with increased levels in the growth rate of *E. coli* in HTST-treated DHM in comparison with raw milk (102, 147), although no differences in the growth of *S. aureus* were observed between raw and HTST-DHM (102). It remains unclear, whether HTST or HoP treatment has a more degradative influence, due to contrasting findings. However, the effect of HTST on the bacteriostatic capacity of milk against *E. coli* is apparent. Further studies are needed to determine which processing technique best preserves the bacteriostatic properties of milk. A depletion in the natural bactericidal capacity of DHM following processing is concerning, as those in receipt of DHM are at increased risk of infection and disease. Minimizing the negative effect of thermal processing on the natural bacteriostatic capacity of raw milk should be considered when selecting an ideal technology for milk processing.

Findings suggest that HTST is superior or at the least equivalent to HoP at retaining some of the bioactive agents of milk. The observed variability in results may be due to the holding time and temperature applied, the chosen method of biochemical analysis, or the apparatus used for HTST processing. For instance, a wide assortment of methodologies has been used to perform HTST including plate heat exchange (141), water bath immersion (122, 144), and various laboratory-scale continuous-flow devices (74, 143, 146). Indeed, the apparatus used to implement HTST may have differing effects on the temperature and cooling profiles of DHM. In particular, temperature distribution may vary with non-continuous-flow instruments, resulting in portions of the sample being exposed to higher thermal conditions for increased periods. Evidently, precise temperature regulation, calibration of instruments, and a uniform approach are critical to gather comparable findings.

### *3.1.3 Microbial inactivation with HTST*

The ability of any milk processing technology to effectively eliminate bacterial growth is critical in order to be considered for a milk bank setting. HTST successfully inhibits the naturally occurring microbial community in DHM (123, 141, 146). The microbial deactivation of DHM following HTST has been further demonstrated with

inoculation studies. Bacterial spiking studies demonstrated the efficacy of HTST against *E. coli* ($10^8$ CFU $mL^{-1}$), *S. aureus* ($10^7$–$10^8$ CFU $mL^{-1}$), *Streptococcus agalactiae* ($10^6$ CFU $mL^{-1}$), *Listeria monocytogenes* ($10^9$ CFU $mL^{-1}$), and *Cronobacter sakazakii* ($10^9$ CFU $mL^{-1}$) (146, 148). However, a critical finding is that HTST was not effective against naturally occurring B. cereus (123). In this study, HTST conditions of 70–75 °C for 5–25 s were tested and none of the temperature–time combinations applied were capable of *B. cereus* eradication. For HTST to be a favourable alternative to HoP, inactivation of *Bacillus* is advisable. Further studies should assess the potential resistance of *B. cereus* to HTST conditions.

Overall, HTST appears equally effective to HoP at eliminating the microbial community of DHM; however, both procedures fail to address the ongoing challenge of *B. cereus* contamination. Furthermore, although widely adopted by the dairy industry, the logistics of implementing HTST in a milk bank setting may be challenging. Aside from high costs, HTST equipment requires cleaning between each pasteurization cycle, making it labour-intensive. Additionally, in comparison with the dairy sector, much smaller volumes are processed by milk banks. Although some of these issues may be overcome, Giribaldi et al. (146) designed a small-scale continuous-flow HTST instrument with the ability to process 0.1–10 L of milk. Such a device would need to be readily available commercially and undergo further testing. Importantly, the need for cleaning between cycles is still required. Evidently, aside from the need for further studies, the practicality and feasibility of downscaling HTST for clinical use warrants further consideration.

### 3.2 Retort processing

Retort processing is a commercial food sterilization technique that applies heat (115–121 °C) and pressure (15–20 psi) to a product for a set holding time before subsequent cooling (149). Recently, retort processing has been utilized for the preservation of DHM, producing a shelf-stable product that is currently available to neonatal intensive care units in the USA (104).

In all studies to date, retort processing of DHM was carried out at 121 °C, at 20 psi, for 5 min using water immersion (40, 104, 150, 151). Retort processing preserved the gross macronutrient composition of DHM (40). However, a reduction in lysine

and thiamine of 20 and 80 %, respectively, was observed. As noted previously, a reduction in lysine could indicate a modification in the protein quality, despite no observed change in total protein levels. A significant reduction in bioactive proteins of milk following retort processing occurs in lactoferrin, IgA, IgG, and IgM of 84 % (150, 151), 88–100 % (104, 150, 151), 77–100% (150, 151), and 100 % (150), respectively. In addition, lysozyme was reduced by 54–89 % (104, 151), although the retention of lysozyme was reported elsewhere (150). Furthermore, retort processing results in almost the complete degradation of vascular endothelial GF (VEGF) and TGF-β2, whereas TGF-β1 is maintained (150). A clear advantage of retort processing is the complete microbial inactivation of DHM, including *B. cereus* (104).

However, it is apparent that the efficiency of microbial inactivation is at a significant nutritional cost. The bioactive properties of DHM are at risk of almost complete destruction. A further advantage associated with retort processing is the ability to be packaged and subsequently stored at room temperature where cold storage facilities are lacking. Despite these advantages, the nutritional and therapeutic value of shelf-stable DHM is significantly diminished and may require fortification post-processing, suggesting it is an inferior alternative to HoP-treated DHM.

### 3.3 Microwave heating

Despite the small number of studies, microwave heating has emerged as a promising novel pasteurization method. Microwave heating relies on the generation of microwaves, which penetrate the food product, where they are rapidly absorbed and dissipate energy in the form of heat (152).

Leite et al. assessed the influence of microwave heating at 60 °C, 65 °C, and 70 °C for 30 s, 15 s, and 10 s, respectively, on certain bioactive factors in DHM (153). The group noted that at all temperature and time combinations, microwave heating did not alter the concentration of oligosaccharides or fatty acids in DHM. Furthermore, microwave heating at 60 °C for 30 s maintained 82, 88, 95, and 87 % of IgA, IgG, IgM, and lactoferrin, respectively. Similarly, no significant reduction in the Ig or lactoferrin content was observed following processing at 65 °C for 15 s. However, microwave heating at 70 °C for 5 s did cause significant reductions in IgG, IgM, and lactoferrin levels of 37–76 %. Comparable results were reported following

microwave heating at 62.5 °C for 5 min (154). High levels of retention were observed for lactoferrin, sIgA, and vitamin C of approximately 60-92 %. Furthermore, the retention of TGF-β2 was documented. However, the study noted a significant depletion of approximately 91 % in basal lipase activity following microwave heating. In addition, a reduction in lysozyme concentration and activity of approximately 56 and 30 %, respectively, occurred.

These findings demonstrate the potential of microwave heating in milk banks with high levels of bioactive factors retained. However, the choice of temperature and length of treatment is critical. Indeed, when Martysiak-Żurowska et al. increased the temperature of microwave heating to 66 °C for 3 min, a significant loss in all of the bioactive components was observed (154). These findings indicate that microwave heating at 60–65 °C allows for optimal retention of bioactive factors of DHM when the treatment duration is short (15–30 s), whereas an increased treatment time of 5 min may deplete levels of lysozyme and lipase.

More recently, when microwave heating with HTST conditions of 72.5 °C for 15 s was applied the macronutrient content and fatty acid profile of DHM were maintained (122). Furthermore, the levels of lactoferrin and lysozyme were unchanged post-processing. However, a reduction in vitamin C and α-amylase of 42.6 and 6 %, respectively, was observed. The retention of lactoferrin and lysozyme is in contrast to previous findings at 66 °C for 3 min and 70 °C for 10 s (153, 154). The contrast in findings calls for further assessment of microwave heating at increased temperatures (>66 °C) on DHM to allow for an optimal time–temperature combination to be determined.

Critically, the ability of microwave heating at 60 °C for 30 s to eradicate the natural microbiota of DHM and inactivate ALP has been demonstrated (124). Moreover, microwave heating achieved a complete reduction of *Salmonella typhimurium* and *S. aureus* inoculated at concentrations of $10^6$ CFU $mL^{-1}$ (124). Despite the encouraging findings, the data on the capacity of microwave heating for microbial inactivation are lacking. Further assessment of the inhibition of thermo-resistant microbes and the pasteurization conditions applied is needed. Once the optimal treatment parameters for microbial inactivation have been established,

subsequent analysis on the macro- and micronutrients of DHM should be performed.

Overall, findings to date on microwave heating are encouraging but preliminary. A key advantage of microwave heating is its ease of operation and accessibility within a milk bank setting. Furthermore, microwave heating requires little space and is timesaving, cost-effective, and low energy. However, the non-uniform transfer of heat during microwave heating may pose a challenge, resulting in the creation of cold spots in the milk (152). Further evaluation of the dielectric properties of human milk and its subsequent interaction with microwave heating is required to ensure consistent results. Indeed, a stirring mechanism or the equivalent should be implemented to prevent this from occurring. In addition, strict monitoring of time and temperature is essential.

### 3.4 Thermo-ultrasonication

The combination of ultrasonication (20–100 kHz) and moderate heat (>50 °C) to induce bacterial inactivation in foodstuffs is known as thermo-ultrasonication (TUS). Ultrasonication occurs following the formation and cavitation of microscopic bubbles. Bubbles are produced as a result of pressure shifts, following ultrasound wave propagation during processing. These bubbles collapse rapidly and with force, producing heat and shockwaves. The consequence of this is cellular membrane disruption and subsequent cell death (155).

Thus far, the limited data indicate that the bioactive properties of DHM undergo moderate reduction following TUS, the extent of which is mainly determined by ultrasound power (W) and also exposure time. For instance, the treatment of DHM at 40 W for 9 min maintained the IgA, lactoferrin, and lysozyme content, although a reduction in BSSL was observed (101). In comparison, when the power was increased to 60 W for a reduced period of 6 min, TUS retained IgA and lactoferrin but reduced lysozyme and BSSL by more than 40 and 70 %, respectively (101). Moreover, following TUS at 60 W for 9 min, a significant reduction in all the examined bioactive proteins occurred and levels of retention were comparable to those observed post-HoP (101). As indicated by the authors, the degree of bioactive protein retention decreased as the power of ultrasound increased. In a separate

study, high levels of insulin retention (97 %) have been reported, with a lower retention rate of 67 % following HoP (156). It appears that TUS has the potential to better preserve some bioactive properties of DHM; however, this is likely dependent on the choice of power, time, and temperature applied.

The ability of TUS to cause microbial deactivation of inoculated strains of *S. epidermis*, *E. coli*, and *S. aureus* has been previously observed (157, 158). Indeed, Kontopodi et al. demonstrated the ability of TUS (40–60 W, 6–9 min) to cause a > 6-log CFU $mL^{-1}$ reduction of inoculated *E. coli*, *S. epidermis*, and *E. cloacae* in DHM (101). These findings were further supported by Mank et al. (156) who recorded a 5-log reduction in the same bacterial strains following TUS at 60 W for 6 min. Overall, TUS appears an effective technique for the microbial inactivation of DHM, with significant reductions of inoculated pathogenic strains reported to date. However, the efficacy of TUS against bacterial spores is yet to be determined. In particular, further evaluation of TUS at 40 W is advisable, due to the optimal retention of bioactive proteins observed at this pasteurization condition.

Uniquely, TUS presents the opportunity to reduce thermal exposure of DHM and consequently may produce superior DHM. Evidently, although the findings to date are encouraging, additional studies are required to establish the efficiency of TUS and identify the optimal treatment parameters for DHM pasteurization. In particular, the macro- and micronutrient composition of DHM following TUS remains unknown.

### 3.5 Experimental temperature and time durations

A number of studies have carried out pasteurization at a range of temperatures and holding times to assess the effects on the nutritional and microbial components of human milk. Due to the experimental processing conditions chosen, these experiments do not fit within the parameters of previously discussed thermal techniques and are presented below, categorized according to thermal exposure. The results from these studies may provide greater insight into the optimal conditions for thermal processing of DHM.

### *3.5.1 Use of temperatures <62.5 °C*

Various studies reduced the pasteurization temperature or holding time below that of HoP (<62.5 °C, <30 min). Czank et al. reported a 90 % retention of sIgA, lactoferrin, and lysozyme following processing at 57°C for 30 min with an experimental pasteurizer (93). Similarly, pasteurization at 40–55 °C for 30 min retained >60, >80, and > 80 % of IgA, lactoferrin, and lysozyme, respectively (159). The observed difference in protein retention, despite the lower thermal conditions of the latter study, may be due to the pasteurizer design. The experimental pasteurizer used by Czank et al. was capable of precise temperature and time regulation.

In contrast, Zhang et al. used a bottle immersion technique. Although this method more closely reflects the technique applied in milk banks, it does not have the same capacity for temperature monitoring. Both groups concluded that the temperature applied during processing, not the length of exposure, was the critical factor for protein retention. Indeed, a 1 °C increase in temperature caused a reduction in the retention of all three proteins; however, a proportionate reduction in exposure time had no effect (93). Moreover, processing at 62.5 °C for 5 s resulted in a much lower retention of lactoferrin (32 %) and lysozyme (72 %) but a similar retention of IgA (83.2–95 %) (143, 160). These findings further suggest that the temperature applied during pasteurization is the key factor influencing bioactive protein retention.

Importantly, exposure to temperatures >50 °C reduced the endogenous bacteria in DHM (3.15–4.78 $\log_{10}$ CFU $mL^{-1}$) to undetectable levels (159). Indeed, pasteurization at 57°C for 30 min was sufficient to eradicate inoculated bacteria at a concentration of $1 \times 10^5$ CFU $mL^{-1}$, including *B. cereus* (93). In contrast, bacterial growth persisted following processing at 62.5 °C for 5 s (143, 160). Additionally, ALP retention of 6–27.3 % suggests that 5 s of treatment is inadequate for DHM pasteurization. Collectively, the findings demonstrate the inactivation of bacterial contamination and the improved retention of bioactive properties of DHM when processed at 55–57 °C for 30 min. Further examination of microbial inactivation at these processing conditions is merited. In particular, the inclusion of heat-resistant bacteria is necessary, as studies of treatment at higher temperatures for longer periods have

noted the persistence of *Bacillus* spores (66, 104, 123). The clinical potential of the improved retention of bioactive factors needs to be balanced with microbial safety.

### *3.5.2 Treatment at 80–100°C*

Multiple experiments with high-intensity treatment conditions of 80–100 °C have been performed. As a whole, findings demonstrate the poor preservation of bioactive factors at this increased temperature range. Low levels of retention of 0–22 % in IgA, IgG, and IgM were observed following treatment at 80–100 °C (97, 143, 144). Similarly, a reduction of 78–85 % in lactoferrin occurred when processed at 81–88 °C for 5 s and levels were undetectable following 100 °C for 5 min (97, 143). Lysozyme demonstrated the greatest resistance to thermal exposure, with no significant reduction following processing at 80 °C for 15 s (144). However, when the length of treatment increased to 10 min, a ~25 % reduction was observed, which further increased following processing at 90 °C (144).

Not surprisingly, the microbial inactivation of DHM occurs following pasteurization at high temperatures. A > 5-log reduction in *C. sakazakii* and *L. monocytogenes* and a > 3-log reduction in *Enterococcus faecalis* were achieved with pasteurization at 81.5 °C for 5 s (143).

Evidently, DHM processing at increased holding temperatures results in a significant decrease in the key bioactive components of DHM. Moreover, at higher temperatures, the length of exposure can further exacerbate the observed degradation. This is evident with the level of lysozyme retention decreasing with an extended processing time, despite the same temperature being applied (144). Overall, there is no demonstrated benefit to the treatment of DHM at these high-temperature conditions. A similar microbial inactivation has been achieved with lower temperatures, allowing for greater retention of bioactive components of milk.

### *3.5.3 Treatment >100 °C*

Heating temperatures of >100 °C are commonly applied in the dairy industry, e.g., in cases of sterilization (~121 °C) or ultra-high temperature (UHT) (135–150 °C) (161, 162). The extreme temperatures used by the dairy industry, when applied to DHM, have an expected negative impact on bioactive properties of milk. Indeed, less than

20 % of IgA and lactoferrin were retained following pasteurization at 127 °C for 15 s (159). Similarly, following UHT, the Ig content of DHM was undetectable and an 84 % reduction in lactoferrin occurred (150). Again, lysozyme was the most resistant to high-temperature processing, with approximately <40 % retention following 127 °C for 15 s (159) and no observed reduction following UHT (142 °C, 6 s) (150).

Furthermore, an effect that has not been seen at lower processing temperatures is the impact of sterilization conditions on the lipid fraction of DHM. Processing at 120 °C for 30 min decreased the available lipid content by ~10 % due to the formation of surface skin and deposit on the inside of the sterilization container (36). Additionally, sterilization reduced the fatty acid content of DHM, specifically linolenic and arachidonic acid (36). Whether these effects occur at sterilization and UHT temperatures, with a reduced processing time, is not known. The degradation of bioactive properties of DHM and its potential influence on the lipid fraction emphasize that the current techniques used by the dairy industry are not suitable for adoption by milk banks.

# 4 Non-thermal processing techniques

Accumulating evidence illustrates the degradation of various DHM components with prolonged thermal exposure. The destruction of the heat-labile components produces DHM with a reduced therapeutic value. As a result, various non-thermal strategies are now being assessed for DHM pasteurization.

## 4.1 High-pressure processing

In recent years, high-pressure processing (HPP) has been shown as a safe, effective, and alternative method of preservation in the food industry. HPP is widely used in a number of foodstuffs, including vegetable, meat, and dairy products, due to its ability to reduce microbial load while retaining high nutritional value and protecting the organoleptic characteristics of foods (163, 164). Furthermore, HPP-treated food is viewed as minimally processed, lending to a high level of consumer acceptability (165). During HPP, food is vacuum-packed and placed into a pressure vessel containing a pressure-transmitting fluid. It is then subjected to isostatic pressure, typically 400–600 MPa at 4–45 °C (164). Pressure is delivered

instantaneously and distributed evenly throughout the product. The primary adjustable conditions that influence the efficacy of HPP are the pressure selected (MPa), the holding time, and the temperature (166). As pressurization does not disrupt covalent bonds, the loss of nutrients and bioactive compounds is minimal. Instead, pressure impacts weaker bonds (Van der Waals forces, electrostatic interactions, and hydrogen bridges) resulting in a loss of cellular function, membrane disruption, and subsequent microbial inactivation (165).

### *4.1.1 HPP and nutritional composition*

Findings to date indicate that HPP at 300–600 MPa for 5–10 min does not cause any change in the macronutrient content of DHM (41, 42, 167). Despite this, a 2.9 % reduction in total carbohydrate levels following HPP at 500 MPa for 8 min was reported (41). However, further supporting the stability of the carbohydrate portion of DHM, Marousex et al. (80) noted the retention of HMOs following HPP at 350 MPa. Similarly, a reduction was reported in some n-3 series PUFAs following HPP for 6 min at 600 MPa (45). However, others note the retention of the lipid profile of DHM following HPP (300–600 MPa), with no reported changes to the fatty acid composition or the triacylglycerol profile (43, 44, 47, 167). Furthermore, following HPP, the total protein profile of DHM was unchanged (76) and more closely resembled that of raw milk than HoP-treated milk (78). Overall, these findings indicate the retention of the macronutrient composition of milk following HPP.

The influence of HPP on the micronutrient content of DHM has been limited to vitamin composition, with varying findings. Tocopherols underwent significant reductions following HPP at 600 MPa of 21–27 %, 44–4 7%, and 25–33 %, in α-, δ-, and γ-tocopherol, respectively (45), whereas pressurization at 400 MPa retained α- and δ-tocopherol but reduced γ-tocopherol by 26–29 % (45). In contrast, a separate study reported the retention of tocopherol levels following HPP at 400–600 MPa for 5 min (44). Similarly, contradictory reports of vitamin C stability have been recorded. Despite a reported reduction in vitamin C content of 75 % following HPP at 500 MPa for 8 min (41), the retention of vitamin C following processing at 400–600 MPa for 5 min has also been demonstrated (44). Folate was unchanged by HPP at 500 MPa for 8 min (41). In addition, one study has reported on the carotenoid

content of DHM; both β-carotene and lycopene, important antioxidants, are preserved following HPP at 450–600 MPa for 10–15 min (47). However, lutein and zeaxanthin underwent reductions of ~60 %.

Collectively, the findings suggest that the effect of pressurization on the vitamin composition of DHM is distinct for each vitamin. Furthermore, the extent of vitamin reduction seemingly increases with an increase in pressure. The impact of holding time remains unclear but may account for the observed discrepancy in vitamin C retention. It is evident that an investigation of a wider variety of vitamins and indeed minerals is required to fully ascertain the influence of HPP on the micronutrient composition of DHM and any subsequent clinical significance.

#### *4.1.2 HPP and bioactives*

To date, there is promising evidence of Ig retention following HPP. Indeed, a number of studies noted the retention of IgG, IgM, and IgA following HPP at 200–450 MPa for 2.5–30 min (94, 96, 98, 101, 168). However, a significant depletion in Ig is observed with an increased pressure of 600 MPa (76, 94, 96, 101, 144, 169). For instance, a reduction in IgA, IgM, and IgG of 20–26 %, 59–60 %, and 35–40 %, respectively, occurred subsequent to HPP at 600 MPa for 15–30 min (98). Although a reduced holding time of 2.5 min maintained IgA levels, losses of 21 % in IgM and IgG still occurred. Clearly, the choice of pressure and exposure time are influential factors.

This was further illustrated by Permanyer et al., who noted that, as pressure increased, an additional reduction in IgA occurred; 100 % retention at 400 MPa, 87.9 % at 500 MPa, and 69.3 % at 600 MPa (94). Similarly, Kontopodi et al. (101) observed a greater reduction in IgA levels with increased treatment time during HPP at 600 MPa, with 55 % retention after 3 min and 47 % after 5 min. On the whole, these findings suggest that HPP can retain the Ig profile of DHM but the selection of processing parameters is a critical determinant. In particular, as the pressure or time exposure increases, so too does the risk of Ig loss. Further studies should ascertain the optimum holding time for HPP treatment, with findings to date suggesting that HPP at <500 MPa is suitable for Ig retention.

The retention of lactoferrin following HPP at 350–600 MPa with assorted holding times has been demonstrated (76, 101, 170, 171). Conversely, a reduction of 25 % following HPP at 500 MPa for 8 min was reported (41). Similar reductions of 35–44 % were observed following HPP depending on the combination of pressure, holding, and resting times applied (169). Importantly, the optimal variant group tested, HPP at 200 MPa for 10 min followed by a 10 min resting period and 400 MPa for 10 min (200 + 400 MPa), did not cause a significant reduction in lactoferrin. Evidently, the HPP specifications applied will influence the degree of lactoferrin denaturation. For example, following 15 min of HPP at 300, 400, 500, or 600 MPa, a reduction in lactoferrin of 9, 23, 34, and 48 %, respectively, occurred (110). Furthermore, the majority of reduction in lactoferrin occurred within the first 5 min of treatment, with only a further 8 % loss incurred between 7 and 30 min of treatment (110). Despite the varying outcomes, the evidence suggests the superior retention of lactoferrin following HPP as opposed to HoP treatment (41, 169, 171). Unlike HoP, which results in the aggregation of disulfide bonds and denaturation of lactoferrin, HPP does not impact covalent bonds (110, 171, 172), thus better preserving the lactoferrin content of DHM. Overall, HPP can maintain lactoferrin, but this is dependent on the pressure applied and to a lesser degree, the holding time.

Although lysozyme is more robust to thermal processing than other bioactive proteins, a degradative effect of heat treatment has been observed (98, 168). In contrast, HPP at 200–600 MPa at various holding times of 2.5–30 min retains the lysozyme levels of DHM (41, 98, 101, 168, 170, 171). Collectively, the available data substantiate the stability of lysozyme during the HPP of DHM.

The influence of HPP on cell signalling molecules is still in its infancy with a small number of studies performed and contrasting data gathered. The content of cytokines and growth factors in DHM following HPP is presented in Table 3. In brief, there is consensus on the stability of IL-6, IL-8, IL-12, and TNF-α following HPP at 400 MPa (45, 99), despite a reported reduction in IL-10 of 31–42% following HPP at 400–600 MPa (45). Franch et al. only noted a reduction in IL-10 when a pressure of 600 MPa was applied (99). Similar disparate findings on TNF-α retention were reported following HPP at 600 MPa (45, 99). A reduction of 22–33 % in monocyte

chemotactic and activating factor occurred following HPP at 400–600 MPa (45). In addition, IFN-γ and IL-17 underwent varying losses, depending on the time–pressure combination applied (45).

A similar variation in GF retention following HPP has been recorded. For instance, HGF levels were either retained or underwent a reduction of 57–64 % following HPP depending on the conditions applied (169). Furthermore, a 21 % reduction in HGF levels occurred following HPP at 450 MPa for 15 min (173). The retention of EGF, TGF-β1, and TGF-β2 following HPP has been recorded (99). Evidently, HPP has variable effects on the cytokine and GF levels of DHM, depending on the HPP conditions applied and the cell signalling molecule in question. It is apparent from the conflicting data that further research is needed to determine the appropriate conditions for cytokine and GF retention. However, the highest retention levels across the widest range of molecules appear to be HPP at 400 MPa for <5 min. It is unlikely that a complete lack of effect on cell signalling molecules is achievable. However, HPP with optimal pasteurization parameters may retain key molecules involved in the inflammatory process and ensure a preferable balance of pro- and anti- inflammatory molecules.

Table 3 Influence of high-pressure processing on the levels of cell signalling molecules in human milk.

| | Effect | Processing conditions | Reference |
|---|---|---|---|
| **Cytokine** | | | |
| IL-6 | – | 400–600 MPa, 3–6 min | (45, 99) |
| IL-8 | – | 400–600 MPa, 3–6 min | (45, 99) |
| | ↑ | 600 MPa, 5 min | (99) |
| TNF-α | – | 400–600 MPa, 3–6 min | (45, 99) |
| | ↓ | 500–600 MPa, 5 min | (99) |
| IL-10 | - | 400–600 MPa, 3–6 min | (45, 99) |
| | ↓ | 400-500 MPa, 5 min | (99) |
| MCAF | ↓ (22–33%) | 400–600 MPa, 3–6 min | (45) |
| IFN-γ | ↓ (90–95%) | 400–600 MPa, 3 min | (45) |
| | – | 400–600 MPa, 6 min | (45) |
| IL-17 | – | 400 MPa, 3 min | (45) |
| | | 600 MPa, 6 min | |
| | ↓ (84–100%) | 400 MPa, 6 min | (45) |
| | | 600 MPa, 3 min | |
| **Growth Factor** | | | |
| HGF | ↓ (64%) | 600 MPa, 10 min | (167) |
| | ↓ (61%) | 100 MPa + 600 MPa | (167) |
| | ↓ (57%) | 200 + 600 MPa | (167) |
| | – | 200 + 400 MPa | (167) |
| | ↓ (21%) | 450 MPa, 15 min | (171) |
| EGF | – | 400–600 MPa, 5 min | (97) |
| TGF-β1 | – | 400–600 MPa, 5 min | (97) |
| TGF-β2 | – | 400–600 MPa, 5 min | (97) |

(↑) A significant increase in level of cytokine/growth factor occurred, (↓) A significant reduction in level of cytokine/growth factor occurred. (–) No significant effect on cytokine/growth factor concentration was observed, (%) The percentage reduction/increase is noted in brackets.

Only one study to date has evaluated the miRNA portion of DHM following HPP treatment. Smyczynska et al. noted that, unlike HoP, HPP did not decrease the miRNA reads of DHM (131). Nevertheless, HPP did result in changes to the composition of the miRNA fraction. In particular, reduced levels of miRNA-29 and miRNA-148a-3p were observed. On the other hand, miRNA-30d-5p appeared highly resistant to pressurized conditions. The authors suggest a protective effect of milk exosomes during pressurization. The consequences of compositional changes on miRNA functionality and subsequent immune responses are not known. The limited

available data suggest that HPP may be less detrimental to the miRNA content of human milk than HoP. However, the modification of DHM miRNA following milk processing and the potential implications this may have for the recipient requires further study.

The degradative effect of thermal processing on human milk lipases has been widely reported. Therefore, the demonstrated potential of HPP to retain high levels of milk lipases is an encouraging prospect. Wesolowska et al. reported a 79–87 % retention of milk lipase activity following HPP at 450 MPa and 200 + 400 MPa (47, 173), whereas HPP at 600 MPa resulted in <17 % retention in lipase activity (47). However, further supporting the maintenance of milk lipase following HPP, LPL was preserved following HPP at 400 MPa for 5 min (171). Furthermore, BSSL is retained following exposure to pressures of 400–600 MPa for 5 min (171), 8 min (41), and 1.5–30 min (101).

The influence of HPP on the hormonal constituents of DHM indicates the retention of some key metabolic hormones. Indeed, both leptin and insulin were retained when processed at 350 MPa (69), 450 MPa (173), and 200 + 400 MPa (169). Similarly, nesfatin-1, cortisol, and GLP-1 were stable after processing at 350 MPa (69). In contrast, HPP resulted in a depletion of adiponectin and apelin of 50–85 and 20 %, respectively (69, 169, 173). It remains unclear why adiponectin and apelin appear more vulnerable to pressurization. However, as hypothesized by Marousez et al., it may be a result of pressure-induced modifications to both the physiochemical properties of milk and the structural composition of apelin and adiponectin (69). Despite these observed reductions, the overall hormonal composition of DHM appears well preserved following HPP, in particular, the higher levels of leptin and insulin when compared with HoP-treated DHM.

Despite the modification in the levels of some bioactive factors in DHM, the bacteriostatic ability of milk is preserved following HPP (101, 170, 174). Indeed, DHM processed at 350 MPa retained its antimicrobial activity when tested against *E. coli* and *S. agalactiae* (170). Similar findings were observed following HPP at 400–500 MPa and HPP at 200 + 400 MPa against *S. aureus* and *E. coli* (101, 174). In contrast, a higher pressurization of 600 MPa for 3–5 min did reduce the inhibition

rate of DHM against *S. aureus* (101). These findings suggest that DHM processed at <500 MPa preserves the bacteriostatic capacity of DHM.

### *4.1.3 HPP and microbial inactivation*

Multiple studies have demonstrated the ability of HPP at 400–600 MPa to reduce the endogenous bacteria of DHM to undetectable levels (42, 94, 168, 171, 175). Importantly, HPP at 300 MPa for up to 15 min did not eliminate the native bacterial population of DHM, deeming it unsuitable for application within the milk bank setting (171, 175). Further studies have assessed the bacterial inactivation of inoculated pathogens following HPP. The first inoculation study was performed by Viazis et al., who reported an 8-log reduction in *L. monocytogenes* and *S. agalactiae*, after HPP at 450 MPa for 2 and 4 min, respectively (95). The group also demonstrated a 6–8 log reduction in *E. coli* and *S. aureus*; however, 30 min of treatment was required. These findings indicate the potential for pressure-resistant microorganisms.

However, subsequent studies have reported the inactivation of *S. aureus* and *E. coli*, following HPP. For instance, 15 min of HPP at 500 MPa was sufficient to reduce *S. aureus* by 5-log CFU $mL^{-1}$ (175). In addition, a complete reduction in *S. aureus, E. coli*, *L. monocytogenes*, *C. sakazakii*, and *B. cereus* was recorded after HPP at 450 MPa for 15 min, with a starting concentration of $10^{5}$–$^{7}$ CFU $mL^{-1}$ (173). Furthermore, a > 7.8-log reduction in *S. epidermis* and *E. cloacae* was reported following HPP at 400–600 MPa for 5 min (101). Clearly, despite the pressure-resistant capacity of some microbes, when optimal HPP parameters are applied, microbial deactivation is achieved. Moreover, a 5-log reduction is the maximum necessary reduction if the acceptable growth limits according to milk banking guidelines are followed (24). However, the available data on the microbial safety of DHM following HPP remain limited in comparison with the extensive studies carried out on HoP-treated DHM. Accordingly, future studies should further endeavour to confirm the capacity of HPP at 400–600 MPa to eradicate microbial growth and determine the minimum treatment time required particularly focusing on those strains with demonstrated pressure resistance and bacterial spores, mainly *B. cereus*.

### *4.1.4 Novel HPP conditions*

Alternative approaches to HPP of DHM have been tested through an optimized HPP system (176) or by combining pressure treatment with extreme temperatures (177, 178). Martysiak-Żurowska et al. combined HPP at 193 MPa with a sub-zero temperature of −20°C (177). The group found that HPP at −20 °C did not alter the FA composition or the concentration of secondary lipid oxidation products. Furthermore, the total vitamin C and TAC levels of DHM were preserved, although a reduction in ascorbic acid of 11 % was observed. The authors hypothesize that extreme temperatures can intensify the effect of pressurization, allowing a lower pressure to be applied. This study presents the potential for the application of lower pressures, enabling greater preservation of bioactive components of milk. However, the data are preliminary and must be further substantiated—in particular, the capacity of lower pressure at sub-zero temperatures to not only retain the nutritional and bioactive content of milk but also ensure microbial inactivation. In contrast, a study that combined pressure with high temperatures (178) found that treatment at 65 °C and 80 °C depleted the α-tocopherol, fatty acid composition, and some cytokines, regardless of the pressure applied. Furthermore, only a pressure of 300 MPa at 50 °C resulted in moderate Ig retention of 48–100 %. Evidently, thermal HPP results in disruption to the nutritional and bioactive components of DHM.

Separately, an optimized, novel HPP system was applied to DHM (176). The group designed a HPP system in which the parameters for pressure delivery are accounted for, including compression rate, decompression rate, and application mode. The process parameters were as follows: pressure (350 MPa), temperature (38 °C), application rate (1 MPa $s^{-1}$), and application mode (four cycles of 4 min duration with 5 min latency time between each cycle). Following processing, high levels of sIgA, lactoferrin, and lipase were retained at 63–64, 93–97, and 80 %, respectively. Critically, the group demonstrated a 6-log CFU $mL^{-1}$ reduction of inoculated *S. aureus* and *B. cereus*. The potential of this optimized HPP system to eradicate bacterial spores emphasizes its potential for milk pasteurization. Indeed, 10 % of donated milk samples in this study were contaminated with *B. cereus* (176).

This approach would produce milk high in nutritional value with improved microbial safety. Implementation of this HPP system should be performed in future studies with further assessment of microbial inactivation and an evaluation of its influence on the nutritional composition of milk.

### 4.2 UV-C irradiation

Ultraviolet (UV)-C irradiation is an emerging innovative alternative technique for milk pasteurization. UV-C (200–280 nm) exposure disrupts the cell's genetic material, interfering with the cell's ability for DNA transcription and replication, resulting in cell death (101). The penetrative ability of UV-C during milk processing faces two challenges. First, the high absorption coefficient of milk due to its opaque appearance (300 cm−1 at 254 nm). In comparison, translucent solutions such as water have a much lower absorption coefficient (0.1 $cm^{-1}$) (179, 180). Second, the total solid concentration of milk, which varies, can further increase the absorption coefficient (181, 182). Despite these challenges, the ability of UV-C to penetrate bovine milk has been achieved by creating a thin film turbulent flow-through of milk, ensuring exposure of the whole sample to the UV source (180, 182, 183). A similar approach has been adopted by studies assessing UV-C processing of DHM.

Only a small number of studies have reported on the macronutrient content of DHM following UV-C. Pitino et al., reported no significant changes in the concentration of carbohydrates, lipid, and protein content of DHM following UV-C treatment (41). Furthermore, UV-C at 4863 J $L^{-1}$ caused no significant changes to the protein concentration and fatty acid profile of DHM, except for an increase in the level of fatty acid C8:0 (101, 179). However, increased levels of lipid oxidation end products following UV-C at a low dosage of 172.9–740 J $L^{-1}$ have been reported (184). Similarly, there has been minimal reporting on the micronutrient composition of DHM following UV-C irradiation. Two studies have recorded reductions in vitamin C and folate of 35–72 and 25 %, respectively (41, 184). Despite this reduction, the TAC of DHM was preserved (184). Overall, the macronutrient composition of UV-C treated DHM appears stable post-processing. However, the observed increase in lipid oxidation end products calls for further evaluation of the lipid fraction post-

processing. Additionally, any potential loss or alteration in the concentration or composition of micronutrients remains relatively unknown.

As this process is non-thermal, heat-induced denaturation of bioactive compounds does not occur. Studies suggest that UV-C preserves key immune factors including lactoferrin, lysozyme, and IgA. Lactoferrin retention of 90–95 % and 80–87 % following UV-C at 2084–3645 J $L^{-1}$ and 4,683–4,863 J $L^{-1}$, respectively, has been reported (101, 103, 181). Similarly, high levels of lysozyme preservation were observed at 84–96 % and 75–80 %, respectively (101, 103, 181), although lower levels of lysozyme retention (~60 %) were reported elsewhere (184). Studies of the Ig profile following UV-C are limited and have focused on the IgA content. IgA levels were preserved at >80 % following UV-C at 2430–4863 J $L^{-1}$ (101). These findings were further supported by sIgA retention of 89–95 % following UV-C at 2084–4683 J $L^{-1}$ (103, 181). Clearly, findings suggest that UV-C may be a suitable non-thermal alternative to HoP that will better preserve key DHM components. Indeed, comparative studies noted much lower levels of retention of lactoferrin, lysozyme, and IgA following HoP of 9–32 %, 41–78, and 40 %, respectively (101, 103, 181). In addition, a number of studies have reported the retention of BSSL following UV-C at 1,100–5,500 J $L^{-1}$ (101, 119, 179). Indeed, a reduction in BSSL of 64.7 % was only observed when a high UV-C dosage of 16,500 J $L^{-1}$ was applied, which further increased to 79.8 % at a dosage of 33,000 J $L^{-1}$ (119). These findings demonstrate that BSSL is stable when exposed to moderate UV-C exposure.

The retention of immune proteins and antioxidant factors is a likely contributor to the preserved bacteriostatic activity of UV-C-treated DHM. Barbarska et al. noted the preserved bactericidal capacity of DHM following UV-C at 6720 J $L^{-1}$. Indeed, untreated DHM caused a 46.6 % reduction in *E. coli* growth, whereas, following UV-C irradiation, *E. coli* growth was reduced by 57.6–62.6 %. In comparison, HoP-treated DHM only reduced growth by 12 % (174). Retention of the antimicrobial activity of DHM following UV-C at 2084–4863 J $L^{-1}$ against *S. aureus* and *E. coli* was also demonstrated elsewhere (101, 103). These findings suggest that UV-C retains not only the bioactive properties but also the subsequent antimicrobial activity of milk.

A number of studies have demonstrated the efficacy of UV-C in ensuring sufficient microbial inactivation of DHM. Christen et al. were the first to note that UV-C at a dosage of 4,836 J $L^{-1}$ results in a 5-log CFU $mL^{-1}$ reduction of *S. epidermis*, *S. aureus*, *E. cloacae*, and *B. cereus* (179). Similarly, at the same dosage, a reduction of 5.78–6.95 log CFU $mL^{-1}$ was achieved in DHM inoculated with a concentration of $10^8$ CFU $mL^{-1}$ *S. epidermis*, *E. cloacae*, and *E. coli* (101). In the same study, a reduced UV-C dosage of 3,645 J $L^{-1}$ and 2,430 J $L^{-1}$ resulted in log reductions of 4.6–5.9 and 4.3–5, respectively (101). These findings were further supported by Almutawif et al. (181) who observed a > 5-log reduction in *S. aureus* when UV-C was applied to DHM at 2259 J $L^{-1}$. In fact, no growth persisted following treatment for 14 days at 4 °C and 18 h at 37 °C.

However, a separate study reported that a higher dosage of 2,750 J $L^{-1}$ resulted in a less than 5-log reduction in *C. sakazakii*, *E. faecium*, *L. monocytogenes*, and *S. aureus* (119). Furthermore, this dosage only resulted in a 2.75 log reduction in bacterial spores of the *Bacillus* and *Paenibacillus* species. The study noted that a high UV-C dosage (8,250 J $L^{-1}$) was required for a > 5-log reduction across all strains tested, including spores. Finally, UV-C treatment at 85–740 J $L^{-1}$ is inadequate for microbial inactivation of DHM, with reductions in *E. faecium* of <1-log following 550 J $L^{-1}$ and < 3-log following 740 J $L^{-1}$ (119, 184). Evidently, UV-C processing of DHM can produce microbiologically safe milk when the correct parameters are applied. Findings thus far indicate that a UV-C dosage of 4,863 J $L^{-1}$ and 8,250 J $L^{-1}$ can inactivate the microbial components of DHM, including bacterial spores.

Although there have been positive indications for UV-C treatment at lower doses (2259–3,645 J $L^{-1}$), further studies are necessary due to the contradictory results reported. In particular, the potential survival of bacterial spores requires further examination. Furthermore, prior to processing the total solid concentration of the milk samples should be quantified. As observed by Christen et al., the required UV-C dosage applied to milk increases linearly with the total solid concentration of milk (179). This may account for the discrepancies noted in findings to date, in addition to the applied turbulent flow rate and pasteurizer design during UV-C processing.

As a whole, the research suggests that UV-C at >4,863 J $L^{-1}$ results in a > 5-log reduction in microbial growth, the maximum allowed growth in milk banks (24). Importantly, significant retention of the bioactive properties of milk is also achieved with these treatment parameters, as summarized in Table 4. The observed variance in bacterial reduction at lower dosages suggests further study is required to ascertain the minimal parameters for UV-C treatment of milk. All studies discussed applied a turbulent flow to milk while undergoing UV-C to expose DHM evenly and overcome the high absorption coefficient of milk. To optimize treatment parameters, future studies should assess the total solid content of milk prior to processing. In addition, further evaluation of the influence of UV-C on other key factors with biologically active roles, including IgG, IgM, cytokines, and hormones, is warranted.

### 4.3 Gamma irradiation

Gamma irradiation is a non-thermal technology that can be used to pasteurize foodstuffs. During processing, the product undergoes controlled exposure to gamma rays from a radioactive source, for example, cobalt-60 or cesium-137. The gamma rays are absorbed by the food product, damaging the genetic material of microbial cells, inducing cell death, and consequently pathogen reduction (185–187). Despite the approval of gamma irradiation use in certain food groups by multiple agencies, including the WHO, the European Food Safety Authority, and the US Food and Drug Administration (188–190), consumer hesitancy persists (191). Consequently, studies of this technology for DHM pasteurization are minimal and its applicability to milk banks may be viewed unfavourably. Gamma irradiation exposure of up to 10 kGy is sufficient to ensure microbial safety, retain the nutritional components of foodstuff, and limit radiolysis (185, 187). Consequently, 10 kGy has mostly been applied as the maximum dose in DHM studies to date and the results are presented herein.

Gamma irradiation of DHM has mainly been applied as part of a hybrid approach with either freeze-drying or the addition of an antimicrobial mixture. For instance, Blackshaw et al. studied the effects of gamma irradiation on freeze-dried DHM. The group suggest that 2–5 kGy is the optimal exposure range for the retention of milk's

nutritional properties (192). Indeed, irradiation at 2 kGy retained the protein profile of DHM and did not increase the levels of lipid oxidation end products. In a separate study, the same group demonstrated the capacity of irradiation at 5 kGy to completely eliminate strains of *S. aureus* and *S. typhimurium* inoculated at a concentration of $10^6$ CFU $mL^{-1}$ (193). Furthermore, the bacteriostatic properties of DHM were retained following irradiation (193). Similarly, Balaji et al. (194) used an alternative hybrid approach on DHM of gamma irradiation at 5 kGy combined with antimicrobial formulations. The hybrid treatment resulted in a 6-log reduction in bacteria, including *B. cereus*. Furthermore, the combined treatment maintained the Ig and lactose levels in DHM. However, protein hydrolysis and a subsequent increase in peptide concentration occurred; while this did not impact the in vitro digestibility of protein.

Few studies have assessed the potential of gamma irradiation as a standalone treatment. That being said, reported reductions of 70.8, 18, and 28 % in BSSL, IgA, and lactoferrin, respectively, occurred following gamma irradiation of DHM (119, 138). Importantly, both studies applied a high dosage of 25 kGy during processing. The increased dosage may explain the depletion in IgA, as opposed to its retention following hybrid IR processing. However, the levels of BSSL and lactoferrin have not been assessed elsewhere, and therefore, the influence of a reduced irradiation dosage remains unknown.

Clearly, there is not enough data to determine the efficacy of irradiation for DHM processing. Further evaluation of the influence of gamma irradiation on the nutritional, bioactive, and microbial composition of DHM is required. Future studies should be carried out at a dosage of approximately 5 kGy to ensure optimal retention and allow for comparison with more recent findings. Overall, based on findings to date and potential consumer perspective issues, gamma irradiation is an unlikely candidate for milk bank implementation. However, the initial results highlight the value of combined hybrid treatments. Indeed, the combination of gamma irradiation and freeze-drying results in effective microbial inactivation and retention of key antimicrobial and nutritional properties of DHM. Advantageously, this hybrid approach would allow for the pasteurization of pre-packaged powdered

DHM with an extended shelf life. Additionally, the use of antimicrobial formulations may contribute to increased shelf stability of DHM. The applicability of freeze-drying or antimicrobials in combination with alternative processing technologies should be further explored.

### 4.4 Pulsed electric field treatment

Pulsed electric field (PEF) treatment is a non-thermal novel processing technique that results in bacterial inactivation whilst preserving the nutritional and sensory characteristics of food (195). During PEF, the food is placed between two electrodes, and short high-voltage (approximately 20–80 kV/cm) pulses are applied (195). Consequently, pore formation occurs in the microbial cell membrane, this is known as electroporation (196). The structural changes to the cell membrane as a result of electroporation cause an imbalance in cell homeostasis and induce cell death (197). To date, only two studies have explored PEF processing of DHM. Sanchaya et al. (198) demonstrated the retention of the physiochemical properties and the lipid content of DHM following PEF. In addition, PEF at 35 kV for 3,000 µs was sufficient to eliminate the naturally occurring microbes of DHM. In contrast, bacterial growth persisted following processing at 25–30 kV for 3,000–4,500 µs.

Separately, Zhang et al. performed nanosecond (ns)-PEF on DHM (199). The group used response surface methodology, a mathematical modelling system, to determine two optimum processing conditions for DHM. These were 15 kV voltage with 6,000 pulses at a frequency of 20 Hz or 50 Hz. Indeed, following nsPEF at 20 Hz, retention of lysozyme, sIgA, and lactoferrin of 76, 108, and 74 %, respectively, was observed. Moreover, nsPEF increased the levels of xanthine oxidase and a greater than 60% retention of the enzyme lactoperoxidase was observed.

Of the sparse data available, the processing of DHM with PEF has encouraging results. However, it is evident that substantial research is still required to fully establish its effect prior to its application in a milk bank setting. In particular, challenges in relation to the scalability, installation and optimisation of a PEF processing system must be overcome to establish PEF as a viable non-thermal processing option. Nonetheless, the accomplished lab scale studies to date, point to the potential processing parameters for future investigations.

# 5 Discussion

Human milk processing is an area of research that has undergone much investigation and progress in recent years. In particular, alternative pasteurization technologies have emerged with the potential for implementation by the milk bank sector. The emerging technologies can be categorized into two groups: thermal and non-thermal processing techniques.

Heat treatment of food is a long-established method for food preservation. Indeed, the concept of heating milk before infant feeding was first suggested in 1824, and by 1889, heat-treated milk dispensaries were conceived (200). Currently, HoP is the widely accepted milk processing technique established within milk banks. However, in recent years, the negative influence of HoP on valuable bioactive components of DHM has been substantiated. Consequently, the appraisal of substitute thermal processing techniques has been explored. Of these, HTST has been the most extensively studied. Despite the potential superior retention of some DHM components, the effect on milk lipases and lactoferrin is degradative. Other components, such as cell signalling molecules and the bacteriostatic capacity of milk, remain unclear. A potential drawback of HTST is its logistical applicability to a milk bank setting.

In contrast, thermal processing by microwave heating has greater accessibility and is less labour-intensive. However, data on this experimental technique remains scarce and an overall impact on DHM components cannot yet be determined. Similarly, although findings on TUS processing are positive, the research is still in its infancy and further studies are necessary to substantiate the results to date on novel technologies and to determine the optimal treatment parameters. Conversely, retort processing and pasteurization at high temperatures (>80 °C) incur significant losses in various nutritional and bioactive factors, suggesting that the required conditions are too harsh for DHM processing.

Non-thermal methods have rightly generated much interest due to the protective capacity of such techniques on the heat-labile compounds of DHM. Of the non-thermal technologies, HPP stands out as the most promising and consequently

most studied. Determining the optimal HPP parameters should therefore be a priority (201). However, neglected areas of research remain, mainly, the impact on micronutrient content of DHM, in particular mineral content and bioactive constituents including miRNA and milk hormones. Furthermore, in comparison with HoP, further study of microbial deactivation with pressurization is required. Although the potential of UV-C has been demonstrated, the limited existing data impedes a determination but does encourage further evaluation of this technique. Similarly, PEF and gamma IR are novel techniques with scarce findings requiring further evaluation. The applicability of these processing techniques in terms of feasibility, implementation, and consumer acceptance should be considered. Unlike newer technologies, HoP has been extensively examined; consequently, an unevenness in the volume and range of data exists. For a direct comparative analysis to be enabled, further research must be carried out on these alternative processing technologies that remain relatively unexplored. In particular, the capacity for bacterial spore inactivation should be a primary focus, as this is a major limitation of the currently favoured HoP technique.

A core issue across all studies using various modes of pasteurization is the variability in study designs. To date, studies have differed vastly in terms of pre-processing collection, handling, and storage of samples. Furthermore, the number of freeze–thaw cycles to which a sample is exposed should be clearly presented where possible. All of these factors may influence the milk constituents and their vulnerability to processing conditions. In addition, both the sample volume and number of donors per pool have differed significantly across studies. These factors can influence the total solids concentration and the required treatment time, potentially impacting both the effectiveness of the technique, the subsequent analysis and the comparability of findings. Similarly, following processing, variation in the analytical methods deployed is common. Collectively, these alternating approaches make it difficult to discern the true effect of processing when contrasting results are reported. Recently, a review by Kontopodi et al. presents the most commonly used analytical methods and suggests a template workflow for evaluating DHM processing (83). Adherence to an accepted workflow and

consideration of the aforementioned factors would increase uniformity and may reduce the variability in findings. Evidently, a standardized approach when assessing milk processing techniques would facilitate future comparative analysis.

Despite the wide range of technologies examined to date, some areas remain relatively unexplored, for instance, biopreservation. The potential use of antimicrobials in food preservation is gaining momentum in dairy and other foodstuffs (202–204). Future studies should endeavour to assess their potential in DHM, in particular, as part of hybrid processing with other technologies. Indeed, hybrid processing, as demonstrated in a small number of studies, may reduce the severity of processing conditions required. In addition, the production of DHM in powdered form may provide greater transport and storage options—in addition to prolonged shelf life. These factors are particularly important in under-resourced areas, where refrigeration storage may not be possible and milk supply is low. Another technique under investigation by both the dairy and infant formula industries is the use of microfiltration as a method of milk pasteurization. This technique is yet to be studied in DHM and should be evaluated in the future. Furthermore, although beyond the scope of this review, DHM may be enhanced by fortification to replace lost nutrients - or indeed, inoculated to allow for the re-introduction of mothers' own milk microbiota (205–210). The combination of optimal processing techniques with the potential for personalized nutrition may produce the highest value DHM.

Human milk is a highly valuable food that influences and shapes the infant's gut, physiological development, and immune system maturation. Breast milk promotes the survival and development of the infant while ensuring tailored nutrition with an ample supply of immune proteins. The impact of alternatively processed DHM on the clinical value for the newborn recipient should be further assessed, and any processing technique under consideration should first be assessed for its efficacy in microbial inactivation. Bacterial spores present a persistent challenge that future technologies should be equipped to overcome.

In conclusion, the heterogeneity in sample history, study design, and analytical methods used have contributed to divergence in the available information for

optimal processing of DHM and prevented a complete understanding of the emerging processing techniques. A uniform approach to future studies and comprehensive information regarding sample history will allow further insight and optimization of milk banking pasteurization. Most importantly, any future technology should mitigate the risk of bacterial contamination whilst promoting the optimal retention of bioactive components, ensuring DHM that confers the highest quality nutrition to the newborn recipient.

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## 7 Author Contributions

RCS: Writing – original draft, Writing – review & editing. RPR: Writing – review & editing. AK: Writing – review & editing. CS: Writing – review & editing.

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# Chapter 2

## Literature Review

### Carotenoids in Milk and the Potential for Dairy Based Functional Foods

Ruth Conboy-Stephenson, R. Paul Ross, Catherine Stanton

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## Abstract

Carotenoids are a family of over 1100 known natural pigments synthesized by plants, algae, fungi and bacteria. Dietary intake of carotenoids is necessary for mammals as they cannot be synthesized in the body. In cows, the nature of the diet consumed strongly influences the composition of milk produced and this includes carotenoid concentration and profile. Fresh forage is the richest source of carotenoids for cows. The main carotenoids identified in forages are lutein, β-carotene, zeaxanthin and epilutein. Manipulating cow feed via carotenoid supplementation increases the carotenoid content of bovine milk. In humans, carotenoids have antioxidant, anti-inflammatory and provitamin A activity. Lutein is a major carotenoid in human milk and the brain tissue of adults and infants. Lutein and zeaxanthin are linked to improved eye health and cognitive function. Traditionally for humans, fruit and vegetables have been the main source of carotenoid intake. Functional foods present an opportunity to incorporate these naturally occurring compounds into milk products for added health benefits, widening the range of dietary sources of carotenoids. We offer an overview of the literature to date on carotenoid-fortified dairy products and infant formula. This review will describe and summarize the key mechanisms by which the carotenoid profile of bovine milk can be manipulated. We present findings on the origin and role of carotenoids in bovine and human milk, outline factors that impact the carotenoid content of milk, evaluate carotenoid-fortified milk products and discuss the associated challenges, such as bioaccessibility and stability.

## 1 Introduction

In mammals, milk is a biological fluid that provides the neonate with the required nutrients for development and immunological protection during the initial critical period of life. Bovine milk is also a popular and nutritious food item available for human consumption. Bovine milk is regularly consumed as part of a balanced diet and is an important source of various macronutrients, including protein (~3.2%), lactose (~4.8%) and fat (~3.5%) [1]. Furthermore, bovine milk also provides micronutrients such as vitamins (A, D, B, C), minerals (calcium, magnesium,

potassium) and trace elements (zinc, iodine, copper) [2]. Milk is also a unique source of bioactive compounds, including carotenoids. Carotenoids can be found within the lipid fraction of milk [3] and contribute the characteristic yellow hue associated with dairy products [4].

Carotenoids are naturally occurring lipid-soluble plant pigments that are only obtained by mammals through their diet. Carotenoids have valuable antioxidant and anti-inflammatory properties [5]. Carotenoid consumption has been linked with various health-promoting effects including improved ocular health and cognitive function [6,7] and provitamin A activity [8]. In the human diet, carotenoid intake is mainly associated with fruit and vegetable consumption. In recent years, there has been increased interest in alternative dietary sources of carotenoids through the development of functional foods. While there is no globally accepted definition for functional foods for the purpose of this review, functional foods are whole, fortified, enriched or enhanced foods that confer positive health effects to the human body [9]. Furthermore, functional foods should take the form of ordinary foods and not that of a pill or capsule [10]. The existing carotenoid content of bovine milk, in combination with its valuable composition of macro- and micronutrients, highlights the potential for a fortified dairy product. Three servings of dairy a day are recommended by the Irish Department of Health as part of a balanced healthy diet [11]. The major barriers to carotenoid fortification include low stability, bioaccessibility and bioavailability and potential sensory changes to the final product [12]. The unique fat composition of dairy may increase the stability and bioavailability of carotenoids. Dairy fortification presents an opportunity for the food and farming industry to target milk for increased nutritional value. When developing a functional food product, it is important to consider consumer opinions, with current preferences for a naturally sourced and nutritionally valuable product [13]. This complements the naturally occurring nature of carotenoids and high nutritional value of milk. Carotenoids are also important bioactive constituents in human milk and have been implicated in infant health and development [14]. It is important after birth that the infant receives the correct nutritional supply to ensure proper development and immunological protection. However, to date, little

emphasis has been placed on carotenoid inclusion in infant formula preparations [15].

This review will discuss the origins of carotenoids in bovine milk and the potential to modify the carotenoid profile of milk for a dairy functional food. The carotenoid content of milk can be manipulated in three ways—firstly, on the farm via choice of feeding system and farm management; secondly, on the farm via supplementation of the dietary feed; and finally, by supplementation of the milk or milk product at a later stage in the manufacturing process. We will present the main findings on carotenoid fortification of milk with reference to the current challenges faced both at the farming level and during product development. We will also examine the potential to fortify infant formula to ensure adequate carotenoid supply for the infant when mothers' own milk may not be available.

## 2 Carotenoids

Carotenoids are a family of over 1100 naturally occurring pigments found in plants, algae, fungi and bacteria [16]. Carotenoids are divided into two classes based on their chemical composition—xanthophylls and carotenes. Carotenes are exclusively composed of hydrogen and carbons, whereas xanthophylls also contain oxygen. Both classes are lipophilic.

In plants, carotenoids have a functional role in photosynthetic processes and confer a yellow-red colour to tissue, acting as attractants in various fruit and flowers [17]. In mammals, carotenoids are associated with various health-promoting effects, mainly as antioxidant [5,18,19], anti-inflammatory [18,20] and immunomodulatory [21,22] compounds. Carotenoids with an unsubstituted β-ionone ring have provitamin A activity, mainly carotenes (β-carotene, α-carotene) but also the xanthophyll β-cryptoxanthin. Vitamin A contributes to vision and growth and is one of the major nutrient deficiencies worldwide, particularly in developing countries [23]. Xanthophylls, particularly lutein and zeaxanthin, have been associated with improved ocular and cognitive health in humans. Lutein and zeaxanthin preferentially accumulate in the macula of the retina with meso-zeaxanthin, i.e., lutein's metabolite, to compose the macular pigment [6]. The

macular pigment acts as an optical filter for blue light (400–500 nm), protecting the retina from photochemical damage [24]. Increased lutein and zeaxanthin intake are associated with reduced risk of age-related macular degeneration, the leading cause of blindness in the Western world [25]. In the adult brain, xanthophylls account for 66–77% of total carotenoids [26,27]. In older adults, these unique bioactive compounds have been linked to improved cognitive health [7,27,28]. Furthermore, reduced macular pigment, lutein and zeaxanthin serum levels were reported in patients with Alzheimer's disease [29].

In mammals, carotenoids cannot be synthesized de novo and are only obtained through diet. For this reason, and taking into account that carotenoids have many biological functions in humans, an adequate concentration in dietary sources is important. In total, 40–50 carotenoids have been identified in common foodstuffs, primarily fruit and vegetables [30]. The major carotenoids in food include lutein, β-carotene, α-carotene, zeaxanthin, β-cryptoxanthin and lycopene [30,31]. Furthermore, violaxanthin, neoxanthin, phytoene and phytofluene have also been detected in a variety of dietary sources [32]. Green vegetables are known for their particularly high lutein concentration, with concentrations of 44–395 and 48.10–119.38 µg/g for kale and spinach, respectively [33,34]. Carotene levels are high in yellow/red fruits and vegetables [30]. Beta-carotene concentrations in pumpkin and carrots are 17–422.6 and 34–182.5 µg/g, respectively [31,34]. Similarly, in carrots, α-carotene levels of 22–106.5 µg/g have been recorded. Lycopene is found in high levels in red fruit, like tomatoes (25–233 µg/g), in addition to phytoene and phytofluene [31,35,36]. Similar to fruit and vegetables, egg yolk has high lutein and zeaxanthin concentrations, with an average total xanthophyll content of 12 µg/g [33]. Ollilainen et al. reported even higher levels of lutein, 15.76 µg/g [37]. Bovine milk is a known source of carotenoids, particularly β-carotene. The concentration of carotenoids in dairy products from Finland was quantified [37]. The β-carotene contents in milk and full fat cream were 16.7 and 186.5 µg/100 g, respectively. In cheese, β-carotene content varied with type, ranging from 13.7 to 186.5 µg/100 g. Lutein was found in trace amounts in all products and the fat content affected β-carotene concentration. As carotenoid intake is only achieved through the diet,

increasing the supply of carotenoids via food fortification is an area of interest, particularly due to their potential health attributes.

## 3 Functional foods

When designing a carotenoid-fortified food, a variety of factors must be considered to ensure optimal carotenoid delivery and a marketable food product with enhanced value for the consumer (Figure 1). The low bioavailability, solubility and stability of carotenoids are key challenges facing the food industry when developing a fortified food product. Bioavailability refers to the "fraction of the nutrient or bioactive compound ingested that is available for use in physiologic functions or to be stored" [38]. The bioavailability of functional foods is generally determined by measuring the carotenoid levels in plasma. Bioavailability is distinguishable from bioaccessibility which is "the fraction of a compound that is released from its matrix in the gastrointestinal (GI) tract and thus becomes available for intestinal absorption" [38]. The bioaccessibility directly impacts the bioavailability and the subsequent bioactivity of carotenoid-fortified products in vivo. In order to design a functional food, an understanding of the challenges and contributors to low carotenoid bioavailability is vital. A detailed review on the absorption of carotenoids and factors limiting bioavailability including, food matrices, carotenoid–nutrient interaction and processing steps has recently been published [12]. This section will give an overview of carotenoid digestion, absorption and metabolism in vivo. It will also look at the importance of the food matrix in the context of dairy, focusing on fat content. The fat content in dairy could be utilized to overcome the challenge of low bioavailability associated with carotenoids in fruit and vegetables. Bovine milk and dairy are commonly consumed and a popular food group with potential for high carotenoid delivery.

### 3.1 Digestion Metabolism and Absorption of Carotenoids

For carotenoids to carry out their biological functions in vivo, these bioactive compounds must successfully reach their target tissue following ingestion. The first step is the release of carotenoids from the food matrix by mastication and enzymatic action in the mouth before undergoing emulsification into lipid droplets

in the stomach [39]. Carotenoids are then transferred into mixed micelles and transported to the apical surface of epithelial cells lining the GI tract, ready for intestinal absorption [12]. The portion of carotenoids incorporated into mixed micelles and ready for uptake by intestinal absorptive cells is known as 'bioaccessibility' [40]. The 'bioavailability' of carotenoids is the level of successfully absorbed carotenoids in the plasma ready for utilization by the body.

Previously, intestinal absorption of carotenoids was thought to occur via passive diffusion. However, in vitro studies suggest the role of various lipid transporters (Table 1) [41]. To date, three lipid transport proteins have been implicated in this process, Scavenger receptor class B type I (SR-BI), Cluster Determinant 36 (CD-36) and Niemann–Pick C1-Like 1 (NPC1L1) [42].

Following transfer to the cell interior, provitamin A carotenoids undergo transformation to retinol or retinyl esters which are packaged into chylomicrons, in addition to non-provitamin A carotenoids [12]. The mechanism of intracellular uptake of carotenoids by chylomicrons remains unclear [51]. Chylomicrons are secreted into lymph before release into plasma. In the plasma, chylomicrons are reduced by lipoprotein lipase to chylomicron remnants and undergo uptake by hepatocytes. In the liver, carotenoids are incorporated into very-low-density lipoproteins (VLDL) particles and secreted back into the plasma, where they are circulated as low-density lipoproteins (LDL) for peripheral distribution to their target tissue via LDL receptors [39].

The steps involved in bioaccessibility and the factors capable of influencing carotenoid bioavailability should be considered when designing a fortified food product to ensure the bioactive potential of carotenoids is fully realized. These factors are represented by the mnemonic SLAMENGHI (S: species of carotenoid; L: molecular linkage; A: amount consumed in a meal; M: matrix used for carotenoid incorporation; E: effectors of absorption and bioconversion; N: nutrient status of the host; G: genetic factors; H: host-related factors; I: interactions) [52]. In particular, this review will examine the choice and quantity of carotenoid species, and their interaction within a dairy based matrix in studies to date.

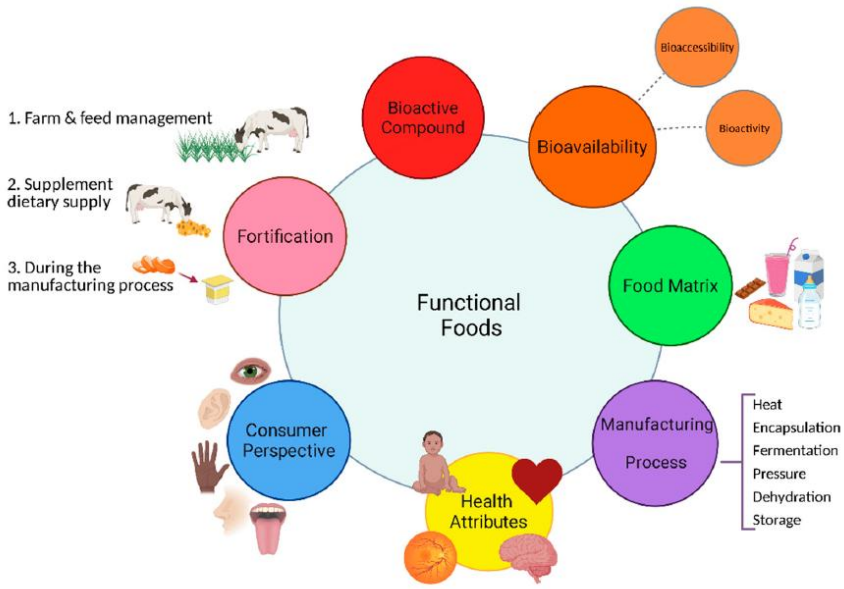



Figure 1: Examples of the key concepts to consider when designing a carotenoid functional food, in particular a dairy product with added value. Each factor can potentially influence another and all these interactions must be considered.

### 3.2 Dietary Factors Impacting Bioavailability

The dietary components of food can significantly affect the absorption and availability of carotenoids. Dietary lipids enhance carotenoid absorption from fruit and vegetables which are naturally low in fat [53,54]. Dairy products are a potential vehicle for increased carotenoid delivery due to the fat content of milk which is composed of 65% saturated fatty acid (SFA), 32% monounsaturated fatty acid (MUFA) and 3% polyunsaturated fatty acid (PUFA) [55].

Table 1: The lipid transporters implicated in the intestinal absorption of carotenoids.

| Carotenoid | Lipid Transporter | References |
|---|---|---|
| β-Carotene | SR-BI, NPC1L1 | [43–45] |
| α-Carotene | SR-BI, NPC1L1 | [43,44] |
| β-Cryptoxanthin | SR-BI, NPC1L1 | [43,44] |
| Lutein | SR-BI, NPC1L1, CD-36 | [44,46–48] |
| Zeaxanthin | SR-BI, NPC1L1 | [44,45] |
| Phytoene | SR-BI | [49] |
| Phytofluene | SR-BI | [49] |
| Lycopene | SR-BI, NPC1L1 *, CD-36 | [44,47,50] |

Asterisk (*) indicates that at least one study has not implicated this lipid transporter.

The co-ingestion of dietary fat facilitates the solubilization of carotenoids following release from the food matrix and enhances carotenoid micellarization [56]. Lipid intake prompts the secretion of bile salt and pancreatic enzymes, which contribute to lipid digestion and micelle formation [41]. Additionally, lipid digestion provides increased digestive products for the structuring of mixed micelles, including lysophospholipids, monoglycerides, and free fatty acids [50]. Overall, dietary lipid intake improves micelle formation and carotenoid uptake, ultimately increasing the bioaccessibility of the micronutrient. A study by Mashurabad et al. [56] examined the effect of vegetable oil intake on carotenoid (lycopene, α-carotene, β-carotene and lutein) micellarization in various fruit and vegetable food matrices (carrot, spinach, drumstick leaves and papaya). They reported that dietary fat intake of 1–2.5% is sufficient to enhance carotenoid micellarization and saturation occurs at 5% [56]. Additionally, dietary fat provides increased products of lipid digestion for chylomicron assembly [51], ultimately enhancing carotenoid transfer to the lymphatic system.

Lipid type with regard to fatty acid chain length and degree of unsaturation is an influencing factor in carotenoid absorption. The micellarization of carotenoids from some vegetables and fruits was 2–3 times higher in fat sources with higher levels of unsaturated fatty acids compared to SFAs [56]. Clark et al. reported higher carotenoid absorption in olive oil (MUFAs) than corn oil (PUFAs) [57]. Furthermore, Borel et al. observed decreased β-carotene and astaxanthin uptake by chylomicrons in the presence of medium-chain triglycerides in comparison with long-chain triglycerides, suggesting decreased chylomicron secretion in the presence of medium-chain triglycerides [58]. In a study with spinach, bioaccessibility of lutein was highest when combined with fat sources rich in medium-chain SFAs and highest for β-carotene in the presence of long-chain MUFAs [59]. These findings highlight the influence of fat type and a specific interaction of carotenoid class on bioaccessibility. Furthermore, the amount of dietary fat intake, the food matrix and the polarity of carotenoids impact carotenoid micellarization and subsequent absorption [56].

Evidently, dietary fat intake, fat type, food matrix and choice of carotenoid should be considered when designing a fortified product for optimized bioactivity in vivo. Specifically for dairy product fortification, the interaction of the class and polarity of carotenoids with the type and amount of fat should be assessed. The unique lipid content of a dairy food-based matrix may alleviate the limiting factors associated with low bioavailability of carotenoids in foodstuffs.

# 4 Bovine Milk

## 4.1 Carotenoids in Bovine Milk

Carotenoids are stored in the lipid fraction of bovine milk. Only a small number of carotenoids have been identified in bovine milk, with β-carotene and lutein representing the two major carotenoids (Figure 2) [60]. Of these, β-carotene is the dominant carotenoid, comprising 75–90% of the total carotenoid concentration [60–62]. Zeaxanthin was identified at low levels in milk [63]. However, it was not identified in a separate study [60].

## 4.2 The Health-Promoting and Sensorial Effects of Carotenoids in Bovine Milk

Beta-carotene, a major milk carotenoid, is a vitamin A precursor [8]. The high concentration of β-carotene enhances milk's natural properties and provides a valuable source of vitamin A to the consumer. In bovine health, carotenoid consumption has been linked with reduced incidence of post-calving reproductive disorders [64] and improved udder health and fertility [65,66].

Carotenoids also influence the sensorial properties of bovine milk in two ways. Firstly, they confer the yellow hue associated with dairy products. The yellow colour is more strongly associated with grass-based feeding systems [4]. The yellow colour of dairy based products is important in terms of consumer preference, traditionally indicating naturally farmed milk. The potential for the yellow colour to act as a biomarker for feed management and as an indicator for carotenoid (mainly β-carotene) concentration has been suggested [67], although other findings suggest that the colour index of bovine plasma, not milk, may be a better indicator [60]. Secondly, the antioxidant capacities associated with carotenoids have protective

effects against oxidation of milk [68]. Carotenoids act as scavengers of singlet oxygen and their ability to act as light filters has previously been demonstrated [69,70]. These findings suggest the potential for β-carotene to protect milk from light-activated oxidation as a result of riboflavin sensibilization. However, Havemose et al. found that increased carotenoid levels did not prevent the sensibilization of riboflavin or reduce lipid oxidation. Instead, higher carotenoid content was associated with lower protein oxidation, which results in the initial off-flavours attributed to light-induced oxidation [71]. Evidently, carotenoids in bovine milk influence the nutritional quality and stability of milk due to their provitamin A action, anti-oxidative capabilities and potentially immune-enhancing effects.

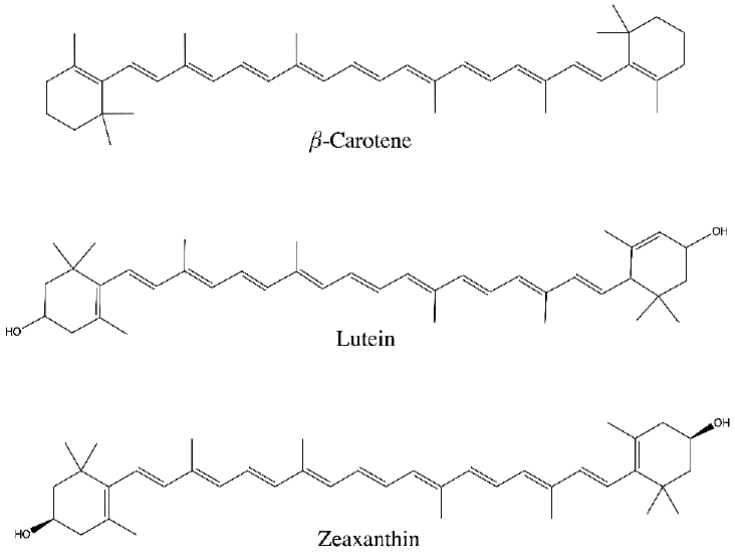



Figure 2: The main carotenoids identified in bovine milk

### 4.3 Factors Influencing the Carotenoid Content of Bovine Milk

As outlined in Section 4.2, milk is a valuable source of carotenoids that contribute to its nutritional and sensorial properties. Bovine milk relies on external dietary sources for carotenoids and the choice of forage influences dietary supply. This section will give an overview of the influencing factors and potential mechanisms which could alter the carotenoid levels of milk and subsequent dairy products at the farm level. A variety of physiological factors are known to impact milk composition including genetics, the health status of the cow, the stage of lactation and parity [1]. These factors have also been associated with influencing carotenoid

uptake and concentration in milk [72]. In particular, an effect of breed has been reported [63,73,74].

### *4.3.1 Dietary Sources for Bovine Carotenoid Consumption*

Bovine carotenoid consumption is entirely dependent on dietary supply, which is mainly forage-based. To date, less than 10 carotenoids associated with forage feed have been identified [72]. These include the β-carotene isomers, 13-*cis* and all-*trans*, in addition to 5 of the xanthophyll class, violaxanthin, antheraxanthin, lutein, zeaxanthin and epilutein, each representing 4, 11, 14, 3, 49, 10 and 9% of total carotenoid content, respectively [61]. Carotenoid levels vary with herbage types. Investigations by Larsen et al. reported higher β-carotene and lutein levels in a red clover mixture (235 and 655 mg/kg of dry matter (DM), respectively) in comparison with a lucerne mixture (148 and 433 mg/kg of DM, respectively) [75]. Although Cardinault et al. reported lower levels of lutein and β-carotene in red clover (136 and 29 of μg/g of DM, respectively) [76], finding by Livingstone et al. support a higher concentration of xanthophylls and carotenes in red clover in comparison with lucerne [77]. Elgersma and colleagues also reported disparity in carotenoid content between forage types ranging from 129 to 206 mg/kg of DM for lutein and 26 to 61 mg/kg of DM for β-carotene [78]. Carotenoid content is lower in preserved forage and relative to the portion of grass-based forage [79,80]. The ensiling process reduced β-carotene in Virginia Fanpetals by 56% [81]. Grass-based silage has higher total carotenoid concentrations than hay, 441 and 165 μg/g of DM, respectively [60]. In the same study, there was a 50% reduction in lutein from hay vs. grass silage [60]. Other factors that influence the carotenoid content of forage are fertilization [82-84], the ratio of leaf to stem [77], diurnal variations [17], cutting height [81], harvest date and maturity [883,85,86].

Clearly, forage choice and feed management can strongly influence the level of carotenoid ingestion and therefore, carotenoid output in bovine milk. An extensive review on carotenoids in forage and their transfer to bovine tissue and milk has previously been carried out [72].

### *4.3.2 Feed Management*

Production conditions influence the nutritional profile of milk. In particular, the effect of different feeding systems on carotenoid concentration has been established. The two main feeding systems utilized by the farming industry are pasture-based feeding (grazing fresh grass and forage) or total mixed ration (TMR) feeding. For a TMR-based feeding system, cows are usually housed indoors and supplied with grass/maize silage supplemented with high levels of concentrate feeds [87]. The feeding system employed by farmers is determined by a number of factors including climate, environmental and economic considerations. A pasture-based feeding system is widely used by the dairy industry in temperate regions like Ireland and New Zealand [88]. Pasture-based feeding has positive effects on the nutritional composition of bovine milk [3]. Additionally, it is a cheaper source of feed than more expensive concentrate feeds. Dillon et al. reported that milk production costs are reduced by 2.5 cent per liter for every 10% increase in the proportion of grazed grass [89]. It is important to note that in the majority of studies to date, the effect of season is often interchangeable with dietary effects. This is a result of indoor housing and preserved feeding during the winter period versus cows grazing outdoors on pasture in the spring and summer months.

As outlined in Section 4.3.1, fresh pasture is the optimal source of carotenoids for ruminants. Feeding fresh herbage increases the carotenoid content of bovine milk, in comparison with conserved forage and concentrate feeds. A study of bulk tank raw milk in the Netherlands demonstrated that winter feeding resulted in a 20% reduction in β-carotene [62]. Furthermore, the total carotenoid levels of raw milk were 16.8 μg/100 g following the winter period as opposed to higher levels of 24.5 μg/100 g recorded following the summer months. This decrease was attributed to cows being fed a mixture of grass and corn ensilage and pellets. Similarly, French bulk tank milk has higher β-carotene and lutein during the summer months with increases of 2.0 and 0.23 μg/g of fat, respectively, in addition to a stronger yellow colour [4]. The highest levels of β-carotene were observed in September (5.3 μg/g fat) and for lutein in July (0.59 μg/g fat). Furthermore, a UK-based study recorded increased lutein and zeaxanthin concentrations in milk from grazing systems with

greater proportions of pasture-based feeding over a 2 year period [63]. Preserved forage feeding decreases the carotenoid content of bovine milk. However, during the winter period, indoor feeding of preserved forage and concentrates is necessary due to poor weather. The proportion of grass-based preserved forage and method of forage conservation impact the carotenoid profile of milk. Calderon et al. demonstrated that a switch in diet from hay to grass ensilage and alfalfa protein feed resulted in increased β-carotene from 2.74 to 4.21 μg/g of fat [60]. The lutein levels remained constant despite dietary changes averaging 0.73 μg/g of fat. Similarly, a shift from grass ensilage to hay resulted in a rapid decrease in β-carotene levels from 0.17 to 0.07 μg/mL [74]. Furthermore, adjusting the ratio of maize to lucerne silage from 5:1 to 2:1 resulted in a 23–27% increase in carotenoid concentration of milk fat [90].

It is apparent that farm management, particularly feeding systems, is an accessible and effective way to modify the quality of milk and dairy products. Thus, the farming and dairy industry can potentially target milk production for the development of milk and dairy products with different nutritional profiles, including carotenoids.

#### *4.3.3 Grassland Management*

Forage maturity influences the carotenoid content of milk. Calderon et al. noted a significant decrease in the carotenoid concentration of milk (5.37 to 3.87 μg/g of fat) during the growth period, which was followed by an increase in early regrowth to 4.91 μg/g [61]. These findings indicate that herbage maturity does affect the carotenoid concentration of milk. This is likely a result of the decreased carotenoid content of maturing forage, as noted in Section 4.1. Additionally, the same group examined the effect of different grazing patterns, rotational vs. strip grazing. Grazing management did not significantly impact the β-carotene concentration in milk, whilst minor reductions in lutein levels were observed following rotational grazing [61]. Although it is not a major factor, forage maturity can be considered when supplying feed to bovines for optimal carotenoid output in milk.

### *4.3.4 Microbial Rumen Degradation*

The effects of rumen digestion on the bioavailability of carotenoids remain unclear. During rumen digestion, carotenoids are released from the plant matrix and incorporated into the rumen liquid phase. This is followed by the absorption and transportation of carotenoids to peripheral tissues for metabolism, which occurs in a tissue-specific manner (adipose storage, provitamin A and antioxidant activity) [72]. The participation of rumen microbes in carotenoid degradation remains uncertain due to varying results from in vivo and in vitro studies, ranging from high levels of degradation to low [72]. In bovines, rumen degradation of carotenoids has been shown to occur at low levels and losses were not associated with attack by rumen micro-organisms [91,92]. Furthermore, studies have suggested a positive interaction between carotenoids and rumen microbes. A study in sheep demonstrated that carotenoid levels (lutein and β-carotene) increased following ruminant digestion [76]. Carotenoid intake was 174, 16.5 and 20.9 mg/day for lutein, 13-*cis*-β-carotene and *trans*-β-carotene, respectively. By the stage of duodenum analysis, levels had increased to 204, 53.4 and 63.9 mg/day, respectively. The authors proposed that increased carotenoid levels occurred as a result of rumen microbes facilitating the release and synthesis of carotenoids in the rumen. As noted in their study, previous findings that micro-organisms have the ability to synthesize carotenes and the presence of β-carotene in fecal samples from calves deprived of carotene, following inoculation with rumen microbes, supports their theory [76]. Additionally, Hino et al. demonstrated the ability of β- carotene to increase bacterial growth following suppression by safflower oil in the ruminal content of goats [93]. Similarly, β-carotene enhanced microbial growth in goat rumen fluid [94]. The potential for a mutualistic relationship between the microbial population in the rumen and carotenoid ingestion exists. Evidently, this relationship warrants further exploration to fully understand the digestion of carotenoids and the potential to increase carotenoid concentration in vivo. Future studies should also examine whether carotenoids influence the transformation of other digestive products. Overall, these findings suggest a positive role of rumen microbes in carotenoid digestion.

#### *4.3.5 Saturation Phenomenon in the Carotenoids Transfer from Plasma to Milk*

The final concentration of carotenoids in bovine milk relies on the transfer from plasma to milk via the mammary gland. This occurs at relatively low levels. Noziere et al. demonstrated low daily extraction rates of β-carotene of 0.008% [74]. Plasma concentration of carotenoids varies but is higher than that of milk. Plasma levels of β-carotene were 5.10 µg/mL and 1.71 µg /mL following grass silage and hay feeding, respectively. In comparison, the levels in milk were 30 and 24 times lower, respectively [74]. Low levels of β-carotene transfer occur even in the presence of a carotenoid-rich diet. Investigations by Calderon et al. noted that saturation is a contributing factor influencing β-carotene uptake under high carotenoid dietary conditions. In this study, high levels of β-carotene feeding resulted in a plateau in bovine milk. The β-carotene concentration of milk fat following 28 days of carotenoid-rich feed did not differ between groups despite varying rations. This trend was not observed in bovine plasma concentrations. The group suggest that β-carotene levels of 5 µg/mL in plasma result in saturation and further increments in β-carotene supply will not increase β-carotene yield in milk [60]. Findings by Agabriel et al. [4] and Larsen et al. [75] support this theory. Agabriel and colleagues, 2007 [4] noted that a shift from a period with low carotenoid intake (February-March) to higher carotenoid intake did result in a significant increase in the milk β-carotene concentrations. However, within the period of higher carotenoid intake (May-September), an increase in the proportion of β-carotene content did not result in any significant changes [4]. Findings suggest that saturation is a limiting step in the uptake of β-carotene in the mammary gland and subsequent secretion into the milk. The exact mechanisms of carotenoid uptake in the mammary gland needs to be explored further. This phenomenon should be considered when manipulating carotenoid concentration, particularly β-carotene, at the farm level.

## 5 Dairy as a Functional Food

Carotenoids are desirable components in bovine milk and subsequent dairy products. The potential for a dairy functional food as a vector for increased carotenoid consumption is a growing area of interest. The ability to manipulate the

carotenoid content and profile of dairy via farm management or supplementation both at the farming and processing stages has been established. Of particular interest are the carotenes with provitamin A activity and lutein and zeaxanthin due to their associated health benefits. When developing a carotenoid-fortified food current safety recommendations should be considered. The European Food Safety Authority (EFSA) has advised an acceptable daily intake (ADI) of 1 and 0.5 mg/kg of body weight for lutein and lycopene, respectively [95,96]. Although there are no current ADIs for β-carotene and zeaxanthin, and EFSA report no safety concerns when daily supplemental intake levels remain under 15 and 0.75 mg/kg of body weight, respectively [97,98]. Furthermore, various carotenoid containing products have been accepted as GRAS (generally recognized as safe) by the US Food and Drug Administration (FDA), these findings have previously been reviewed [99]. The advised levels are higher than the average daily consumption of carotenoid (5.42–15.44 mg/day) and indicate the potential for carotenoid fortification of food products with safe and beneficial outcomes [22].

The modification of the carotenoid profile of milk and dairy products via farm feeding practices has been reported. Butter produced from pasture-based milk had higher levels of *trans*-β-carotene than butter derived from TMR-based milk, 5.16 and 2.27 mg/kg, respectively [100]. Additionally, the pasture-based butter had enhanced sensorial properties (flavour and appearance). This highlights the potential to naturally fortify milk through farm management without negatively impacting the sensorial attributes of the dairy product. This is supported by the previously discussed findings that dietary supplementation and farm management practices can influence the carotenoid content of milk [4,62]. Lutein supplementation of cow feed increased the lutein content of milk [101,102]. Xu et al. reported that the incorporation of lutein into TMR at 0–4 g/day per head resulted in the lutein concentration of milk increasing from 0.59 to 1.50 μg/100 mL. Lutein supplementation also improved milk yield and quality [101]. Furthermore, supplementation of bovine feed with lutein and lutein co-supplemented with vitamin E increased the lutein levels of milk by 3.8 μg/L and 5.66 μg/L, respectively [102]. In this case, lutein supplementation had positive sensory effects on texture,

flavour and aroma with slightly lower scores for appearance. Dietary supplementation on farm is a viable option and warrants further exploration to elevate milk carotenoid content and to determine sensory attributes, optimal concentration and stability.

Dairy products can be considered for functional food development. However, in order to design a product that optimizes carotenoid bioactivity, the barriers in terms of bioaccessibility, bioavailability, safety, stability and sensorial properties need to be overcome. These challenges in relation to the fortification of dairy products to date will be discussed in this section. Although it is not within the scope of this review, plant-based milk alternatives originating from soy, oat, coconut, hemp, rice and nuts have grown in popularity in recent years [103]. Despite attempts to mimic the nutritional and sensorial properties of bovine milk, plant-based alternatives often have poorer nutritional value and are highly variable [104,105]. For instance, the average protein content in 17 plant-based alternatives was only 48% that of bovine milk [104]. Additionally, in an Australian-based cross-sectional survey, only 1/3 of plant-based alternatives had similar calcium concentrations to bovine milk [106]. The carotenoid content and profile of plant-based milk has largely been overlooked in studies to date despite its nutritional benefits and unique sensorial contribution to bovine milk. Future analysis and development of plant-based milk should examine the inclusion of carotenoids and their behaviour in a plant-based matrix.

### 5.1 Bioaccessibility

Establishing the potential bioaccessibility of a bioactive compound in a food product should be of priority when designing a fortified food. The nature of the lipid in dairy makes it an ideal candidate for increasing carotenoid transfer to micelles and subsequent absorption in vivo. To date, information on carotenoid bioaccessibility in dairy based produce is limited but promising.

Xavier and colleagues investigated the in vitro bioaccessibility of lutein esters in milk and yogurt [107]. Lutein esters were added to whole, semi-skimmed and skimmed milk, in addition to their yogurt counterparts. The study reported similar

findings for both milk and yogurt products. The bioaccessibility of lutein was 38.3–47.5% in whole and semi-skimmed dairy products, with significantly lower accessibility in skimmed products (17.8%). Furthermore, low levels of carotenoid micellarization were observed in skimmed milk (19.7%) in comparison with whole and semi-skimmed, 46.5% and 45.8%, respectively. The authors proposed a minimal fat requirement of 1.55% for carotenoid micellarization and absorption. Similarly, when persimmon fruit was added to yogurt as a source of carotenoids, bioaccessibility improved by 21% in the presence of a higher fat content of 3.6% vs. 0.25%. In the presence of whole milk, provitamin A carotenoids had a bioaccessibility of 38% [108]. This highlights the potential to combine carotenoid-rich fruit with dairy products for a fortified food product. Furthermore, improved bioaccessibility of total carotenoids and β-carotene, β-cryptoxanthin, zeaxanthin and lutein were reported in a whole milk-fruit juice combination, with reductions of 72–17% in the skimmed milk alternatives [109]. In a similar study, a fruit juice-whole milk combination had higher bioaccessibility, 23.5%, than the soy- or water-based substitutes, 15.9 and 12.9%, respectively [110]. Again, this is likely due to the higher fat content in a milk-based food matrix (3.6%), in comparison with soy (1.6%) and water (0%).

Overall, studies to date demonstrate good bioaccessibility of carotenoids in a dairy based food matrix, ranging from 23.5 to 47.5%. Findings suggest that the lipid portion of dairy is a key factor contributing to improved carotenoid bioaccessibility. Based on the findings above, a fat content of approximately 1.55% is sufficient to ensure optimal carotenoid bioaccessibility. When targeting consumers conscious of the natural origins of food products, the fortification of dairy with the naturally occurring carotenoids in fruit and vegetables is ideal. Similarly, increasing the carotenoid content through farming practices may also be viewed favorably. Future studies should examine the bioaccessibility of fortified dairy products following modification at the farm level.

### 5.2 Stability

The stability of carotenoids in dairy is a concern when designing and marketing a fortified food product. It is important that the levels of carotenoid remain constant

during storage prior to consumption and that the sensorial properties are unaffected. In a study with strawberry yogurt, it was indicated that up to 3 mg of lutein can be incorporated in to a 170 g portion of yogurt without any significant change in the sensory characteristics of the fortified product [111]. Furthermore, the physicochemical properties including pH, viscosity, flavour and texture remained unchanged. A slight decrease in lutein was observed over a 5 week storage period at 5 °C [111,112]. In contrast, Domingo and colleagues demonstrated that lutein added to yogurt remained stable during storage for 35 days at refrigeration temperatures [113]. Furthermore, lutein also protected the yogurt product from photo-oxidation during storage and scored well during sensory evaluation. Findings indicate that lutein is stable as part of a fortified yogurt product. The incorporation of lutein into cheese has also been evaluated. Lutein was added to Cheddar cheese at 1, 3 and 6 mg per serving size (28 g). No lutein degradation was observed over 24 weeks of ripening at 4.5 °C, confirming the stability of lutein during the aging process [114]. Although no differences in body/texture and sensory/colour were noted during sensory evaluation, lutein fortification did result in a distinct bitter flavour and changes in appearance [114]. Similarly, the successful fortification of Prato cheese with lutein has been demonstrated with varying sensorial effects [112,115]. Lutein remained stable during the cheese-making process, with high levels of lutein recovery (95.2%) [112]. Kubo et al. reported an intense yellow-orange colour and bitter flavour following fortification. In contrast, Sobral and colleagues noted that lutein supplementation of Prato cheese without annatto remained stable during ripening for 60 days at 10 °C with acceptable sensory scores [115].

These findings suggest that the fortification of dairy with lutein is viable. However, sensorial effects may present a challenge for marketing. Further studies should be performed in a variety of food matrices with different types of carotenoids and at varying concentrations to see if negative sensory effects can be prevented. Consideration of the type of dairy product is important, particularly with regard to colour and taste. Sensory changes appear to be less noticeable in fortified yogurt than cheese. The stability of lutein is promising and suggests dairy is an ideal vehicle

for the retention and delivery of carotenoids to the consumer. However, sensory-related challenges are apparent and must be overcome to ensure consumer interest.

### 5.3 Potential for Encapsulation in Dairy

The encapsulation of carotenoids in food as a method to optimize carotenoid delivery has recently been explored. Encapsulation presents the opportunity for a hydrophobic compound to be dispersed in a hydrophilic environment. Encapsulated delivery systems include biopolymeric and lipid-based nanocarriers (emulsions, nanoparticles, liposomes and hydrogels) in addition to microencapsulation techniques, spray and freeze drying [116,117]. This increases the type of food matrices available for functional food development, enabling foods with higher water content to be utilized [118]. Furthermore, encapsulation could enhance the stability, bioavailability and release of bioactive compounds [119]. Campo et al. examined the influence of carotenoid nanoencapsulation (nanoparticles and nanoemulsions) on the stability and sensory properties of yogurt [118]. The group demonstrated that zeaxanthin nanoparticles remained stable during storage. Zeaxanthin nanoparticles had greater retention, ~22%, than zeaxanthin nanoemulsions, ~16%. Furthermore, although bioavailability was higher in nanoemulsions, the authors suggest that the nanoparticles confer a greater advantage, attributing the low bioavailability of nanoparticles to a controlled release of zeaxanthin [118]. As noted by McClements et al. [119], a controlled or targeted release is a desirable characteristic of an encapsulated delivery system. Neither approach negatively impacted the sensory or physicochemical properties of the yogurt. Similarly, yogurt fortified with porcine gelatin encapsulated carotenoid extract from melons demonstrated β-carotene stability during storage [120]. Furthermore, the encapsulation of β-carotene in gelatin also promoted carotenoid solubility and contributed a positively perceived yellow colour to the yogurt. The use of a water dispersible β-carotene and a spray-dried β-carotene powder in yogurt and pudding demonstrated the importance of the food matrix [121]. Micellarization was significantly higher for both forms in pudding (13.1 and 17%, respectively), than in yogurt (0.8 and 5.5%, respectively). However,

encapsulation significantly reduced the incorporation into micelles regardless of the food matrix [121].

Encapsulated delivery of carotenoids could provide increased solubility and carotenoid stability with reduced adverse effects on the food sensory properties. However, the level of bioavailability remains low. This can in part be attributed to the choice of structuring material. Donhowe et al. noted that alginate and chitosan significantly reduced carotenoid micellarization in comparison with spray-dried β-carotene [121]. Similarly, Medeiros et al. reported superior solubility and stability following nanoencapsulation with porcine gelatin than whey protein isolate or whey protein concentrate [120]. The choice of structuring material and method of delivery should be considered when designing alternative carotenoid delivery systems with high bioavailability. Additionally, the interaction with the chosen food matrix must be evaluated. Consideration should be given to the loading capacity, safety and the commercial suitability of a proposed delivery system. Further exploration of the bioavailability of carotenoids in a dairy based food matrix following fortification with encapsulated carotenoids versus carotenoids in their free form should be carried out. Detailed reviews of alternative delivery systems of bioactive compounds and suggested systematic approaches to designing such delivery systems are available [119,122].

### 5.4 Agri-Food Waste – A Future Source for Dairy Fortification?

The by-products generated during the processing of fruit and vegetables are often discarded as waste despite being a rich source of bioactive compounds, including carotenoids [123]. The processing of fruit and vegetables in the United States and China results in approximately 15.0 and 32.0 million tons, respectively, of waste annually [124]. In recent years, the use of fruit and vegetable by-products, including the peel, pulp, pomace and seeds, has been identified as a novel source of carotenoids [125]. For instance, tomato peel contains lycopene, phytoene, phytofluene, β-carotene and lutein [126]. Furthermore, a concentration of 611.10 mg/100 g of lycopene has been reported from tomato pomace [127]. The major carotenoids reported in citrus fruit peel are α-carotene, β-carotene, lutein, zeaxanthin and β-cryptoxanthin at varying concentrations (11–204 mg/100 g) [128].

When considering food waste as a source of bioactive compounds, the choice of extraction technique is important. Oftentimes, particularly with lipophilic compounds such as carotenoids, toxic solvents are used as part of the extraction process [128]. Current studies are looking to shift to non-toxic solvents and even non-solvent-based techniques. Putnik et al. and Pattnaik et al. have recently reviewed the challenges facing extraction design and the current alternative methods under consideration [128,129].

There are limited studies to date on the fortification of dairy based foods with carotenoids from food waste by-products. However, the fortification of traditional Tunisian butter with lycopene from tomatoes waste (peel and seeds) has been demonstrated. The study found that fortification of butter with 400 mg of tomato waste by-product per kg reduced peroxide values during 60 days of storage at 4 °C. The fortified butter had improved shelf life and reduced lipid oxidation. Although, in the same study fortification at 800 mg/kg resulted in pro-oxidant activity [130]. A separate study by Rizk et al. noted that ice cream fortified with carotenoid from tomato peel waste improved antioxidant activity [126]. Furthermore, the ice cream fortified with 3% carotenoid extract had the highest scores during sensory evaluation (flavour, body and texture, melting and colour) in comparison with both the control and other fortification concentration (1, 2, 4 and 5%). It is important to note that fortification at 4% and above resulted in a decline across all sensory scores [126]. As discussed in Section 5.3 the labile nature of carotenoids and the risk of degradation during processing has led to the encapsulation of carotenoids for improved stability and delivery. Šeregelj et al. has recently reported the encapsulation of carotenoids from carrot waste in alginate beads as part of a yogurt product at concentrations of 2.5 and 5 g per 100 g [131]. The yogurt was stored for 28 days at 4 °C and showed improved antioxidant activity whilst preserving the microbial and physiochemical properties of the yogurt.

The compositional value of fruit and vegetable waste by-products poses an opportunity for the farming, food and dairy industries to recycle waste products for functional food development with added nutritional value from a more sustainable source. Although limited, the findings to date suggest the potential for dairy

fortification with carotenoids from food waste products due to their antioxidant properties. However, further research is warranted to establish the sensorial effects. The combination of agri-food waste with encapsulation techniques is a promising area for future consideration. As far as we are aware, no study has examined the bioaccessibility and bioavailability of such foods, this should be explored in future work.

### 5.5 The Effect of Processing on the Carotenoid Content and Bioaccessibility of Dairy Foods

Dairy products undergo various manufacturing and processing steps depending on the end product. The manufacturing process can alter the microstructure of food, influencing the retention of bioactive compounds (including carotenoids) and their bioaccessibility. The effect of processing on various dairy based food matrices is summarized in Table 2.

Hulshof et al. assessed the retention of β-carotene in Gouda and Edammer cheeses that were not artificially fortified. In Gouda, the β-carotene retention was 38% [62]. The β-carotene concentration of Gouda was significantly reduced during the first 12 days of the cheese-making process but was unaffected by ripening. In contrast, Edammer cheese had greater retention during the initial cheese making at 70%, than the ripening stage (40%) [62]. As previously mentioned (Section 5.2), the supplementation of Prato cheese with lutein survived the cheese-making process with high levels of retention (>90%) [112,115]. In Cheddar cheese, the lutein was added at the hooping stage to avoid potential degradation during processing but proved robust to the maturation stage [114]. Carotenoid retention during cheese making varies from 38 to 92.5%. Influencing factors include cheese and carotenoid type, supplementation or natural modification and the point of fortification. In semi-liquid dairy products, the naturally-derived carotenoid content had high levels of retention in yogurt at 92%, and similar high levels were observed for custard, buttermilk and cream [62]. Furthermore, 100% retention was observed in pasteurized full-fat milk. The fortification of yogurt with freeze-dried papaya and melon extract as carotenoid source was unaffected by fermentation but incurred a 20–27% reduction following pasteurization [132]. The retention of carotenoids in

dairy products appears to be greater in semi-liquid dairy products and soft cheese as opposed to hard cheese. This is likely due to a combination of food matrix and processing conditions.

In bovine milk fortified with lutein via dietary supply, the effect of ultra-high-temperature (UHT) and high-temperature short-time (HTST) processing on lutein content was investigated. Findings by Wang et al. showed little effect of HTST on the lutein content of milk, whereas UHT resulted in reductions of 8%. Reductions were somewhat mitigated when lutein was co-supplemented with vitamin E. However, losses of 5% were still observed [102]. Thermal-based treatments are widely used for dairy processing as they are effective against microorganisms. These treatments alter the nutritional and sensorial properties of food products, therefore non-thermal alternatives are being considered. High-pressure processing (HPP) and high-intensity pulsed electric fields (HIPEF) of a functional beverage showed promising results, with greater carotenoid bioaccessibility than thermal treatment [110]. Thermal treatment resulted in reductions in bioaccessibility of up to 63%, regardless of the food matrix (milk, soy or water). In contrast, HIPEF of a milk-based functional beverage increased the carotenoid bioaccessibility by 15%. However, in a water-based matrix, all three treatments resulted in reductions, highlighting the influence of the food matrix.

During development, functional foods will undergo various manufacturing steps and processing techniques. The impact of processing on the carotenoid content of food varies. When designing a fortified food, the stability of carotenoids within the food matrix and the subsequent bioavailability should be examined. The point of fortification and approach (natural modification, supplementation at farm level or during processing) and their interaction with processing should be investigated.

Table 2: The effect of processing on the carotenoid content of dairy products

| Food Matrix | Carotenoid | Processing Technique | Carotenoid Retention (%) | Reference |
|---|---|---|---|---|
| Gouda cheese | β-Carotene | Cheese making (initial 12 days) | ↓ 62% | [62] |
| | | Ripening (26 weeks) | ↓ 9% | |
| Edammer cheese | β-Carotene | Cheese making (initial 12 days) | ↓ 30% | [62] |
| | | Ripening (20 weeks) | ↓ 60% | |
| Prato cheese | Lutein | Prato cheese making (after milk pasteurization and at milk coagulation) | ↓ <10% | [112,115] |
| Cheddar cheese | Lutein | Ripening (24 weeks) | x | [114] |
| Yogurt | β-Carotene, β-Cryptoxanthin, Lycopene | Pasteurization | ↓ 20–27% | [132] |
| | | Fermentation | ↓ <3% | |
| Milk | Lutein | HTST | ↓ <3% | [102] |
| | | UHT | ↓ 8% | |
| Milk | Lutein + Vitamin E | HTST | ↓ <3% | [102] |
| | | UHT | ↓ 3% | |
| Milk | β-Carotene | Pasteurization | x | [62] |

(↓) indicates reduction. (x) indicates no change, 100% retention. HTST = High-temperature short-time. UHT = Ultra-high-temperature.

# 6 Carotenoids in Human Milk and Infant Formula

## 6.1 Carotenoid Content of Human Milk

In contrast to bovine milk, a greater variety of carotenoids that are more evenly distributed exist in human milk. In human milk, the major carotenoids are lutein, zeaxanthin, β-carotene, lycopene, α-carotene and β-cryptoxanthin [133–135]. Carotenoids are stored in the fat fraction. A number of influencing factors including inter- and intra-subject variation, milk type and diurnal variations have been reported [134]. Colostrum has 5 times the concentration of carotenoids as mature milk [136]. Hindmilk carotenoid content is 25% richer than mid- or foremilk [133]. Although not significant, a trend for higher levels in the morning than evening has been observed [133,134]. A multi-geographical study demonstrated that carotenoid profile is unique to country [137]. The variations observed between subjects, country and milk type are strongly associated with fat content and dietary effects. Factors that have no effect include BMI, number of years post-menarche and stage of lactation [135]. Similar to adults, carotenoid consumption has been linked with a number of health benefits in infants. In total, 50% of the carotenoid content in milk has provitamin A activity (α-carotene, β-carotene and β-cryptoxanthin) [137]. Lutein and zeaxanthin have been implicated in ocular development [138]. A correlation between infant serum zeaxanthin and infant macular pigment optical density has

been demonstrated [139]. More recently, lutein has been identified as the predominant (59%) carotenoid in the infant brain. Furthermore, lutein levels were much lower in preterm infants, suggesting accumulation of lutein in the brain during the later stages of maturation in the womb [140]. These findings underline the importance of carotenoid consumption in infants. In the early stages of life, carotenoid ingestion is fully reliant on mothers' own milk or formula alternatives. The influential nature of carotenoids on infant health stresses the importance of adequate dietary supply of carotenoids in infant formula.

### 6.2 Carotenoid Content of Infant Formula

When formula feeding is required, it is important that the nutritional composition of the formula mimics that of human milk. The carotenoid concentrations in non-fortified infant formulas vary, but are mainly low and not representative of human milk. Sommerburg et al. reported that the carotenoid profiles of infant formulas were much lower than that of human milk [136]. Only four out of eight formula preparations examined had any carotenoid content, with β-carotene in four and β-cryptoxanthin in three [136]. A higher concentration of carotenoids was recorded in infant formulas in Northern Ireland [141]. Lutein and zeaxanthin levels were present in 5 out of 6 formulas, although their levels varied greatly from 0.7 to 9.7 and 0.1 to 1.2 nmol/g fat, respectively. The authors attributed the high levels of lutein and zeaxanthin observed in some of the formulas to the fat source used, e.g., egg yolk, which has high xanthophyll content [141]. The low levels of carotenoids in formulas are reflected in infant plasma. Formula-fed infants had a median carotenoid plasma concentration of 14 μg/L in comparison with higher levels at birth of 24 μg/L or in the plasma of breastfed infants, at 32 μg/L [136]. It is apparent that carotenoid inclusion during the design of infant formula is not always considered. This can result in lower intake and accumulation of carotenoids in formula-fed infants. Due to the known bioactivity of carotenoids and their potential role in infant development, fortification of infant formula should be considered in future.

### 6.3 Fortified Infant Formula

In this section, we will discuss the development of fortified formula in terms of the bioaccessibility, bioavailability, safety and potential effects on infant development.

It is important when designing infant formula that it is representative of the nutritional composition of breast milk. The primary carotenoids of interest for optimized infant nutrition are those with provitamin A activity or the xanthophylls, lutein and zeaxanthin, due to their implication in ocular and neural development.

### *6.3.1 Bioaccessibility and Bioavailability of Fortified Infant Formula*

Both human milk and lutein-fortified infant formula have similar bioaccessibility, 29 and 36%, respectively. However, the bioavailability of human milk was 4 times greater than the lutein-fortified formula [142]. These findings are supported by the higher plasma levels of carotenoids observed in breastfed infants in comparison with infants from a formula-fed group [15,143]. Bettler et al. reported that after 12 weeks, breastfed infants had mean lutein plasma levels of 69.3 µg/L in comparison with 11.3 µg/ L in the unfortified formula-fed group [15]. Furthermore, lutein-fortified formula resulted in an increase in the lutein serum levels of infants in a dose-dependent manner. Similarly, Mackey and colleagues reported higher carotenoid plasma concentrations in breastfed and fortified formula-fed infants, in comparison with the unfortified formula group [143]. Bettler et al. suggest that fortified infant formula needs 4 times the levels of lutein observed in human milk to have similar circulating levels in infant plasma [15]. Unfortified formula in both studies had low lutein levels of 13.7–20 µg/L. The successful fortification of infant formula with carotenoids has been demonstrated. However, infant formula has lower bioavailability than human milk. This should be taken into consideration when designing a formula to reflect the nutritive value of breast milk. The exact mechanisms responsible for higher bioavailability of human milk, despite similar bioaccessibility remain unclear. Perhaps, specific nutritional components or factors unique to human milk facilitate the optimized absorption and transportation of carotenoids. Greater understanding of the properties of human milk that allow for superior carotenoid delivery is needed to optimize a fortified formula product.

### *6.3.2 Safety and Efficacy?*

When designing a fortified product for infant nutrition and health, the product must undergo the appropriate safety evaluations. Growth studies are key to evaluating

the clinical suitability of infant formula and should monitor weight, length, GI tolerance and food intake [144].

The safety of lutein-fortified formula at concentrations of 200 µg/L has previously been reported [145]. Safety was evaluated through monitoring the occurrence of study events and blood chemistry analysis; all results fell within the normal range. The anthropometric results indicate similar growth effects in fortified and unfortified formulas, with regard to length, weight and head circumference. Overall, the lutein-fortified formula was well tolerated. Similarly, two fortified infant formulas with different concentrations of β-carotene, lycopene and lutein also proved safe and adequately supported growth in infants [143]. The total carotenoid concentration of the fortified formulas were 129.5 and 225.8 µg/L in comparison with much lower levels in the unfortified control, 27.8 µg/L. This study supports the safety and efficacy of a multi-carotenoid formula. An experimental formula fortified with lutein (11.4 µg/100 mL) supported the normal growth of infants [144]. Although the overall outcome was determined safe, three adverse side effects (rash; colic and abdominal pain; constipation) were observed in the experimental formula-fed group. However, the safety of lutein fortification at higher levels in other studies points to a different contributing factor of the experimental composition of the formula [144]. A study by Rubin et al. fortified an infant formula with β-carotene, lycopene and lutein. Again, the fortified formula was safe and effective for infant development [14]. Furthermore, lower plasma C-reactive protein (CRP) levels and greater rod photoreceptor sensitivity were observed in the fortified infant group in comparison with the control. These findings highlight a potential link between carotenoid intake and decreased inflammation. CRP is a commonly used marker of inflammation. Reductions in CRP following carotenoid supplementation suggest antioxidant and immunomodulatory effects of carotenoids. Additionally, findings implicate lutein in enhanced visual development of infants as supplemented formula improved rod photoreceptor sensitivity [14]. Clearly, the fortification of infant formula with carotenoids can produce a formula preparation that is safe, tolerated, and effective. Further clinical evaluations are required to confirm the potential health benefits of carotenoids during infant development.

## 7 Future Considerations

Overall, there is potential for a fortified milk product due to the associated health effects of carotenoids and the agreeable nature of the dairy food matrix. The carotenoid content of milk and milk products can be enhanced in three key ways, (1) naturally via farm management; (2) through supplementation of bovine feed; (3) by supplementation at the processing stage. The majority of studies to date have focused on fortification of dairy at the point of production. This strategy has the advantage of being able to incorporate the chosen carotenoid in a controlled and quantified manner. This mechanism may be further optimized by the inclusion of encapsulation techniques or recovering carotenoids from agri-food waste. The fortification of dairy at the point of farming has the advantage of being widely accessible to the farming industry, viewed more naturally by the consumer and is economically viable. However, there are limited studies to date on this mode of fortification and further evaluation in terms of bioaccessibility, bioavailability and stability following processing must be determined. As outlined by the findings presented in this review, the key factors that should be considered when developing a carotenoid-fortified dairy product include the method and point of milk fortification, the manufacturing and processing conditions required and perhaps most importantly, the composition and choice of food matrix. Additionally, the selection of carotenoid is not only important in terms of health associations but also due to differing polarity and interactions within the food structure. The bioaccessibility and bioavailability are primary concerns with promising but varying findings to date. Further scientific studies should establish the adequate dosage, the optimal delivery system and verify the stability and safety of carotenoid-fortified dairy products. Additionally, the effects of fortification on the nutritional value, sensory properties and bioactivity vary. Evidently, these properties and interactions must be assessed in a product-specific manner when designing a functional food suitable for commercial production.

Furthermore, carotenoids are of value in human milk with potential health benefits for the infant. Human milk and infant formula are the sole source of carotenoids for the developing infant during the first few months of life. The low levels of

carotenoids associated with the available infant formulas highlights the need for fortification. Carotenoid fortification of infant formula provides a more representative source of nutrition for the infant when mother's milk is unavailable. To date, findings show fortification is a safe and viable option that supports the normal growth of the infant. Further examination of fortification at high levels to achieve a similar serum level to breastfed infants should be performed.

This review highlights the growing body of evidence that the carotenoid content and profile of bovine milk can be modified for a product with increased nutritional and marketable value. Additionally, the potential to develop an infant formula that is more representative of human milk whilst preserving the safety and efficacy of the formula is apparent. However, further research is required to establish the clinical benefits associated with a fortified formula or a dairy functional food.

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## 9 Author Contributions

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# Chapter 3

## The microbial composition of Irish bovine colostrum as influenced by antibiotic treatment

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## Abstract

Bovine colostrum, the initial milk produced by cows postpartum, contains an array of key nutritional, immune, and microbial components that support the calf's physiological development, immune maturation, and intestinal colonization. This study investigated the microbial profile of Irish bovine colostrum and the influence of antibiotic therapy and parity. Bovine colostrum samples were collected from five Irish dairy farms that implemented different methods of dry cow therapy (DCT): natural or blanket. For blanket DCT, four of the five farms administered intramammary antibiotics at the start of the drying off period. Two farms administered a fourth-generation cephalosporin, cefquinome, and two farms used an antibiotic of the penicillin class, with the active ingredients consisting of procaine benzylpenicillin, penethamate hydriodide, and framycetin sulphate. One farm did not administer antibiotics but applied a teat sealant (natural DCT). Following calving, colostrum samples from 90 healthy dairy cows were analysed. 16S rRNA sequence analysis revealed Firmicutes, Actinobacteriota, Bacteroidota, and Proteobacteria as the most abundant phyla across all treatment groups, with *Acinetobacter*, *Corynebacterium*, *Facklamia*, *Jeotgalicoccus*, *Lactococcus*, *Leuconostoc*, *Psychrobacter*, and *Staphylococcus* dominating at genus level. Parity did not significantly affect the microbial composition in this study, but antibiotic treatment did. Cows receiving no antibiotics showed distinct microbial clustering compared with antibiotic-treated cows (β-diversity, $p < 0.001$). Microbial diversity also differed between the antibiotic-treated groups, with significant changes in both α-diversity ($p < 0.01$) and β-diversity ($p < 0.001$), suggesting that the choice of antibiotic may also influence the microbiota. An influence of farm was also observed. Differential abundance analysis showed no increase in mastitis-associated genera in colostrum following natural DCT, although increased abundance was demonstrated with blanket DCT. Our findings substantiate the diverse and unique microbial composition of bovine colostrum. The data indicate that the microbial profile of bovine colostrum is influenced by antibiotic treatment administered during the dry period and affirms the latest policies inhibiting prophylactic antibiotic administration. Future studies should elucidate strain level

changes in the colostrum microbiota following on-farm antibiotic use, assess the associated risks of antimicrobial resistance, and explore non-antibiotic alternatives for drying off cows. Evidently, the microbial composition of bovine colostrum is influenced by farm management strategies and optimizing these measures may further increase the valuable constituents of bovine colostrum and confer added health benefits to the new-born calf.

## 1 Introduction

Colostrum is the initial milk produced by mammals postpartum. Bovine colostrum is a complete source of nutrition for the new-born calf, meeting full nutritional and energy requirements. Colostrum is rich in macro- and micro-nutrients, in addition to a range of bioactive compounds and a unique community of microorganisms [1,2]. Bovine colostrum is compositionally unique to mature milk, with elevated levels of protein (15% vs. 3%), fat (6–7 vs. 3–4%), and lower carbohydrate levels (2.5%) [3]. In addition, a range of essential minerals and vitamins are higher in colostrum [1], including calcium, phosphorus, sodium, chloride, and magnesium [3–5], whilst concentrations of potassium and manganese are lower [4,5]. Similarly, a greater concentration of vitamins B2, B12, A, E, and D have been observed in colostrum [3,5]. Importantly, the immunoglobulin (Ig) content is markedly richer, comprising 70–80% of colostrum's total protein content [1,6]. IgG is the most abundant class, accounting for 80–90% of the total Ig content [3,7]. In addition, colostrum is known for its high availability of bioactive compounds including lactoferrin, lysozyme, and lactoperoxidase [1,3,4,8,9]. These compounds have protective functions for the calf, due to their bacteriostatic and germicidal activity [9]. Patently, colostrum provides increased nutritional value. Bovine colostrum facilitates basic cell function, thermoregulation, maturation of the immune response, and gastrointestinal and muscle development [4,10]. Colostrum consumption in calves is associated with reduced incidences of infection, diarrhoea, respiratory disorder, and mortality [11,12], and increased immune protection, growth performance, and milk yield [11–13]. Indeed, the new-born calf is reliant on colostrum to acquire immune protection through passive transfer of

immunoglobulins [14–16]. Thus, receipt of colostrum is essential for the development and survival of the new-born calf.

In addition to being nutrient dense, colostrum is also host to a rich microbial population, which is passed to the new-born upon ingestion, initiating gut colonization [8,17]. Intestinal colonization by colostrum consumption increased levels of *Bifidobacterium* in the colon and reduced levels of the pathogenic microbes *Escherichia coli* and *Escherichia-shigella*, highlighting its role in immune defence [18]. Although still under investigation, the origin of the bovine colostrum microbiota has mainly been linked with the udder skin and teat canal, and the surrounding farm environment [19–21]. In addition, the existence of an entero-mammary pathway has been suggested; however, this is yet to be established [21–24]. To date, studies are largely in agreement that colostrum is dominated by the same four phyla; Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria [25]. However, at lower taxonomic levels, greater discordance exists. A recent review identifies *Acinetobacter*, *Pseudomonas*, and *Staphylococcus* as the most commonly identified bacterial genera in colostrum [4]. Even greater variability exists at the species level. Recent developments in sequencing technology have enabled a more comprehensive insight into the microbial population associated with the bovine mammary gland. Indeed, shotgun sequencing has confirmed the presence of microbes at the species level in bovine colostrum, with both pathogenic and probiotic potential [26]. These include bacteria with beneficial properties widely used as starter cultures in the food industry, such as *Leuconostoc mesenteroides*, *Lactococcus lactis*, and *Lacticaseibacillus paracasei*, and known pathogenic strains, *Pseudomonas aeruginosa*, *E. coli,* and *Listeria monocytogenes* [27–30]. The bacterial content of colostrum is often screened as an indicator of microbial quality and infection status. In colostrum, a total plate count (TPC) and total coliform count (TCC) of <100,000 CFU $mL^{-1}$ and <10,000 CFU $mL^{-1}$, respectively, are the acceptable thresholds [4]. In bulk tank milk, a somatic cell count (SCC) > 400,000 cells $mL^{-1}$ exceeds the limit for milk intended for human consumption according to European Union (EU) legislation [31]. It is widely accepted that a SCC > 200,000 cells $mL^{-1}$ at herd level is indicative of intermammary infection [32]. Although

current legislation refers to bovine milk, SCC is also an effective way of determining the quality of colostrum [33].

The nutritional and bioactive composition of colostrum not only facilitates adequate growth and development in the offspring, but also provides immune protection [9,34]. Van Hese et al. suggests 4 L of high quality colostrum for new-born calves within 6 h of parturition will confer sufficient serum IgG levels (10 g/L) to ensure passive immunity [17]. When the offspring does not receive adequate colostrum of high quality, failed transfer of Igs can occur, decreasing the chance of survival. In addition, high quality colostrum influences intestinal colonization of the neonate, promoting the growth of beneficial bacteria [8]. Clearly, high quality colostrum in the initial critical period ensures successful passive transfer and results in improved health of the calf. However, in the United States and Brazil, only 39% and 22.6% of bovine colostrum samples, respectively, met the industry recommendations for both IgG and microbial growth [35,36]. Furthermore, in Northern Ireland, 44% of colostrum samples had <50 mg $mL^{-1}$ IgG and 81% had a TPC > 100,000 cfu $mL^{-1}$ [14]. Evidently, further optimization of farm management strategies is needed to ensure the production of bovine colostrum with satisfactory microbial and nutritional value.

A multitude of factors can influence the composition and quality of bovine colostrum, including seasonal variation [6,37,38], maternal nutrition [14,39], breed [40,41], and herd size [42]. In addition, parity is known to influence the nutritional and immunological quality of colostrum [6,14,37,38,40,41,43,44]. However, evidence regarding its impact on the colostrum microbiota remains inconsistent [17,45,46]. Furthermore, although some influential factors are predetermined, others can be modified with farm management practices [38], in particular, DCT [38,43]. The dry period is a non-lactating period, referring to the time between the last lactation pre-calving and the first lactation post-calving. This interval is essential for the optimization of mammary gland health and milk production, facilitating the regeneration and repair of mammary epithelial cells [45]. DCT frequently includes the administration of intramammary antibiotics. In addition, an intramammary teat sealant is often used in combination with antibiotics or

independently (natural DCT), mimicking the naturally formed keratin plug in ruminants. Teat sealant is usually composed of bismuth subnitrate and forms a physical barrier to protect against invading pathogens [47]. There are two established protocols to antibiotic DCT—selective and blanket. Selective DCT limits antibiotic administration to cows with an ongoing infection or at imminent risk of infection during the dry period. Blanket DCT refers to the prophylactic administration of antibiotics, regardless of the cow's infection status. However, as of 2020, an EU directive has prohibited prophylactic antibiotic administration on farms due to growing concerns regarding the rise in antibiotic resistance [48]. Conversely, blanket DCT is still practiced in other parts of the world [49–51]. The primary infection driving the need for antibiotic administration is bovine mastitis, which is the inflammation of the mammary parenchyma due to bacterial infection. Mastitis is a known economic burden to the dairy sector and an ongoing challenge for farmers and herd health [52]. The main mastitis-causing pathogens include *Corynebacterium bovis*, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae,* and *Streptococcus agalactiae* [47,53]. The use of antibiotics when treating intramammary infection is considered a key contributor to the growing emergence and spread of antimicrobial resistance. Indeed, antibiotic-resistant bacteria have previously been isolated from bovine milk [54,55]. Moreover, bovine colostrum and milk are suggested reservoirs for antibiotic-resistant genes [26,56,57]. The potential vertical transfer of antimicrobial resistance to the environment and food-chain makes antibiotic administration on dairy farms a One Health issue.

In Ireland, dairy farming contributes EUR 16 billion to the Irish economy and is responsible for 85,000 jobs [58,59]. In addition, the number of dairy cows in Ireland increased by 34.8% in 2020 since 2013, accounting for over 1.5 million dairy cows [60]. As a global leader in the dairy industry, it is of vital importance to not only protect but also optimize the farm and dairy industry through the prioritisation of animal health and milk quality. A greater understanding of the farm management practices that influence the microbial quality of bovine colostrum will enable improved on-site management practices and herd health. In this study, we

investigated the microbial composition of bovine colostrum from healthy cohorts of Irish dairy cows. Our primary objective was to compare the effect of blanket and natural DCT on the microbial profile of bovine colostrum. We also determined the effect of parity and individual farm variation.

## 2 Methods

### 2.1 Study Population

Colostrum samples were collected from five dairy farms in the same locality of North Cork in February 2020, with an approximate herd size of 75 cows per farm. Experimental cows were selected at random and any cows presenting signs of mastitis or other infection during the dry or sampling period were excluded. A total of 90 Holstein Friesian cows were selected for this study, providing a total of 90 colostrum samples. The farms included in this study were identified as; Farm C, Farm D, Farm L, Farm P and Farm T. All cows were housed indoors during the winter until calving (November-February). A dry period of 6-8 weeks was implemented. On all farms, cows were treated with teat sealant (Boviseal). In addition, four of the five farms administered broad spectrum intra-mammary antibiotics to multiparous cows at the start of the drying off period (blanket DCT). Two of the farms (L and T) used 'Ubro Red Dry Cow Intramammary Suspension' and two of the farms (D and P) used 'Cephaguard DC 150 mg intramammary ointment' at drying off. Ubro Red contains the active ingredients Framycetin Sulfate (100 mg), Penethamate Hydriodide (100 mg) and Procaine Penicillin (300 mg). For Cephaguard, the active ingredient is Cefquinome (150 mg), a fourth-generation cephalosporin. In order to evaluate the influence of antibiotic therapy, colostrum samples from these farms were designated as UBRO group and CEF group, respectively. One farm (C) did not use any antibiotic therapy (natural drying off) and was grouped as, no antibiotic (NOAB) group. The NOAB group also included samples from primiparous cows of farms D, L, P and T. For parity analysis, colostrum samples were grouped according to the number of previous gestations; 0 (first gestation), 1, 2, 3, 4+. See Table 1 for metadata on the experimental cows and farms used in this study.

## 2.2 Sample Collection

All farms and personnel who participated in sample collection were quality-assurance-certified members of the Irish Food Board (Bord Bia). Farmers completed a questionnaire for each sample providing information on the identification number, age, infection status, antibiotic history and parity of the cow and the date of calving. All farmers were provided with and followed instructions for sterile sample collection, in accordance with the recommendations from the National Mastitis Council's Laboratory Handbook on bovine mastitis (60). In brief, colostrum samples were collected at the first milking within 1 h of calving. To ensure mammary gland stimulation, the first streams of colostrum from each mammary quarter were discarded (fore-stripping). Pre-dipping was then carried out by dipping the teats in iodine tincture. Teats were dried and scrubbed with wipes soaked in 70 % alcohol. Finally, 15 mL of colostrum were collected in sterile falcon tubes (Sarstedt). Each sample was labelled with the cow's identification number and the date of calving. Samples were stored immediately at -20 °C in a chest freezer on farm for a maximum of one week. Finally, samples underwent cold storage and were transported to Teagasc, Moorepark and stored in a -80 °C freezer, awaiting analysis.

## 2.3 DNA Extraction

Prior to extraction, samples were removed from the -80 °C freezer and allowed to thaw at 4 °C overnight. Microbial DNA was extracted following a modified protocol for the DNeasy Powerfood Microbial kit (Qiagen, UK) as previously described (61). In brief, 12 mL of sample was transferred to a sterile falcon, homogenized by inverting multiple times and centrifuged at 4,000 *g* for 30 min at 4 °C. The fat layer was removed using a sterile cotton swab (Thermo Fisher Scientific Inc) and the supernatant containing protein was decanted. The remaining cell pellet was re-suspended in 1 mL of sterile phosphate-buffered saline (PBS) (Sigma Aldrich) and transferred to a sterile 1.5 mL tube and centrifuged at 13,000 *g* for 1 min at room temperature. The supernatant was discarded. This wash step was repeated until the supernatant was no longer cloudy. The pellet was re-suspended in 1 mL of PBS and treated with 90 µL of 50 mg/mL of lysozyme from chicken egg white (Sigma Aldrich) and 50 µL of 5 KU/mL Mutanolysin from *Streptomyces globisporus* ATCC

21553 (Sigma Aldrich). The samples were incubated at 55 °C for 15 min and bump vortexed at 3 min intervals. Samples were removed from heat and 28 µL of proteinase K (Sigma Aldrich) was added before further incubation at 55°C for 15 min followed by centrifugation at 14,000 *g* for 10 min at 4 °C. The supernatant was discarded. Finally, DNA extraction was completed using the DNeasy Microbial Powerfood Kit following the manufacturer's instructions. A negative control using sterile molecular grade water (VWR) was included. Following extraction, samples were stored at -20 °C awaiting further analysis.

### 2.4 Preparation of DNA for Sequencing

PCR amplification of the V3-V4 hypervariable region of the 16S rRNA gene was carried out using the 16S metagenomic sequencing library protocol (Illumina). For amplification, template DNA was amplified using specific V3-V4 region primers; forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; and reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

The PCR reaction was composed of 2.5 µL of template DNA, 5 µL forward primer (5 µM), 5 µL reverse primer (5 µM), 12.5 µL 2X KAPA HiFi Hotstart ready mix (Anachem) and molecular grade water (VWR). The applied PCR conditions were an initial denaturing step of 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final elongation step of 72 °C for 5 min. To confirm amplification, gel electrophoresis (1X TAE buffer, 1.5% agarose, 100V for 30 min) was used for visualization of the PCR product. For purification of the amplicons, AMPure XP magnetic bead-based purification (Labplan) was used followed by a second PCR reaction. For indexing, dual indices and Illumina sequencing adapters (Illumina Nextera XT indexing primers) were added to the DNA product (5 µL). Samples were quantified using the Qubit (Bio-Sciences) and the high specificity DNA quantification assay kit (BioSciences). Following quantification, samples were pooled in equal molar concentrations. For quality analysis, the pooled samples were run on the Agilent Bioanalyser and prepared for sequencing in accordance with Illumina guidelines. Samples were sequenced on the MiSeq sequencing

platform in the Teagasc Sequencing facility, using a 2 x 300 cycle V3 kit, following standard Illumina sequencing protocols.

### 2.5 Bioinformatic and Statistical Analysis

The raw sequencing data were processed using the DADA2 pipeline (version 1.16) as previously described [139]. Specifications for optimal data refinement were applied, including maxN = 0, maxEE = c (2, 2), rm.phix = TRUE, truncQ = 2, trimLeft = c (17, 21), and truncLen = c (280, 210). The selected parameters ensured rigorous quality control and accurate sequence inference. Using the processed data, an Amplicon Sequence Variant (ASV) table was generated. Taxonomic rank was assigned using the SILVA v138.1 database [140]. Finally, to enable downstream analysis, a Phyloseq object was created through the combination of sample metadata and OTU table and taxonomic data [141]. Statistical analysis was performed using R (version 4.3.2). Statistical significance was accepted as $p < 0.05$. Relative abundances were calculated and visualized using the R software packages phyloseq, microbiomeutilities, microbiome, and ggplot2. Pairwise comparison was performed using the Wilcoxon signed-rank test with the command 'geom_pwc', and significant *p*-values were adjusted using the Benjamin Hochberg method. Alpha diversity (within sample variation) was determined using Chao1 and Shannon indices. Beta diversity (between sample variations) was measured based on the Bray–Curtis distance matrix and visualized using Principal Co-ordinate Analysis (PCoA). Significance was obtained using Permutational Analysis of Variance (PERMANOVA). Differential abundance analysis was performed at a genus level using the *MaAsLin2* package in R. The general linear model, log transformation, and CLR normalization were the applied parameters. Antibiotic-treated groups (UBRORED and CEF) were selected as fixed effects and NOAB was chosen as the reference group, unless stated otherwise.

Table 1: Metadata on the colostrum samples. All cows were housed indoors during the winter period until calving (November – February). The breed of cows was the same on all farms (Holstein Friesian).

| Sample ID | Farm | Parity Status | Age (years) | Antibiotic Treatment |
|---|---|---|---|---|
| **C10** | Farm C | 2 | 4 | None |
| **C11** | Farm C | 4+ | 6 | None |
| **C12** | Farm C | 1 | 3 | None |
| **C13** | Farm C | 1 | 3 | None |
| **C14** | Farm C | 4+ | 7 | None |
| **C15** | Farm C | 2 | 4 | None |
| **C16** | Farm C | 0 | 2 | None |
| **C17** | Farm C | 4+ | 8 | None |
| **C18** | Farm C | 4+ | 6 | None |
| **C19** | Farm C | 0 | 2 | None |
| **C1** | Farm C | 1 | 3 | None |
| **C20** | Farm C | 4+ | 6 | None |
| **C2** | Farm C | 0 | 2 | None |
| **C3** | Farm C | 1 | 3 | None |
| **C5** | Farm C | 0 | 2 | None |
| **C6** | Farm C | 3 | 5 | None |
| **C7** | Farm C | 1 | 3 | None |
| **C8** | Farm C | 4+ | 6 | None |
| **C9** | Farm C | 4+ | 6 | None |
| **D10** | Farm D | 4+ | 10 | Cefquinome |
| **D11** | Farm D | 2 | 4 | Cefquinome |
| **D12** | Farm D | 4+ | 6 | Cefquinome |
| **D13** | Farm D | 1 | 3 | Cefquinome |
| **D14** | Farm D | 1 | 3 | Cefquinome |
| **D15** | Farm D | 3 | 5 | Cefquinome |
| **D17** | Farm D | 2 | 4 | Cefquinome |
| **D18** | Farm D | 4+ | 9 | Cefquinome |
| **D19** | Farm D | 4+ | 6 | Cefquinome |
| **D1** | Farm D | 4+ | 10 | Cefquinome |
| **D20** | Farm D | 0 | 2 | None |
| **D2** | Farm D | 3 | 5 | Cefquinome |
| **D3** | Farm D | 2 | 4 | Cefquinome |
| **D5** | Farm D | 4+ | 9 | Cefquinome |
| **D6** | Farm D | 4+ | 6 | Cefquinome |
| **D7** | Farm D | 3 | 5 | Cefquinome |
| **D9** | Farm D | 1 | 3 | Cefquinome |
| **L10** | Farm L | 3 | 5 | Ubro Red |
| **L11** | Farm L | 3 | 5 | Ubro Red |

| | | | | |
|---|---|---|---|---|
| **L12** | Farm L | 4+ | 7 | Ubro Red |
| **L13** | Farm L | 1 | 3 | Ubro Red |
| **L14** | Farm L | 4+ | 8 | Ubro Red |
| **L15** | Farm L | 0 | 2 | None |
| **L16** | Farm L | 3 | 5 | Ubro Red |
| **L18** | Farm L | 4+ | 9 | Ubro Red |
| **L19** | Farm L | 2 | 4 | Ubro Red |
| **L20** | Farm L | 2 | 4 | Ubro Red |
| **L2** | Farm L | 3 | 5 | Ubro Red |
| **L3** | Farm L | 1 | 3 | Ubro Red |
| **L5** | Farm L | 1 | 3 | Ubro Red |
| **L6** | Farm L | 1 | 3 | Ubro Red |
| **L7** | Farm L | 2 | 4 | Ubro Red |
| **L8** | Farm L | 4+ | 7 | Ubro Red |
| **L9** | Farm L | 4+ | 9 | Ubro Red |
| **P10** | Farm P | 1 | 3 | Cefquinome |
| **P11** | Farm P | 4+ | 8 | Cefquinome |
| **P12** | Farm P | 2 | 4 | Cefquinome |
| **P13** | Farm P | 4+ | 7 | Cefquinome |
| **P14** | Farm P | 0 | 2 | None |
| **P15** | Farm P | 4+ | 9 | Ubro Red |
| **P16** | Farm P | 0 | 2 | None |
| **P18** | Farm P | 0 | 2 | None |
| **P19** | Farm P | 1 | 3 | Cefquinome |
| **P1** | Farm P | 4+ | 6 | Cefquinome |
| **P20** | Farm P | 1 | 3 | Cefquinome |
| **P2** | Farm P | 2 | 4 | Cefquinome |
| **P3** | Farm P | 4+ | 6 | Cefquinome |
| **P4** | Farm P | 4+ | 8 | Cefquinome |
| **P5** | Farm P | 4+ | 7 | Cefquinome |
| **P6** | Farm P | 4+ | 7 | Cefquinome |
| **P7** | Farm P | 4+ | 6 | Cefquinome |
| **P8** | Farm P | 2 | 4 | Cefquinome |
| **P9** | Farm P | 1 | 3 | Cefquinome |
| **T10** | Farm T | 3 | 5 | Ubro Red |
| **T11** | Farm T | 0 | 2 | None |
| **T12** | Farm T | 4+ | 13 | Ubro Red |
| **T13** | Farm T | 4+ | 9 | Ubro Red |
| **T15** | Farm T | 4+ | 8 | Ubro Red |
| **T16** | Farm T | 3 | 5 | Ubro Red |
| **T17** | Farm T | 1 | 3 | Ubro Red |
| **T18** | Farm T | 2 | 4 | Ubro Red |
| **T19** | Farm T | 2 | 4 | Ubro Red |
| **T1** | Farm T | 4+ | 7 | Ubro Red |
| **T20** | Farm T | 2 | 4 | Ubro Red |

| | | | | |
|---|---|---|---|---|
| **T2** | Farm T | 4+ | 6 | Ubro Red |
| **T4** | Farm T | 1 | 3 | Ubro Red |
| **T5** | Farm T | 2 | 4 | Ubro Red |
| **T6** | Farm T | 0 | 2 | None |
| **T7** | Farm T | 4+ | 9 | Ubro Red |
| **T8** | Farm T | 3 | 5 | Ubro Red |
| **T9** | Farm T | 0 | 2 | None |

# 3 Results

In this study, microbial DNA was extracted from bovine colostrum samples. Samples were grouped according to antibiotic treatment. 16S rRNA sequencing was performed to determine the effect of DCT (blanket vs natural) on the microbial profile of colostrum.

## 3.1 Microbial composition of bovine colostrum following dry cow therapy

Relative abundance analysis revealed a total of 12 phyla in bovine colostrum. Across all three treatment groups (CEF, NOAB and UBRORED) the most prevalent phyla were identified as; Firmicutes (48 %), Actinobacteriota (22 %), Bacteroidota (7 %), Proteobacteria (21 %), and Patescibacteria (1 %) (see Figure 1). The remaining phyla were identified at abundances of <0.5% and considered to be of low abundance and grouped as other.

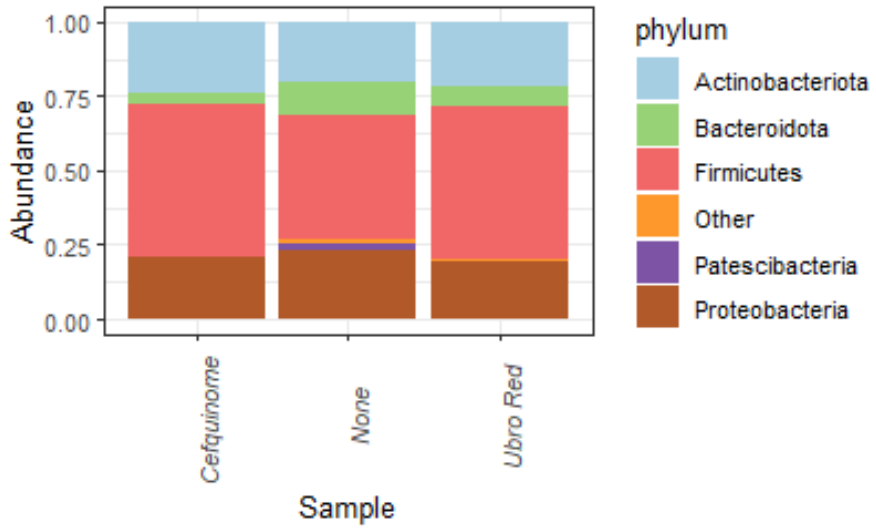



Figure 1: The 5 most prevalent phyla in bovine colostrum grouped according to antibiotic treatment.

Pairwise comparisons of the distribution of relative abundance at phylum level between DCT treatment groups revealed significant differences in the abundance of Firmicutes, Actinobacteriota and Patescibacteria (see Figure 2). A lower abundance of Firmicutes was observed in the NOAB group when compared with both the CEF and UBRORED groups ($P < 0.05$). The NOAB group had a higher abundance of Bacteroidota than the CEF ($p < 0.001$) and UBRORED ($P < 0.05$) treated groups. In addition, differences in the abundance of Bacteroidota was observed between the two antibiotic treated groups, with a higher abundance in UBRORED than CEF treated colostrum ($p < 0.05$). Similarly, the lowest abundance of Patescibacteria was observed in both the antibiotic treated groups when compared with the NOAB group (CEF $p < 0.0001$; UBRORED $p < 0.001$) and a significantly lower abundance was observed in the CEF treated samples when compared with the UBRORED group ($p < 0.01$).

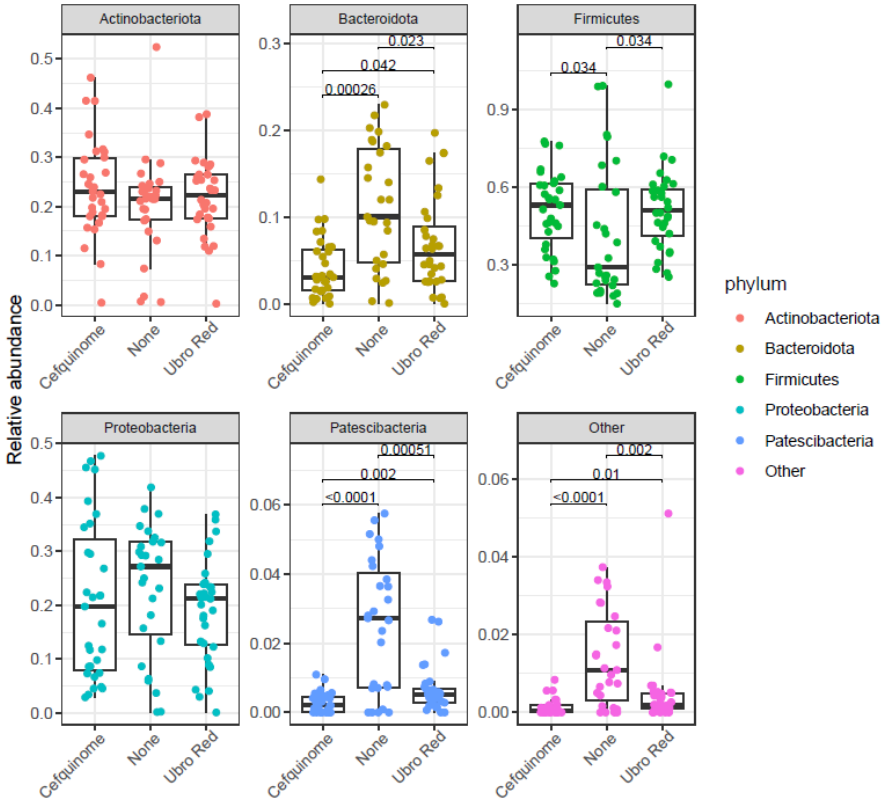



Figure 2: The distribution of abundance of phyla according to dry cow therapy. Significance was determined using ggpubr for pairwise comparison. P.adj values <0.05 were considered significant.

The choice of DCT also influenced the microbial composition at genus level. A total of 227 genera were identified in bovine colostrum and unknown sequences accounted for 24 %. The 8 most predominant genera, representing 40 % of the total relative abundance, were identified as *Acinetobacter*, *Corynebacterium*, *Facklamia*, *Jeotgalicoccus*, *Lactococcus*, *Leuconostoc*, *Psychrobacter* and *Staphylococcus* (Figure 3).

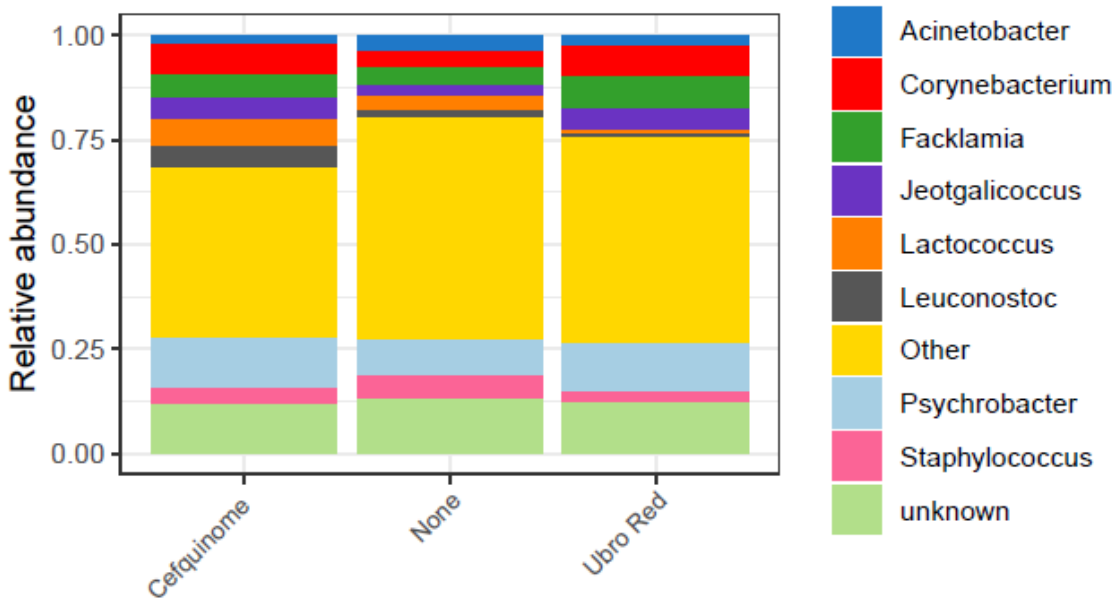



Figure 3: The relative abundance of the top 10 genera identified in bovine colostrum from all three DCT treatment groups.

### 3.2 Differential Abundance Analysis

To examine genus level changes associated with antibiotic treatment, differential abundance analysis was performed with MaAslin2. Of the 227 genera detected in bovine colostrum, 224 were differentially abundant when the effect of antibiotic treatment was considered with NOAB as the reference group. A total of 111 and 113 features (genera) were significant in the CEF and UBRORED group, respectively. A positive coefficient indicates a higher abundance of a genus in the specified antibiotic-treated group, whereas a negative coefficient reflects a lower abundance relative to the reference (NOAB group). To determine which features may be causing differences between DCT treatment groups, a heat map of the top 50 differentially abundant genera was created (see Figure 4). Of the top 50 features, *Rhodococcus*,

*Paracoccus,* and *Sphingomonas* had increased abundance in both of the antibiotic-treated groups when compared with the NOAB group.

Differential analysis of the ten most dominant genera further identified significant genus level shifts associated with antibiotic treatment (Table 2). *Corynebacterium* was most abundant in the UBRORED group and was significant when compared with the NOAB group (β = 1.327, q = 0.0251). Similarly, *Facklamia* and *Jeotgalicococcus* were elevated in UBRORED relative to NOAB (β = 1.36, q = 0.0184 and β = 1.580, q = 0.0241, respectively). *Staphylococcus* showed the lowest abundance in NOAB, differing significantly from both the CEF (B = 3.002, q value = 0.003) and UBRORED (β = 2.546, q = 0.0115) groups. *Leuconostoc* was significantly enriched in the CEF group when compared with NOAB (β = 3.766, q = 0.0099). No significant differences were detected for *Acinetobacter*, *Lactococcus,* or *Psychrobacter* across the DCT treatment groups (q > 0.05). Differential analysis between the antibiotic-treated groups revealed no significant associations among the ten dominant genera when comparing CEF and UBRORED directly.

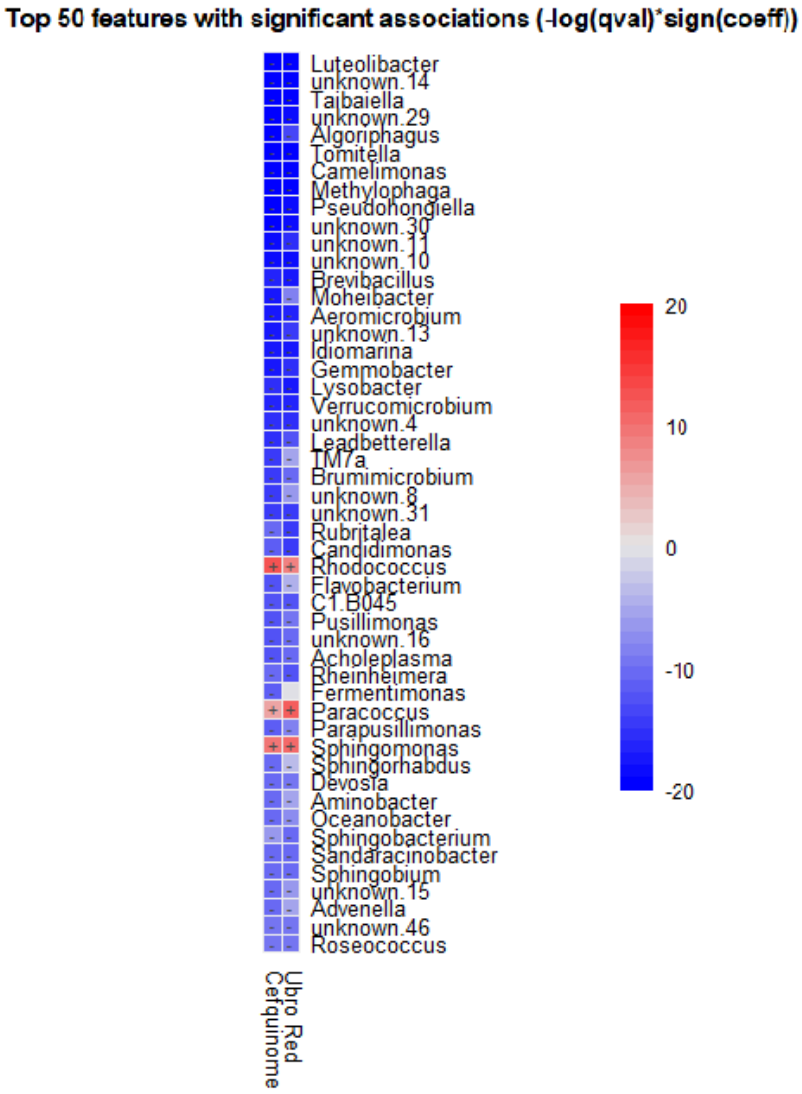



Figure 4: A heat map showing the top 50 taxa identified as differentially abundant in antibiotic-treated groups (CEF and UBRORED) relative to the NOAB reference group using MaAslin2. Blue tiles indicate a negative association (depletion relative to NOAB) and red tiles indicate a positive association (enrichment).

In addition, a number of mastitis-associated genera were significantly different depending on antibiotic treatment received. In the CEF group, the abundance of *Pseudomonas* (β = 2.593, q = 0.0017), *Staphylococcus* (β = 3.002, q = 0.0033), and *Enterococcus* (β = 2.167, q = 0.0309) was higher when compared with NOAB (Figure 5). Similarly, in the UBRORED samples, an increased abundance of *Corynebacterium* (β = 1.327, q = 0.0251) and *Staphylococcus* (β = 2.546, q = 0.0115) was observed (Figure 5). Furthermore, direct comparison of the antibiotic-treated

groups revealed significantly lower abundance of *Pseudomonas* in the UBRORED group compared with CEF (β = −3.8312, q = 2.9667).

Table 2. Significant associations among the ten most abundant genera in colostrum. Differential abundance analysis was conducted using MaAslin2, comparing antibiotic-treated groups (UBRORED or CEF) with the NOAB group as reference. Reported values include the model coefficient (β), standard error (SE), *p*-value, FDR adjusted q-value, total sample size (N), and the number of samples with non-zero abundance (N.not.0).

| Feature | Value | coef (β) | se | *p*-value | q-value | n | n.not.0 |
|---|---|---|---|---|---|---|---|
| ***Corynebacterium*** | Ubro Red | 1.327 | 0.494 | 0.0086 | 0.0251 | 90 | 89 |
| ***Facklamia*** | Ubro Red | 1.360 | 0.566 | 0.0184 | 0.0462 | 90 | 86 |
| ***Jeotgaliococcus*** | Ubro Red | 1.580 | 0.584 | 0.0082 | 0.0241 | 90 | 86 |
| ***Leuconostoc*** | Cefquinome | 3.766 | 1.231 | 0.0029 | 0.0099 | 90 | 43 |
| ***Staphylococcus*** | Cefquinome | 3.002 | 0.857 | 0.0007 | 0.0030 | 90 | 72 |
| ***Staphylococcus*** | Ubro Red | 2.546 | 0.851 | 0.0036 | 0.0115 | 90 | 72 |

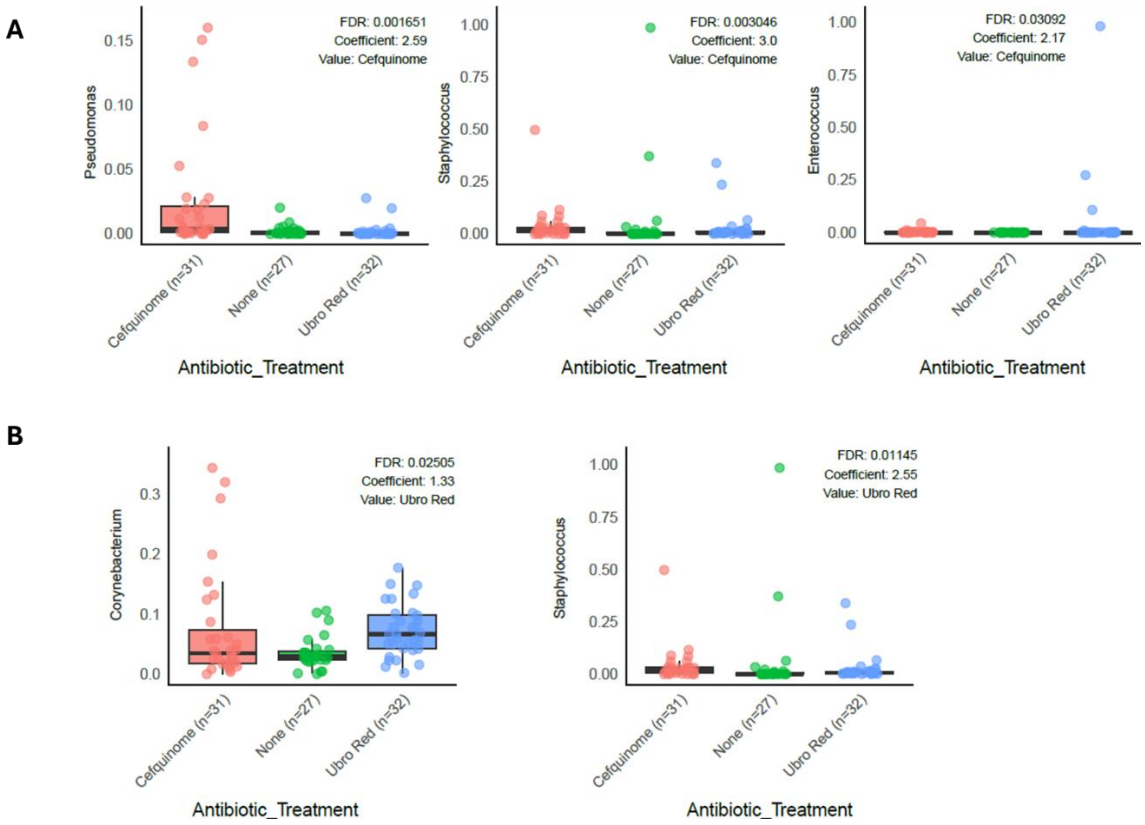



Figure 5: Differential abundance analysis of mastitis-associated pathogens between the antibiotic-treated groups and the NOAB group: (**A**) *Pseudomonas*, *Staphylococcus,* and *Enterococcus* demonstrated higher abundance in the CEF group relative to the NOAB group. (**B**) *Staphylococcus* and *Corynebacterium* were significantly enriched in the UBRORED group relative to the NOAB group.

## 3.3 The influence of dry cow therapy on the microbial diversity of bovine colostrum

### *3.3.1 Alpha Diversity*

The alpha diversity indices Chao1 and Shannon were used to determine intra-sample variation in all three DCT treatment groups (see Figure 6). The Chao1 index assess the richness of bovine colostrum samples, whereas the Shannon index estimates both richness and evenness. To determine if there was any significant differences in α-diversity between the treatment groups, the Wilcoxon signed-rank test was performed. According to both measures, the highest α-diversity was observed in the NOAB group (NOAB vs CEF: Chao1 $p < 0.01$, Shannon $p < 0.01$; for NOAB vs UBRO: Shannon $p < 0.05$). Furthermore, comparison of the diversity indices indicate that the microbiota within the CEF-group has lower species

richness (CEF vs UBRO: Chao1 $p < 0.05$) and less diversity (Shannon $p < 0.01$) in comparison with colostrum from the UBRORED group.

### *3.3.2 Beta Diversity*

Beta-diversity was used to measure dissimilarity in the microbial diversity of colostrum between the three DCT treatment groups. To determine if significant differences in β-diversity existed, a PCoA plot based on the Bray-Curtis distance matrix was constructed and PERMANOVA was performed. As shown in Figure 7, β-diversity analysis revealed the distinct clustering of the colostrum microbiota in the NOAB group. PERMANOVA showed the β-diversity of the NOAB group was significant against the CEF ($p = 0.001$) and UBRO ($p = 0.001$) groups. In addition, discrete clustering of the colostrum microbiota between the two antibiotic treated groups occurred and significance was confirmed (CEF vs UBRORED $p=0.001$).

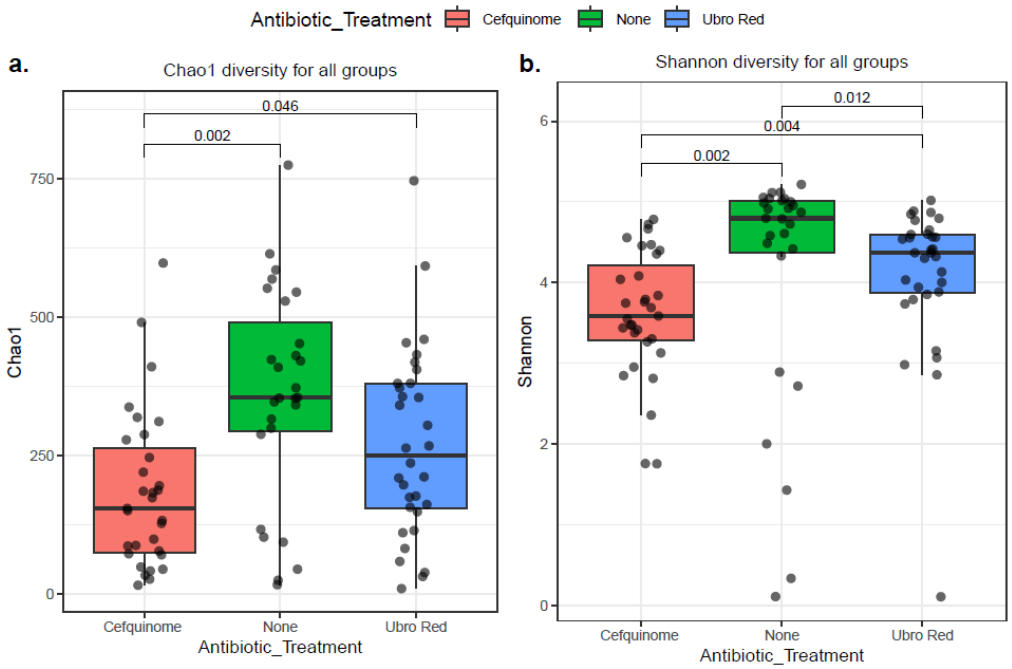



Figure 6: Alpha diversity indices were calculated for bovine colostrum samples grouped according to dry cow therapy treatment. (**A**) Chao1 index, reflecting species richness and (**B**) Shannon index, reflecting species richness and evenness. Alpha diversity differed between treatment groups, with NOAB exhibiting the highest richness and diversity across both indices. Significance was accepted if $p < 0.05$.

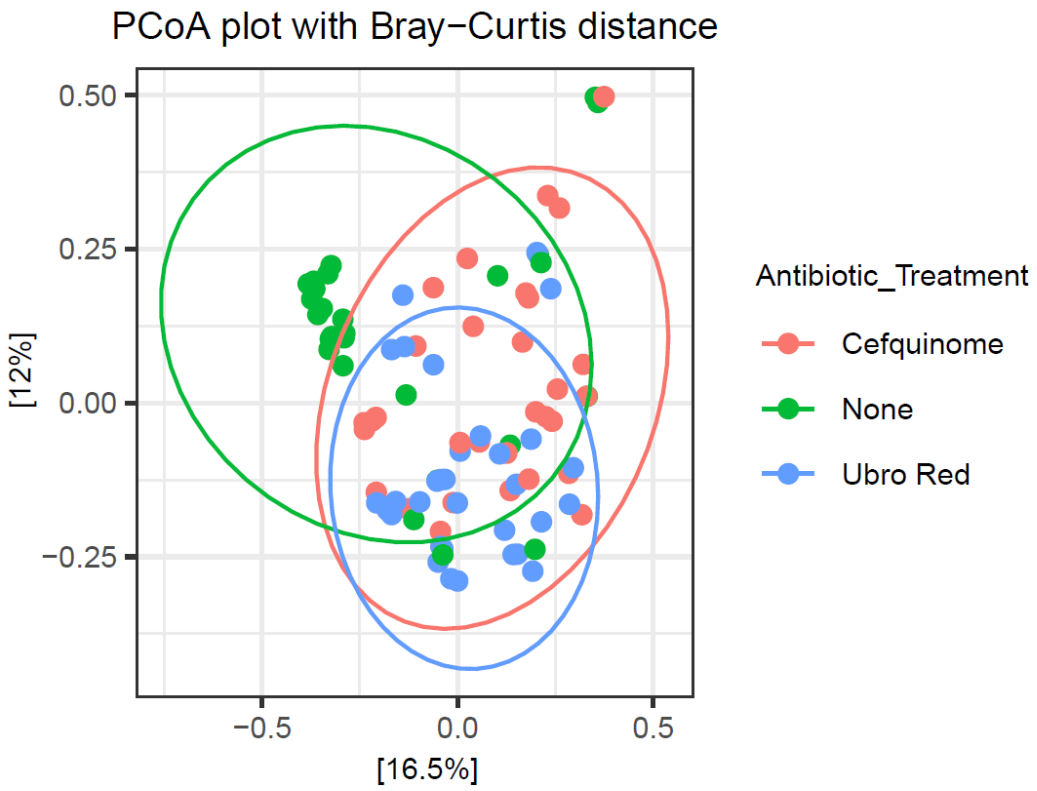



Figure 7: A PCoA plot based on the Bray-Curtis distance dissimilarity matrix of the inter-sample variation according to Dry Cow Therapy treatment. The plot reveals the discrete clustering of the NOAB group when compared with CEF and UBRORED groups. A significant difference in the colostrum microbiota β-diversity of the NOAB group in comparison with both antibiotic treated groups was determined with pairwise Adonis test (NOAB vs CEF p=0.001; NOAB vs UBRORED p = 0.001). Separate grouping also occurred between the two antibiotic treated groups and significance was confirmed (CEF vs UBRO p = 0.001).

### 3.4 The influence of individual farm variation on bovine colostrum

Alpha diversity indices Chao1 and Shannon revealed significant variation in microbial richness and diversity at farm level (Figure 8). The highest α-diversity was observed in farm C. Chao1 estimated richness was significantly higher in farm C when compared with farm D ($p < 0.0001$) and farm P ($p < 0.01$). The lowest α-diversity was observed in farm D. The Chao1 index for farm D was significantly lower to farm L ($p < 0.001$), farm P ($p < 0.01$) and farm T ($p < 0.05$). Furthermore, using the Shannon index, significant variations in microbial richness and uniformity were observed in all five farms and significance was confirmed with the Wilcoxon test. With regards to β-diversity, PCoA based on Bray-Curtis distance matrix revealed

colostrum microbiota differs significantly according to farm. As depicted in Figure 9, discrete microbial grouping occurred between all five farms and significance was obtained with PERMANOVA (Farm C vs all farms $p < 0.01$; Farm D vs Farm L $p < 0.001$; Farm D vs Farm P $p < 0.01$; Farm D vs Farm T $p < 0.05$).

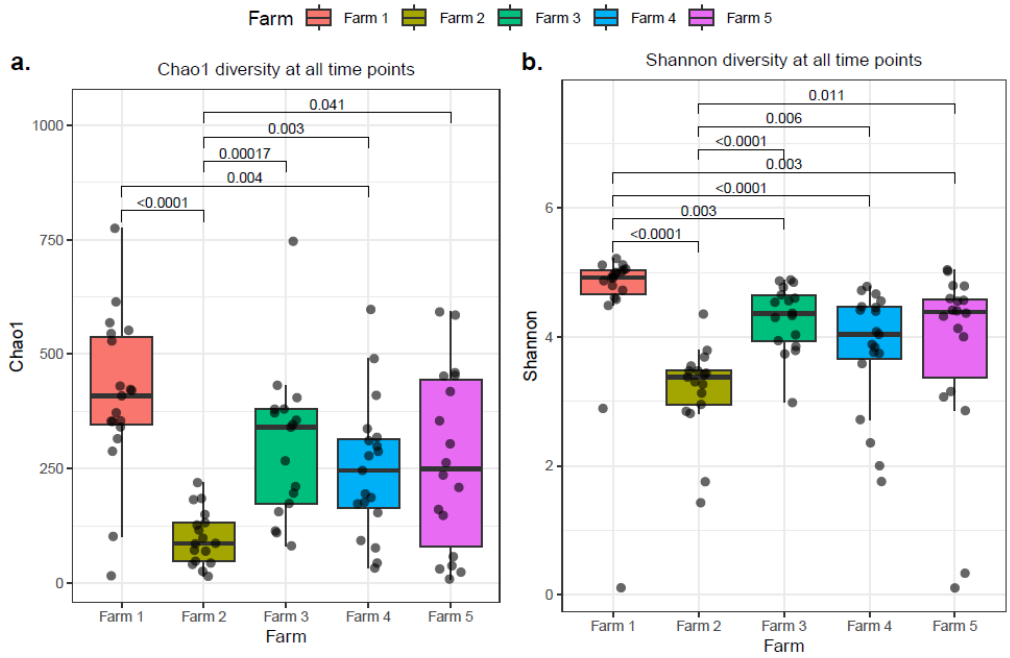



Figure 8: When colostrum samples were grouped according to farm significant differences in the alpha diversity were observed with the Chao1 and Shannon index.

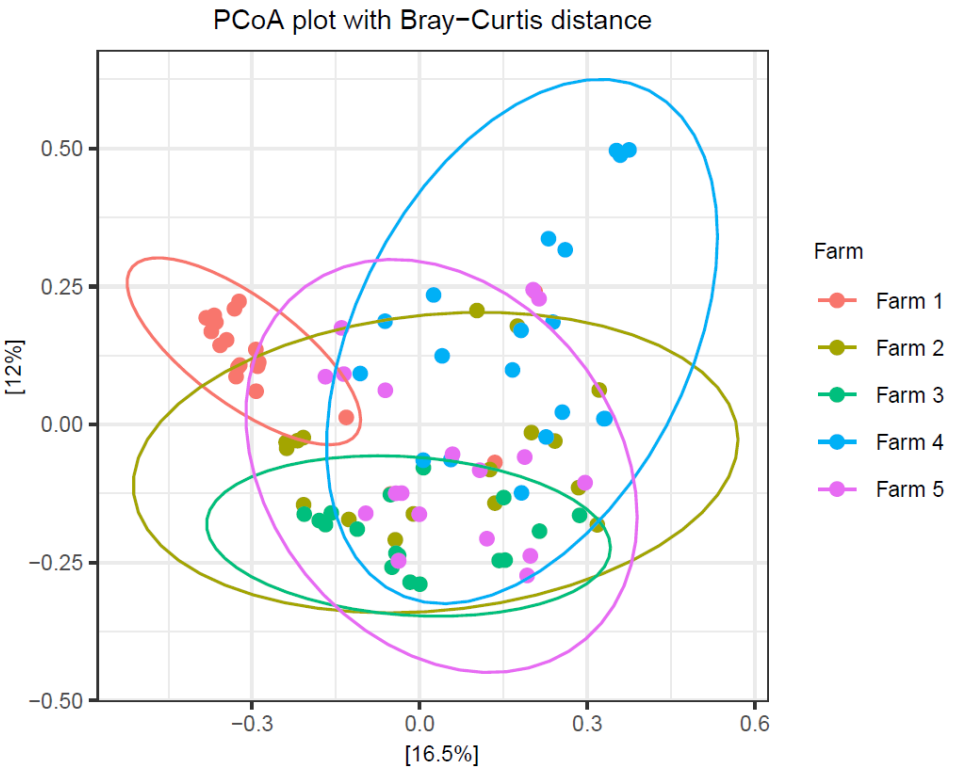



Figure 9: β-diversity analysis showed discrete grouping at farm level. Microbial diversity was significant between farms ($p < 0.05$). Highest significance was observed in Farm C ($p < 0.01$).

### 3.5 Parity and the microbial composition of bovine colostrum

When colostrum samples were grouped according to their parity status (0, 1, 2, 3, 4+), α-diversity metrics revealed no significant differences in richness and evenness ($p > 0.05$) (Figure 10). Similarly, β-diversity analysis showed no distinct grouping or significance in the microbial diversity between colostrum samples when grouped according to parity ($p > 0.05$) (Figure 11).

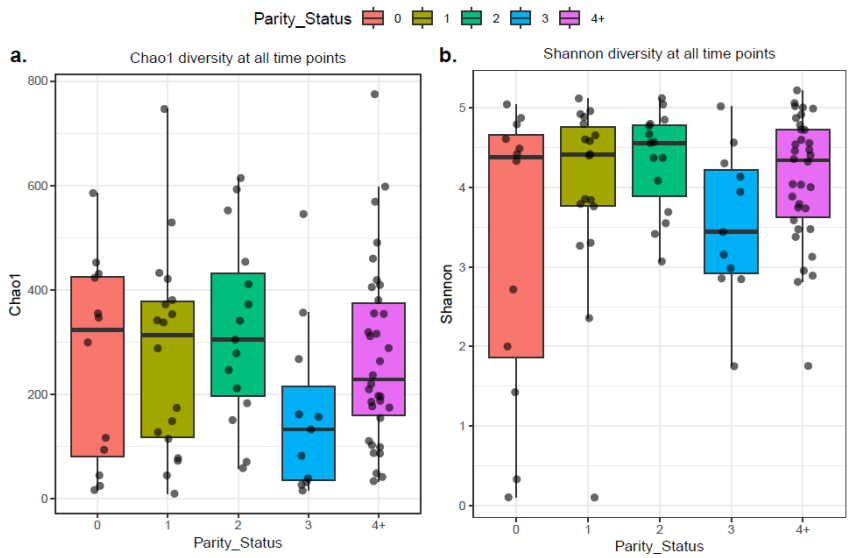



Figure 10: Colostrum samples grouped according to parity status (0, 1, 2, 3, 4+) showed no significant differences in alpha diversity. Both (**a**) Chao1 (estimated richness) and (**b**) Shannon (richness and evenness) indices were not significantly different across parity groups based on the Wilcoxon test. The *p*.adj values < 0.05 were considered significant.

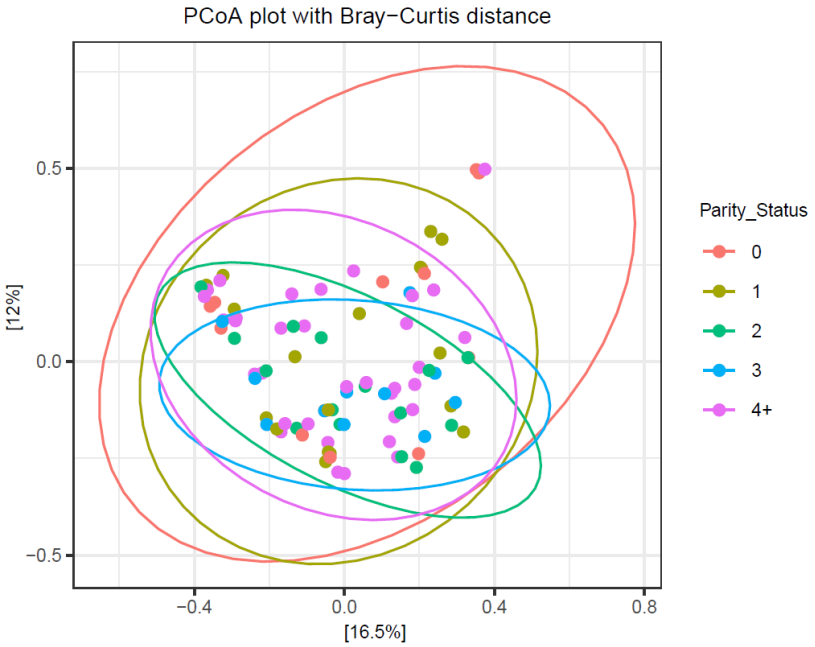



Figure 11: No significance in β-diversity was observed among colostrum samples when grouped by parity ($p > 0.05$).

# 4 Discussion

Extensive data are available on the microbial profile of bovine milk [61]; however, less is known on the influence of antibiotic treatment on bovine colostrum, particularly from healthy cows. Current EU legislation prohibits prophylactic antibiotic therapy in dairy cows [48], which has raised concerns amongst farmers for animal health during lactation [62]. In the present study, we sought to not only identify the microbial composition of bovine colostrum in Irish dairy cows but also assess the effect of antibiotic administration. The samples used in this study were collected prior to the implementation of the ban on Blanket DCT (February 2020). Thus, this study examined the influence of three methods of DCT on the microbiota of bovine colostrum. Finally, we also accounted for the influence of additional factors, including parity and inter-farm variation.

Our study confirms the presence of a highly diverse and rich microbial population in bovine colostrum that is subject to variation with DCT. In this study, the abundance of several phyla was established, with Firmicutes (48%), Actinobacteria (22%), Proteobacteria (21%), and Bacteroidota (7%) representing the predominant phyla, regardless of the chosen DCT method. Thus far, there has been broad agreement at the phylum level, and our findings align with previous studies [17,34,45,46,63]. The distribution of the predominant phyla in both milk and colostrum has been shown to vary with Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, accounting for 23–84%, 16–68%, 3–25%, and <0.1–8%, respectively [26,64–66]. Although variations in the relative frequencies of the dominant phyla have been established, the driving cause for such fluctuations is not fully understood. In our study, the phylum Firmicutes was dominant across all treatment groups. However, the relative abundance of Firmicutes was significantly lower in the NOAB group as opposed to both antibiotic-treated groups. Similarly, Bacteroidota was influenced by antibiotic administration, with the highest relative abundance in the NOAB group. Furthermore, the choice of antibiotic proved significant, with increased relative abundance in the UBRORED group. In contrast, a previous study noted no change in Bacteroidota abundance at the phylum level when comparing DCT with a teat sealant alone or a teat sealant with antibiotic

treatment [67]. The lower abundance of Firmicutes in the NOAB group is unexpected and the cause remains unclear. However, this may be a result of a more balanced community profile, allowing competing Bacteroidota to occupy a greater proportion of the colostrum microbiota. Additionally, farm level factors may contribute to the observed pattern, as Firmicutes are known to vary with housing, hygiene, and farm management conditions. Previous studies suggest that the relative proportions of phyla may be an indicator of the health status of the cow. For instance, an increased relative abundance of Proteobacteria was established in healthy cows, whereas in cows with subclinical mastitis, Firmicutes was predominant [66]. Moreover, Kaczorowski et al. suggest that the ratio of phyla abundance is key, with Firmicutes and Proteobacteria occurring in approximately equal amounts in a healthy cow [68], whereas the occurrence of subclinical mastitis creates a shift, dependent on the causative pathogen, allowing one phylum to dominate. Indeed, other studies have reported similar trends; with Proteobacteria dominating cases of *E. coli*-associated mastitis (98%), whereas Firmicutes were more abundant in *Streptococcus*-associated mastitis (69.6%) [69]. However, in healthy cows, the accuracy of the equal ratio remains unclear. Indeed, the same study noted abundances of 57.7% and 26% for Firmicutes and Proteobacteria in healthy dairy cows, respectively. These proportions are similar to those observed in our study, where the cohort of experimental cows were infection-free. Evidently, our findings substantiate existing data on the predominant phyla in bovine colostrum but also highlight fluctuations in their proportions as a result of antibiotic administration during drying off. The potential influence of such changes in relative abundance at the phylum level on ruminant health remains unclear.

In previous studies, much greater variation in relative abundances at lower taxonomic levels has been demonstrated, including genera. Of the wide variety of bacterial genera identified in bovine colostrum and milk, some include members with known probiotic, spoilage, and infectious properties. Commonly identified genera include *Acinetobacter*, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Pseudomonas*, and *Escherichia-Shigella* in both bovine colostrum [17,26,34,45,65,70] and milk [66,68,71,72]. In our study, the predominant genera

were *Psychrobacter* (10.75%), *Corynebacterium* (6.21%), *Facklamia* (6.06%), *Jeotgaliococcus* (4.21%), *Staphylococcus* (4.08%), *Lactococcus* (3.77%), *Acinetobacter* (2.96%), and *Leuconostoc* (2.34%). Unknown genera accounted for 23.86% and all other genera had an abundance of less than 2%.

We observed significant variations in the abundance of different genera as a result of DCT treatment. Our study demonstrated increased abundance of the *Facklamia* genus as a result of UBRORED administration when compared to NOAB-treated samples. Members of the genus have previously been associated with the bovine female genitourinary tract [73] and identified as a predominant member of the teat apex, cistern, and canal of healthy dairy cows [74,75]. Recently, *Facklamia* was identified as a predominant genus in raw bulk tank milk and an increased abundance was associated with high feed efficiency [76]. Additionally, two novel strains were identified in raw bulk tank milk, suggesting *Facklamia* may be a putative member of the milk microbiota [77]. Elsewhere, a study noted the highest abundance of *Facklamia* was associated with subclinical mastitis, followed by healthy cows, and lastly cows with clinical mastitis [78]. Overall, the genus is regarded as a commensal bacteria in the bovine milk microbiota [79]. However, its isolation from infected sources demands further exploration of its potential pathogenesis. Similarly, an increased abundance of *Jeotgaliococcus* was observed in the UBRORED group when compared with colostrum following natural DCT (NOAB). This genus was first isolated from a traditional Korean fermented seafood, jeotgal [80], and has since been identified in a wide range of sources including a poultry house, marine animals and environments, urban air samples, and goats milk [81–86]. *Jeotgaliococcus* was also identified in the bovine teat canal and on the bovine foot skin [74,87]. Recently, the genus has been identified in both healthy and subclinical mastitis milk [88]. Little is known of its functional role in milk, specifically regarding the potential pathogenesis and resistome profile of the genus, although resistance to heavy metals has been demonstrated [89]. Our results are consistent with *Facklamia* and *Jeotgaliococcus* being commensal bacteria of bovine colostrum, with increased abundance as a result of DCT with β-lactam antibiotics of the penicillin class.

In agreement with our findings, *Staphylococcus* has previously been identified as a dominant genus in milk samples from healthy cows, as well as subclinical and clinical mastitis milk samples [53,68,90]. Staphylococci are a primary cause of mastitis in dairy cows worldwide [78], with mastitis-associated staphylococci divided into two groups: *S. aureus*, a major mastitis causing pathogen, and coagulase negative (CNS) staphylococci, considered opportunistic mastitis pathogens, for example, *S. chromogenes*, *S. epidermis*, *S. haemolyticus*, *S. simulans,* and *S. xylosus* [91]. Both groups of *Staphylococcus* have been identified in milk from subclinical mastitis, clinical mastitis, and healthy cows [78,90,92,93]. Furthermore, staphylococci have been isolated from extra-mammary sites including the perineum skin, udder skin, teat apices, and teat canal of lactating cows, in addition to the farm environment [92,94,95]. In this study, the abundance of *Staphylococcus* was increased as a result of antibiotic treatment, regardless of the class of antibiotic administered. In contrast, a previous study noted the abundance of *Staphylococcus* was reduced following antibiotic treatment in cows with subclinical and clinical mastitis [78]. The observed differences between the two studies may be attributed to variations in the health status of the cow (healthy vs. clinical/subclinical), sampling matrix (milk vs. colostrum), mode, timing and class of antibiotic treatment (intracisternal administration of a broad-spectrum tetracycline) and analytical approach. The reason for increased abundance of *Staphylococcus* with antibiotic treatment in our study is unclear; however, it may be a result of disruption to the natural community of microbes in colostrum, facilitating increased *Staphylococcus* dominance. Despite the acknowledged existence of staphylococci as a commonly occurring microbe in colostrum and milk, its increased prevalence following antibiotic administration during DCT is a concern and warrants further research, due to the pathogenic nature of various members of the genus. The *Staphylococcus* genus is known to have both intrinsic and acquired virulence factors that contribute to their efficiency as a pathogenic organism and challenge the dairy industry at controlling their spread [91,96]. In addition, further contributing to this challenge and increasing the risk to animal welfare is the rising resistance to β-lactam antibiotics observed in both *S. aureus* and CNS *Staphylococcus* strains [91,97].

The highest prevalence of the genus *Corynebacterium* was observed in the UBRORED group, with a significantly lower abundance in the NOAB group, although the same was not observed in the CEF-treated colostrum. *Corynebacterium* is frequently isolated from milk, with members of the genus having both pathogenic and non-pathogenic roles. The prevalence in bovine milk is believed to be due to contamination on farms, facilitating the uptake of opportunistic pathogenic members and non-pathogenic colonizers. Previously, *Corynebacterium* was identified as a dominant genus in milk from Holstein dairy cows, with an increased abundance in winter milk samples when compared with summer sampling [76]. This may explain its high abundance across all samples in our study, as sample collection occurred following winter housing months. The genus *Corynebacterium* represents species frequently associated with mastitis, most notably *C. bovis*. Indeed, *C. bovis* was identified as the dominant species in subclinical mastitis milk and was associated with an increase in SCC [98]. Similarly, *C. bovis* was the predominant species in both healthy and infected samples and associated with an increased SCC [99]. Although *C. amycolatum* was also identified, it is believed to be less clinically relevant [99]. Likewise, other members of the genus were infrequently isolated in subclinical mastitis samples, and were not associated with increased SCC [98]. Indeed, in addition to the presence of the genus in clinically infected milk, it has also been established in healthy milk [68,78,99]. These findings indicate that although *C. bovis* is likely the predominant species from the genus in bovine milk, other members of the genus are present and their pathogenicity is unknown. Interestingly, a previous study noted increased levels of *Corynebacterium* following antibiotic treatment in both clinical and subclinical groups compared with pre-antibiotic administration [78]. Additionally, a lower abundance of *Corynebacterium* in clinical mastitis samples both before and after treatment was observed when compared with samples from the healthy cohort. *Leuconostoc* are facultative anaerobic lactic acid bacteria with a well-established role in food fermentation and technology [22,100]. They are commonly found in plants, dairy products, meats, and fermented foods [27], and are frequently identified in bovine milk [46,101]. Several species, including *L. mesenteroides* and *Leuconostoc lactis*, have been identified in bovine milk [102]. Despite their

technological value, members of the genus have previously exhibited high levels of resistance to vancomycin [103]. Furthermore, multiple *Leuconostoc* spp. have demonstrated multi-drug resistance, including resistance to tetracycline, chloramphenicol, trimethoprim, and vancomycin [104]. The increased abundance of *Leuconostoc* in the CEF-treated colostrum is unexpected; however, it may reflect suppression of more susceptible taxa by cefquinome, enabling *Leuconostoc* to persist or proliferate. Notably, this pattern was not observed in the UBRORED group, suggesting a treatment-specific effect. The potential antimicrobial resistance traits of *Leuconostoc* warrant further investigation. Future work should evaluate any implications for udder or calf health and characterize the antimicrobial-resistant capacities associated with *Leuconostoc*. We observed a high degree of commonality in the abundance of *Acinetobacter,* regardless of DCT approach. The high abundance of *Acinetobacter* in colostrum is not surprising, as it is commonly found in nature and frequently identified in soil and water-based ecological niches [105]. The genus has previously been identified in milk from healthy [68], subclinical [68,78], and clinical mastitis [57] cows. Of the genus, *Acinetobacter baumannii* has garnered the most attention due to its role in nosocomial infection, intrinsic antibiotic resistance, and ability to adapt to unfavourable environmental conditions [106]. Its presence in milk is linked with environmental contamination via the farm. Indeed, *A. baumannii* isolates were present in 21.2% of 280 dairy cows and identified on the bovine nasal area, the farm floor, and rectal swabs [107]. However, the genus consists of over 80 species [108]. Indeed, in bulk tank milk, 176 *Acinetobacter* species were isolated, with *A. baumannii* accounting for only 32% [109]. Separately, 27 strains of *Acinetobacter* were isolated from milk [57]. Evidently, colostrum is host to a highly diverse population of *Acinetobacter*. Although members of the genus have previously shown resistance to antibiotics of both the cephalosporin and penicillin class, their susceptibility has also been demonstrated [107,109]. The high abundance in the NOAB group and persistent abundance in the antibiotic-treated group is likely due to the role of *Acinetobacter* as an innocuous component of the bovine milk microbiota. In contrast to the findings presented here, a previous study noted highest levels of *A. baumannii* isolation from milk when a cephalosporin had been

used on the farm in the preceding six months [107]. Despite a growing understanding of the pathogenic members of the genus, little remains known of the non-pathogenic species. A recent review suggests the dualistic nature of *Acinetobacter*, acknowledging the pathogenicity of some whilst highlighting the potential role of the genus in biotechnological processes [105]. Evidently, further research is required of *Acinetobacter* species identified in milk to fully ascertain their role as part of the wider bovine colostrum microbiota network. Other predominant genera identified in our study include *Psychrobacter* and *Lactococcus*; no significant variations in their relative abundance occurred as a result of DCT.

Importantly, in our study, genera commonly associated with mastitis, including *Staphylococcus* and *Corynebacterium*, were higher in colostrum samples following blanket DCT than natural DCT. Additionally, a positive association was observed in *Pseudomonas* and *Enterococcus* abundance in the CEF group following differential analysis, with NOAB as the reference. Both *Pseudomonas* and *Enterococcus* have previously been identified as genera representing strains with pathogenic capabilities, namely *P. aeruginosa* and *E. faecalis*, respectively [110–112]. Evidently, the treatment of cows with antibiotics during DCT does impact the microbial composition of colostrum, with an increase in mastitis-associated genera observed. This may be due to the presence of other non-pathogenic species belonging to the same genus but could also indicate a disruption to the microbial population, resulting in increased abundances of genera with known virulence factors. Future studies should investigate the observed disruption by employing techniques such as shot-gun sequencing, which will allow for greater understanding of strain level disruption and the implication for long-term animal health and productivity. In particular, the potential presence of antibiotic-resistant bacteria and genes should be investigated. Critically, natural DCT did not result in increased pathogenic microbes, suggesting its potential as a farm management strategy.

Studies to date indicate a higher α-diversity for healthy milk samples and lower levels demonstrated in mastitis milk [45,71,113–115]. This is likely the result of

dominance by the causative pathogenic strain during infection, although the opposite has also been reported [79,90]. In the majority of studies to date, α-diversity following antibiotic administration was assessed in mastitic cows. For example, no significant variation was observed in the α-diversity of bovine milk samples from subclinical and clinical mastitis groups, in receipt of antibiotics, when compared with untreated healthy milk samples [78]. Furthermore, when comparing a mastitis group in receipt of antibiotics with a mastitis positive group not receiving antibiotic therapy, no difference in α-diversity was observed [115,116]. However, the effect of antibiotics alone cannot be determined due to the presence and potential influence of infection status, whereas in this study milk was collected from healthy cows who received prophylactic antibiotic administration. However, two previous studies examined the Chao1 and Shannon indices of colostrum samples from a cohort of healthy cows following antibiotic treatment or teat sealant alone and found no significant difference [64,72]. These findings are in contrast to those presented here. We identified a significant increase in microbial richness and evenness in colostrum samples from cows that received no antibiotic treatment during DCT, whilst the lowest was seen in the CEF-treated group. With regards to β-diversity, a previous study noted no significant difference between milk samples of infected cows in receipt of a broad spectrum antibiotic and the healthy group [78]. Furthermore, in a study similar to our own, β-diversity was not significant between those in receipt of antibiotics and those with teat sealant alone in a healthy cohort of cows [72]. Contrarily, for β-diversity analysis, we demonstrate a clear separation of the microbial diversity of the 'NOAB'-treated colostrum samples from those following either UBRORED or CEF administration during the dry period. Additionally, a longitudinal study carried out by our group on a subset of the same samples analysed in this study demonstrated that distinct microbial clustering of the NOAB group persisted in samples even at 2 months post-DCT [56]. This emphasises the long-lasting effects antibiotic administration can have on the microbial profile of bovine colostrum and mature milk. Furthermore, the same study noted an increased abundance of antibiotic-resistant genes in bacterial strains following CEF and UBRORED DCT. These findings lend to the growing concern of the rapid emergence and proliferation of antimicrobial resistance due to antibiotic therapy

during the drying off period. Our findings indicate that colostrum samples from cows that are treated naturally during DCT have a compositionally distinct microbiota to those following antibiotic therapy. Despite the high degree of commonality observed at phylum and genus levels, antibiotic administration does shift the microbial composition of colostrum. The separate grouping between treatment groups suggests potential dysbiosis in the microbial profile of bovine colostrum as a result of antibiotic administration. Dysbiosis has been suggested as a facilitating factor in the development of intramammary infections; however, whether mammary dysbiosis is the result of infection or the causative link remains unclear [21,23,24]. In addition, we also identified significant dissimilarity in microbial diversity between the two antibiotic-treated groups, Cephagaurd and Ubro Red, suggesting that the choice of antibiotic can further impact the colostrum microbiota profile. In this instance, both are broad spectrum β-lactam antibiotics. Cephagaurd is known to target mastitis-causing agents, including *S. aureus*, CNS *Staphylococcus,* and *Streptococcus* spp., including *S. uberis*, *S. dysgalactiae,* and *S. agalactiae*. Similarly, UBRORED also targets *Staphylococcus* and *Streptococcus* spp., in addition to *Corynebacterium*, *E. coli*, *Klebsiella*, and *Pseudomonas*. Bacteria exist within an interconnected and diverse microbial network. Fluctuations in the presence of one species can modify the functionality of the community, influencing the host and surrounding environment [95]. Further analysis of microbial disparity at the strain level will allow for a greater understanding of the influence of antibiotic therapy on bovine colostrum.

It is important to recognize that antimicrobials are a valuable tool in the treatment of disease amongst animals and humans. However, indiscriminate overuse has driven the need for a reduction in antimicrobial administration. The resulting EU ban on blanket DCT has led to the routine implementation of selective DCT on dairy farms. Previous studies have demonstrated the efficacy of selective DCT in treating and limiting the spread of infection, reducing the use of antimicrobials, without compromising animal health [117,118]. Similarly, teat sealant alone (natural DCT) has proven safe in low infection risk animals [67,119], further supporting the cessation of prophylactic antibiotic administration. Despite such findings,

scepticism persists, with concerns for long-term animal health and productivity, in addition to potential antibiotic requirements during lactation [118]. Alternative therapies have been extensively reviewed previously [112,120]. Of these, *L. lactis* DPC3147 proved equally effective to conventional antibiotic therapy in treating subclinical and clinical mastitis [121] through the stimulation of the host intramammary immune response [122,123]. A variety of probiotic-based mitigation strategies are now being explored. For example, a *Lactobacillus*-based teat dip proved effective in reducing mastitis-associated bacteria, suggesting an alternative to current chemical-based teat disinfectants [124]. In addition, bacteriocins are proposed as promising antimicrobials to be included in teat seal and udder preparation formulations for mastitis prevention. The potential of a germicidal sanitizer containing Nisin (AMBICINN®) was previously demonstrated against mastitis agents and was comparable to an iodine teat dip [125]. Indeed, WipeOut dairy wipes (ImmuCell Corporation) are now commercially available. Impressively, the inclusion of lacticin 3147 in teat seal formulations proved effective for mastitis prevention [126–129]. Furthermore, an intramammary infusion of Nisin Z had therapeutic effects in cows with subclinical mastitis, with a high recovery rate in *S. agalactiae* cases, although lower recovery was observed in *Staphylococcus*-based infections [130]. Other novel therapies currently under investigation include plant-derived compounds, postbiotics, and bacteriophage therapy [131–133]. Evidently, the development of alternative treatment and mitigation options has received considerable interest, although further establishment of their efficacy is still required. The development and implementation of novel therapies, in addition to a reduction in on-site antibiotic use, will ensure the continued efficacy of antibiotic therapy and poses a reduced threat at farm and environmental levels.

Parity exerted no impact on the microbial composition or diversity of bovine colostrum in our study and others [17]. An increase in microbial richness (Chao1 index) of colostrum from primiparous cows has been reported elsewhere [45]. However, when analysed for richness and evenness (Shannon index), no significance was observed. Separately, no significant difference in α-diversity of primiparous and multiparous cows was observed; however, β-diversity analysis

revealed distinct clustering of bovine milk from multiparous cows in early lactation [46]. Additionally, unlike our findings, both studies noted changes in relative abundances at the genus level. Despite conflicting reports regarding parity and the microbial composition, greater consensus exists in relation to parity and its influence on colostrum quality, determined by colostral Ig levels. Indeed, colostrum quality is known to increase with higher levels of parity [14,38,40,43]. In fact, a number of studies have noted that Ig content is highest in parity levels >3 [6,17,41,44]. This is most likely a result of the more mature and experienced immune system in comparison to the younger naïve immune system. Furthermore, nutritional components may also be influenced by parity, with higher fat and lactose levels in lower parity cows [14]. In contrast to microbial studies, findings to date are in agreement that parity does significantly affect the nutritional composition of colostrum; thus, it is likely that parity has greater influence on colostrum's nutritional and bioactive components rather than the microbial population.

At a farm level, significant variation in Shannon index and β-diversity was observed, with significance demonstrated between all farms. Antibiotic therapy is likely the primary cause for the observed differences; however, significance was still observed in α-diversity (Shannon index) between farm D and P, despite the same antibiotic (Cephagaurd) being used. Similarly, for β-diversity, despite the same choice of antibiotic, differences were observed between farm D and P (Cephagaurd) and farms L and T (Ubro Red). Therefore, our findings suggest that additional factors influence the microbial profile of bovine colostrum. As we can account for breed, parity, and season, we know these factors are not the cause in our study (see Supplementary Table S1). However, we suggest that farm management practices, the farm environment, and choice of feed may explain the difference found between the dairy farms evaluated. Indeed, a previous study of colostrum from farms in China noted significant variations in the microbial structure of colostrum between two farms at the genus level, despite a high degree of commonality in the predominant phyla [34]. Similarly, the group suggests that feed and farm management practices were responsible for the observed differences. Overall, we note variation in microbial diversity between farms, in addition to the influence of

antibiotic therapy. However, further investigation of genetic, environmental, and management factors need to be performed to determine the degree of influence on the bovine colostrum microbial population.

A key limitation in this study is the use of 16S rRNA sequencing at a single time point. Future studies should incorporate longitudinal sampling and higher resolution sequencing approaches. Shotgun metagenomics or whole-genome sequencing (WGS) would provide more detailed insights into the influence of DCT, strengthen the data on on-farm antibiotic usage, provide greater insight into the potential resistome of bovine colostrum, and enable a greater understanding of species level variability and functionality. Identifying the source of ARGs on dairy farms is critical to mitigate the global spread of antibiotic resistance. Further investigations are needed to determine the origin and mechanism of transmission, including horizontal and vertical gene transfer. To this end, the inclusion of both environmental (bedding, water, soil) and faecal samples would provide a more comprehensive insight into resistome dynamics on farms. In addition, quantitative techniques such as flow-cytometry-based methods, including Flow-FISH, could complement sequencing data by providing direct measurements of absolute bacterial loads in colostrum. Furthermore, the inclusion of an additional treatment group of cows with a positive infection status would indicate how infection modulates microbial responses to drying off. Future studies should monitor SCC and IgG levels to confirm udder health and evaluate any secondary effect of DCT on colostrum quality. IgG testing can be performed on-farm using a Brix refractometer; however, for improved accuracy, laboratory-based methods such as the radial immunodiffusion assay or ELISA can be performed [134,135]. For SCC testing, several on-farm techniques are available, including the California Mastitis Test, the Porta SCC test, and the Delaval Cell Counter [136]. In addition, an automated fluorescence-based cell counter, the Fossomatic, is widely used and provides high-throughput, accurate results. Finally, across microbiota studies to date, a number of different sampling, DNA extraction, and sequencing methods have been employed, potentially introducing variations in the data gathered following milk microbiota analysis.

This study provides new insight into how different DCT strategies influence the microbial composition of bovine colostrum under commercial dairy farm conditions. Our findings demonstrate that natural drying off is associated with a distinct colostrum microbiota, without an increased abundance of mastitis-associated genera. These results have practical implications for farm management practices, supporting the current move towards reduced prophylactic antibiotic administration and highlighting the need for further development of targeted non-antibiotic alternatives to maintain udder health.

## 5 Conclusion

These data indicate no increased abundance in mastitis associated genera with natural DCT. Mammary gland health is irrevocably linked with animal productivity, well-being and end product quality. We suggest non-antibiotic alternatives could be a superior long-term solution due to increased bacterial dysbiosis and resistance associated with prophylactic antibiotic administration. This study may have practical implications for farm management strategies during the dry period and lends support to the recent EU ban of blanket DCT. Rapidly evolving antimicrobial resistance is a global health concern and a reduction in the use of anti-microbials in dairy farming would have undeniable benefits both economically and from a One Health perspective and therefore the future development of targeted novel therapeutic strategies is critical.

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# Chapter 4

## Potential of lactic acid bacteria from bovine colostrum for food and health applications

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## Abstract

Lactic acid bacteria (LAB) are a phylogenetically and functionally diverse family of gram-positive micro-organisms, known to possess beneficial probiotic-like properties. Owing to their ability to produce a wide range of bioactive metabolites and their well-established safety profile, LAB have broad application in the biomedical and food sectors. LAB are widely distributed in nature, particularly in ecological niches associated with humans, animals and fermented foods. In this study, we screened 166 bacterial isolates from ten dairy farms in Ireland for a number of characteristics. Bacterial isolates underwent a series of identification and differentiation steps, resulting in the selection of 30 LAB strains identified with 16S rRNA Sanger sequencing. The identified LAB strains were further subjected to phenotypic characterisation for bacteriocin, exopolysaccharide (EPS) and bile salt hydrolase (BSH) production, with 13 %, 87 % and 17 % exhibiting positive phenotypes, respectively. Based on their phenotypic characteristic and taxonomic classification, ten colostrum-derived strains belonging to the genera *Loigolactobacillus*, *Levilactobacillus*, *Limsoilactobacillus*, *Lactiplantibacillus*, *Lacticaseibacillus*, *Lentilactobacillus* and *Lactococcus* were selected for whole genome sequencing (WGS) and subsequent analysis. Genomic mining identified the presence of a putative plasmid-borne bacteriocin gene cluster in a *Lacticaseibacillus paracasei* strain, encoding the bacteriocins Acidocin 8912 and BacSJ. This strain also demonstrated *in vitro* inhibitory activity against *Enterococcus thailandicus* and showed potential immunity to the anti-microbial effects of *Lactococcus lactis* and nisin A. Furthermore, a separate putative bacteriocin gene cluster encoding bacteriocin LSEI_2386 was identified in the genome of the same *Lc. paracasei* strain, as well as in two additional *Lc. paracasei* strains. However, due to the diffuse nature of the inhibition zones observed against *Lactobacillus delbrueckii subsp. bulgaricus*, and the absence of a corresponding mass peak with colony mass spectrometry, further investigation is needed to confirm their antimicrobial potential and verify the functionality of the predicted bacteriocin cluster. In addition, we identified a putative γ-Aminobutyric acid (GABA)-producing *Levilactobacillus brevis* strain and a BSH-producing

*Limsoilactobacillus mucosae* strain, both of which exhibited a positive EPS phenotype. Evidently, the bovine colostrum environment harbours a rich and heterogenous community of LAB with health-promoting properties, including anti-microbial production. Herein, we present bovine colostrum-derived LAB strains exhibiting promising characteristics ideal for potential development and application in the pharmaceutical and food industries. This diverse bank of colostrum-derived microbes warrants further exploration to fully characterize their functional potential and to determine optimal conditions for metabolite production.

## 1 Introduction

Bacterial strains of animal origin are numerous and often exhibit host-specific adaptions; they include bacteria with pathogenic and probiotic properties. Bacteria capable of producing metabolic by-products with health-promoting properties, including bacteriostatic activity, serve as ideal candidates as biopresevatives, food culture starters and even probiotic supplements (1). The microbial population associated with bovines is diverse, site specific and influenced by environmental factors. Previously the bacterial genera *Alistipes*, *Bacteroides* and *Clostridium* were identified in bovine faeces, *Fibrobacter*, *Prevotella* and *Ruminococcus* in the rumen and *Acinetobacter*, *Pseudomonas* and *Staphylococcus* in bovine colostrum (2-4). Bovine colostrum is a highly nutritious fluid consumed by the newborn calf, host to an array of bioactive compounds and a diverse microbial community (5). Colostrum composition is distinct from that of mature milk, with known specific nutritional and bioactive properties that do not extend to mature milk. The pH, temperature and nutritional constituents of bovine colostrum and milk make it an ideal environment for bacterial colonization and proliferation (6). Thus, the isolation and characterisation of bacterial strains from bovine colostrum as potential health-promoting bacteria is a promising strategy. Indeed, previous studies have reported the probiotic properties of LAB from both bovine milk (7) and colostrum (8).

Since the early 1900s, the recognition of bacteria as producers of bioactive and anti-microbial compounds, has revolutionized the study of ecological niches and resulted in the emergence of alternative, novel and naturally occurring therapeutic

agents (9, 10). Specifically, the application of bacteria or compounds derived from bacteria to develop probiotics, bio-preservative agents or act as starter cultures has been established (11). Probiotics were officially defined in 2001 by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) as '*live micro-organisms, which when consumed in adequate amounts confer a health benefit on the host*' (12). Biopreservation refers to the addition of bacteria with anti-microbial activity or competitive advantage, to improve food safety and extend the shelf life of the product by inhibiting spoilage or pathogenic microbes (1). The search for safe, naturally-occurring therapeutic and food agents requires the discovery of novel bacterial compounds with promising health and technological properties (13). In particular, lactic acid bacteria (LAB) have attracted considerable commercial and research interest. Indeed, members of the former *Lactobacillus* genus - recently reclassified into multiple genera following taxonomic revision (14) - are widely regarded as ideal probiotic candidates, due to their established health-promoting attributes and generally recognized as safe (GRAS) or qualified presumption of safety (QPS) status (15, 16). A recent study demonstrated that in patients with small intestinal bacterial overgrowth (SIBO) who suffered from depression and diabetes, treatment with a LAB capsule combined with escitalopram reduced depressive like symptoms, when compared with the control group (escitalopram alone). In addition, supplementation improved immune function, reduced inflammatory markers and reduced incidence of adverse side-effects to treatment (17). Separately, in patients with diarrhoea dominant irritable bowel syndrome, a probiotic formulation (*Lacticaseibacillus rhamnosus*, *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Bifidobacterium longum*, *Bifidobacterium bifidum* and fructooligosaccharides) gastrointestinal symptoms were significantly improved and the treatment was well tolerated (18). Moreover, in food and agricultural applications, LAB are often used as bio-preservatives or in the production of fermented foods as functional starter cultures, contributing to product flavour, texture and extending the shelf life (19-21). Indeed, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were successfully used as a starter cultures for yogurt fermentation, reducing syneresis whilst improving product texture and

sensorial properties (22). Evidently, the manipulation of bacteria producing bioactive metabolites is a promising strategy for the health and food industries. However, it is important to note that the possession of probiotic-like activity is strain dependent. As such, the establishment and validation of probiotic capacity, mode of action and optimal working parameters is necessary. Furthermore, any potential probiotic or live biotherapeutic strain of interest must undergo stringent testing, to ensure compliance with the safety requirements established by the European Food Safety Authority (EFSA) and the U.S Food and Drug Administration (FDA) (23).

Further driving the development of naturally-occurring biotherapeutic agents is the emergence and rapid proliferation of antibiotic-resistant and multi-resistant bacteria in the environment (24). LAB are avid producers of anti-microbial compounds, also known as bacteriocins (24). Bacteriocins are ribosomally synthesised anti-microbial peptides of bacterial origin with inhibitory activity against other sensitive bacteria (25). Bacteriocins are most commonly isolated from gram-positive bacteria with activity against closely related species (26). Their production provides bacteria with competitive advantage and improved survival in microbe rich matrices (24, 25). Most often, the inhibitory action of bacteriocins occurs through membrane permeabilization and pore formation of the target bacteria and disruption of cellular constituents, ultimately resulting in cell death (26). At present, bacteriocins are most commonly grouped into three major classes (I-III), according to their primary structures (27). Class I (lantibiotics) are post translationally modified peptides with characteristic ring forming lanthionine or methyllanthionine residues. Class II bacteriocins are small (< 10 k Da), unmodified, heat-stable peptides. Class III are large (> 30 k Da), unmodified, heat-labile proteins (20, 27). Class II bacteriocins can be further subdivided into four categories; IIa cationic one peptide pediocin-like bacteriocins, IIb consists of two complementary peptides, IIc bacteriocins with a cyclic structure and IId unmodified, linear, non-pediocin like bacteriocins (20, 27). The potential of bacteriocins to inhibit microbial growth, remain non-toxic and be compatible with the host microbiota, suggests that bacteriocins may be promising alternative therapeutic agents, helping to curb the spread of antibiotic resistance (28). In addition, an increase in consumer demand

for natural, non-chemical and safe additives presents bacteriocin-producing strains as ideal candidates for industrial development. For example, bacteriocin-producing *Lc. paracasei* strains isolated from fermented milk, inhibited the spread of pathogenic bacteria (*Listeria monocytogenes* and *Bacillus cereus*) during cheese ripening, whilst also improving cheese texture and flavour (29). Furthermore, in phase I clinical trials, children suffering re-current acute otitis media demonstrated an 84 % reduction in incidences when treated with a live biotherapeutic formulation of *Ligilactobacillus salivarius* PS7 (~1 × $10^8$ CFU), a producer of the bacteriocin Abp-118 (30). Moreover, bacteriocin formulations are now commercially available. These include, Nisin A produced by *Lactococcus lactis* subsp. *lactis*, a bacteriocin with broad spectrum of activity against gram-positive bacteria, as Nisaplin® (Danisco, Dupont) (31). In addition, the anti-listerial bacteriocin, Pediocin PA-1, produced by *Pediococcus acidilactici*, is now available as a fermentate, ALTA™ 2431 (Kerry Group PLC) (32).

Other advantageous and desirable technological properties that are simultaneously protective for the bacterial strain within the host environment includes EPS production and BSH activity. Several LAB produce polysaccharides that are released from the cell and excreted into the extracellular matrix as EPS (33). EPS are natural, non-toxic by products with diverse biological functions (34). EPS synthesis and secretion plays a role in cellular recognition, biofilm formation and surface adhesion (33). In addition, EPS production serves as a protective mechanism, shielding bacteria from hostile environmental conditions, including low pH, osmotic stress and anti-microbials (35). Furthermore, EPS-producing LAB have wide application in the development of functional and fermented food products, acting as thickeners, emulsifiers and stabilizers—ultimately improving food quality and texture (36). In addition, EPS-producing bacteria are considered ideal for probiotic development due to their antioxidant, immunomodulating capacity, anti-viral and anti-bacterial activity (36-38). Additionally, the emergence of BSH-producing LAB has garnered much interest. BSHs are a diverse family of intracellular enzymes, responsible for catalysing the hydrolysis of amide bonds in bile acids, resulting in the release of free bile acids and amino acids (39-41). BSH

enzymes have an influential role on both microbial and host physiology. BSH is associated with enhanced bile tolerance, acting as a colonization aid for the bacteria, ultimately enabling transit through the gastrointestinal tract (42, 43). Critically, BSH activity is associated with cholesterol lowering effects. Indeed, the deconjugation of bile acids by BSH-positive bacteria increases their hydrophobicity, reducing their absorption by enterocytes and in turn increases faecal excretion of cholesterol rich bile acids (41, 43, 44). Bacteria with BSH activity are considered to play a role in metabolic processes, including cholesterol, metabolism and inflammatory regulation (41, 44, 45). Furthermore, BSH activity in bacteria has been used as a functional marker for gut adapted strains. As such, BSH activity is oftentimes included in the selection criteria for probiotics (43).

In the present study, the antimicrobial capacity of LAB isolated from pooled bovine colostrum was examined. To this aim, bacterial strains underwent a series of identification and differentiation tests to enable a preliminary selection of strains, followed by phenotypic characterisation, including testing for potential bacteriocin, EPS and BSH production. Following *in vitro* evaluation, selected strains of interest underwent WGS and *in silico* analysis, enabling further characterisation of potential probiotic-like attributes.

## 2 Methods

### 2.1 Experimental design & sample collection

To develop a bank of colostrum milk microbes, colostrum samples were collected from 10 farms located across Southern Ireland (County Limerick, Cork and Tipperary). Daily sample collections were carried out from the 1st of February 2019, for 26 days. Herd size across the 10 farms varied, with an average herd size of 304. The total number of cows on all 10 farms was 3,040. The average collection per cow was 5.1 L. Colostrum samples for microbial analysis were collected in 100 mL sterile containers (Sarstedt) within 24 h of parturition. Colostrum samples were pooled prior to transportation. Following collection, samples were stored on ice and transferred to Teagasc, Moorepark on a daily basis. Samples for microbial composition analysis were labelled and stored at -80°C awaiting further analysis

(n=179), a minimum of one sample per farm, per week, was analysed. Sample analysis included fresh culturing for isolation, followed by a series of strain identification and characterisation steps to investigate the potential health-promoting attributes of the bacterial isolates.

## 2.2 Bacterial Recovery

For bacterial isolation, a 10-fold serial dilution was carried out on each sample. 1 mL of colostrum sample was diluted in 9 mL of maximum recovery diluent (MRD) (Oxoid Ltd). 100 µL of each serial dilution was spread-plated on to selective media. In this study, the following media was used: *Lactobacillus* selection (LBS) agar (Difco) supplemented with glacial acetic acid (Sigma Aldrich) and de Man, Rogosa, Sharpe (MRS: Difco) agar supplemented with 0.05% L-cysteine hydrochloride (Sigma Aldrich) both for the selection of lactobacilli. In addition, Reinforced Clostridial Agar (RCA) (Millipore) was prepared. All media were prepared according to manufacturer's instructions and supplemented with 50 U $mL^{-1}$ nystatin. All plates were incubated anaerobically at 37 °C for 48 h. Following incubation, individual colonies (3-5) were chosen from each plate and re-streaked on fresh plates of the appropriate media. Plates were incubated anaerobically for a further 48 h. Pure colonies were picked from each plate with a sterile loop and incubated anaerobically in overnight culture. Following incubation, 700 µL of culture was stocked in glycerol and stored at -80 °C awaiting further analysis.

## 2.3 Differentiation and Identification

### *2.3.1 Morphological Characterization and Catalase Activity Testing*

For light microscopy visualization, isolates were streaked onto the appropriate media and incubated anaerobically for 48 h. Using a sterile loop, a colony of interest was picked from the agar plate and transferred to a microscopic slide. A drop of sterile water was added to the slide and mixed with the colony before placing a cover slide on top. Individual colonies were viewed under the microscope to characterize their cell morphology. In addition, a catalase test was performed for further differentiation. A colony was selected and placed on to a clean, dry microscopic slide. A drop of 3 % $H_20_2$ (Hydrogen peroxide) was added directly to the slide with a transfer-pipet (Thermo Fisher Scientific). An isolate was deemed

catalase positive if the production of bubbles was observed within 30 s, if no bubbles were produced the isolate was catalase negative.

### *2.3.2 Random Amplified Polymorphic DNA (RAPD) PCR*

For RAPD PCR, isolates were re-streaked onto the appropriate media and incubated under anaerobic conditions for 48 h. Using a sterile loop, a single colony was picked from the plate and placed into a 2 mL collection tube with 15 µL of lysis buffer (100 µL triton x-100 (Sigma Aldrich), 200 µL Tris-EDTA 100x (Sigma Aldrich) and 9.7 mL deionised water). The sample was incubated at 95 °C for 10 min followed by centrifugation at 16,444 *g* x 10 min. Following centrifugation, the supernatant (10 µL) was transferred to a fresh collection tube and the cell debris was discarded. A 25 µL PCR reaction composed of 1 µL of supernatant (DNA), 12.5 µL of BioMix Red (Bioline), 1 µL GTG primer (1 µM) and 10.5 µL of PCR grade water was prepared. The PCR conditions for amplification were an initial denaturation step of 94 °C for 10 min followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final step of 72 °C for 10 min. Following RAPD PCR, the PCR product of each isolate was grouped according to farm and visualized using gel electrophoresis at 100 V for 30 min (1X TAE buffer and 1.5% agarose (Sigma Aldrich)). Isolates with identical band patterns, from the same farm were accepted as the same strain and only one was selected for further identification and characterisation steps.

## 2.4 Species determination

Bacterial isolates were grown anaerobically in overnight culture at 37 °C. Genomic DNA was extracted using the GenElute Bacterial Genomic DNA kit (Sigma Aldrich) in accordance with the manufacturer's protocol. Following genomic extraction, samples were prepared for PCR amplification. Each 25 µL PCR reaction contained 1 µL of supernatant (DNA), 12.5 µL of BioMix Red (Bioline), 1 µL 27F and 1 µL 1472R primers (1 µM) and 9.5 µL of sterile PCR grade water (Thermo fisher scientific). The 16S rRNA genes were amplified using the primers 27F (5'-GAG AGT TTG ATC CTG GCT CAG-3') and 1472R (5'-TAC CTT GTT ACG ACT T-3'). The PCR conditions for amplification were an initial denaturation step of 94 °C for 2 min followed by 35 cycles of 94 °C for 20 s, 55 °C for 30 s, 72 °C for 1 min 30 s and a final elongation step of 72 °C for 10 min. The resulting amplicons were visualized using gel

electrophoresis (1X TAE buffer, 1.5% agarose at 100 V). The PCR amplicons were purified using the GenElute PCR Clean-Up Kit (Sigma Aldrich) in accordance with manufacturers guidelines. Following purification, samples were sent to GENEWIZ, Inc. (Takeley, Essex, UK) for 16S Sanger sequencing. Once sequence data was obtained, the FASTA file of each isolate was uploaded onto the National Centre for Biotechnology Information (NCBI) through the nucleotide basic local alignment search tool (BLASTn) database. Bacterial isolates were assigned to a species level.

## 2.5 Characterisation of Lactic Acid Bacteria from Bovine Colostrum

### *2.5.1 Bile-salt hydrolase plate assay*

To assess the ability of strains to deconjugate bile acids, a BSH activity assay was performed as previously described (46). 5 µL of fresh overnight culture was spotted onto mMRS (MRS, Difco) agar containing filter sterilised 0.037% (w/v) calcium chloride ($CaCl_2$) (Sigma Aldrich) and 0.5% (w/v) taurodeoxycholic acid (TDCA) (Sigma Aldrich). Plates were incubated anaerobically at 37 °C for 48 h. All plating was performed in duplicate. *Lm. mucosae* DPC 6426 was used as a positive control (44). A bacterial strain was deemed positive for BSH activity if an opaque halo-like ring formed surrounding the spot culture. BSH activity assays were performed in triplicate and results recorded with activity level scored +, ++, +++, ++++ (least to most).

### *2.5.2 Exopolysaccharide production*

To determine the ability of bacterial strains to produce EPS, a loop touch test was performed. 5 µL of fresh overnight culture was spotted on MRS agar from first principles as previously described and supplemented with 7% (w/v) glucose (Sigma Aldrich) (47). Plates were incubated anaerobically at 37 °C for 48 h. All plating was performed in duplicate. Following incubation, a sterile loop was touched against an individual colony and slowly pulled away. An observable string or filament between the loop and colony was recorded as positive EPS production and the level of production was scored (48). *Lm. mucosae* DPC 6426 was used as a positive control (44). EPS production assays were performed in triplicate.

### *2.5.3 In vitro anti-microbial activity*

#### 2.5.3.1 Deferred antagonism assay

To screen for bacteriocin production by colostrum-derived strains, an agar-based deferred antagonism assay was performed. *L. delbrueckii* subsp. *bulgaricus* LMG 6901 was used as an indicator strain. Bacterial strains were streaked onto mMRS agar and incubated anaerobically at 37 °C for 48 h. A single colony was picked with a sterile loop and inoculated in mMRS broth and incubated anaerobically for 16-20 h. Following incubation, 3 µL of culture was spotted onto mMRS agar and allowed to dry. Plates were overlaid with 50 mL of tempered (50 °C) sloppy mMRS agar (0.75% w/v), seeded with a 1 % inoculum (500 µL) of the indicator strain culture and allowed to dry. Once dry, the plates were placed under UV light for 20 min at maximum strength. Plates were incubated anaerobically at 37 °C overnight. Following incubation, plates were examined for the presence of a zone of clearing. *L. lactis* was used as a positive control.

#### 2.5.3.2 Well diffusion assay

As the zones observed in deferred antagonism assays can be the result of acid production, bacterial strains were further characterized with a well diffusion assay (WDA). A zone of clearing following a WDA indicates a degree of microbial inhibition between the producer (isolate of interest) and the indicator (49). Both the cell-free supernatant (CFS) and cell-bound activity was tested. Bacterial strains were streaked onto mMRS agar and incubated anaerobically at 37 °C for 48 h. Following incubation, pure colonies were inoculated in 10 mL of mMRS broth and incubated anaerobically at 37 °C for 16-20 h. Plates were prepared by adding a 1 % (500 µL) inoculum of the indicator strain to 50 mL of tempered mMRS agar (1% w/v), the mixture was briefly swirled and poured rapidly. Once the plate had set, using sterile glass Pasteur pipettes, 6 mm wide wells were bored into the mMRS agar. The overnight cultures were removed from the incubator and vortexed gently for 2 min. 1 mL of overnight culture was transferred to a 2 mL Eppendorf tube and centrifuged at maximum speed 16,444 *g* for 10 min at 4 °C. The supernatant (CFS) was carefully removed and transferred to a fresh Eppendorf tube. 50 µL of CFS was transferred into the wells of 1% mMRS agar. The remaining cell pellet was resuspended in 1 mL of 70 % Isopropyl alcohol (IPA) and transferred to the wells of a separate 1% mMRS

agar plate to examine cell-bound activity. Plates were incubated anaerobically at 37 °C overnight. Two indicator strains were used for the WDAs (*L. delbrueckii* subsp. *bulgaricus* LMG 6901 and *Enterococcus thailandicus*). All well diffusion assays were performed in triplicate. Two positive controls were used: *L. lactis* and Nisin A. Bacterial strains were deemed positive for potential anti-microbial activity with the presence of a circular zone of inhibition surrounding the culture of interest following incubation.

#### 2.5.3.3 Protease sensitivity

A crescent moon protease assay was performed to determine the susceptibility of anti-microbials produced by the putative bacteriocin-producing colostrum strains to the proteolytic enzyme, proteinase K, as previously reported (50). 20 µL of overnight culture of the strain under investigation was spotted onto the centre of a fresh mMRS agar plate and allowed to dry. Plates were incubated anaerobically at 37 °C for 24 h. Following incubation, the plate was placed under UV light for 20 min at maximum strength. Following UV treatment, 20 µL of proteinase K (20 mg $mL^{-1}$) (Sigma Aldrich) was spotted next to colony and allowed to dry, before incubating anaerobically for 1 h at 37 °C. 10 mL of tempered (50 °C) soft mMRS agar (0.75% w/v) was seeded with a 1 % inoculum of the indicator strain and poured over the mMRS agar plate with the spotted culture and enzyme. Plate was incubated anaerobically at 37 °C overnight. Following incubation, the plates were inspected for a zone of inhibition in the growth of the indicator strain. If the zone of inhibition was circular, this suggests that the anti-microbial produced is insensitive to protease digestion. An indentation in the zone of inhibition resulting in a “crescent moon shape”, indicates the anti-microbial produced is sensitive to protease digestion and proteinaceous in nature.

#### 2.5.3.4 MALDI TOF Mass Spectrometry analysis

Bacterial strains were streaked on mMRS agar and incubated anaerobically at 37 °C for 48 h. For colony mass spectrometry, bacterial colonies were collected from the plate with a sterile loop and mixed with 50 µL of 70 % Propan-2-ol containing 0.1% trifluoroacetic acid (TFA). The suspension was vortexed and centrifuged using a benchtop centrifuge (19,066 *g* x 30 s). Cell extracts were analysed for masses of

interest in positive ion linear mode on an iD Plus Performance MALDI TOF Mass spectrometer (Shimadzu Europa GmbH, Duisburg, Germany) and the supernatant was retained for subsequent analysis. For supernatant analysis, MALDI-TOF mass spectrometry was performed with an Axima TOF$^2$ MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5 µL aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10 mg mL$^{-1}$ in 50 % acetonitrile 0.1 % TFA (v/v) was placed onto the target and left for 20 s before being removed. The residual solution was allowed to air dry and the sample solution deposited on to the pre-coated sample spot. 0.5 µL of matrix solution was added to the sample and allowed to air dry. The sample was subsequently analysed in positive ion reflection mode (adapted from Field et al. (51)).

#### *2.5.4 Haemolytic activity*

Columbia Agar (Sigma Aldrich) with 5 % (v/v) sterile defibrinated sheep blood (EO labs) was prepared in accordance with manufacturer's instructions. To prevent coagulation, defibrinated sheep blood was allowed warm to room temperature before it was added to autoclaved Columbia agar. The Columbia blood agar was inverted several times to ensure a homogeneous mixture, poured gently and allowed to set. Plates were streaked with strains of interest and incubated anaerobically at 37 °C for 48 h. Following incubation, plates were examined for haemolytic activity. Haemolytic activity was characterized as β-haemolysis (lysis of blood cells, clear zone), α-haemolysis (green discolouration around colonies) and γ-haemolysis (no haemolysis, colonies remained unchanged).

### 2.6 Whole genome sequencing

Following preliminary 16S rRNA identification and phenotypic characterisation, 10 colostrum-derived strains with promising probiotic properties were selected for WGS with MicrobesNG (Birmingham, UK). Bacterial strains were grown in mMRS broth at 37 °C to achieve bacterial growth with an optical density of 600 nm (OD600). 1 mL of culture was transferred to an Eppendorf tube containing 500 µL of DNA/RNA shield (Zymo Research). Samples were shipped to MicrobesNG for WGS. In brief, genomic libraries were prepared using the Nextera XT Library Prep Kit (Illumina, San Diego, USA) in accordance with manufacturer guidelines, with two modifications,

input DNA was increased 2-fold and PCR elongation was increased by 45 s. Libraries were sequenced using an Illumina NovaSeq 6000 (Illumina) with a 250 bp paired end protocol. The removal of adapter sequences and low-quality reads was performed using the software Trimmomatic v 0.30 (52) with a sliding window quality cut off of Q15. The quality of reads was assessed with in-house MicrobesNG scripts combined with the software: Samtools, BedTools and bwa-mem (53-55). *De novo* assembly was achieved using SPAdes (v3.7.0) (56). Enhanced contig FASTA files (.fna) generated through hybrid sequencing by MicrobesNG were used for downstream analysis. Genome annotation was performed using Bakta (v1.9.3) with default parameters.

### 2.7 Average Nucleotide Identity

Complete reference genomes for each of the 10 species of interest (*Lc. paracasei, L. lactis, Loigolactobacillus coryniformis, Lm. mucosae*, *Lv. brevis*, *Lentilactobacillus buchneri* and *Lactiplantibacillus plantarum*) were downloaded from the NCBI RefSeq database using the NCBI Datasets command-line interface (v14.33.0). Only assemblies at the chromosome or complete level were retrieved using the --assembly-level chromosome, complete and --include genome parameters to ensure high-quality genome data. Downloaded datasets were initially in dehydrated format and were rehydrated using the datasets rehydrate command following extraction with unzip. In total 117, 113, 7, 38, 9, 4, 338 RefSeq genomes for *Lc. paracasei*, *L. lactis*, *Lm. mucosae*, *Lv. brevis*, *Lt. buchneri, Lg. coryniformis* and *Lp. plantarum* were downloaded, respectively. Taxonomic identification of the downloaded reference genomes was done using the Genome Taxonomy Database (GTDB) (v2.4.0) to ensure accurate species-level classification. The resulting FASTA (.fna) files were then used for Average Nucleotide Identity (ANI) analysis to assess genomic relatedness. ANI values between each genome of interest and its corresponding species-level RefSeq genomes were calculated using FastANI (v1.33) with default parameters.

In addition, the fastANI tool within the Proksee platform was used for ANI analysis of query strains against selected reference genomes (57). The FASTA files of reference genomes, *Lv. brevis* DPC 6108, also known as Lbr-6108

(GCA_001722065.1) and *Lm. mucosae* DPC 6426 (GCF_000789175.1) were downloaded from the NCBI database and uploaded to Proksee to enable ANI (%) comparison with the query strains. Furthermore, the same process was repeated to perform ANI comparison between the two *Lp. plantarum* and three *Lc. paracasei* strains.

### 2.8 Phylogenetic comparison

Phylogenetic relationships between the bacterial genomes and their respective RefSeq reference genomes (outlined in section 2.7) were inferred using PhyloPhlAn v3.0.3 (58). Species-level phylogenies were constructed using species-specific marker genes. The appropriate marker gene database was first initialized using the phylophlan_setup_database command with the -g option set to the target species (e.g., s__Lactobacillus_plantarum). A custom configuration file was generated using phylophlan_write_config_file, specifying key parameters such as mafft for multiple sequence alignment, trimal for alignment trimming, and both fasttree and raxml for tree inference. The phylogenetic tree was constructed using the phylophlan command with the species-specific database generated with parameters optimized for species-level resolution, including --not_variant_threshold 0.99, --diversity low, and --force_nucleotides.

### 2.9 *In silico* prediction of bacteriocin gene clusters

Annotated BAKTA genome files (.GFF) of colostrum strains with positive WDA results were uploaded to the online bacteriocin mining tools BAGEL4 (59) and antiSMASH7.0 (60) to identify putative bacteriocin gene clusters. To confirm the identity of genes, the amino acid sequences of predicted core and accessory genes were uploaded to the NCBI BLASTprotein (BLASTp) database. Further identification steps included the BLAST tool on the Collection of Anti-Microbial Peptides (CAMPR4) database for the amino acid analysis of predicted core peptides (61) and the Interpro 106.0 database for domain analysis (62). For genomic comparisons of putative clusters against known bacteriocin operons, the genbank output files from antiSMASH were uploaded to clinker (63). Similarly, clinker was used for the visualization of bacteriocin gene clusters. Pairwise and Multiple sequence alignment and percentage identity was performed with Clustal Omega (64).

### 2.10 Prediction of GABA production

A search was performed using the NCBI database for complete CDS sequences of gad related genes. Search inputs were: “gadA and Lactobacillus’, “gadB and Lactobacillus”, “gadC and Lactobacillus”, “gadR and Lactobacillus”. This search was repeated but the genus of interest was replaced with ‘Lactococcus’. The FASTA files of all complete CDS sequences from NCBI were downloaded and combined into one file using cat. Partial sequences and truncated genes were excluded to minimize false positives. To search for homologous sequences in the genomes of strains, a BLAST database was created for each whole genome assembly using makeblastdb (BLAST+ v2.14.1) with the nucleotide option. Each genome was queried against the combined GABA gene database using BLASTn, with parameters set to an e-value threshold of 1e-10. BLAST output files were imported into R (v4.3.2) for downstream filtering and visualization using the dplyr, tidyr, and pheatmap packages. Only high-confidence hits were retained, defined by a minimum percent identity of ≥ 90 % and alignment length ≥ 1000 bp. A heatmap was generated to visualize the maximum percent identity for each gene hit per genome, using colour gradients to reflect sequence similarity.

### 2.11 Genotype Analysis of Antibiotic Resistance Genes

The software resistance gene identifier (RGI) v6.0.3 based on the comprehensive antibiotic resistance database (CARD) was used to identify the presence of antibiotic-resistance genes in the genomes of the LAB (65). The summary output file was uploaded to R and for each predicted antimicrobial resistant gene percentage identity and coverage (i.e., percentage of reference sequence length) were extracted and used for further analysis and heatmaps were generated for visualization.

### 2.12 Analysis of Carbohydrate Active Enzymes

Carbohydrate-active enzymes (CAZymes) were identified using the dbCAN (v4.1.4), which integrates three tools: HMMER, DIAMOND, and Hotpep (dbCAN-sub). Genomes were submitted in .fna format and analysed using run_dbcan in prokaryotic mode. Output files from dbCAN was uploaded to R. Only gene families supported by at least two of the three tools were retained. To focus on key CAZyme

types, only glycoside hydrolases (GH) and glycosyltransferases (GT) were included in the downstream analysis. Using the pheatmap package, a heatmap was created clustering the Glycosyltransferases (GTs) and Glycoside hydrolases (GHs) of interest ("GH54", "GT31", "GH13", "GH19", "GH23", "GH24", "GH73", "GH1", "GH25", "GT2", "GT4", "GT32", "GT14"). These families were selected based on previous literature reporting their roles in EPS production in LAB (35, 66).

Table 1: The number of colonies (n) derived from bovine colostrum on selective media at each stage of differentiation and identification.

| Media | Fresh from culturing | Post-microscopy | Post-RAPD PCR |
|---|---|---|---|
| RCA | 82 | 6 | 5 |
| LBS | 47 | 24 | 22 |
| mMRS | 70 | 38 | 17 |
| **Total** | 199 | 68 | 44 |

# 3 Results

## 3.1 Identification of putative Lactobacillus from pooled colostrum

A total of 166 bacterial isolates were isolated from pooled bovine colostrum emanating from 10 different Irish dairy farms. Using selective media RCA, LBS and mMRS, a total of 82, 70 and 47 bacterial isolates were collected, respectively. All isolates were visualised using light microscopy to determine their morphological features. A total of 68 isolates were identified as putative *Lactobacillus* based on their rod shape morphology and a catalase negative test (Table 1). Visualization of RAPD PCR products by gel electrophoresis allowed for the comparison of band patterns of individual bovine colostrum isolates (Figure 1). Isolates with identical band patterns, originating from the same farm, were suspected to be the same strain and only one was brought forward for further characterisation to avoid duplication. Following RAPD PCR, 44 isolates of interest remained. Following the initial differentiation steps, isolates were subjected to 16S rRNA Sanger sequencing and identified in accordance with the NCBI BLASTn database. A total of 29 sequenced colostrum-derived strains were identified (97-99% similarity) as members of the Lactobacillaceae family, namely *Lc. paracasei* and one strain was

identified as a member of the Streptococcaceae family, *L. lactis*. A comprehensive breakdown of strain identities following 16S rRNA Sanger sequencing is given in Table 2.

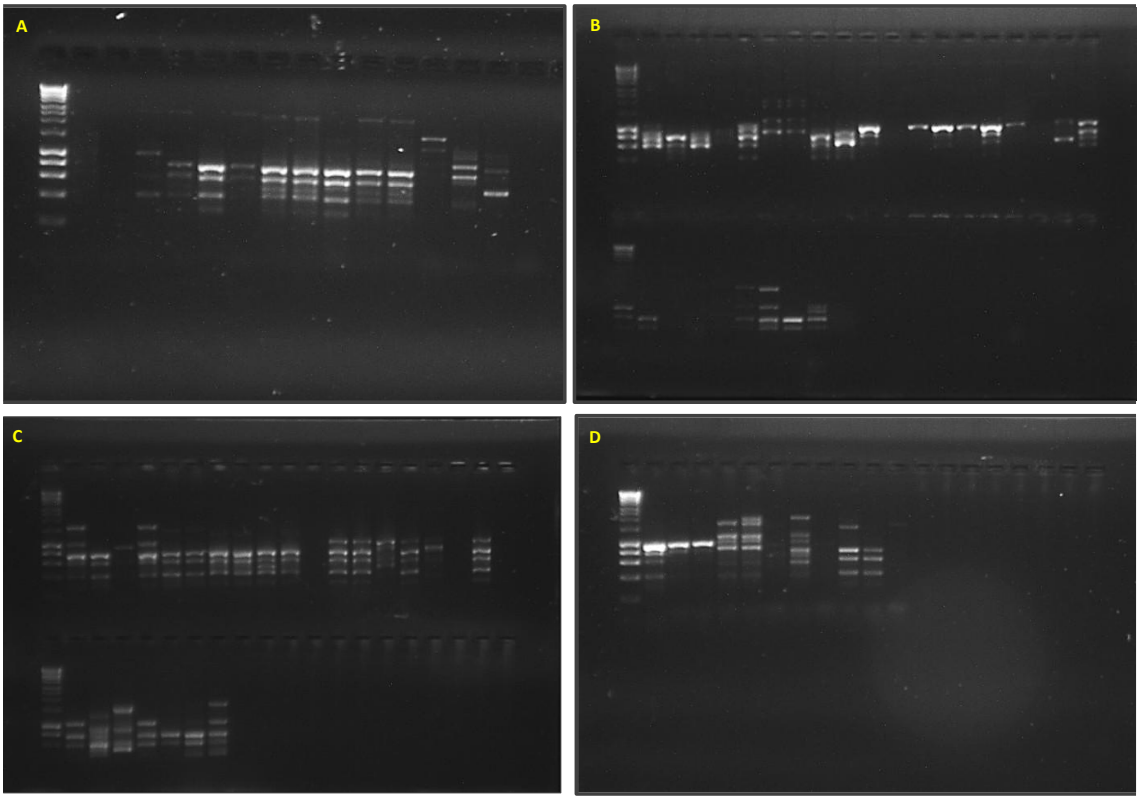



Figure 1: RAPD PCR visualisation with gel electrophoresis. (**A**) Farm 1 and Farm 2 isolates (**B**) Farm 3, 4 and 5 isolates (**C**) Farm 6-10 isolates (**D**) a re-run of isolates that produced no band pattern on gels A-C. Isolates with identical band patterns, originating from the same farm, were suspected to be the same strain and only one was brought forward for further characterisation.

Table 2: Species classification of LAB (n=30) from bovine colostrum following 16S rRNA Sanger sequencing according to the NCBI BLASTn database.

| SAMPLE NAME | MEDIA | CLASSIFICATION OF CLOSEST SPECIES | ACCESSION NUMBER | IDENTITY (%) |
|---|---|---|---|---|
| **F2.C16.2R** | RCA | *Lactococcus lactis* subsp. *cremoris* | CP003132.1 | 98 |
| **F5.C9.2** | mMRS | *Lactobacillus coryniformis* subsp. *torquens* | MT538965.1 | 97 |
| **F6.C16.1** | LBS | *Lactobacillus casei* | MF191597.1 | 96 |
| **F5.C3.3** | LBS | *Lentilactobacillus buchneri* | OR029295.1 | 98 |
| **F5.C22.1** | LBS | *Lactobacillus paracasei* | KR006277.1 | 99 |
| **F5.C3.2** | mMRS | *Lentilactobacillus buchneri* subsp. *silagei* | AP028329.1 | 97 |
| **F5.C22.2** | LBS | *Lactobacillus paracasei* | KR006277.1 | 97 |
| **F1.C8.1** | mMRS | *Lactobacillus parabuchneri* | MT211334.1 | 98 |
| **F1.C8.2** | mMRS | *Lactobacillus paracasei* | MT611809.1 | 97 |
| **F5.C3.1** | mMRS | *Lentilactobacillus buchneri* subsp. *silagei* | AP028329.1 | 98 |
| **F7.C5.3** | mMRS | *Lactobacillus paracasei* | MH656945.1 | 97 |
| **F1.C16.1** | mMRS | *Lactobacillus casei* | MF191597.1 | 97 |
| **F5.C9.1** | mMRS | *Levilactobacillus brevis* | OR857375.1 | 98 |
| **F9.C7.1** | LBS | *Lactobacillus casei* | MF191597.1 | 96 |
| **F4.C3.1** | LBS | *Lactobacillus paracasei* subsp. *tolerans* | MK774566.1 | 98 |
| **F9.C11.1** | LBS | *Lentilactobacillus buchneri* subsp. *silagei* | AP028329.1 | 97 |
| **F7.C16.2** | LBS | *Lactobacillus buchneri* | MT604653.1 | 98 |
| **F3.C16.1** | LBS | *Lentilactobacillus buchneri* subsp. *silagei* | AP028329.1 | 97 |
| **F3.C16.1M** | mMRS | *Lactobacillus paracasei* subsp. *tolerans* | MK774566.1 | 98 |
| **F10.C11.1** | LBS | *Lacticaseibacillus paracasei* | CP035563.1 | 97 |
| **F5.C16.2** | LBS | *Lactobacillus casei* | MF191597.1 | 97 |
| **F3.C2.2** | LBS | *Lactobacillus casei* | MF191597.1 | 97 |
| **F3.C2.1** | mMRS | *Lactiplantibacillus plantarum* | ON799439.1 | 95 |
| **F3.C2.1M** | mMRS | *Lactiplantibacillus plantarum* | OQ096578.1 | 98 |
| **F1.C1.2** | LBS | *Lactobacillus casei* | MF191597.1 | 96 |
| **F3.C16.3** | LBS | *Lentilactobacillus buchneri* subsp. *silagei* | AP028329.1 | 99 |
| **F1.C1.1** | LBS | *Lactobacillus paracasei* | MT463626.1 | 99 |
| **F9.C7.2** | LBS | *Lactobacillus paracasei* | MT463428.1 | 98 |
| **F2.C1.1** | LBS | *Lactobacillus parabuchneri* | MT211334.1 | 97 |
| **F2.C16.2** | LBS | *Lactobacillus mucosae* | MT613556.1 | 98 |

## 3.2 Microbial assays for the characterisation of lactic acid bacteria from bovine colostrum

### *3.2.1 Exopolysaccharide production*

A loop touch test was employed to detect bacterial strains with an EPS phenotype on mMRS agar plates supplemented with 7% (w/v) glucose. High levels of EPS production were observed in the bank of microbes, a total of 26 strains (87 %) produced ropy/slimy colonies, indicative of EPS production. The level of EPS production varied across LAB, indeed of the 30 strains screened, 3 were categorised as "++++" and 10 were categorised as "+", indicating high to low levels of EPS production, respectively (see Table 3). The most pronounced EPS production was observed in *Lt. buchneri* and *Lm. mucosae* (Figure 2).

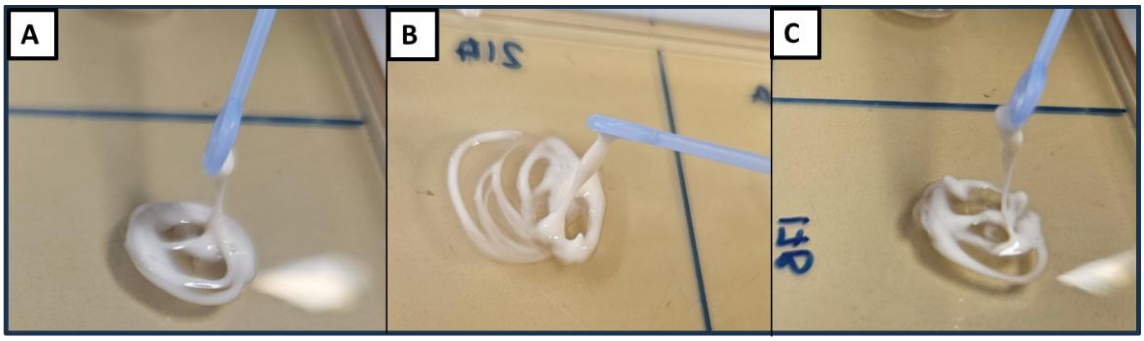



Figure 2: A loop touch test to examine EPS production. Strong EPS production in bacterial strains isolated from bovine colostrum (**A**) *Lm. mucosae* F2.C16.2 (**B**) *Lt. buchneri* F3.C16.1 (**C**) *Lt. buchneri* F9.C11.1

### *3.2.2 Bile salt hydrolase assay*

BSH activity was determined with the formation of an opaque halo-like precipitation zone around the colony when spotted on mMRS + $CaCl_2$ plates. A precipitation zone indicated the ability of a strain to hydrolyse the TDCA sodium salt present in the agar medium. In general, low levels of BSH activity was observed in colostrum strains, with only 5 of 30 scored as positive for BSH activity (see Table 3). The strongest BSH activity was observed in *Lt. buchneri* and *Lm. mucosae* (see Figure 3).

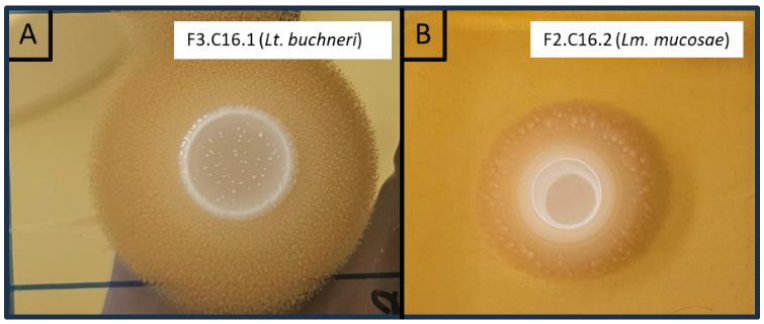



Figure 3: BSH production was determined on mMRS + $CaCl_2$ plates with the formation of a precipitation zone. Strong bile salt hydrolase activity in *Lt. buchneri* F3.C16.1 and *Lm. mucosae* F2.C16.1

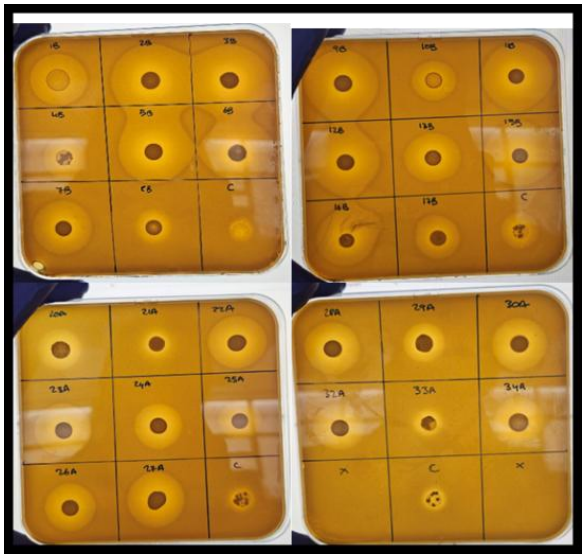


Figure 4: Deferred antagonism assay of colostrum-derived microbes with indicator *L. bulgaricus*

### *3.2.3 Potential bacteriocin production by LAB*

A total of 30 LAB were screened for bacteriocin production. All colonies displayed zones of clearing against *L. bulgaricus* LMG 6901 in the agar-based deferred antagonism assay (see Figure 4). As such, all strains underwent further screening and were subjected to a WDA with *L. bulgaricus* LMG 6901 as an indicator strain. The CFS of 3 strains (10 %) produced putative zones of inhibition, *Lc. paracasei* F7.C5.3, *Lc. paracasei* F10.C11.1 and *Lp. plantarum* F3.C2.1. The zones of clearing were consistently active in three separate assays. However, the zone boundary was diffuse in nature, which may be indicative of acid production, as CFS was not neutralised (Figure 5). In addition, in both an overlay and WDA, the observed zone

of clearing were unaffected by proteinase K treatment. Therefore, the observed zones may be the consequence of organic acid production, nevertheless, the bacterial strains were selected for WGS to allow further exploration of their genomes and the potential presence of biosynthetic gene clusters.

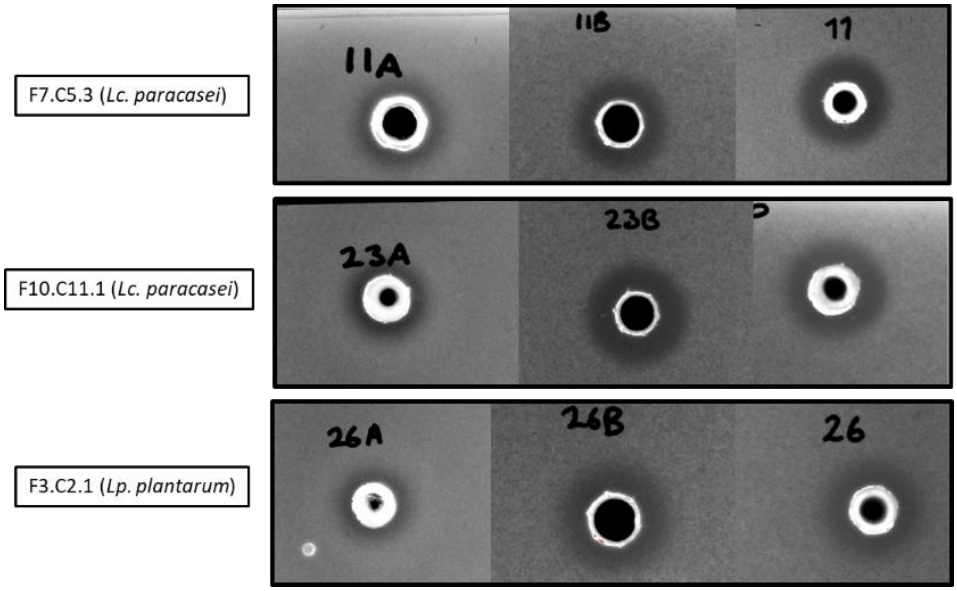



Figure 5: Well diffusion assay with a 1% inoculum of L. bulgaricus demonstrated zones with a diffuse boundary for two *Lc. paracasei* strains (F7.C5.3; F10.C11.1) and a *Lp. plantarum* strain (F3.C2.1)

In a further WDA with *E. thailandicus* as the indicator, the CFS of one strain (*Lc. paracasei* F5.C22.2) exhibited a zone of inhibition with defined edges suggestive of bacteriocin production (Figure 6). The remaining 29 strains displayed no level of inhibition against *E. thailandicus*. The anti-microbial activity of *Lc. paracasei* F5.C22.2 was reproducible in three separate well-diffusion assays, and was affected following treatment with proteinase K, suggesting the presence of a soluble and proteinaceous antimicrobial peptide (Figure 7). In addition, the potential for cell-bound bacteriocin activity was examined in all colostrum strains in WDAs with both indicators and no evidence of anti-microbial activity was observed.

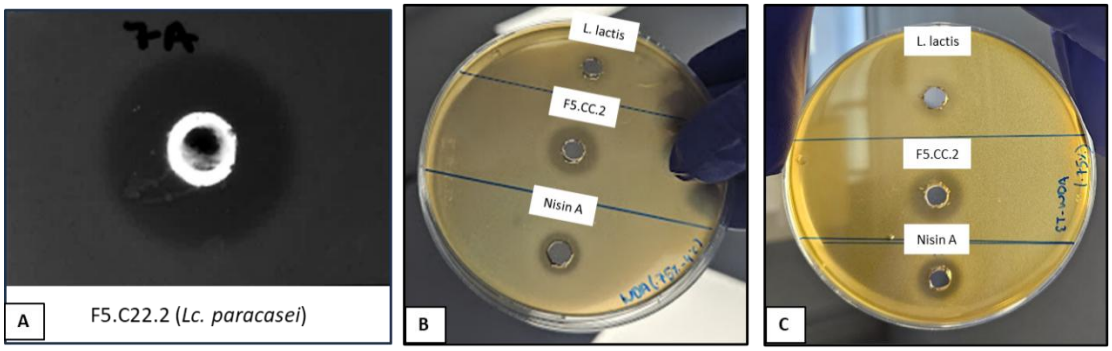



Figure 6: (**A**) Zone of inhibition by *Lc. paracasei* F5.C22.2 against *E. thailandicus* in a well diffusion assay. (**B-C**) Zone of inhibition in comparison with known antimicrobial producer *L. lactis* and bacteriocin Nisin A.

Finally, a series of WDAs to test for cross immunity between the bacterial strains isolated from bovine colostrum in our study was performed. Each colostrum strain was used as an indicator in separate WDAs, no anti-microbial activity was observed between the LAB from bovine colostrum. However, when *Lc. paracasei* F5.C22.2 was used as an indicator, the positive controls, *L. lactis* and Nisin A did not produce zones of inhibition, suggesting the potential immune capacity of *Lc. paracasei* F5.C22.2, against two lantibiotic-based antimicrobials (Figure 8). Of the 30 LAB strains screened, a total of 4 were categorised as putative bacteriocin producers warranting further investigation at a genomic level.

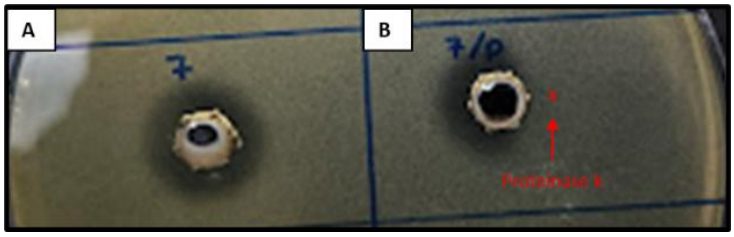



Figure 7: Testing the protease susceptibility of *Lc. paracasei* F5.C22.2: (**A**) Well diffusion assay with *E. thailandicus* inoculum in the absence of proteinase K. (**B**) *Lc. paracasei* F5.C22.2 demonstrates sensitivity to Proteinase K

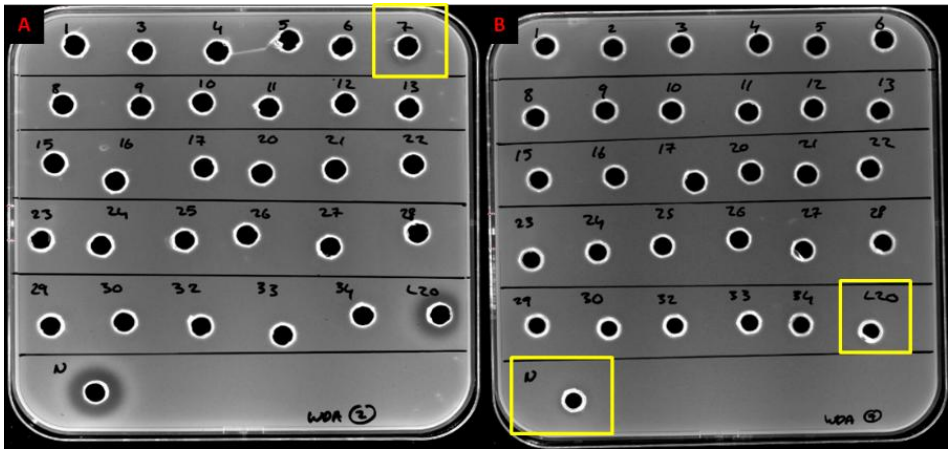



Figure 8: Bacterial strains derived from bovine colostrum used as the indicator strain in well diffusion assays to test for cross immunity between strains. (**A**) Well diffusion assay with *Lg. coryniformis* (F5.C9.2) as the indicator strain, no zone of inhibition with sharp or clear boundary observed. (**B**) Well diffusion assay with *Lc. paracasei* (F5.C22.2) as the indicator strain, no zone of inhibition with colostrum-derived strains or known anti-microbial Nisin A (N) and *L. lactis* (L20).

Table 3: Summary of phenotypic traits of lactic acid bacteria from bovine colostrum following *in vitro* characterization; EPS production, BSH activity; bacteriocin activity with WDA; haemolytic activity. (-) = negative, (+, ++, +++, ++++) = positive; weak to strong activity. Bacterial strains highlighted in yellow were selected for WGS.

| SAMPLE ID | SPECIES | EPS | BSH | WDA | HA |
|---|---|---|---|---|---|
| F2.C16.2R | *L. lactis* | + | - | - | γ- |
| F5.C9.2 | *Lg. coryniformis* | ++ | - | - | γ- |
| F6.C16.1 | *Lc. casei* | ++ | - | + | γ- |
| F5.C3.3 | *Lt. buchneri* | - | - | - | γ- |
| F5.C22.1 | *Lc. paracasei* | ++ | - | - | γ- |
| F5.C3.2 | *Lt. buchneri* | +++ | + | - | γ- |
| F5.C22.2 | *Lc. paracasei* | + | - | + | γ- |
| F1.C8.1 | *Lt. parabuchneri* | - | - | - | γ- |
| F1.C8.2 | *Lc. paracasei* | ++ | - | - | γ- |
| F5.C3.1 | *Lt. buchneri* | - | - | - | γ- |
| F7.C5.3 | *Lc. paracasei* | + | - | + | γ- |
| F1.C16.1 | *Lc. casei* | ++ | - | - | γ- |
| F5.C9.1 | *Lv. brevis* | ++ | - | - | γ- |

| | | | | | |
|---|---|---|---|---|---|
| F9.C7.1 | *Lc. casei* | + | ++ | - | γ- |
| F4.C3.1 | *Lc. paracasei* | + | - | - | γ- |
| F9.C11.1 | *Lt. buchneri* | ++++ | +++ | - | γ- |
| F7.C16.2 | *Lt. buchneri* | + | + | - | γ- |
| F3.C16.1 | *Lt. buchneri* | ++++ | ++++ | - | γ- |
| F3.C16.1M | *Lc. paracasei* | + | - | - | γ- |
| F10.C11.1 | *Lc. paracasei* | ++ | - | + | γ- |
| F5.C16.2 | *Lc. casei* | ++ | - | - | γ- |
| F3.C2.2 | *Lc. casei* | ++ | - | - | γ- |
| F3.C2.1 | *Lp. plantarum* | + | - | + | γ- |
| F3.C2.1M | *Lp. plantarum* | + | - | + | γ- |
| F1.C1.2 | *Lc. casei* | +++ | - | - | γ- |
| F3.C16.3 | *Lt. buchneri* | ++++ | - | - | γ- |
| F1.C1.1 | *Lc. paracasei* | ++ | - | - | γ- |
| F9.C7.2 | *Lc. paracasei* | + | - | - | γ- |
| F2.C1.1 | *Lt. parabuchneri* | - | - | - | γ- |
| F2.C16.2 | *Lm. mucosae* | +++ | ++++ | - | γ- |

### 3.3 Haemolytic activity

All colostrum-derived strains were evaluated for the ability to lyse red blood cells (haemolytic activity). Haemolytic activity can be categorised as complete (β-haemolysis), partial (α-haemolysis) or absent (γ-haemolysis). The colostrum microbes did not produce any zone of clearing or discolouration suggestive of haemolysis. As such, all colostrum-derived strains were deemed non-haemolytic (γ-haemolysis).

Table 4: Summary of the genomic properties of colostrum-derived microbes following annotation of whole genome sequences with BAKTA. bp = base pair, GC = Guanine and Cytosine (%), CDS = Coding DNA sequence.

| Sample | Genome Length (bp) | Contigs | GC (%) | N50 | CDSs | tRNAs | rRNAs | Hypotheticals | Coding Density (%) |
|---|---|---|---|---|---|---|---|---|---|
| **F2.C16.2R** | 2,738,042 | 2 | 35.5 | 2,716,747 | 2,725 | 67 | 19 | 173 | 87.5 |
| **F5.C9.2** | 3,070,515 | 3 | 43.0 | 2,965,018 | 2,961 | 64 | 15 | 221 | 86.7 |
| **F5.C22.2** | 3,108,650 | 17 | 46.4 | 2,965,900 | 3,014 | 59 | 15 | 224 | 85.6 |
| **F7.C5.3** | 3,102,760 | 9 | 46.4 | 3,030,163 | 2,983 | 59 | 15 | 209 | 86.0 |
| **F5.C9.1** | 2,506,710 | 8 | 45.7 | 2,334,770 | 2,460 | 63 | 15 | 167 | 86.6 |
| **F3.C16.1** | 2,529,124 | 3 | 44.3 | 2,456,233 | 2,362 | 61 | 15 | 112 | 88.0 |
| **F10.C11.1** | 3,272,576 | 4 | 46.2 | 3,207,069 | 3,128 | 59 | 15 | 221 | 85.9 |
| **F3.C2.1** | 3,353,923 | 8 | 44.4 | 3,109,748 | 3,218 | 70 | 16 | 194 | 84.8 |
| **F2.C16.2** | 2,061,189 | 2 | 46.8 | 1,996,990 | 1,875 | 92 | 24 | 119 | 87.1 |

## 3.4 In silico analysis of probiotic traits in lactic acid bacteria from bovine colostrum

### *3.4.1 Whole genome sequencing of colostrum isolates*

In this study, a total of 10 bacterial strains isolated from bovine colostrum were selected for WGS based on their classification following 16S rRNA Sanger sequencing and demonstrated probiotic-like properties in a series of lab-based assays (see Table 3). All colostrum strains were LAB belonging to the genera *Loigolactobacillus*, *Levilactobacillus*, *Limsoilactobacillus*, *Lactiplantibacillus*, *Lacticaseibacillus*, *Lentilactobacillus* and *Lactococcus*. WGS confirmed that, *Lc. paracasei* was the most frequent species under investigation accounting for 30 % of the colostrum-derived microbes. The draft genomes were annotated with BAKTA and their characteristics are outlined in Table 4. The genome length ranged from 2,061,189 – 3,353,923 bp, and the average size was 2,860,388 bp. The average G + C content and number of coding sequences was 43 % and 2747, respectively.

### *3.4.2 Average nucleotide identity*

Comparative genome analysis was performed based on the ANI of all 10 strains following WGS against high quality RefSeq genomes using the NCBI database. An ANI value higher than 95 % suggests the query and reference genome are of the same species (67). Furthermore, for an ANI value to be indicative of the same strain, a threshold of > 99.99 % is suggested. Thus, where the ANI (%) was > 95 %, the strain was confirmed as the same species. All strains aligned with previously identified species classification following 16S rRNA sequencing and BLASTn classification,

except for *L. lactis* F2.C16.2R, which was identified as belonging to the *Lactococcus cremoris* species. Thus, ANI analysis confirms the species identification of our strains as *L. cremoris F2.C16.2R, Lg. coryniformis* F5.C9.2*, Lv. brevis* F5.C9.1*, Lp. plantarum* F3.C2.1*, Lm. mucosae* F2.C16.2 and three *Lc. paracasei* strains, F5.C22.2*,* F7.C5.3 *and* F10.C11.1. One strain (*Lt. buchneri* F3.C16.1) had an ANI value > 99.9 % indicating it was likely *Lt. buchneri subsp. silagei MGR2-32* and was therefore assigned to strain level identification. The details of the RefSeq genomes with the three highest ANI values (%) for each colostrum strain are provided in Table 5.

FastANI analysis was performed on the three *Lc. paracasei* (F5.C22.2, F7.C5.3 and F10.C11.1) strains and separate strain identity was confirmed. Indeed, *Lc. paracasei* F5.C22.2 had an ANI of 98.22 % and 98.12 % when compared against F7.C5.3 and F10.C11.1. In addition, an ANI of 98.27 % was observed between *Lc. paracasei* F7.C5.3 *and* F10.C11.1. ANI analysis of *Lp. plantarum* F3.C2.1 and *Lp. plantarum* F3.C2.1M confirmed they were the same strain (> 99.99 %) and as such only *Lp. plantarum* F3.C2.1 was considered for further analysis. Due to the observed EPS and BSH activity *in vitro*, fastANI analysis of *Lm. mucosae* F2.C16.2 was performed against an established *Lm. mucosae* strain with strong probiotic characteristics (*Lm. mucosae* DPC 6426) (44, 45). ANI analysis revealed an ANI of 98.01 %. Likewise, ANI analysis of a previously identified *Lv. brevis* strain with health-promoting attributes, *Lv. brevis* DPC 6801, demonstrated an ANI of 97.24 % when compared against the strain *Lv. brevis* F5.C9.1 (68, 69). ANI analysis suggests a high degree of genetic similarity between the strains *Lm. mucosae* F2.C16.2 and *Lv. brevis* F5.C9.1 and the probiotic strains *Lm. mucosae* DPC 6426 and *Lv. brevis* DPC 6801, respectively (Figure 9).

Table 5: Comparative genome analysis was performed using the NCBI database. ANI (%) analysis of bacterial strains to the closest RefSeq genome. Top three ANI (%) of RefSeq genomes and the source of bacterial strain. An ANI value higher than 95 % suggests the query and reference genome are of the same species

| Sample | RefSeq genome | ANI (%) | Source |
|---|---|---|---|
| **F2.C16.2R** | *Lactococcus cremoris* subsp. *cremoris* IBB477<br>*Lactococcus cremoris* 71<br>*Lactococcus cremoris* 71b | 99.644<br>99.3017<br>99.0013 | Raw milk<br>-<br>Pecorino cheese |
| **F5.C9.2** | *Loigolactobacillus coryniformis* subsp. *coryniformis* DSM 20001<br>*L. coryniformis* subsp. *coryniformis* CBA3616<br>*L. coryniformis* subsp. *torquens* DSM 20004 | 97.7902<br>97.5844<br>97.1519 | silage<br>Kimchi<br>Cow shed air |
| **F5.C22.2** | *Lacticaseibacillus paracasei* KL1<br>*Lacticaseibacillus paracasei* JCM 8130<br>*Lacticaseibacillus paracasei* LMG 19719 | 99.5399<br>99.3665<br>99.3235 | Milk<br>-<br>Blood culture |
| **F7.C5.3** | *Lacticaseibacillus paracasei* TK1501<br>*Lacticaseibacillus paracasei* Lpc10<br>*Lacticaseibacillus paracasei* subsp. *paracasei* 8700:2 | 99.3089<br>99.2602<br>99.2039 | Congee<br>Wine<br>Terrestrial environment |
| **F5.C9.1** | *Levilactobacillus brevis* H8<br>*Levilactobacillus brevis* UCCLB556<br>*Levilactobacillus brevis* TCI988 | 99.4859<br>99.4668<br>99.428 | Korean Beef<br>Brewery environment<br>Tempeh |
| **F3.C16.1** | *Lentilactobacillus buchneri* subsp. *silagei* MGR2-32<br>*Lentilactobacillus buchneri* CIRM-BIA2511<br>*Lentilactobacillus buchneri* subsp. *silagei* CD034 | 99.9422<br>99.8671<br>99.8621 | Grass silage<br>Fermented vegetable<br>Grass silage |
| **F10.C11.1** | *Lacticaseibacillus paracasei* LC355<br>*Lacticaseibacillus paracasei* subsp. *tolerans* SNB<br>*Lacticaseibacillus paracasei* SMN-LBK | 99.6926<br>99.6097<br>99.6092 | Cream products<br>Fermented milk<br>Koumiss |
| **F3.C2.1** | *Lactiplantibacillus plantarum* SHY21-2<br>*Lactiplantibacillus plantarum* C2<br>*Lactiplantibacillus plantarum* SRCM103303 | 99.5582<br>99.3848<br>99.2675 | Yak yogurt<br>Plant<br>Food |
| **F2.C16.2** | *Limosilactobacillus mucosae* Q2<br>*Limosilactobacillus mucosae* L1<br>*Limosilactobacillus mucosae* SLAM-JK01 | 97.9187<br>97.8613<br>97.6868 | Sheep rumen<br>Bovine rectum<br>Holstein faeces |

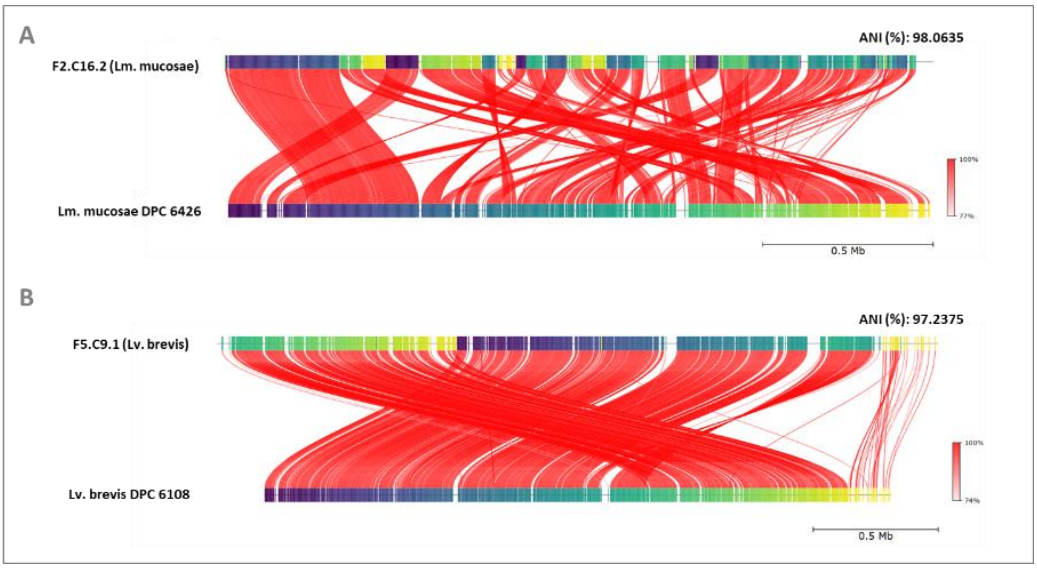



Figure 9: ANI analysis of bovine colostrum strains against known bacterial strains of the same species with similar phenotypic characteristics **(A)** Bacterial strain F2.C16.2 (*Lm. mucosae*) vs *Lm. mucosae* DPC 6426 (GCF_000789175.1). Query Sequence Fragments: 659, Orthologous Matches: 611. (**B**) Bacterial strain F5.C9.1 (*Lv. brevis*) vs *Lv. brevis* DPC 6108 (GCA_001722065.1). Query Sequence Fragments: 956, Orthologous Matches: 757

### *3.4.3 Phylogeny of LAB colostrum microbes*

A phylogenetic tree was created based on the RefSeq genomes of 117 *Lc. paracasei* strains from the NCBI database and the draft genomes of the three *Lc. paracasei* strains isolated from bovine colostrum (F5.C22.2, F7.C5.3 *and* F10.C11.1) to establish the phylogenetic relationship of *Lc. paracasei* strains (Figure 10). The phylogenetic tree revealed *Lc. paracasei* F5.C22.2 and *Lc. paracasei* F7.C5.3 cluster closely on the same clade, suggesting a high degree of genetic relatedness. Whereas *Lc. paracasei* F10.C11.1 is positioned on a separate clade, indicating it is more distantly related.

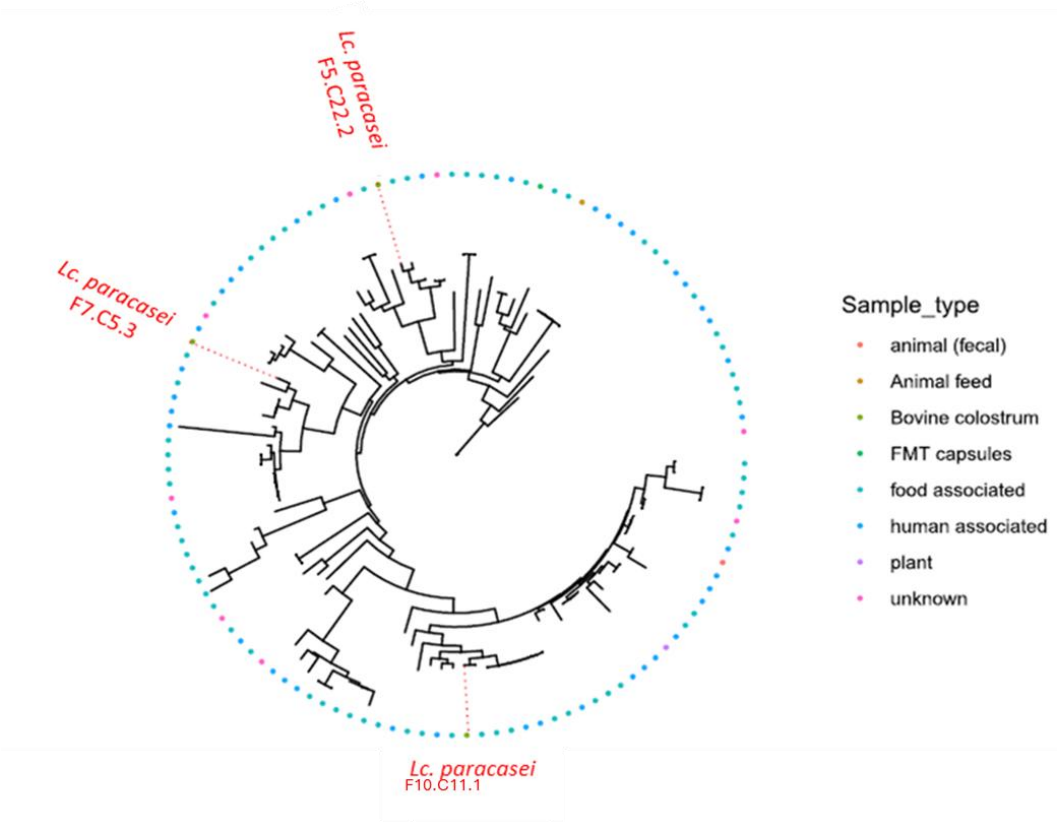



Figure 10: Phylogenetic tree demonstrating the evolutionary relationship of the three *Lc. paracasei* strains F5.C22.2, F7.C5.3 and F3.C16.1 derived from bovine colostrum in this study. The phylogenetic tree was created based on the RefSeq genomes of 117 *Lc. paracasei* strains from the NCBI database

### *3.4.4 Predicting bacteriocin gene clusters in colostrum microbes*

To investigate the presence of bacteriocin gene clusters in colostrum strains that had previously exhibited potential anti-microbial activity against *L. bulgaricus* and *E. thailandicus* in WDAs, the genomes of four LAB colostrum strains were mined using the online databases BAGEL4 and antiSMASH. Furthermore, the identity of all core peptides and accessory genes present underwent further identification using the NCBI BLASTp, the CAMPR4 and InterPro databases. Potential bacteriocin gene clusters were identified within all four draft genomes, with a total of five predicted bacteriocin gene clusters. One potential bacteriocin gene cluster was identified in *Lc. paracasei* F7.C5.3, *Lc. paracasei* F10.C11.1 and *Lp. plantarum* F3.C2.1. Genomic mining revealed the presence of two putative bacteriocin gene clusters in *Lc. paracasei* F5.C22.2.

Putative bacteriocin gene clusters with similar structural organization and the same core peptide were identified in the genomes of all three *Lc. paracasei* strains (F5.C22.2, F7.C5.3 and F10.C11.1). The core peptide identified in all three gene clusters shared strong sequence homology with LSEI_2386, a putative class II bacteriocin (see Figure 11). Multiple sequence analysis revealed a 100 % identity between LSEI_2386 (accession number: ABJ71126.1) and the amino acid sequence of the predicted core peptides in *Lc. paracasei* F5.C22.2 and F7.C5.3 (Figure 12A). A lower identity of 97.78 % was demonstrated for the predicted core peptide in *Lc. paracasei* F10.C11.1, due to an amino acid substitution at position 36. In addition, putative regulatory and immunity genes were identified upstream of the core peptide, while transport genes were located downstream. In all three clusters, LSEI_2386 was assigned the likely core peptide due to its position within the cluster (flanked by accessory genes) and high sequence homology with the experimentally validated bacteriocin LSEI_2386. Furthermore, the amino acid sequence of the predicted LSEI_2386 peptide from all three *Lc. paracasei* were compared. A 100% identity was observed between *Lc. paracasei* F5.C22.2 and F7.C5.3, while *Lc. paracasei* F10.C11.1 shared a 98.41 % identity with both. Colony mass spectrometry did not detect a peak for LSEI_2386 (molecular mass 2698.01 Da) in all three clusters (data not shown). As such, despite the presence of a bacteriocin gene cluster with similar homology to a previously identified and experimentally validated cluster, the active production of bacteriocin LSEI_2386 in all three genomes is unclear and requires further examination. In addition, BAGEL4 and antiSMASH predicted additional open reading frames (orfs) as core peptides upstream of the putative LSEI_2386 cluster in *Lc. paracasei* F7.C5.3 (orf_13, orf_23, orf_24) and *Lc. paracasei* F10.C11.1 (orf_17, orf_28, orf_29). Sequence analysis revealed an exact match of orf_13, orf_23 and orf_24 in F7.C5.3 with orf_17, orf_28 and orf_29 in F10.C11.1, respectively. BAGEL4 annotated orf_13 (F7.C5.3) and orf_17 (F10.C11.1) as the class IIb bacteriocin, Enterocin-x-β. However, amino acid sequence analysis with CAMPR4 revealed only a 46% identity over 28 amino acids. For the same orfs (orf_13 and orf_17), antiSMASH assigned them as class IIb bacteriocins of the lactobin A/cerein 7B family. However, the top BLASTp hit for both orfs was to a hypothetical protein from *Lc. paracasei*, with unknown functional

properties (Accession number: AKU60537.1, 100 % identity). Similarly, orf_23/24 of F7.C5.3 and orf_28/29 of F10.C11.1 were annotated as class II bacteriocins by both BAGEL and antiSMASH, but BLAST analysis using the CAMPR4 and NCBI databases was unable to determine a match to any known anti-microbials. Although, domain analysis confirmed the presence of a GG-motif (PF10439.12). In addition, a fourth predicted core peptide (orf_22) was identified in the cluster of *Lc. paracasei* F10.C11.1. Again, BAGEL4 identified the orf as Enterocin-x-β, however, domain and sequence analysis failed to support this.

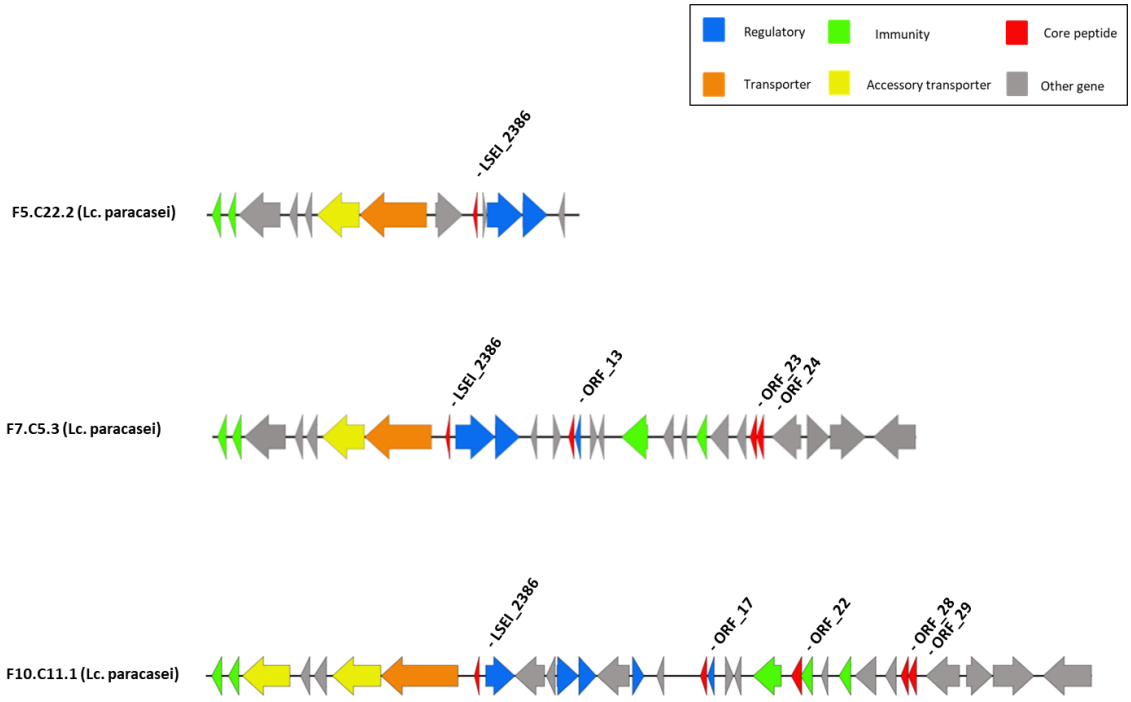



Figure 11: Genomic mining of colostrum strains with antiSMASH and BAGEL identified three putative bacteriocin gene cluster in *Lc. paracasei* strains (F5.C22.2, F7.C5.3, F10.C11.1) with the core peptide LSEI_2386

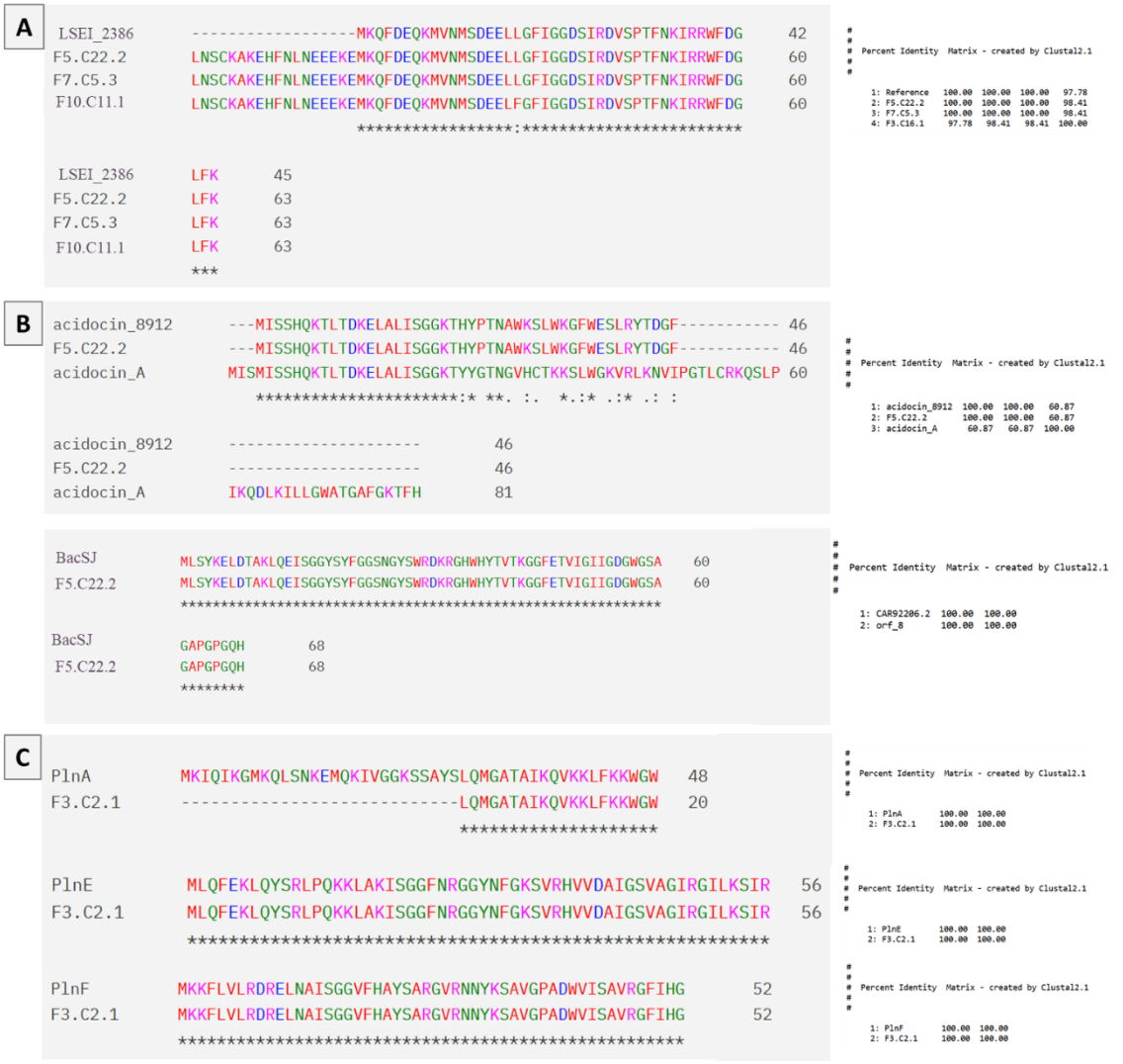



Figure 12: Pairwise and multiple sequence alignment of putative core peptides and their percentage identity (**A**) *Lc. paracasei* strains, F5.C22.2, F7.C5.3 and F10.C11.1 against the reference LSEI_2386 from *Lc. paracasei* ATCC 334 (CP000423.1) (**B**) *Lc. paracasei* F5.C22.2 vs reference Acidocin 8912 from *L. acidophilus* TK8912 (AB081463.1) and Acidocin A from *L. acidophilus* TK9201. *Lc. paracasei* F5.C22.2 of second putative core peptide vs BacSJ (CAR92206.2) from *Lc. paracasei* subsp. *paracasei* BGSJ2-8 (**C**) Predicted PlnA, PlnE and PlnF gene of colostrum strain *Lp. plantarum* F3.C2.1 vs. *Lp. plantarum* C11 (X94434.2)

A second bacteriocin gene cluster was identified in *Lc. paracasei* F5.C22.2 on contig 2 in the region of 25,683-37,842 bp. The predicted cluster contained the presence of two class II bacteriocins, Acidocin 8912 and Bac-SJ, with a 100 % identity (Figure 12B). Putative immunity and transport genes were located between

the two core peptides; no regulatory genes were identified (Figure 13). Interpro domain analysis identified a putative LciA-like (Lactoccin A) immunity protein. BLAST and domain analysis confirmed the presence of an ABC transporter and a HlyD-family accessory transporter. Genomic comparative analysis revealed strong similarity between cluster 2 in *Lc. paracasei* F5.C22.2 and the Bac-SJ bacteriocin cluster from *Lc. paracasei* subsp. *paracasei* BGSJ2-8, located on the plasmid PsJ2-8 (accession number: FM246455) (Figure 14) (70). Further supporting the bacteriocin activity of cluster 2 in *Lc. paracasei* F5.C22.2, colony mass spectrometry analysis identified a peak at 3220 Da corresponding to the molecular mass of the bacteriocin Acidocin 8912 (3219 Da) (see Figure 15).

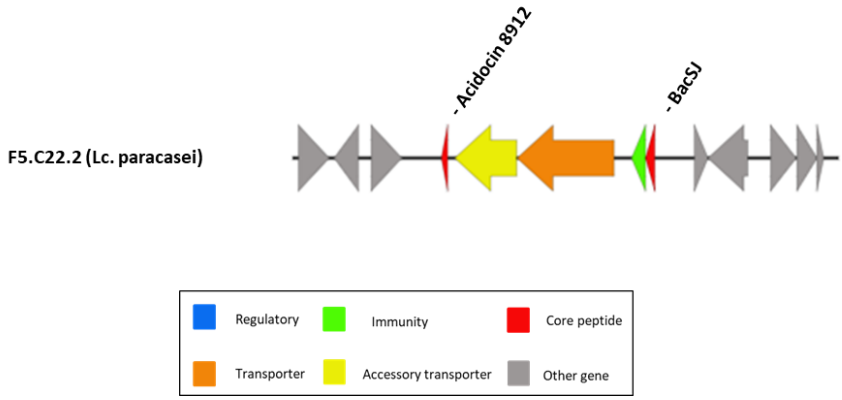



Figure 13: Putative plasmid based bacteriocin gene cluster (cluster 2) identified in *Lc. paracasei* F5.C22.2 with core peptides: Acidocin 8912 and BacSJ.

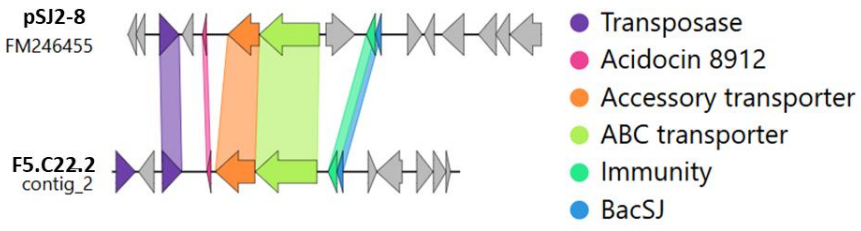



Figure 14: Genomic comparison of plasmid pSJ2-8 from *Lc. paracasei* subsp. *paracasei* BGSJ2-8 (containing the two bacteriocins Acidocin 8912 and BacSJ) against the predicted bacteriocin gene cluster of *Lc. paracasei* F5.C22.2.

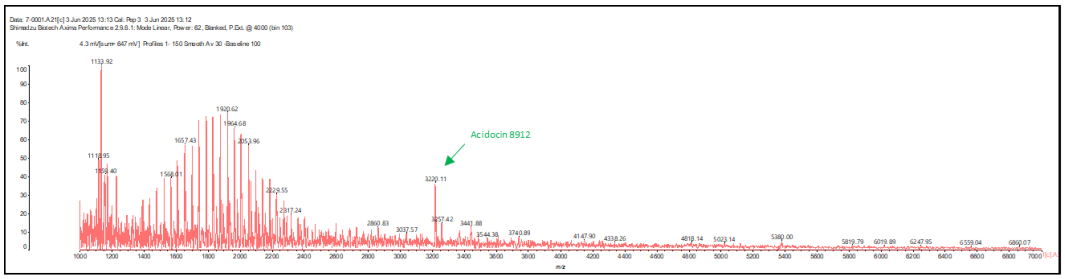



Figure 15: MALDI TOF Mass Spectrometry; Colony Mass Spectrometry of *Lc. paracasei* F5.C22.2 revealed a mass peak at 3220 Da which correlates with the Acidocin 8912 - molecular mass 3219 Da

The final putative cluster was identified within *Lp. plantarum* F3.C2.1 in the region of 367942 – 3800929 bp (Figure 16). In strain F3.C2.1, comparative genomic analysis of the bacteriocin-like region identified a high degree of structural conservation with the established *pln* locus from *Lp. plantarum* C11 (71). Indeed, *Lp. plantarum* F3.C2.1 harboured the genes encoding the class IIb bacteriocins PlnE and PlnF, with 100 % identity (Figure 12). In addition, the predicted immunity protein, shared 100% sequence homology with PlnI. Similarly, the predicted ABC transporter and accessory transporter gene shared a 100% identity to PlnG and PlnH. However, despite the presence and sequence homology of PlnT, PlnU and PlnV, the operon was disrupted by two orfs in the expected position of PlnS. Both orfs were identified as transposase of the IS30 family. Furthermore, the predicted proteins PlnB, PlnC and PlnD shared 100% amino acid sequence identity with the ABCD operon of *Lp. plantarum* C11. However, the amino acid sequence of PlnA in strain F3.C2.1 demonstrated only 40 % coverage of the full PlnA sequence. The aligned region, from position 31 to 50, shared 100% (Figure 12C). Furthermore, colony mass spectrometry did not detect a peak for PlnE and PlnF (molecular mass: 3545.12 Da and 3703 Da, respectively) (data not shown).

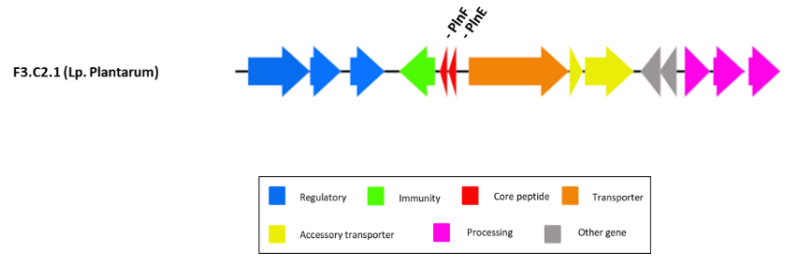



Figure 16: Predicted bacteriocin gene cluster identified in Lp. plantarum F3.C2.1 with core peptides PlnE and PlnF

### *3.4.5 GABA production in Lv. brevis*

Genomic analysis of draft genomes revealed the presence of four key genes for GABA biosynthesis in *Lv. brevis* F5.C9.1. The genes *gadA*, *gadB*, *gadC* and *gadR* had a percentage identity of 98.72 %, 99.27 %, 99.93 % and 98.48 %, respectively, in the genome of *Lv. brevis* F5.C9.1 (Figure 17). The presence of *gadB* was also observed in *L. cremoris* F2.C16.2R (96 % identity) and *Lp. plantarum* F3.C2.1 (98.64 % identity). However, both strains lacked *gadA*. Furthermore, *gadC* and *gadR* were only present in *L. cremoris* with a lower identity of 95.83 % and 98 %. Both *gadC* and *gadR* were absent in *Lp. plantarum* F2.C16.2. No GABA related genes were identified in the remaining seven strains. Due to the presence of key genes involved in the GABA biosynthesis pathway, *Lv. brevis* F5.C9.1 was identified as a potential GABA producing bacterial strain.

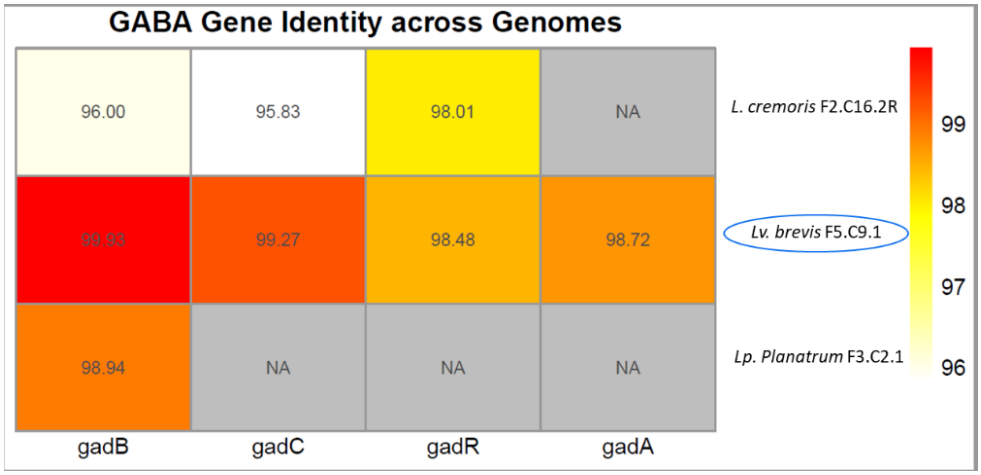



Figure 17: Genomic analysis of draft genomes for the presence of key genes for GABA biosynthesis (*gadA*, *gadB*, *gadC* and *gadR*) in bovine colostrum-derived bacteria, demonstrating *Lv. brevis* F5.C9.1 is a potential GABA producer

### *3.4.6 Carbohydrate-active enZymes in bovine colostrum isolates*

In this study, dbCAN and the CAZy database were used to analyse the genomes of LAB isolated from bovine colostrum to identify the presence of CAZyme genes. Given their role in the EPS biosynthesis gene cluster, the analysis was filtered to focus on enzymes from the GHs and GTs families (Figure 18). The presence of two key genes *GT2* and *GT4*, were detected in all strains. Similarly, *GH13*, *GH25* and *GH73* were present in all strains, albeit at lower levels. The *GT14* gene was exclusively identified in *Lm. mucosae* F2.C16.2. In contrast, *GH1* was absent in both *Lm. mucosae* F2.C16.2 and *Lt. buchneri* F3.C16.1. The *GT32* gene was detected at low levels in *Lc. paracasei*, F5.C22.2 and F7.C5.3, *Lv. brevis* F5.C9.1 and *Lt. buchneri* F3.C16.1. Meanwhile, *GT32* showed moderate identity in *Lc. paracasei* F10.C11.1 and was absent in the remaining strains.

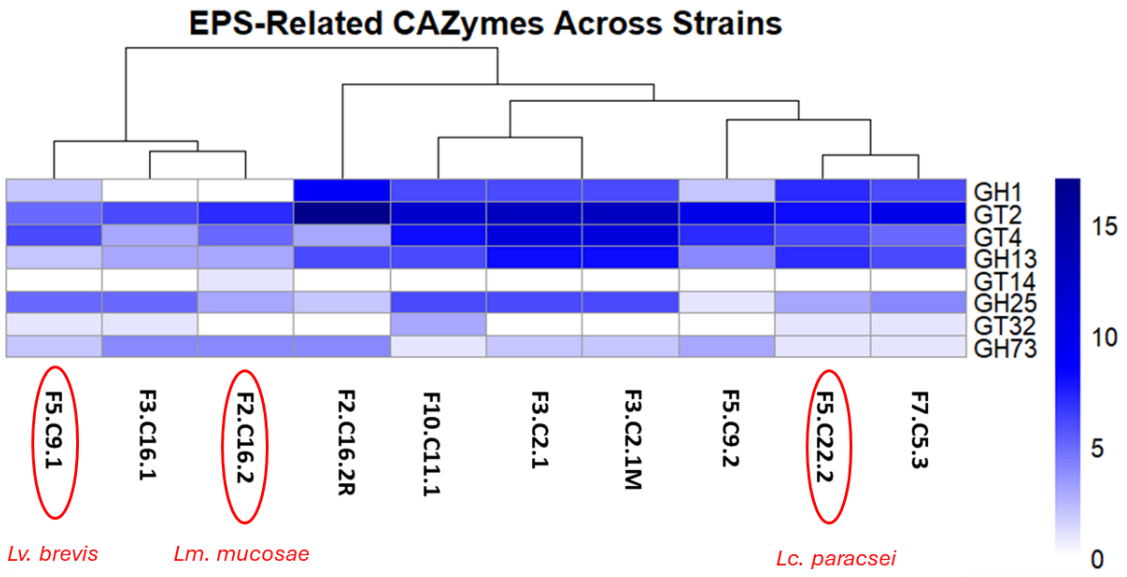



Figure 18: Carbohydrate-active enzymes were identified in bovine colostrum strains using the dbCAN. Highest identity was seen for GT2 and GT4 families.

### *3.4.7 Safety evaluation of colostrum microbes*

To determine whether any of the bacterial strains isolated from bovine colostrum harboured antibiotic-resistant genes, the genomes of all strains were screened for the presence of genes with known resistance against various antibiotic classes including, lincosamide, vancomycin, tetracycline and metronidazole. Only one of the bacterial strains under genomic investigation (*L. cremoris* F2.C16.2R) was found to carry an antibiotic-resistant gene. Indeed, *L. cremoris* F2.C16.2R exhibited

100 % identity to the *lmrD* gene, which confers resistance to lincosamides. Thus, *L. cremoris* F2.C16.2R likely harbours a functional resistance mechanism to lincosamide (Figure 19). *L. cremoris* F2.C16.2R was not positive for any other antibiotic-resistant genes. Furthermore, no antibiotic-resistant genes were identified in the remaining nine genomes.

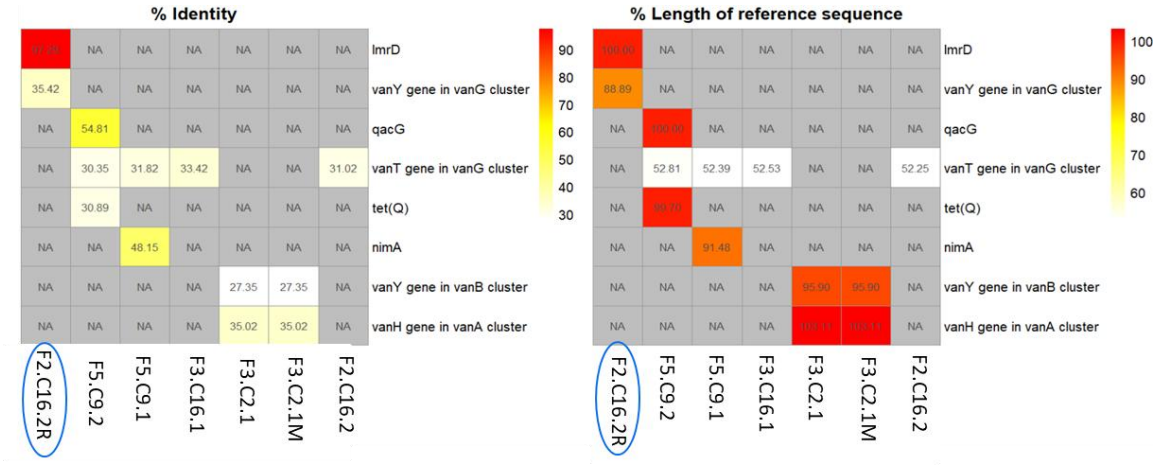



Figure 19: Genomic screening for the presence of antibiotic resistance genes in bovine colostrum-derived LAB was performed using RGI based on the CARD database. *L. cremoris* F2.C16.2R exhibited 100 % identity to the *lmrD* gene. No antibiotic-resistant genes were identified in the remaining nine genomes.

## 4 Discussion

Bacterial screening of bovine colostrum revealed the presence of a diverse and numerous population of LAB in this study. In total, 30 LAB were selected following 16S rRNA sanger sequencing and species classification was assigned with BLAST. Microbiological *in vitro* assays screened the strains for their phenotypic characteristics, demonstrating a high level of EPS production amongst strains (87 %) and moderate bacteriocin and BSH activity (13 % and 17%, respectively). Bovine colostrum presents as an ecological niche rich in LAB with desirable probiotic-like properties. Following a series of lab based microbiological assays, a total of 10 LAB derived from bovine colostrum were selected for WGS, to confirm species classification and explore their phylogenetic relationship, safety characteristics and presence of genes encoding for probiotic attributes, including bacteriocin gene

clusters, GABA production and antibiotic resistance. These results demonstrate the presence of bacterial strains in bovine colostrum with properties ideal for further study and potential industrial applications.

Both phenotypic and genetic assessment of bacterial strains derived from bovine colostrum demonstrated bacteriocinogenic potential. WDAs indicated the potential inhibitory action of four colostrum strains. To gain insights into the genetic elements responsible for this action and confirm the presence of bacteriocin gene clusters, genome mining of the draft genomes following WGS was performed. In order to establish the presence of a putative bacteriocin gene cluster, the presence of a bacteriocin (core peptide) alone is not sufficient, indeed, a number of accessory genes are required, otherwise, the bacteriocin is unlikely to be functioning, but is instead suggestive of an artefact or remnant. The required accessory genes are usually localized within a cluster and include; an immunity protein and a transporter protein and its accessory protein (72). In addition, the presence of regulatory genes is desirable, although not a necessity. An immunity protein protects the producer bacteria against its own anti-microbial activity. The transporter protein, most commonly, an ATP-binding cassette (ABC) transporter and its accessory protein, are responsible for the exportation of the bacteriocin.

In this study, both BAGEL4 and antiSMASH predictions revealed the presence of three putative bacteriocin gene clusters in three separate *Lc. paracasei* strains, linked to the production of a small cationic peptide, LSEI_2386. First identified in *Lacticaseibacillus casei* ATCC334, LSEI_2386 was described as a putative pheromone peptide (20). LSEI_2386 encodes a pre-peptide, 45 amino acid long with a GG-motif and a mature peptide, 22 amino acids long (20). The peptide is heat-stable and has established anti-microbial activities against a number of *Listeria* species including *L. innocua*, *L. monocytogenes*, *L. welshemeri* and *L. seetigeri* (20). Furthermore, a LSEI_2386 producing strain, *Lc. paracasei* SP5, inhibited *Escherichia coli* and *Staphylococcus aureus* (24). In contrast, no inhibition was observed against more closely related strains, including *L. rhamonosus* (ATCC 21052/14957), *Lp. plantarum* (ATCC 8014) and *Lc. casei* (ATCC 393/27139) (20).

In this study, LSEI_2386 was identified in three phylogenetically related *Lc. paracasei* strains isolated from different farms, F5.C22.2, F7.C5.3 and F10.C11.1. Despite the established anti-microbial activity of peptide LSEI_2386 by previous studies, the bacteriocin gene cluster lacks comprehensive functional annotation. The putative bacteriocin cluster of *Lc. paracasei* F5.C22.2, contained the core peptide LSEI_2386, flanked by accessory genes for immunity, transport and regulation. The immunity genes positioned downstream of the core peptide contain the domain PF0895 and PF0895.13, confirming their function as a bacteriocin immunity protein, potentially of the EntA immunity family. In addition, domain and BLAST analysis of the transporter proteins revealed a complete bacteriocin secretion system, including a primary ABC transporter and an accessory transporter of the HlyD family, consistent with bacteriocin export. Finally, a two-component regulatory system was identified upstream of the core peptide, composed of a histidine kinase and a response regulator of the LyTR-family, likely functioning in response to environmental cues and mediating the expression of bacteriocin associated genes.

A similar structural organization and functional prediction was observed in the putative bacteriocin clusters of strains F7.C5.3 and F10.C11.1. However, genome mining of both strains revealed the presence of additional core peptides upstream of the putative LSEI_2386 bacteriocin in both strains with similar homology. However, BLAST and domain analysis failed to support their role as an antimicrobial peptide. As such, the identity and functionality of the orfs located in the clusters of both strains remains unknown. The annotations predicted by BAGEL and antiSMASH may be due to the presence of a GG-motif in the sequences. A similar BAGEL output of multi-core peptide bacteriocin cluster in the presence of LSEI_2386 has previously been reported in a number of *Lc. paracasei* strains (CZ-6, 8 and 14) and a *L. rhamonosus* strain (29, 73, 74). Nonetheless, whether the orfs are in fact core peptides with anti-microbial activity remains to be determined. In the predicted clusters of both F7.C5.3 and F10.C11.1, the flanking of LSEI_2386 by accessory genes indicates it is likely the active core peptide. In this study, although the observed zone of inhibition had a diffuse boundary following WDA and no peak

was revealed with colony mass spectrometry, the predicted presence of LSEI_2386 and a complete set of accessory genes, located in all three clusters is indicative of a functioning bacteriocin gene cluster. Furthermore, as noted in previous studies, LSEI_2386 has shown greater inhibition against *Listeria sp., E. coli* and *S. aureus*, rather than more closely related *Lactobacillus* genes (20, 24). As such, future *in vitro* study of the *Lc. paracasei* strains should test against a broader range of indicators, such as *Staphylococcus*, *Listeria* and *Bacillus*, to confirm the presence of a functioning bacteriocin cluster and determine the spectrum inhibition. The presence of multiple core peptides in the clusters may be the result of horizontal gene transfer or evolutionary remnants. Future work, including purification of the core peptide will confirm if LSEI_2386 is the functioning bacteriocin. Furthermore, the presence of numerous putative core peptides, without identification, should be explored to determine and accurately define their antimicrobial potential, if any.

A second plasmid borne bacteriocin gene cluster was predicted in *Lc. paracasei* F5.C22.2. The putative bacteriocin cluster contained a gene encoding for the core peptide Acidocin 8912 and a second predicted core peptide, BacSJ. The putative bacteriocin cluster was only identified in the strain, *Lc. paracasei* (F5.C22.2). As such, we suggest the microbial inhibition observed in the WDA against *E. thailandicus* by *Lc. paracasei F5.C22.2* is a result of cluster 2, not cluster 1. Indeed, the inhibition of *E. thailandicus* was not observed with strains, *Lc. paracasei* F7.C5.3 and F3.C16.1, suggesting the LSEI_2386 cluster, which is present in all three strains was not responsible. Further experimental work is required to confirm this theory. The antibacterial activity of the small cationic heat stable peptide, Acidocin 8912, was first identified and described in *L. acidophilus* TK8912 (75, 76). Acidocin 8912, has a bactericidal effect against target cells through the disruption of the outer cytoplasmic membrane, by dissipating the proton motive force and the formation of voltage independent pores (76). Acidocin 8912 has demonstrated inhibition against gram-positive bacteria of the *Lactococcus*, *Enterococcus*, *Bacillus*, *Mycobacterium* and *Micrococcus* genera and gram-negative *E. coli* and fungi (26). The bacteriocin BacSJ was first identified in *Lc. paracasei* subsp. *paracasei* BGSJ2-8 isolated from artisanal semi-hard homemade cheese (77).

BacSJ is a heat-stable, class IId bacteriocin, 68 amino acids in length with a double glycine leader peptide and a predicted 50 amino acids mature peptide and is active over a broad pH range of 2-11 (70, 78). BacSJ has demonstrated broad anti-microbial activity against gram positive bacteria, including *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Pediococcus* and *Listeria* (78). The bacteriocin acts through a receptor-dependent mechanism, binding to Mannose-specific phosphotransferase system receptors and inducing cell membrane disruption (78). A study by Kojic et al. (70) demonstrated that *Lc. paracasei* subsp. *paracasei* BGSJ2-8, harbours the plasmid PsJ2-8 which contains the two bacteriocins, Acidocin 8912 and BacSJ, in addition to transporter and immunity genes. Furthermore, the group established the functioning of the gene cluster in the absence of regulatory genes. The group suggest that both bacteriocins use the same ABC-transporter, although the mechanism of this remains to be established.

Genomic comparative analysis revealed strong structural and functional similarity between cluster 2 of *Lc. paracasei* F5.C22.2 and the BacSJ bacteriocin cluster from *Lc. paracasei* subsp. *paracasei* BGSJ2-8, located on the plasmid PsJ2-8. Indeed, the predicted core peptide BacSJ and the immunity protein share 100% homology with those of the BacSJ cluster. Furthermore, the associated accessory and ABC transporter revealed 99.78 % and 98.85 % identity, respectively. The sequence conservation suggests the cluster is functionally equivalent to the BacSJ bacteriocin system, producing Acidocin 8912 and BacSJ as active core peptides. The observed differences in gene organization and the presence of transposases within the cluster of strain *Lc. paracasei* F5.C22.2 are likely a result of horizontal gene transfer. Often times bacteriocins are located on mobile genetic elements, enabling the transfer of plasmids and their associated genes. Indeed, the homology between plasmid PsJ2-8 and that of plasmid pLA103 of *L. acidophilus* TK8912 encoding Acidocin 8912 has previously been demonstrated, further supporting the occurrence of horizontal gene transfer between bacteria within the same genera (70). Future work should investigate the presence of mobilization proteins in contig 2 of strain *Lc. paracasei* F5.C22.2 and assess the functionality of the transposase genes identified within cluster 2 to confirm potential horizontal gene transfer.

A separate study screened *Lc. casei* and *Lc. paracasei* isolates from various ecological niches for the presence of the BacSJ/Acidocin 8912 cluster (72). The group reported that 40 out of 52 isolates harboured both the *bacSJ* and *acidocin 8912* genes. However, only seven isolates possessed a complete bacteriocin cluster, moreover, just four demonstrated functional bacteriocin production. However, in that study, microbial inhibition assays were limited to closely related species (*Lactobacillus spp.* and *L. lactis*) as indicator strains. In contrast, findings from this study demonstrated that microbial inhibition by *Lc. paracasei* F5.C22.2 was only achieved against *E. thailandicus*, emphasizing the importance of using a broader range of indicator strains to accurately assess the inhibitory potential of this gene cluster.

The location of the predicted bacteriocin cluster 2 from *Lc. paracasei* F5.C22.2 on a plasmid is a desirable trait. Within bacterial communities, plasmid borne bacteriocins are more readily transferred to new host organisms and are capable of rapid dissemination. From a biotechnological perspective, these self-contained clusters are particularly amenable for cloning, transfer and expression in selected targets. Notably, the homologous plasmid PsJ2-8 has previously been expressed in a cloning vector, pA13, and introduced into *E. coli, Lactobacillus* and *Lactococcus* strains, exhibiting high segregational and structural stability (70). Similarly, BacSJ inhibition was achieved in previously non-functioning BacSJ producing strains when introduced into a plasmid with the required ABC and accessory transporter genes (72). The potential broad spectrum of antimicrobial activity of the putative bacteriocin cluster producing Acidocin 8912 and BacSJ, make it a promising strain for further research as an anti-bacterial agent. Moreover, preliminary safety evaluation of the strain *Lc. paracasei* F5.C22.2 suggests it is safe with respect to haemolytic activity and does not harbour antibiotic-resistant genes. However, further characterisation of the cluster must first be performed to determine its full inhibitory potential, additional safety characteristics, and robustness and stability under processing conditions.

The final putative cluster was identified within the draft genome of *Lp. plantarum* F3.C2.1. Previous studies have noted the ability of *Lp. plantarum* strains to produce

different anti-microbial compounds, with inhibitory activity against pathogenic and spoilage microorganisms (79). The bacteriocins produced by the *Lp. plantarum* species are commonly known as plantaricins, these include the class II bacteriocins, two peptide PlnJ/K and planatricin NC8. Genome mining of *Lp. plantarum* F3.C2.1 predicted the presence of two core peptides, Plantaricin EF (PlnEF). The locus responsible for PlnEF production was first identified and characterized in *Lp. plantarum* C11 (71, 80). The actions of PlnEF are facilitated by the complementary action of three operons, *plnABCD*, *plnEFI* and *plnGHSTUV*. The operon *plnABCD* contains regulatory genes, where *plnA* encodes an autoinducer, enabling the transcription of downstream *pln* genes; *plnB* encodes a sensory histidine kinase and *plnCD* are response regulators, with *plnD* forming a two-component regulatory system with *plnB*, activating bacteriocin gene expression (79, 81, 82). The core peptides, PlnE and PlnF are encoded by the *plnEFI* operon, in addition to an immunity protein, PlnI. The final operon is *plnGHSTUV,* where *plnG* and *plnH* encode an ABC and an accessory transporter protein, respectively, enabling the processing and export of the two core peptides (71).

The optimum anti-microbial activity of PlnEF relies on the presence of both peptides in equimolar amounts, when only one peptide is present, significantly lower microbial inhibition has been reported (83). Both, PlnE and PlnF, contain GG motifs facilitating helix–helix interactions between the complementary set of peptides, enabling membrane permeabilization of the target cell wall (84, 85). Indeed, the mechanism of cell wall disruption by PlnEF was confirmed by Zhang et al. (85), the peptides act synergistically to induce ion leakage, proton motive force, ATP depletion and structural destruction. Plantaricins have significant antimicrobial capacity against gram-positive bacteria, particularly closely related strains, with little inhibition observed against gram-negative bacteria. A previous study demonstrated the antimicrobial activity of PlnEF by *Lp. planatrum* PUK6 against *Lg. coryniformis subsp. coryniformis* JCM 1164T and *L. lactis subsp. lactis* ATCC 19435 (81), whereas gram-negative *E. coli* JM109 appeared impervious to the bacteriocin activity of *Lp. plantarum* PUK6. However, inhibition *of E. coli* has been achieved when *E. coli* was pre-treated with exposure to unfavourable conditions, including

the chelating agent EDTA (86). This additional step weakens the outer membrane of gram-negative bacteria, destabilizing the target cell enabling bacteriocin efficacy.

Comparative genomic analysis of *Lp. plantarum* F3.C2.1, revealed a high degree of structural conservation with the established *pln* locus. However, amino acid sequence analysis of PlnA in the regulatory *plnABCD* operon, revealed only partial coverage (40 %). As PlnA is a signal peptide, responsible for inducing gene expression of the regulatory operon and subsequent bacteriocin production, the presence of an incomplete *plnA* sequence suggests the bacteriocin cluster is likely non-functional under current investigative conditions. In addition, *plnGHSTUV*, was interrupted by the presence of two orfs, identified as transposases, in the expected position of *plnS*. Again, this disruption to the transport and processing operon is likely to impair the function of the bacteriocin cluster. Thus, despite the complete *plnEFI* operon and presence of numerous accessory genes, the predicted *plnEF* cluster of *Lp. plantarum* F3.C2.1 should be considered non-active. As such, the observed diffuse inhibition of F3.C2.1 against *L. bulgaricus* is likely a result of acid production. Bacteriocin production can rely on a highly regulated co-ordinated system, therefore, the extraction of the bacterial strain from its native environment and exposure to cultivating conditions may have resulted in stress induced changes and a loss of bacteriocin function (50, 87). However, this is unlikely to result in gene deletion as seen in *Lp. plantarum* F3.C2.1, but instead gene silencing. Restoration of *plnA* or *plnS* genes to re-establish cluster functionality could be explored through genetic cloning in the future.

Interestingly, ANI analysis of the strain *Lp. plantarum* F3.C2.1 identified *Lp. planatrum* SHY 21-2 (MW405451.1) as the closest match, with an ANI of 99.56 %. *Lp. planatrum* SHY 21-2 was originally isolated from a fermented yak yogurt (88). The strain has recently been shown to produce a novel class IId bacteriocin designated plantaricin LP 21-2, with broad antimicrobial activity against gram-positive and gram-negative bacteria, and fungi. Currently, we are unable to confirm the presence of the bacteriocin LP 21-2 in strain *Lp. plantarum* F3.C2.1, as the reference sequences for LP 21-2 is protected under a patent and is not publicly accessible for genomic comparison. However, the high ANI (%) with SHY 21-2 and

the presence of a *plnEF* artefact is encouraging for the antimicrobial nature of *Lp. plantarum* F3.C2.1. We suggest strain F3.C2.1 warrants further mining of the whole genome sequence, with a focus on GG and GG-like motifs and identification of small orfs flanked by proteins with conserved domains for immunity and transport, to determine the presence of potentially novel bacteriocins.

In this study, preliminary data on bovine colostrum strains with putative EPS and BSH phenotypes are presented. Of the colostrum-derived microbes screened, a total of 26 out of 30 strains demonstrated a positive phenotype for EPS production *in vitro*. Moreover, the four strains characterized as potential bacteriocin producers following positive WDA results, also demonstrated potential EPS activity. In addition, EPS activity was observed in *Lv. brevis* F5.C9.1*, Lm. mucosae* F3.C16.1 and *Lt. buchneri* F2.C16.1. Indeed, following the EPS activity assay the strains displayed an EPS phenotype with a 'ropy', 'slimy' and 'mucoid' appearance.

From a biotechnological standpoint, bacterial isolates with a high EPS yield are a desirable technological property for commercial applications. EPS can enable encapsulation improving the shelf life of a product, enhance the texture and viscosity of fermented dairy products, act as a thickener with prebiotic capacity in functional foods and also protect against harsh processing conditions during product development (89). However, it is important to note that a complex and complete genetic cluster is required for EPS biosynthesis and microbial assays alone cannot confirm EPS production. The EPS gene cluster typically includes a regulatory region (*epsAB*), precursor biosynthesis genes (*epsCD*), one or more GT genes for polymer assembly, and genes involved in polymerization and export (*wzy* polymerase and *wzx* flippase) (35, 66). Bacterial strains may contain one or multiple EPS clusters, whose organization can be generic or non-generic. High variability of EPS gene clusters has previously been demonstrated (35). In this study, although mining for EPS clusters was not undertaken, preliminary *in silico* analysis using dbCAN revealed the presence of multiple GTs and GHs, suggesting the potential presence of an EPS region that warrants further investigation. Notably, the identification of genes encoding core GTs (from GT2 and GT4 families) often associated with EPS biosynthesis and the production of EPS *in vitro* provides

promising indicators of potential EPS clusters in the colostrum-derived strains, including *Lv. brevis* F5.C9.1 and *Lm. mucosae* F3.C16.1 (35). Further genomic analysis is required to confirm the presence of a complete and operational EPS cluster. In addition, further *in vitro* screening of the LAB strains may help elucidate the optimal conditions for high EPS yield. Indeed, culturing conditions including the type and concentration of the selected substrate (glucose, glycerol, sucrose) can influence EPS production (15, 36).

In addition to its potential EPS production, genomic characterisation of *Lv. brevis* F5.C9.1 revealed the presence of four key genes involved in GABA biosynthesis, *gadA*, *gadB*, *gadC* and *gadR* suggesting the strain may be a functioning GABA producer. GABA is a major inhibitory neurotransmitter in the central nervous system and has been associated with potential anti-depressant, anti-hypertensive and anti-diabetic effects in humans (90). Several LAB are known to produce GABA due to the activity of the enzyme, glutamic acid decarboxylase (GAD) (91). For instance, *Lv. brevis* DPC 6108 is a well characterized GABA-producing strain (68, 92), with demonstrated probiotic potential to alleviate hyperglycaemia and improve metabolic and depressive-like behaviour in murine models (69, 93). ANI analysis revealed 97 % ANI between *Lv. brevis* F5.C9.1 and *Lv. brevis* DPC 6108, indicating a high degree of genetic similarity. Additionally, *Lv. brevis* H8 was identified as the top RefSeq genome match. This strain has established antifungal activity against *Aspergillus* and *Penicillium* species when used as a biopreservative in dry-cured ham (94). Taken together, these findings indicate the promising potential of *Lv. brevis* F5.C9.1 as a GABA-producing strain with advantageous properties. Further *in vitro* and *in silico* analysis of *Lv. brevis* F5.C9.1 are warranted to elucidate the strains' full spectrum of health-promoting attributes and potential pharmaceutical application.

Furthermore, in addition to its potential EPS production, microbial assays revealed strong BSH activity in *Lm. mucosae* F3.C16.1. BSH activity is commonly associated with gut adapted strains and may confer competitive advantages *in situ*. Overall, screening of colostrum-derived microbes revealed a low frequency of strains with the ability to deconjugate bile salts, in this case TDCA. This was not surprising as

incidences of BSH positive strains is not common amongst non-intestinal LAB, whereas it is widely distributed amongst strains isolated from the gut (39, 95). Notably, *Lm. mucosae* F3.C16.1 exhibited strong bile acid precipitation halos *in vitro*. However, as genomic mining was not performed in this study, the results remain putative. Further *in silico* analysis is required to establish the presence and functionality of BSH encoding genes. In addition, BSH assays were only performed using the bile salt, TDCA, future screening should expand to include glycocholic acid, glycochenodeoxycholic acid or taurocholic acid. A previous study of *Lm. mucosae* DPC 6426, demonstrated the EPS and BSH activity of the strain, with reported cardioprotective effects (44). Furthermore, in a mouse model fed a high cholesterol, high fat diet, *Lm. mucosae* DPC 6426 supplementation significantly reduced serum and liver cholesterol as well as serum triglyceride concentrations (45). Comparative genomic analysis revealed a 98 % ANI between DPC 6426 and *Lm. mucosae* F3.C16.1, a positive indication of potential genomic overlap. However, as the production and functionality of bioactive metabolites produced by bacteria is strain specific, further genomic characterization of *Lm. mucosae* F3.C16.1 is necessary to confirm its probiotic properties.

Future studies of both colostrum-derived strains (*Lv. brevis* F5.C9.1 and *Lm. mucosae* F3.C16.1) should include genome annotation and pangenome analysis against the reference strains to elucidate shared genetic and metabolic traits, as well as to identify unique genomic features that may confer strain-specific properties.

The phenotypic and genotypic characterisation of LAB isolated from bovine colostrum has identified promising strains with potential probiotic or food bio-preservative traits. However, to be considered as bio-therapeutic candidates, the safety of these strains must first be established (13). A pre-liminary safety assessment was conducted, focusing on the presence of antibiotic-resistant genes and haemolytic activity. This screening confirmed the absence of antibiotic-resistant genes in nine colostrum-derived strains, and no haemolytic activity was observed in any of the strains. While these results are encouraging, further evaluation is required to confirm the safety and suitability of strains from a

consumer perspective and their ability to withstand potential industrial processing conditions. Key characteristics warranting further study include the potential production of virulence factors, resistance to oxidative stress resistance, pH tolerability, and adhesion capacity.

## 5 Conclusion

This study provides valuable insights into the phenotypic and genotypic characteristics of bovine colostrum-derived LAB strains with probiotic potential for application in the food and health sectors. We present the complete genome sequences of LAB from bovine colostrum, offering insight into their genetic diversity and their capacity to produce strain-specific metabolic by-products. Genome sequencing and genetic analysis enabled the identification of a putative class II bacteriocin gene cluster on a plasmid in *Lc. paracasei* F5.C22.2, supported by *in vitro* antimicrobial activity against *E. thailandicus*. Additionally, *Lm. mucosae* and *Lv. brevis* strains exhibiting positive EPS phenotypes were identified as potential BSH and GABA producers, respectively. A culture bank of bovine colostrum-derived bacterial strains were characterised and are available for further identification, characterisation and genetic analysis. Overall, these findings suggest that LAB from bovine colostrum possess features compatible with probiotic and biopreservation applications, supported by preliminary safety evaluations. However, further studies are necessary to elucidate additional health-related properties, assess their resilience to potential processing and scalability conditions, and fully confirm they meet safety thresholds.

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# Chapter 5

## The influence of Holder pasteurization and High-pressure processing on the microbial safety of donor human milk for an extended period of cold storage

Ruth Conboy-Stephenson, Fanyu Meng, Alan L. Kelly, R. Paul Ross, Catherine Stanton.

## Abstract

Human milk banks are responsible for the safe and controlled distribution of donor milk. Holder pasteurization is the current pasteurization technique used by milk banks. Although effective in reducing microbial counts, holder pasteurization can have undesirable effects on the nutritional quality of milk. Current milk banking protocols require milk to be used within 24 hours of thawing, resulting in the potential waste of a valuable fluid often in short supply. The aim of this study was to firstly determine the efficacy of holder pasteurization in reducing the microbial growth in human milk and its ability to sustain microbial safety over an extended storage period of 7 days. Secondly, it evaluated the efficacy of a new processing technique, high pressure processing and assessed the microbial safety of donor milk over the same extended storage period. Milk underwent holder pasteurization or high-pressure processing at 200, 400 or 600 MPa. Bacterial screening on non-selective media demonstrated that holder pasteurized milk remained within acceptable growth limits (<10 CFU $mL^{-1}$) when stored at 4 °C for 7 days, while HPP at 600 MPa reduced microbial counts to undetectable levels, which lasted for the 7 days of cold storage. Immediately following HPP at 400 mPa a 3.45-log reduction in microbial counts was observed, with undetectable growth at subsequent time-points. Additionally, HPP at 400 MPa demonstrated an immediate and complete reduction in *Staphylococcus*, *Pseudomonas* and *Streptococcus* growth. Our findings show the microbial safety of holder pasteurised milk when stored at 4 °C for 7 days and that high pressure processing of donor milk is a potential non-thermal alternative to holder pasteurization.

## 1 Introduction

Human milk is an unmatched source of nutrients in the initial critical period of a new-born's development, conferring bioactive and immune compounds, including cytokines, growth factors and oligosaccharides (1, 2). Fresh mother's own milk is regarded as the optimum nutrition for the new-born, promoting and aiding infant growth and development. However, when mother's own milk is unavailable, donor

human milk (DHM) is recommended as the best alternative by the World Health Organization (WHO) (3). Human milk banks are responsible for the collection, processing and storage of DHM for infants who are unable to access mothers' own milk. Currently, there are 282 human milk banks providing this essential service in Europe, with a further 18 planned (4). Preterm and low birth weight infants are the primary recipients of DHM, emphasising the significant role of milk banks in health-care settings (5-7).

Human milk banks follow strict protocols to ensure the safety and prevent waste of this valuable liquid. Following collection, processing steps include pooling, pasteurisation and bacteriological screening of DHM (8, 9). The microbiological quality and safety of DHM is a primary concern for human milk banks. In addition to the naturally occurring microbiota of breast milk (10), DHM is at risk of exogenous contamination during the handling and processing steps (11). To ensure the inactivation of potentially harmful bacterial agents and viruses, Holder Pasteurisation (HoP), also known as low temperature long time (LTLT) pasteurisation, is performed (12). HoP is a heat-based treatment, subjecting milk samples to a temperature of 62.5 °C for 30 min followed by the rapid cooling of milk to 4 °C (9, 13). Following processing, milk is frozen at -20 °C awaiting further use. In the United Kingdom, which provides milk banking services to Ireland, pasteurized DHM has a maximum frozen shelf-life of 6 months (14). When thawed for use, the batch of DHM is refrigerated for a maximum of 24 hours, after which it must be disposed of. Dispensed DHM should be within the advised bacterial growth limits of <1 CFU/100µL (14, 15). Studies to date demonstrate the efficacy of HoP in reducing the microbial load and eliminating potentially harmful bacteria in DHM (16, 17).

Unlike bacterial screening, nutritional monitoring of DHM is not a mandatory step in DHM processing (14, 15). The nutritional composition of DHM is influenced by a range of factors including the number of donors per pool, the number of previous freeze thaw cycles and the selected method of pasteurization. In particular, thermal processing of DHM has degradative effects on some micronutrients and bioactive compounds in DHM when compared with unpasteurized milk (12, 18). These

include water-soluble vitamins, immunoglobulins, lactoferrin and bile salt stimulated lipase. Clearly, a pasteurization technique that ensures microbial safety without compromising the nutritional value of DHM is desirable. As a result, a number of alternative techniques are being explored, such as high temperature short time (HTST) pasteurization (19), where the product is exposed to higher temperatures over a shorter time period (72-75 °C for 5-15 s) (20). Furthermore, in recent years, non-thermal approaches are of particular interest due to the known degradative effect of thermal exposure on the proteinaceous structure of some bioactive constituents. Perhaps the most promising is the emergence of high-pressure processing (HPP). HPP exposes the target product to isostatic pressure, ranging from 100-900 MPa over a chosen time period, typically at a temperature of 4-45 °C (13, 21, 22). Viazis and colleagues were the first to demonstrate the ability of HPP to successfully pasteurize DHM, which has since been further substantiated (23-25). Moreover, HPP appears to better preserve nutritional constituents of DHM when compared with HoP (26, 27). These findings highlight the potential of HPP to produce a safe and high-quality milk for infants.

The aim of this study was to investigate the efficacy of HoP and HPP at (i) eliminating the bacterial population of DHM and (ii) sustaining the microbial reduction over the course of 7 days when stored at 4 °C. This study will further validate the efficacy of HoP and HPP as pasteurization techniques and investigate the potential to produce DHM with an increased shelf life, without a reduction in microbial safety and nutritional value. As current milk banking guidelines recommend the disposal of DHM within 24 hours of thawing (NICE, 2010), the potential for a safe and prolonged storage period will prevent the waste of a critical product, often in short supply, in the neonatal health care setting.

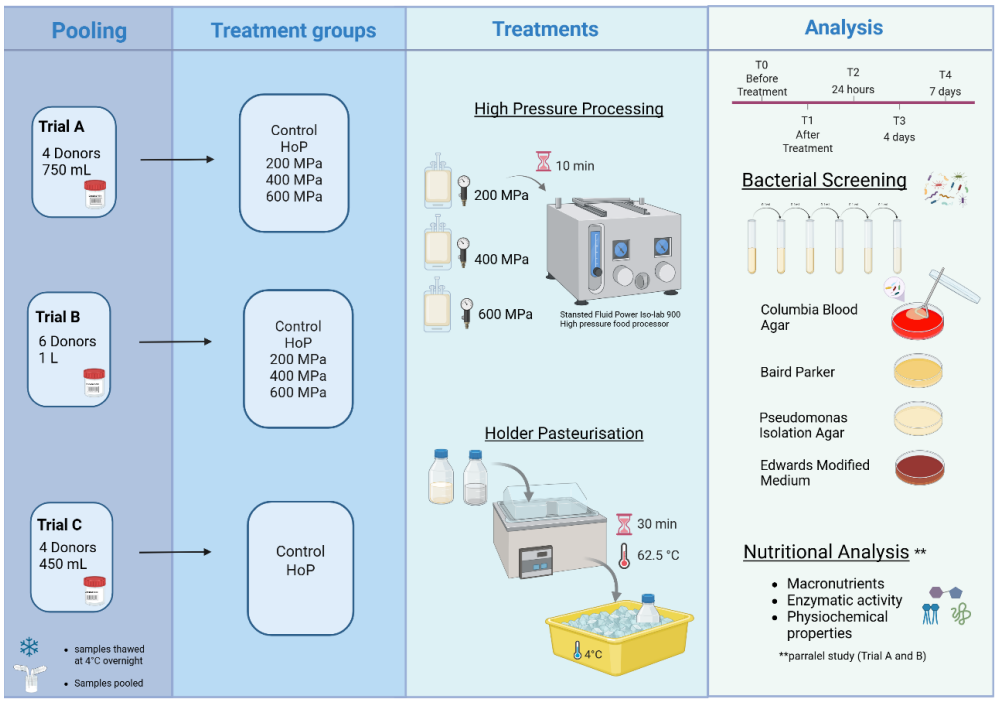



Figure 1: An overview of the experimental design for trials A-C. For HoP, treatment was performed in triplicate in three separate trials (A, B and C) using independent pooled samples. The pooled samples used in Trial A and B were also used for HPP treatment, which was only performed in duplicate (Trial A and B).

# 2 Methods

## 2.1 Experimental Design

To evaluate the microbial safety of donor human milk, samples were subjected to pasteurization treatments and underwent microbial analysis. Microbial analysis was carried out at 5 time-points; before treatment, after treatment, after 24 hours, 4 days and 7 days (T0, T1, T2, T3 and T4, respectively). Between time-points, samples were stored at 4 °C. There were 4 treatment groups: HoP, HPP at 200 MPa, HPP at 400 MPa and HPP at 600 MPa, in addition to an untreated control group. HoP was performed in triplicate (Trial A, B and C). HPP treatments were performed in duplicate (Trial A and B). Nutritional monitoring of the macronutrient content and enzymatic activity of milk was carried out on milk samples from Trial A and Trial B only (see Figure 1).

## 2.2 Human milk samples

Samples of human milk used in this study were unpasteurised milk samples, collected from Cork University Maternity Hospital and one sample was donated with consent. The use of samples in this study was approved by Cork Research Ethics Committee (ethical approval number: ECM (rr) 21/03/17). Following collection, all samples were stored at -20 °C awaiting use. Prior to treatment, milk samples were removed from the -20 °C freezer and allowed to thaw at 4 °C overnight. Once thawed, samples were pooled in a 1 L Duran bottle under sterile conditions. The pooled sample was inverted multiple times to ensure they were evenly mixed. In the first trial, milk samples from 4 donors were pooled for a total volume of 750 mL. In the second trial, 6 donor samples were pooled to achieve a total volume of 1 L. In the third trial, 4 donors contributed to achieve a final volume of 450 mL. Once pooled, the milk sample were divided into five aliquots: control (untreated), HoP, HPP 200, HPP 400 and HPP 600 MPa and underwent the appropriate treatment or were placed directly in storage at 4 °C. Following treatment, each sample was aseptically divided for nutritional and microbial analysis.

## 2.3 Treatments

### *2.3.1 Holder Pasteurisation*

For HoP treatment, human milk (150-200 mL) was transferred into a sterile Duran bottle and placed into a preheated water bath (Grant Instruments Ltd, Cambridgeshire, UK). A control bottle, containing a thermometer and filled with an equal volume of water to the test bottle, was used to monitor the temperature. Once the temperature of the control bottle reached 62.5 °C, both bottles were held for 30 min. Next, the treatment and control bottles were rapidly submerged in an ice bath and allowed to cool to 4 °C.

### *2.3.2 High-pressure processing*

Samples were exposed to three different pressures (200, 400 and 600 MPa) for a holding time of 10 min. Human milk samples (100-150 mL) were vacuum-packed into sterile stomacher bags. The sterile stomacher bag was then placed into a polyethylene bag and vacuum sealed. This process was repeated 3 times. HPP was

performed using a Stansted Fluid Power Iso-lab 900 High Pressure Food Processor (Stansted Fluid Power, Stansted, Essex, UK). A 90:10 mixture of ethanol and castor oil was used as pressurising fluid. Pressure was increased at an average rate of 120 MPa $min^{-1}$ to a value in the range 200–600 MPa and maintained at the desired pressure for 10 min. Detailed profiles of the temperature and pressure during treatment are shown in Figure 2.

## 2.4 Bacterial Screening

For bacterial screening, a 10-fold serial dilution was carried out on each sample. One mL of breast milk sample was diluted in 9 mL of maximum recovery diluent (Oxoid Ltd, Basingstoke, Hampshire, UK). Plate counts were determined by spread plating 100 µL of each serial dilution on selective and non-selective plates at each time point. In this study the following media was used: Columbia Agar (Sigma Aldrich, Dublin, Ireland) supplemented with 5% defibrinated sheep blood (Sigma Aldrich) as a non-selective media, Baird Parker Agar (Sigma Aldrich) supplemented with egg yolk tellurite emulsion (Oxoid Ltd) for the selection of *Staphylococcus*, Edwards Modified Medium (Oxoid Ltd) supplemented with 5 % defibrinated sheep blood (Sigma Aldrich) for the selection of *Streptococcus* and Pseudomonas Isolation Agar (Sigma Aldrich) for the enumeration of *Pseudomonas*. All media was prepared according to manufacturer's instructions. All plates were incubated aerobically at 37 °C for 48 h. Following incubation, bacterial colonies were counted. Plates with a range of 30-300 colonies were selected for counting. When the only available plate count was <30 colonies, this plate was selected and count recorded. Bacterial counts were recorded as colony forming units (CFU) per mL of breast milk. (28).

## 2.5 Nutritional Analysis

### *2.5.1 Macronutrients*

The macronutrient (fat, protein and carbohydrate) composition of human milk was determined using a human milk analyser (MIRIS, Uppsala, Sweden) based on mid-infrared transmission spectroscopy principles.

### *2.5.2 Enzyme Activity*

For enzyme analysis, milk samples were centrifuged at 6,000 *g* for 20 min at 4 °C and the resulting skimmed milk was used to test for lysozyme and lactoperoxidase activity.

Lysozyme activity was determined using a *Micrococcus luteus* assay as described previously by Shugar et al. (28). Briefly, the milk serum phase was combined with a 0.015 % (w/v) cell suspension of *M. luteus* ATC 4698. The optical density was measured using a spectrophotometer at 450 nm (Varian cary 50 UV-VIS, Agilent, Santa Clara, CA). Lysozyme activity was calculated as the amount of lysozyme needed to reduce A450 by 0.001 per min at a pH of 6.25 and 25 °C with *M. luteus* as the substrate.

Lactoperoxidase activity was measured using a method described previously by Zarei et al. (29). In brief, 210 µL of 100 mM potassium phosphate buffer (pH 5.5), 70 µL of 10 mM 2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 10 µL of hydrogen peroxide (0.025%) was combined with 10 µL of skimmed milk in a 96-well UV transparent microplate (VWR International Ltd, Luttervorth, UK). Absorbance was measured using a microplate reader (SpectrafluorPlus, Tecan, Männedorf, Switzerland) at A463 nm for 5 min. One unit of lactoperoxidase activity was determined at the amount of enzyme required to oxidize 1 µmol ABTS $min^{-1}$.

## 2.6 Data Analysis

Following plate counts, CFU $mL^{-1}$ was calculated. Raw CFU $mL^{-1}$ values were converted to log values: $\log_{10}$ (CFU $mL^{-1}$). When a plate count had no detectable bacterial growth (0 CFU $mL^{-1}$), the addition of 1 was used to enable log calculation: $\log_{10}$ ($0x10^0$ CFU $mL^{-1}$ + 1). A data frame consisting of media type, treatment group, trial number and $\log_{10}$ (CFU $mL^{-1}$) was imported into the software package R (version 4.3.2). The packages ggplot and dplyr were used to calculate the mean $\log_{10}$ (CFU $mL^{-1}$) and standard deviation. All graphs were generated on R using ggplot. Statistical analysis was performed with a t-test between HoP treated samples and the control sample at the same time-point. Significance was accepted when $p < 0.05$. Log reduction was calculated: (initial mean $\log_{10}$ (CFU $mL^{-1}$)) - (final mean $\log_{10}$ (CFU $mL^{-1}$)). The percentage reduction was calculated using raw CFU $mL^{-1}$ values

with the formula: 100 x (initial CFU $mL^{-1}$ – final CFU $mL^{-1}$ /initial CFU $mL^{-1}$). For both calculations, the initial value was taken as the control sample and the final value was the treated group, at the same time-point. For HoP treatment results, trial 1, 2 and 3 average counts were used. For HPP treated results, trial A and B were used for average counts (see Figure 1). Control samples were the same for HoP and HPP in trial A and B.

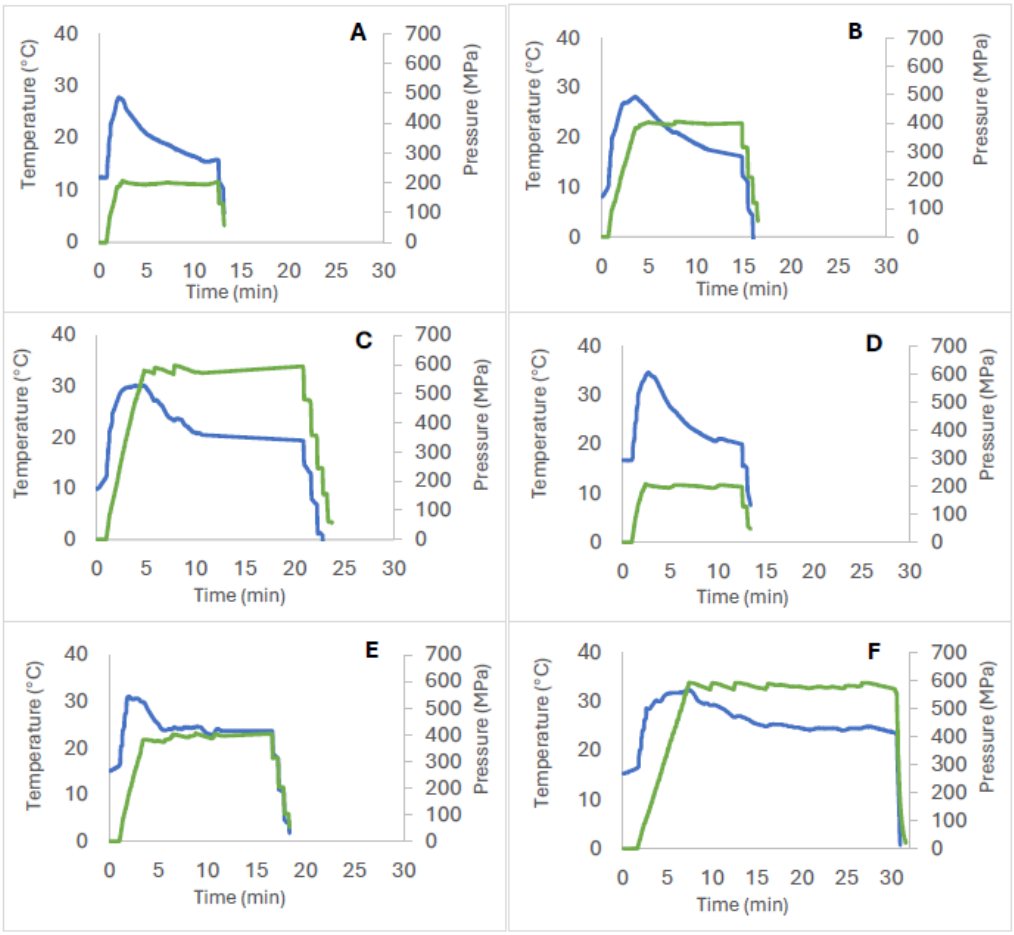



Figure 2: The temperature vs. pressure profiles of HPP treatment in Trial A (A, B, C) and Trial B (D, E, F) at 200, 400 and 600 MPa, respectively. Temperature = ( ▬ ), Pressure = (▬)

# 3 Results

## 3.1 Bacterial Screening following Holder Pasteurization

### *3.1.1 Starting Microbial Counts*

Bacterial screening of raw pooled human milk showed mean bacterial counts of 4.38 $\log_{10}$ CFU $mL^{-1}$ on unselective media (see Table 1). Bacterial growth was also observed in untreated milk samples when cultured on selective media. Before treatment (T0), mean bacterial counts for *Staphylococcus*, *Pseudomonas* and *Streptococcus* were 4.17 $\log_{10}$ CFU $mL^{-1}$, 3.78 $\log_{10}$ CFU $mL^{-1}$ and 3.38 $\log_{10}$ CFU $mL^{-1}$ respectively.

Table 1: The mean bacterial counts ($\log_{10}$ CFU $mL^{-1}$) of donor human milk following holder pasteurisation when stored at 4 °C for 7 days across all media types. All averaged data is represented as mean (SD). C = untreated control, HoP = Holder pasteurised, T0 = before treatment, T1 =after treatment, T2 = after 24 hours, T3 = after 4 days, T4 = after 7 days. p-value determined with t-test. Significance accepted when $p < 0.05$.

| | T0 | T1 | | | T2 | | | T3 | | | T4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | C | HoP | P-value | C | HoP | P-value | C | HoP | P-value | C | HoP | P-value |
| **Total Microbial Count** | 4.38 (0.81) | 4.27 (0.87) | 0 (0) | 0.001 | 3.48 (1.49) | 0 (0) | 0.015 | 3.56 (1.09) | 0 (0) | 0.005 | 3.43 (1.67) | 0 (0) | 0.024 |
| **Staphylococcus** | 4.17 (1.15) | 4.17 (0.86) | 0 (0) | 0.001 | 3.4 (1.16) | 0 (0) | 0.007 | 2.62 (2.28) | 0 (0) | 0.117 | 2.32 (2.05) | 0 (0) | 0.122 |
| **Pseudomonas** | 3.78 (1.01) | 3.88 (1.22) | 1.37 (1.39) | 0.077 | 3.58 (0.64) | 0 (0) | 0.0006 | 2.37 (2.05) | 0 (0) | 0.116 | 2.7 (2.51) | 0 (0) | 0.135 |
| **Streptococcus** | 3.38 (0.86) | 3.32 (0.90) | 0 (0) | 0.003 | 3.10 (0.84) | 0 (0) | 0.003 | 3.34 (0.39) | 0 (0) | 0.0001 | 3.51 (1.56) | 0 (0) | 0.018 |

### *3.1.2 Total Microbial Count*

Following HoP, a significant reduction in the total microbial count occurred with a mean bacterial count of 0 $\log_{10}$ CFU $mL^{-1}$, in comparison with 4.27 $\log_{10}$ CFU $mL^{-1}$ in the untreated control ($p = 0.001$) (Table 1). These findings persisted, with no detectable growth observed after 24 hours (T2), 4 days (T3) and 7 days (T4) of storage at 4 °C (Figure 3).

### *3.1.3 Selective media counts*

On Baird parker media, targeting *Staphylococcus* growth, a > 4-log reduction was observed following HoP and was statistically significant when compared with untreated DHM ($p < 0.01$) (see Table 1). *Staphylococcus* growth in HoP-treated DHM remained undetectable for up to 7 days of cold storage (Figure 4A). Immediately following HoP, a mean bacterial count of 1.37 $log_{10}$ CFU $mL^{-1}$ was observed on Pseudomonas isolation agar, representing a 2.51 log reduction. After 24 hours, no bacterial growth was observed, and was significantly different from the control ($p < 0.001$) and remained undetectable at all subsequent time-points (Figure 4B). Bacterial culturing on Edwards modified medium demonstrated a significant reduction in *Streptococcus* counts following HoP (0 $log_{10}$ CFU $mL^{-1}$; $p < 0.01$) (Table 1). Furthermore, no bacterial growth was detected over 7 days of storage at 4 °C (Figure 4C).

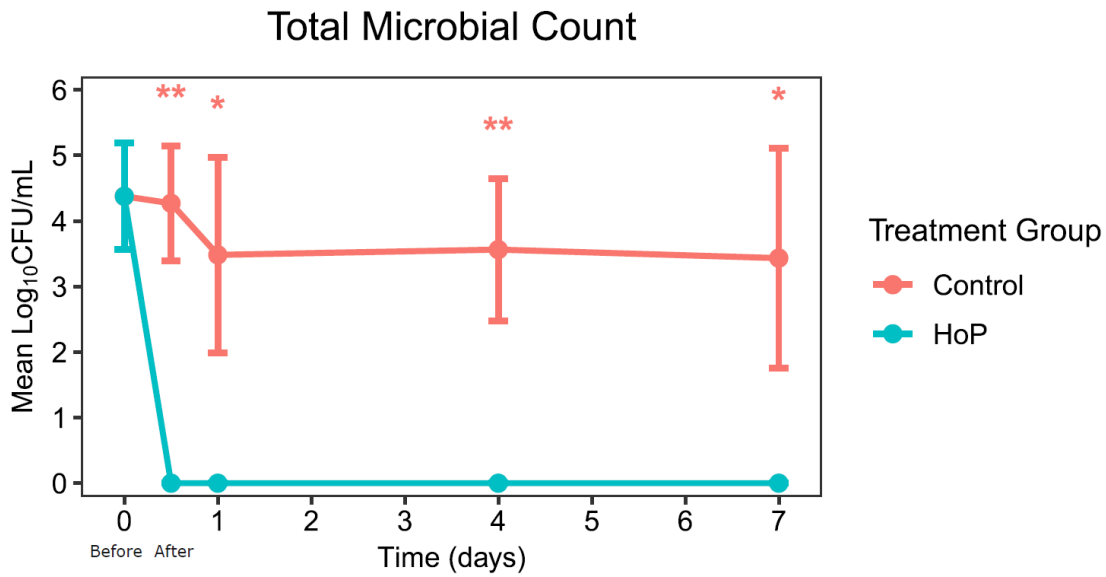



Figure 3: The mean total microbial count in raw and holder pasteurised donor human milk at each time-point, * $p < 0.05$, ** $p < 0.01$. C= control, HoP = Holder Pasteurisation

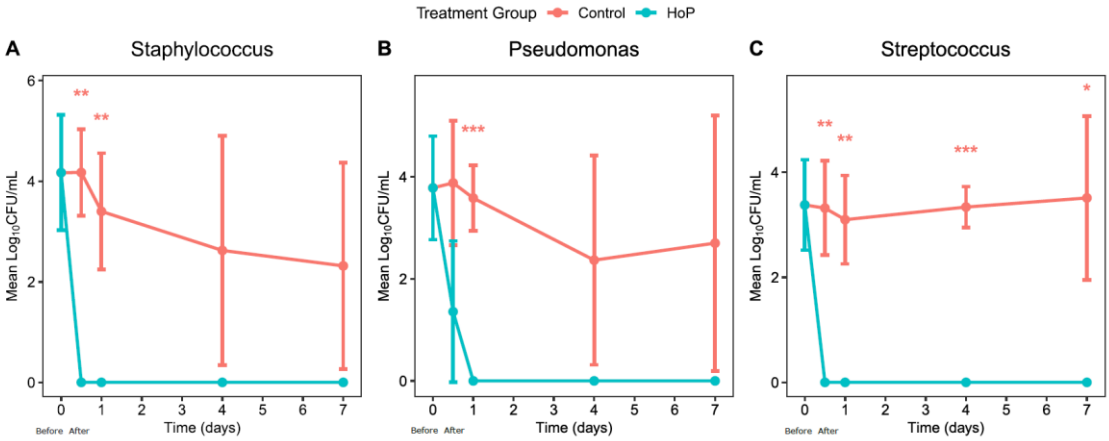



Figure 4: The mean $\log_{10}$ CFU $mL^{-1}$ of holder pasteurised milk in comparison with untreated raw milk across all time-points on (**A**) Baird parker media (**B**) Pseudomonas isolation agar and (**C**) Edwards modified medium. Significance was recorded when $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

## 3.2 High Pressure Processing

### *3.2.1 High Pressure Processing at 200 MPa*

Before HPP the mean starting bacterial counts across all media was 3.77-4.85 $\log_{10}$ CFU $mL^{-1}$. Following HPP at 200 MPa, a total microbial count of 4.55 $\log_{10}$ CFU $mL^{-1}$ was observed. By T4 the total microbial count was 3.39 $\log_{10}$ CFU $mL^{-1}$. Across all time-points, HPP at 200MPa resulted in <1-log reduction in the total microbial count of DHM (Table 2). Similarly, on selective media targeting *Staphylococcus* growth, a bacterial count of 4.4 $\log_{10}$ CFU $mL^{-1}$ was observed in DHM at T1 with a final count of 3.12 $\log_{10}$ CFU $mL^{-1}$ by T4. Across all time-points, HPP at 200 MPa resulted in < 0.5-log reduction in *Staphylococcus* levels when compared with untreated DHM (Table 2). Likewise, *Pseudomonas* persisted with levels of 3.7 $\log_{10}$ CFU $mL^{-1}$ immediately following treatment at 200 MPa. The greatest reduction in *Pseudomonas* occurred at T4 with 3.01 $\log_{10}$ CFU $mL^{-1}$ representing a 1.04-log reduction in comparison with the control (Table 2). HPP at 200 MPa did not cause a reduction in *Streptococcus* growth at T1 and T2. At T1, HPP treated DHM had 4.73 $\log_{10}$ CFU $mL^{-1}$ in comparison with 3.71 $\log_{10}$ CFU $mL^{-1}$ in the control. A similar trend was observed following 24 hours, with 3.67 $\log_{10}$ CFU $mL^{-1}$ and 3.57 $\log_{10}$ CFU $mL^{-1}$ in the HPP-treated DHM and control, respectively. A very slight log reduction of 0.16 in *Streptococcus* occurred at T3, which increased by T4 to a log reduction of 1.04 and a final bacterial count of

3.01 $log_{10}$ CFU $mL^{-1}$ (Table 2). Overall, across all media and time-points, HPP at 200 MPa resulted in a minimal reduction in the bacterial population of DHM when compared with the untreated control (Figure 5).

Table 2: The bacterial counts of DHM following HPP at 200 MPa represented as mean $log_{10}$ CFU $mL^{-1}$ (SD) at each time-point. In addition, the log reduction and percentage. T0 = before treatment, T1 =after treatment, T2 = after 24 hours, T3 = after 4 days, T4 = after 7 days.

| | **T0** | **T1** | | **T2** | | **T3** | | **T4** | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Log (%) reduction | Mean (SD) | Log (%) reduction | Mean (SD) | Log (%) reduction | Mean (SD) | Log (%) reduction |
| **Non-selective** | 4.85 (0.01) | 4.55 (0.13) | 0.23 (37.29) | 3.86 (0.52) | 0.42 (74.36) | 3.78 (0.21) | 0.41 (59.38) | 3.39 (0.13) | 0.91 (95.61) |
| ***Staphylococcus*** | 4.83 (0.14) | 4.4 (0.17) | 0.27 (46.39) | 3.96 (0.70) | 0.04 (11.93) | 3.56 (0.25) | 0.38 (59.38) | 3.12 (0.56) | 0.36 (59.35) |
| ***Pseudomonas*** | 4.36 (0.25) | 3.70 (0.12) | 0.88 (88.19) | 3.48 (0.07) | 0.39 (72.02) | 3.39 (0.09) | 0.16 (30.99) | 3.01 (0.25) | 1.04 (97.62) |
| ***Streptococcus*** | 3.77 (0.76) | 4.73 (0.38) | - | 3.67 (0.52) | - | 3.31 (0.43) | 0.25 (31.08) | 3.19 (0.38) | 1.17 (95.38) |

### *3.2.2 High Pressure Processing at 400 MPa*

Prior to HPP treatment, milk samples had a mean bacterial count of 4.85 $log_{10}$ CFU $mL^{-1}$, 4.83 $log_{10}$ CFU $mL^{-1}$, 4.36 $log_{10}$ CFU $mL^{-1}$ and 3.77 $log_{10}$ CFU $mL^{-1}$ for total, *Staphylococcus*, *Pseudomonas* and *Streptococcus* bacteria, respectively. An immediate reduction in bacterial growth was observed following HPP at 400 MPa for

10 min (Figure 5). Following treatment, DHM had a total microbial count of 1.33 $log_{10}$ CFU $mL^{-1}$, representing a log reduction of 3.45. After 24 hours, no bacterial growth was detected, and the observed reduction persisted for up to 7 days of cold storage. On selective media for *Staphylococcus*, *Pseudomonas* and *Streptococcus*, bacterial growth was undetectable with 0 $log_{10}$ CFU $mL^{-1}$ at all time-points (T1, T2, T3, and T4). Furthermore, HPP at 400 MPa resulted in a > 99.99 % reduction on selective media when compared with untreated DHM (Table 3). The greatest log reduction was observed for *Staphylococcus* growth at T1 of 4.67.

Table 3: The bacterial counts of DHM following HPP at 400 MPa represented as mean $log_{10}$ CFU $mL^{-1}$ (SD) at each time-point. In addition, the log reduction and percentage reduction in the HoP-treated milk when compared with the control sample at the same time-point are shown. T0 = before treatment, T1 =after treatment, T2 = after 24 hours, T3 = after 4 days, T4 = after 7 days.

| | **T0** | **T1** | | **T2** | | **T3** | | **T4** | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Log (%) reduction | Mean (SD) | Log (%) reduction | Mean (SD) | Log (%) reduction | Mean (SD) | Log (%) reduction |
| **Non-selective** | 4.85 (0.01) | 1.33 (1.88) | 3.45 (99.61) | 0 (0) | 4.28 (>99.99) | 0 (0) | 4.19 (>99.99) | 0 (0) | 4.3 (>99.99) |
| ***Staphylococcus*** | 4.83 (0.14) | 0 (0) | 4.67 (>99.99) | 0 (0) | 4 (>99.99) | 0 (0) | 3.94 (>99.99) | 0 (0) | 3.48 (>99.99) |
| ***Pseudomonas*** | 4.36 (0.25) | 0 (0) | 4.58 (>99.99) | 0 (0) | 3.87 (>99.99) | 0 (0) | 3.55 (>99.99) | 0 (0) | 4.05 (>99.99) |
| ***Streptococcus*** | 3.77 (0.76) | 0 (0) | 3.71 (>99.99) | 0 (0) | 3.57 (>99.99) | 0 (0) | 3.56 (>99.99) | 0 (0) | 4.36 (>99.99) |

Table 4: The bacterial counts of DHM following HPP at 600 MPa represented as mean $log_{10}$ c CFU $mL^{-1}$ (SD) at each time-point. In addition, the log reduction and

percentage reduction in the HoP-treated milk when compared with the control sample at the same time-point are shown. T0 = before treatment, T1 =after treatment, T2 = after 24 hours, T3 = after 4 days, T4 = after 7 days.

| | T0 | T1 | | T2 | | T3 | | T4 | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Log (%) reduction | Mean (SD) | Log (%) reduction | Mean (SD) | Log (%) reduction | Mean (SD) | Log (%) reduction |
| **Non-selective** | 4.85 (0.01) | 0 (0) | 4.78 (>99.99) | 0 (0) | 4.28 (>99.99) | 0 (0) | 4.19 (>99.99) | 0 (0) | 4.3 (>99.99) |
| ***Staphylococcus*** | 4.83 (0.14) | 0 (0) | 4.67 (>99.99) | 0 (0) | 4 (>99.99) | 0 (0) | 3.94 (>99.99) | 0 (0) | 3.48 (>99.99) |
| ***Pseudomonas*** | 4.36 (0.25) | 0 (0) | 4.58 (>99.99) | 0 (0) | 3.87 (>99.99) | 0 (0) | 3.55 (>99.99) | 0 (0) | 4.05 (>99.99) |
| ***Streptococcus*** | 3.77 (0.76) | 0 (0) | 3.71 (>99.99) | 0 (0) | 3.57 (>99.99) | 0 (0) | 3.56 (>99.99) | 0 (0) | 4.36 (>99.99) |

### *3.2.3 High Pressure Processing at 600 MPa*

Before treatment, pooled human milk had a mean bacterial count of 4.85 $\log_{10}$ CFU $mL^{-1}$, 4.83 $\log_{10}$ CFU $mL^{-1}$, 4.36 $\log_{10}$ CFU $mL^{-1}$ and 3.77 $\log_{10}$ CFU $mL^{-1}$ for total, *Staphylococcus*, *Pseudomonas* and *Streptococcus* bacteria, respectively. Samples subjected to high pressure treatment at 600 MPa for 10 min, underwent a > 99.99 % reduction in bacterial growth. A substantial reduction in bacterial counts was demonstrated with undetectable levels across all media and time-points following processing at 600 MPa (Figure 5). The maximum possible log reduction was achieved in all samples, with the highest log reduction observed in mean total microbial counts at T1 of 4.78 (Table 4).

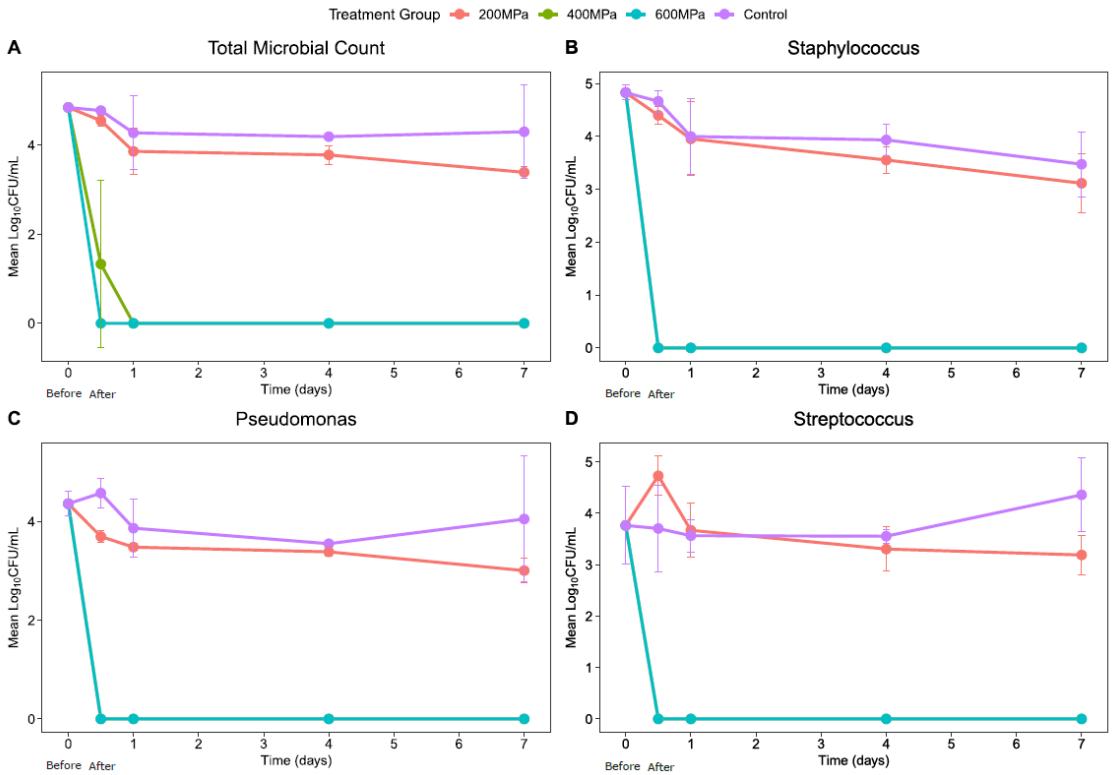



Figure 5: The bacterial counts of DHM following HPP treatment at 200, 400 and 600 MPa in comparison with the untreated control over 7 days of storage at 4 °C.

## 3.3 Nutritional Analysis

### *3.3.1 Macronutrient composition*

At T0 (before treatment), the fat, carbohydrate and protein content of milk was 3.4 (0.25) %, 7.1 (0.07) % and 1.4 (0.04) %, respectively. Immediately following treatments (T1), the fat, protein and carbohydrate content of milk remained stable in HoP-treated milk, with a fat, carbohydrate and protein content of 4.8 (1.68) %, 6.9 (0.21) % and 1.4 (0.18) %, respectively (Table 5). Similarly, in HPP-treated samples, the fat content was 3.5 (0.39) %, 3.9 (0.78) % and 3.8 (0.64) %, following processing at 200 MPa, 400 MPa and 600 MPa, respectively. Moreover, carbohydrate and protein levels were stable and within a range of 7-7.1 % and 1.4-1.5 %, respectively, following HPP treatment. Indeed, the levels of carbohydrate and protein in milk following all treatments were unchanged during storage at 4 °C for 7 days. A minor decrease in the fat content of milk was observed during storage in the control, HoP, HPP 200 MPa and HPP 400 MPa treated samples. No change in the fat content of milk treated at 600 MPa was observed during cold storage for 1 week.

Table 5: The macronutrient composition of DHM at T0 (before treatment) and T1 (after treatment) for every treatment group represented as mean (SD).

| | Fat (%) | Carbohydrate (%) | Protein (%) | Energy (Kcal $dL^{-1}$) | pH |
|---|---|---|---|---|---|
| Before treatment (T0) | | | | | |
| | 3.4 (0.25) | 7.1 (0.07) | 1.4 (0.04) | 66.5 (2.8) | 6.5 (0.06) |
| After treatment (T1) | | | | | |
| Control | 3.4 (0.25) | 7.1 (0.07) | 1.4 (0.04) | 66.5 (2.8) | 6.4 (0.04) |
| HoP | 4.8 (1.63) | 6.9 (0.21) | 1.4 (0.18) | 77.5 (14.8) | 6.6 (0.02) |
| 200 MPa | 3.5 (0.39) | 7.1 (0.04) | 1.5 (0.14) | 67 (4.2) | 6.4 (0.03) |
| 400 MPa | 3.9 (0.78) | 7.0 (0) | 1.5 (0.21) | 69.8 (8.1) | 6.5 (0.03) |
| 600 MPa | 3.8 (0.64) | 7.0 (0.04) | 1.4 (0.18) | 69.3 (7.4) | 6.5 (0.02) |

### *3.3.2 Enzyme activity in milk*

Prior to treatment, human milk samples had low initial levels of lactoperoxidase (4.95 U $L^{-1}$). Following HoP, lactoperoxidase activity was lost (0.11 U $L^{-1}$) but was retained in milk samples following HPP at 200-600 MPa for 10 min. Indeed, milk treated with HPP at 200, 400 and 600 MPa had a lactoperoxidase activity of 7.22 U $L^{-1}$, 3.47 U $L^{-1}$ and 3.29 U $L^{-1}$, respectively. A reduction in lactoperoxidase activity was observed in all samples during refrigerated storage, with lowest levels observed in HoP (0 U $L^{-1}$) and the control (0.11 U $L^{-1}$) milk. Lactoperoxidase activity was highest in HPP treated samples following 7 days of refrigerated storage with levels of 1.05 U $L^{-1}$, 4.72 U $L^{-1}$ and 1.42 U $L^{-1}$, following processing at 200, 400 and 600 MPa for 10 min, respectively.

Before treatment, human milk had an average lysozyme activity of 4048.9 U $mL^{-1}$. A reduction in lysozyme activity was observed following HoP and HPP at 600 MPa for 10 min, with 3607.2 U $mL^{-1}$ and 3768.6 U $mL^{-1}$, respectively. During cold storage, a decline in the level of lysozyme was observed in the untreated milk sample, with an activity of 3347.7 U $mL^{-1}$ by the $7^{th}$ day of cold storage. A similar level of lysozyme activity was observed in the HoP-treated milk (3165.5 U $mL^{-1}$) following cold storage. In contrast, an increase in lysozyme activity was observed in the HPP-treated milk, with the highest levels observed at T3 of 4879.4 U $mL^{-1}$, 5901.7 U $mL^{-1}$ and 5642.7 U

$mL^{-1}$, following processing at 200 MPa, 400 MPa and 600 MPa, respectively. By T4, all samples exhibited similar levels of activity, except for milk treated with HPP at 400 MPa, which had the highest levels of lysozyme, 4757.8 U $mL^{-1}$.

## 4 Discussion

Previous studies have established the ability of HoP to reduce the microbial count of DHM to sufficient safety levels. Similarly, in this study, HoP treated DHM resulted in a complete reduction in the total microbial count. Less extensive research has been carried out on the alternative processing technique, HPP. In our study, HPP at 200 MPa was inadequate for reducing the microbial load of DHM. However, pressure treatment at both 400 and 600 MPa for 10 min resulted in substantial reductions. We further demonstrated that the microbial safety of DHM is maintained for 7 days of storage at 4 °C following HoP and HPP at 400-600 MPa.

Current guidelines regarding bacterial screening by milk banks vary and no unified international acceptance criteria exist. Bacterial screening can be carried out either pre- or post-pasteurisation, or in some cases both. However, best practice guidelines recommend the screening of samples prior to pasteurisation and their disposal if the total microbial counts exceed $10^5$ CFU $mL^{-1}$ (9, 14). Furthermore, counts of $10^4$ CFU $mL^{-1}$ or higher of *Enterobacteriaceae* or *Staphylococcus aureus* are unacceptable and, following pasteurisation, no bacterial growth in milk is acceptable, with an advised threshold of <10 CFU $mL^{-1}$ (14). The handling and processing methods used in this study aimed to mimic that of a milk bank setting, with samples stored at -20 °C upon collection and thawed at 4 °C prior to treatment. Additionally, aseptic techniques were employed throughout the handling, processing and screening steps. The total bacterial count in our study was evaluated by growth on the non-selective media, Columbia Blood Agar. The pre-treatment time-point (T0), demonstrates that prior to pasteurisation, pooled raw milk samples had an average total bacterial count of $10^4$ CFU $mL^{-1}$, indicating an acceptable initial milk quality within milk banks for the DHM samples used in this study. The predominance of *Staphylococcus*, *Pseudomonas* and *Streptococcus*

underscores the importance of strict hygiene and processing practices during milk bank processing. These genera are commonly associated with the skin, environment and handling contamination during milk expression, collection, storage and pooling and are known to persist under refrigeration conditions. Additionally, although CBA is frequently used as a non-selective medium for aerobic bacterial growth, in this study the combined counts obtained on selective media exceeded the total bacterial count observed following culturing on CBA. This suggests that CBA may not adequately support the growth of all cultivable bacteria present in human milk. As such, future studies should evaluate alternative non-selective media, for example tryptic soy agar, plate count agar and brain heart infusion agar to more accurately capture the total cultivable human milk microbiota.

The HoP method is the chosen pasteurisation technique utilized by milk banks worldwide to remove pathogenic microbes that could potentially infect an infant upon consumption. Previous studies have demonstrated the ability of HoP to eliminate the endogenous bacterial population of DHM (16, 17, 30, 31). In addition, studies have demonstrated the ability of HoP to eradicate microbial growth following the deliberate contamination of DHM with artificially inoculated *Escherichia coli, S. aureus, Staphylococcus epidermis* and *Enterobacter cloacae* (32, 33). In this study, as expected, HoP treatment resulted in an immediate total microbial count reduction, in addition to a total reduction of *Streptococcus* and *Staphylococcus* growth. These findings were significant when compared with the untreated control ($p=<0.05$). However, *Pseudomonas* growth of 1.37 $\log_{10}$ CFU $mL^{-1}$ remained following treatment. This finding was unexpected as previous studies have demonstrated the ability of HoP to eradicate *Pseudomonas* growth (17). Additionally, in this study, at subsequent time-points no *Pseudomonas* growth was detected. Therefore, we suggest the observed *Pseudomonas* growth is likely a result of post-pasteurization contamination.

Cold storage is a necessary and frequent activity throughout the milk handling and processing steps from donation to distribution. Due to safety concerns regarding the risk of bacterial contamination and growth of pathogenic microbes during

storage, current guidelines require the disposal of thawed pasteurized DHM following a maximum of 24 hours of cold storage (34). We demonstrated the microbial safety of pasteurized DHM when stored at 4 °C for up to 7 days following HoP. Indeed, previous investigations support these findings, with the microbial safety of DHM maintained for 4 days (35), 5 days (31), 9 days (36) and 14 days (37) at refrigeration temperatures. The findings presented in this study indicate the microbial integrity of DHM following HoP, which is preserved when stored at 4 °C for 7 days, suggesting the potential for current storage limits of DHM to be increased without risk to the recipient.

Furthermore, in a parallel study using the same samples, nutritional analysis confirmed the integrity of DHM following HoP, with no significant changes in the fat, protein and lipid portion of DHM. Indeed, the conservation of DHM macronutrients has been widely reported (38-42). Furthermore, macronutrient levels were stable throughout the 7 days of cold storage which has also been demonstrated elsewhere (43). However, HoP treatment of DHM has been shown to degrade a number of the bioactive components including the immunoglobulins, lactoferrin, lysozyme and bile salt stimulated lipase (BSSL) (44-50). Moreover, despite some conflicting reports, HoP may also influence the levels of micronutrients, more specifically vitamins A, D and E (51-55). Indeed, nutritional monitoring in this study noted the loss of lactoperoxidase and reduction in lysozyme following HoP. The potential loss of irreplaceable bioactive agents and micronutrients is a considerable limitation associated with HoP due to their bacteriostatic capacity and role in physiological development (1, 12, 49). As such, the development and implementation of alternative processing techniques with less negative outcomes is desirable. Indeed, as a result of the damaging effect of heat exposure on the proteinaceous structure of bioactive compounds in DHM, a particular interest in non-thermal processing techniques has emerged (46, 49, 56).

The potential for the increased availability of optimized DHM for vulnerable neonates demands further exploration. As such, the prospect of HPP, an established method of preservation in the food industry, is of increasing interest. In this study, HPP was performed at 200, 400 and 600 MPa for 10 min. Nutritional

analysis confirmed the preservation of macronutrients and lactoperoxidase, although a reduction in lysozyme occurred following HPP at 600 MPa. However, the levels remained higher than those observed post-HoP. Similarly, previous investigations noted the conservation of DHM macronutrients following HPP exposure to 300-600 MPa for 5-10 min (41, 42, 57). Thus far, the literature supports the superior retention of DHM's biologically active components with HPP than thermal pasteurization. For instance, immunoglobulin levels are retained at a pressure 450 MPa, however, reductions do occur at 600 MPa (45, 46, 48, 58). Moreover, the improved retention of lactoferrin, lysozyme and bile salt-stimulated lipase following HPP was demonstrated previously (48, 59, 60). It appears that degree of degradation of bioactive components is dependent on the HPP parameters applied. Overall, despite some reported reductions, the bioactive properties of DHM are better preserved with HPP (26).

To the best of our knowledge, this is the first study to examine the effects of HPP at 200 MPa on the microbial quality of DHM. Our findings demonstrate that HPP at 200 MPa does not result in a DHM that would be considered microbiologically safe, as post pasteurisation levels of bacterial survival were above the recommended threshold of bacterial growth for milk banks (9, 14). After treatment at 200 MPa, mean bacterial growth of 3.7 – 4.73 $\log_{10}$ CFU $mL^{-1}$ remained. Evidently, 200 MPa is too low a pressure to ensure the required safety standards of DHM. Indeed, a previous investigation noted that processing conditions of 300 MPa for 15 min did not reduce bacteria to undetectable levels (25, 60). In contrast, we demonstrated that HPP treatment at 400 or 600 MPa for 10 min resulted in a total reduction of *Staphylococcus*, *Streptococcus* and *Pseudomonas* growth. These findings highlight the ability of HPP at 400-600 MPa to eradicate pathogenic and spoilage microbes associated with DHM. These finding are supported by previous studies where HPP at 400-600 MPa for varying exposure times was effective in eliminating the native microbial population of DHM with varying starting bacterial counts (25, 42, 58, 60, 61).

Thus far, microbial inactivation studies for HPP and DHM have mainly been performed following the artificial inoculation of pathogenic strains. The first study

of its kind by Viazis et al. (23) demonstrated that HPP at 400 MPa successfully eliminated the bacterial growth of 5 artificially inoculated pathogens in DHM. The group noted an 8-log reduction in *Listeria monocytogenes* and *Streptococcus agalactiae* following 2-4 min of pressure treatment, whereas 30 min of exposure to the same pressure was required to reduce *E. coli* and *S. aureus* by 6-8 log. A similar study reported that 15 min of HPP at 500 MPa at 4 °C was required for a 5-log reduction of *S. aureus* (25). In HPP studies, *S. aureus* is a concern due to the pressure-resistant nature of the microorganism (62), which is also associated with mastitis (63). As these samples were artificially inoculated, the starting levels of *S. aureus* ($10^8$ CFU $mL^{-1}$) and the observed log reduction is higher than what would be required for DHM in a milk bank setting if the initial milk quality guidelines are adhered too. In this study, total *Staphylococcus* growth was reduced to undetectable levels following 10 min of treatment at both 400 and 600 MPa. These findings demonstrate that 10 min of HPP at either 400 or 600 MPa results in a > 4-log reduction of *Staphylococcus* growth. This was the maximum possible reduction and is likely sufficient for the naturally occurring *Staphylococcus* levels of fresh DHM.

To date, contrasting findings have been reported regarding the appropriate exposure time required for bacterial eradication. Previous studies noted a 5-7 log reduction following HPP at 450-500 MPa for 15 min in *S. aureus*, *E. coli*, *L. monocytogenes* and *Cronobacter sakazakii* (25, 64). Additionally, *S. epidermis* and *E. cloacae* were reduced by >7-log following 5 min of treatment at 400-600 MPa (48). Going forward, the identification of a minimum pressure and exposure time to ensure the optimum nutritional and microbial composition of DHM will enable greater efficiency in the processing of DHM. Findings from this study show that treatment at 400-600 MPa is a potential non-thermal alternative to the currently favoured treatment, HoP. However, it is important to note that after HPP at 400 MPa, only a 3.45-log reduction in total microbial counts was observed, although the injured bacterial cells in 400 MPa treated DHM did not recover during subsequent handling and cold storage, with undetectable growth after 24 hours up until day 7. As post-processing growth was only seen in our second trial and treatment at 400

MPa did eliminate selected pathogenic growth, HPP at 400 MPa remains promising and should be explored further for an exposure of 10-15 min. Conversely, when DHM underwent HPP at 600 MPa, an immediate and complete reduction in all bacterial growth was observed. Evidently, 10 min of treatment at 600 MPa results in a microbiologically safe end product.

The prolonged storage of DHM at refrigeration temperatures following HPP has not been widely examined to date. To the best of our knowledge, no previous study has investigated the microbial quality of HPP-treated DHM beyond the initial 24 hours post-treatment. HPP at either 400 or 600 MPa successfully retained the achieved microbial safety of DHM over an extended refrigeration period of 7 days. Furthermore, these findings highlight that cold storage of HPP-treated DHM may be capable of preventing the recovery of pressure-resistant microorganisms. Indeed, the remaining injured bacterial cells in 400 MPa treated DHM at T1 did not recover when exposed to subsequent handling and cold storage protocols, with a 4-log and > 99.99 % reduction noted after 24 hours of storage that persisted to the final sampling time-point. The potential for HPP-treated samples to remain microbiologically safe and nutritionally valuable would result in the reduced waste and increased availability of DHM in the health care setting. In addition, HPP-DHM may be of superior nutritional quality due to the potential for greater retention of milk's bioactive agents including immunoglobulins, lactoferrin and lysozyme, when compared with HoP-treated DHM (46, 48, 60).

An ongoing challenge for the milk banking industry is the contamination of DHM with bacterial spores. The scope of this study did not include culturing for the presence of bacterial spores; however, plates were visually inspected as is commonly practiced by milk banks and no growth of bacterial spores was evident in this study, pre- or post-pasteurisation. It is generally accepted that HoP is ineffective at eliminating bacterial spores, particularly those of *Bacillus cereus* (16, 17, 30, 65). Furthermore, enhanced growth of *B. cereus* following exposure to thermal pasteurisation conditions is a concern. Previous studies have reported an increase in *B. cereus* contamination post-HoP, suggesting bacterial sporulation as a result of thermal based pasteurization techniques (16, 17). Currently, milk banks rely on a

strict adherence to post-processing handling and cold storage guidelines to minimize the risk of bacterial sporulation (14). More recently, the potential for HPP to control bacterial spores in DHM has been demonstrated. For instance, an optimized novel HPP system, in which the parameters for pressure delivery are accounted for successfully eradicated the spore population in DHM with HPP at 350 MPa (24). Usually, HPP is applied with three key controlling parameters: pressure, temperature and duration of the treatment (66). However, Demazeau and colleagues expanded on this approach by defining six parameters for the delivery of high pressure: pressure (350 MPa), temperature (38 °C), application rate (1 MPa $sec^{-1}$), application mode (cyclic), duration of cycle (5 min) and latency time between cycles (5 min) (24). Separately, a standard HPP system successfully inactivated artificially inoculated *B. cereus* following processing at 450 MPa for 15 min (64). Evidently, the persistent issue of bacterial spores in milk banking environments needs to be a primary area of focus for future studies. HPP-treatment presents as a potential solution achieving microbial deactivation of bacterial spores when optimal HPP parameters are applied, however, the available data remain limited and warrant further investigation. In the meantime, milk banks should continue to adhere to a policy of screening for the presence of *B. cereus* pre-treatment and disposing of any contaminated samples.

A further consideration for the implementation of HPP in human milk banks is the potential cost associated with its installation and operation. As HPP is still in the early stages of development, limited research on the economic costs, scalability and practicalities of HPP within a milk bank setting have been performed. However, a previous cost-consequence analysis noted that HPP was 7 times more expensive than HoP in a milk bank setting, with annual costs of €35,750 and €5,066, respectively (67). The authors noted that acquiring a HPP device was the biggest reason for the disparity in costs. A daily cost of €162.87 and €60.13, for HPP and HoP respectively, was noted in the same study indicating a ~2.8 times cost difference per day. Furthermore, the cost analysis did not include the costs associated with implementation of a HPP system within a milk bank setting. The logistics require careful consideration, as HPP devices must be installed in a

separate room, with thick, load bearing walls, to comply with manufacturers safety recommendations. That being said, the group suggest that HPP-treated DHM was 55 % more beneficial than HoP-treated DHM. However, this determination was limited as it relied on the measurement of only six bioactive components (leptin, insulin, hepatocyte growth factor (HGF), interleukin (IL)-1, IL-6, erythropoietin). Future analysis should expand to include a wider range of bioactives as well as clinical outcomes. Additionally, longitudinal studies evaluating the cost vs. benefit of HPP are warranted. To determine whether HPP is superior to HoP for DHM processing, future studies need to consider the practical challenges of establishing a HPP system in a milk bank setting, potential scalability issues due to low milk volume, and whether improved clinical outcomes could offset higher upfront costs through reduced long-term healthcare expenditure.

An unavoidable effect of pasteurisation is the loss of the naturally occurring beneficial microbiota in breast milk. Milk banks in Norway use DHM that does not undergo pasteurisation (68). This results in infants consuming DHM that retains the bioactive constituents in addition to the beneficial human milk-associated microbes. In order to prevent the potential transfer of harmful pathogenic microbes to the infant, strict bacterial screening is in place, and milk with bacterial counts of $> 10^5$ CFU $mL^{-1}$ are discarded. However, this is still viewed as an unfavourable option by milk banks in other parts of the world due to the perceived increased risk of transferring pathogenic microbes (13). Therefore, infants receiving pasteurized DHM are not exposed to the mother's milk microbiota, which is key in establishing a healthy infant intestinal microbiome and is associated with various health benefits, including improved protection against respiratory and diarrheal illnesses (69). A potential solution under investigation is the reintroduction of the mother's milk microbiota via inoculation enabling the personalization of DHM for the pre-term recipient (70-73). Evidently, the potential combination of optimal pasteurisation techniques with the artificial inoculation of beneficial microbes warrants further attention and could result in DHM with superior bioactivity, nutritive value and microbial safety.

## 5 Conclusion

The data presented in this chapter add to the growing evidence that HPP treatment may be a superior pasteurisation technique to the current HoP method. HPP at 400 and 600 MPa effectively eliminated potentially pathogenic microbes, in addition to preserving the nutritional components of DHM. We recommend future investigations should assess the ability of HPP at 400 MPa for a longer exposure time of approximately 15 min to eliminate total bacterial growth. Alternatively, the use of HPP at 600 MPa for a shorter exposure duration (<10 min) may yield improved outcomes for the bioactive profile of DHM and maintain microbial safety. HPP is potentially a faster and a more effective method of pasteurisation for milk banks and warrants further investigation. Furthermore, this study demonstrated the maintained microbial safety of DHM when stored at 4 °C for 7 days following either HoP or HPP at 400-600 MPa, which suggests the potential to safely increase the current cold storage limits. An increased storage period can be implemented easily within the milk bank setting and would result in the increased availability of DHM, which is often in short supply. These findings are significant for the vulnerable demographic in receipt of DHM, therefore, this study is important for milk banks and the health-care sector to consider when facilitating the supply of safe and nutritionally valuable DHM to infants.

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## 7 Contributions

The parallel nutritional analysis referred to in this study was performed by Dr. Fanyu Meng as part of a collaboration with the School of Food and Nutritional Sciences.

Dr. Fanyu Meng and Jim McNamara were also involved in the running of treatment trials outlined in this study.

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# Chapter 6

## Novel methods for the processing of donor human milk

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## Abstract

Donor human milk is a valuable resource within the health care sector, providing nutrient dense milk to vulnerable infants, when mothers own milk is not available. The distribution of donor human milk is facilitated by milk banks who oversee the safe processing and storage of this invaluable fluid. In recent years, the optimization and development of alternative processing techniques are of increasing interest due to the negative impact of holder pasteurisation (62.5 °C for 30 min) on the bioactive properties of milk. The aim of this study was to evaluate novel non-thermal processing techniques for the production of donor human milk within acceptable microbial safety standards. Milk underwent a series of novel non-thermal processing techniques; including the application of the bacteriocin, nisin A, and enzyme, lactose oxidase. In addition, de-fatted human milk was subjected to microfiltration. Bacterial screening was performed immediately following treatment and monitored for a further 7 days of refrigerated storage. Prior to treatment, total bacterial counts were high (6.68 $\log_{10}$ CFU $mL^{-1}$) due to a pre-treatment incubation period of 7 days at 10 °C. Immediately following treatment, a complete reduction in microbial growth (> 6-log) on non-selective media was observed following microfiltration or nisin A combined with holder pasteurisation. Similarly, a > 6-log reduction on media targeting *Pseudomonas* and coliforms was observed in both groups. Low levels of microbial inhibition were observed with the direct inclusion of either nisin A or lactose oxidase across the different media and time-points. These findings demonstrate the potential for the incorporation of non-conventional processing techniques. In particular, microfiltration or a hybrid approach of holder pasteurisation and nisin A demonstrated a greater reduction in total bacterial growth, when compared with holder pasteurization alone. This study paves the way for alternative approaches to human milk processing and future studies should further evaluate and optimize these techniques to confirm their suitability for donor human milk processing.

# 1 Introduction

Donor Human Milk (DHM), the recommended alternative when mother's own milk is not available (1), is a crucial tool for preterm vulnerable infants, facilitated by the carefully controlled practices of human milk banks. The ever growing demand for donor human milk within the medical sector has prompted the development of milk processing conditions that will result in the optimum end product (2). Milk banking methods currently rely on the thermal treatment, holder pasteurization (HoP), to ensure milk safety and shelf life stability (3). During HoP treatment, milk is exposed to heat at 62.5 °C for 30 min. However, thermal processing has an established degradative effect on the bioactive components of DHM (4). In this regard, researchers now seek non-thermal alternatives that ensure the retention of milks nutritional properties and guarantee microbial safety, in place of the conventional thermal pasteurization. Thus far, novel techniques used to control microbial contamination of DHM include high-pressure processing, ultraviolet-C irradiation and pulsed electric field treatment (5). To date, the use of bio-preservation techniques for the processing of DHM has been unexplored, despite their growing applicability and success in the food preservation and biomedical sectors (6, 7).

Anti-microbial peptides, such as bacteriocins, have been of particular interest due to their successful applicability as food bio-preservatives (8). Most notably, nisin, a broad spectrum lantibiotic produced by *Lactococcus lactis*, has gained attention as it is non-toxic, flavourless, heat-stable and has established functional diversity (9-13). Nisin remains the most well studied bacteriocin; the anti-microbial effects of nisin was first observed in 1928 (14), was named in 1947 (15) and nisin was accepted as safe for food use by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) in 1969 and approved as a food preservative with GRAS (generally recognized as safe) status for human consumption by the Food and Drug Administration (FDA) in 1988 (16). This designation spurred on its widespread use in food preservation due to its broad anti-microbial capacity with demonstrated microbial inhibition against the foodborne pathogens *Listeria monocytogenes* and *Bacillus cereus* and the gram-positive human pathogens *Staphylococcus aureus* (17, 18). In addition to food preservation,

nisin is garnering attention as a potential for the treatment of infectious diseases (7), with demonstrated efficacy in the treatment of women suffering from mastitis (19). Furthermore, recent strategies include the use of nisin in combination with conventional processing technologies including heat treatment at 80-117 °C and high-pressure processing (HPP) at 200-500 MPa (20), allowing the anti-microbial action of nisin and the unfavourable environmental conditions created by processing to work synergistically and eliminate microbial growth.

The proteinaceous and enzymatic components of milk contribute to the established bacteriostatic capacity of milk (21). One such contributor is the lactoperoxidase (LPO) system, which is naturally occurring in milk, and has a role in protecting the mammary gland from infection and acts as an intrinsic method of microbial inhibition in milk (22). The LPO system has three essential components, LPO enzyme, thiocyanate ion and hydrogen peroxide (23). LPO catalyses the oxidation of thiocyanate by hydrogen peroxide, producing hypothiocyanite, an anti-microbial compound (24). The LPO system has demonstrated anti-microbial activity against both pathogenic and spoilage organisms (25, 26), with greater inhibitory action observed against gram-negative bacteria (24). Hydrogen peroxide is naturally occurring in milk at very low levels; however, the direct addition of hydrogen peroxide to foodstuffs can be viewed unfavourably due to its associated toxicity at higher concentrations (27, 28). Thus, lactose oxidase represents a naturally derived alternative for the exogenous activation of the LPO system (22). In milk, lactose oxidase reduces lactose into lactobionic acid and hydrogen peroxide, providing the necessary levels of hydrogen peroxide for activation of the LPO system (29). Previous studies have demonstrated the capacity of lactose oxidase to reduce microbial population of dairy foods (30, 31), although further studies are still needed to ascertain the potential of lactose oxidase within different food matrices and optimize its effect against different microbial targets. Nevertheless, the potential for increased activation of the LPO system through the addition of lactose oxidase is a promising and favourable alternative to conventional thermal processing of DHM.

Recent strategies for the non-thermal processing of liquid foodstuffs include microfiltration. Microfiltration physically separates bacterial cells from the liquid food matrix, by preventing the passage of bacteria across the filter membrane (32). Size exclusion, determined by the membrane pore size, is the critical factor for achieving the desired separation of target particles from the food matrix (33). A number of studies have demonstrated the microbial safety and improved shelf life of bovine milk following microfiltration (34, 35). In addition, microfiltration has previously been used for the development of infant formula with a greater retention of native proteins (36, 37) or as a method for extracting the bioactive components from milk, colostrum or whey (38). The success and suitability of microfiltration is dependent on three key factors, the membrane pore size, the surface charge of both the membrane surface and the targeted molecule to be filtered, and the hydrophobicity or hydrophilicity of both the membrane surface and filter target (33). The interaction of these factors with the food matrix being processed will determine the success of microbial filtration and the likelihood of the occurrence of membrane fouling, which refers to the accumulation of solids on the membrane surface and within the membrane pores, reducing membrane permeability and selectivity (39). As such, the microfiltration of milk is prone to fouling due to the accumulation of protein and fat molecules at the membrane surface and the low pore size required for bacterial exclusion (33). Hence, microfiltration is mainly applied on skimmed milk, with the potential re-addition of pasteurized fat content post-processing (40-42).

Given the effectiveness of bio-preservation and microfiltration for industrial and therapeutic applications, the objective of this study was to assess their applicability for the human milk banking sector. We investigated the ability of novel processing techniques to eliminate bacterial growth in DHM. In addition, we investigated the potential of HoP in combination with Nisin A to further enhance the currently used technique.

# 2 Methods

## 2.1 Determining experimental conditions

Prior to the commencement of this study, a preliminary cold storage study (Incubation A) was performed to determine the optimum incubation conditions to achieve high starting microbial count (> $10^5$ CFU $mL^{-1}$) in pooled human milk. Hence, a pooled milk sample of 40 mL from two donors was thawed overnight a 4 °C. Milk was inverted to ensure a homogenous mixture. 1 mL of milk was aseptically collected before the remainder of the sample was placed in a 10 °C incubator. The 1 mL of milk underwent a 10-fold serial dilution with maximum recovery diluent (MRD, Oxoid Ltd, Basingstoke, Hampshire, UK) and 100 µL was spread plated on both non-selective and selective media, as described in Section 2.5. This process was repeated daily for 10 days. The sample was continuously stored at 10 °C between sampling points. Based on the bacterial counts obtained in this cold storage study, an incubation period of 7 days at 10 °C was selected as the pre-treatment incubation conditions (Incubation B) to ensure adequate starting microbial counts.

## 2.2 Experimental Design

To evaluate the efficacy of novel milk treatments, pooled human milk was subjected to processing and underwent bacterial screening. The use of all milk samples in this study was approved by Cork Research Ethics Committee (ethics number: ECM (rr) 21/03/17). To ensure a high starting bacterial count that would allow the efficacy of treatments to be determined, a pre-treatment incubation step (Incubation B) was performed (7 days storage at 10 °C). Following incubation, samples underwent treatment and microbial analysis was carried out at 5 time-points; before treatment, immediately after treatment, after 24 hours, 4 days and 7 days (T0, T1, T2, T3 and T4, respectively). All samples were stored at 4 °C between time-points. Six different treatment groups were included in this study: HoP, HoP combined with Nisin A (HoP + Nisin A), Microfiltration, Lactose Oxidase, Nisin A and an untreated control (C). This study was performed in duplicate. A parallel study involving nutritional analysis of milk composition was also performed.

## 2.3 Pre-treatment handling of human milk samples

Samples of human milk used in this study were unpasteurised milk samples from two donors. Prior to use, all samples were stored at -20 °C. Samples were thawed at 4 °C overnight and pooled under sterile conditions. The pooled sample was inverted multiple times to ensure a homogenous mixture. Once pooled, the milk underwent cold storage at 10 °C for 7 days (Incubation B). Incubation conditions were monitored daily with a thermometer. During incubation, 1 mL of sample was taken aseptically at day 0 and day 4 to monitor bacterial growth. Following incubation, the milk sample was divided into six aliquots: C, HoP, HoP + Nisin A, Microfiltration, Lactose Oxidase and Nisin A and underwent the appropriate treatment or were placed directly in storage at 4 °C. Following treatment, each sample was further divided for nutritional and microbial analysis under sterile conditions.

## 2.4 Treatments

### *2.4.1 Holder Pasteurisation*

For HoP treatment, human milk was transferred into a sterile Duran bottle. The sample was placed into a preheated (62.5 °C) water bath (Grant Instruments Ltd, Cambridgeshire, UK). A control bottle, containing a thermometer and filled with an equal volume of water to the test bottle, was used to monitor the temperature. Once the temperature of the control bottle reached 62.5 °C, both bottles were held in the water bath for 30 min. Next, the treatment and control bottle were rapidly submerged in an ice bath and allowed to cool to 4 °C.

### *2.4.2 Holder Pasteurisation + Nisin A*

For HoP + Nisin A treatment, the milk sample underwent HoP as previously described in Section 2.4.1. Following HoP, 1 g $L^{-1}$ of NisinA® (Handary, Brussels, Belgium) was added to 1 mL of the pasteurized milk sample and re-suspended until fully dissolved. Finally, the 1 mL of milk containing the re-suspended nisin A was transferred to the whole sample and vortexed at low speed for 5 min.

### *2.4.3 Nisin A*

For the Nisin A treated milk, NisinA® was added to 1 mL of milk sample, re-suspended with a pipette until fully dissolved and added to sample before vortexing gently for 5 min to achieve a final concentration of 1 g $L^{-1}$.

### *2.4.4 Microfiltration*

For microfiltration treatment, milk was centrifuged at 6,000 *g* for 30 min and the lipid layer was removed using a sterile cotton swab (Thermo Fisher Scientific Inc). The de-fatted milk was filtered through a membrane filtration unit with a pore size of 0.45 µm (VWR International Ltd, Dublin, Ireland). A Buchi V-700 Vacuum pump (Büchi Labortechnik AG, Flawil, Switzerland) was used to generate vacuum. To increase the vacuum, the pressure was gradually decreased from 500 to 200 mbar, allowing the flow of skim milk through the membrane.

### *2.4.5 Lactose Oxidase*

In lactose oxidase-treated milk, lactose oxidase was added directly to milk and vortexed at low speed for 5 min to achieve a final concentration of 1.2 g $L^{-1}$.

## 2.5 Bacterial Screening

For bacterial analysis, milk samples underwent a 10-fold serial dilution with MRD (Oxoid Ltd, Basingstoke, Hampshire, UK). Plate counts were determined by spread plating 100 µL of each serial dilution on selective and non-selective plates at each time point. In this study, the following media was used: Columbia Agar (Sigma Aldrich, Dublin, Ireland) supplemented with 5 % defibrinated sheep blood (Sigma Aldrich) as a non-selective media, Baird Parker Agar (Sigma Aldrich) supplemented with egg yolk tellurite emulsion (Oxoid Ltd) for the selection of *Staphylococcus*, Edwards Modified Medium (Oxoid Ltd) supplemented with 5 % defibrinated sheep blood (Sigma Aldrich) for the selection of *Streptococcus* and Pseudomonas Isolation Agar (Sigma Aldrich) for the enumeration of *Pseudomonas*. MacConkey's agar was used for the identification of coliform growth. All media were prepared according to manufacturer's instructions. All plates were incubated aerobically at 37 °C for 48 h and following incubation, bacterial colonies were counted. Plates with a range of 30-300 colonies were selected for counting. When the only available plate count was < 30 colonies, this plate was selected and counts recorded. All

plate counts were performed in duplicate and the averaged data was used to record bacterial counts as colony forming units (CFU) per mL of milk. These steps were also used for microbial monitoring of bacterial growth at day 0 and day 4 of incubation B prior to treatments.

### 2.6 Nutritional Analysis

The macronutrient content of milk was analyzed immediately after treatment (Day 0) and on the final day of cold storage (Day 4). The total fat, protein and carbohydrate content of milk was determined using a human milk analyzer (MIRIS, Upsala, Sweden) based on mid-infrared transmission spectroscopy. The pH values of milk were monitored at every time-point using a FiveEasy Plus Benchtop FP20 pH/mV Standard Kit (Mettler Toledo, Leicester, UK) at 4 °C.

### 2.7 Data Analysis

Following plate counts, CFU $mL^{-1}$ was calculated. Raw CFU $mL^{-1}$ values were converted to log values: $\log_{10}$ (CFU $mL^{-1}$). When a plate count had no detectable bacterial growth (0 CFU $mL^{-1}$), the addition of 1 was used to enable log calculation: $\log_{10}$ ($0x10^0$ CFU $mL^{-1}$ + 1). A data frame consisting of media type, treatment group, trial number and $\log_{10}$ (CFU $mL^{-1}$) was imported into the software package R (version 4.3.2). The package ggplot and dplyr were used to calculate the mean $\log_{10}$ (CFU $mL^{-1}$) and standard deviation. Similarly, the package ggplot was used for the generation of all graphs. Log reduction was calculated: (initial mean $\log_{10}$ (CFU $mL^{-1}$)) - (final mean $\log_{10}$ (CFU $mL^{-1}$)). The percentage reduction was calculated using raw CFU $mL^{-1}$ values with the formula: 100 x (initial CFU $mL^{-1}$ – final CFU $mL^{-1}$ /initial CFU $mL^{-1}$). For both calculations, the initial value was taken as the control sample and the final value was the treated group, at the same time-point.

## 3 Results

### 3.1 Preliminary cold storage incubation study

To determine incubation conditions that would result in a high starting microbial count, a separate preliminary cold storage study was performed (Incubation A). Pooled human milk stored at 10 °C for 10 days resulted in high levels of bacterial

growth, with an 8.13 $log_{10}$ CFU $mL^{-1}$ total bacterial count (Figure 1). Prior to incubation, milk was sampled and had total *Pseudomonas*, Coliform, *Staphylococcus* and *Streptococcus* counts of 3.1, 1.48, 2.56, 3.26 and 1.48 $log_{10}$ CFU $mL^{-1}$, respectively. By day 7 of incubation, bacterial growth on non-selective media had increased to 6.29 $log_{10}$ CFU $mL^{-1}$. Similarly, bacterial growth of 5.93, 6.27 and 6.26 $log_{10}$ CFU $mL^{-1}$ was observed for *Pseudomonas*, *Streptococcus* and coliforms. *Staphylococcus* growth on Baird parker was undetectable by day 5 of incubation, and no growth was observed at subsequent time-points. Based on the high bacterial growth achieved by 7 days of incubation at 10 °C, these incubation parameters were selected for pre-treatment incubation of the milk samples to ensure sufficient starting counts allowing an effect of treatment to be determined (Incubation B).

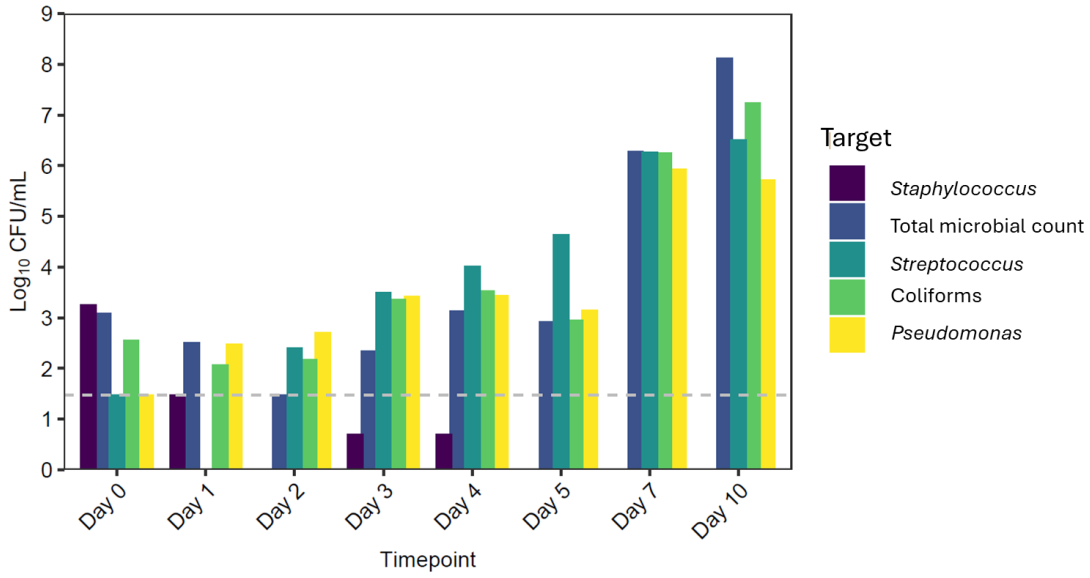



Figure 1: Pre-liminary incubation study (Incubation A) to determine incubation conditions to use in this study: the bacterial counts ($log_{10}$ CFU $mL^{-1}$) when human milk was stored at 10 °C for 10 days following culturing on non-selective and selective media.

## 3.2 Bacterial Screening following treatments

### *3.2.1 Starting microbial counts*

Following incubation at 10 °C for 7 days (Incubation B), bacterial screening revealed a mean starting total microbial count of 6.37 $log_{10}$ CFU $mL^{-1}$ on unselective media (see Table 1). Additionally, before all treatments (T0), mean bacterial counts for *Pseudomonas,* coliforms*, Staphylococcus* and *Streptococcus* were 7.58 $log_{10}$ CFU $mL^{-1}$, 7.74 $log_{10}$ CFU $mL^{-1}$, 1.53 $log_{10}$ CFU $mL^{-1}$ and 4.6 $log_{10}$ CFU $mL^{-1}$ respectively. Additionally, monitoring of bacterial growth during incubation B at 10 °C, revealed bacterial counts of 2.28-3.01 and 3.18-3.69 $log_{10}$ CFU $mL^{-1}$ at day 0 and day 4, respectively (Table 2). Starting counts were in line with expected levels following incubation and were sufficient to allow an effect of treatment to be determined.

### *3.2.2 Total Microbial Counts*

Using milk stored for 7 days at 10°C, and following HoP treatment, a substantial reduction in bacterial levels was observed across time-points. Immediately after HoP treatment (T1), a total microbial count of 1.45 $log_{10}$ CFU $mL^{-1}$, representing a log reduction of 5.23 was observed (see Table 1). Furthermore, no bacteria was detected at all subsequent time-points, with a greater than 7-log reduction. The greatest reduction in bacterial counts was observed in milk following microfiltration or HoP + Nisin A treatment, with undetectable levels at T1, representing a 6.68-log reduction. Indeed, for HoP + Nisin A-treated milk, this finding persisted with no detectable growth for 7 days of storage at 4 °C (see Figure 2). However, in the microfiltered milk, low levels of bacterial growth of 1.1 $log_{10}$ CFU $mL^{-}1$ and 1.05 $log_{10}$ CFU $mL^{-1}$, were observed at T2 and T3, respectively. Nonetheless, by T4 bacterial levels were undetectable. In contrast, bacterial survived following treatment with lactose oxidase and Nisin A, with bacterial counts of 5.77 $log_{10}$ CFU $mL^{-1}$ and 6.77 $log_{10}$ CFU $mL^{-1}$ respectively at T1. Indeed, Nisin A treated milk showed a slight increase in bacterial counts when compared to untreated control milk (6.68 $log_{10}$ CFU $mL^{-1}$). Despite the initial increase, after 24 hours a 2.27-log reduction was observed in the Nisin A treated milk, with similar log reductions observed at T3 (4 days) and T4 (7 days). Across all time-points, lactose oxidase treated milk resulted in less than a 2-log reduction. Both Nisin A and Lactose oxidase demonstrated the

least efficacy against the total aerobic microbial population of pooled human milk in this study.

Table 1: Mean total microbial counts following treatment at each time-point. Total microbial counts as per growth on non-selective Columbia blood agar. Averaged data is presented as mean $\log_{10}$ CFU $mL^{-1}$ (standard deviation). Log reduction and percentage reduction presented in brackets are based off the treatment group when compared with the untreated control at the same time-point. T0 = before treatment, T1 =after treatment, T2 = after 24 hours, T3 = after 4 days, T4 = after 7 days.

| | **T0** | **T1** | | **T2** | | **T3** | | **T4** | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatment group** | Mean (SD) | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction |
| **Control** | 6.37 (0.29) | 6.68 (0.11) | - | 7.13 (0.46) | - | 7.4 (0.74) | - | 7.62 (0.57) | - |
| **HoP** | 6.37 (0.29) | 1.45 (2.05) | 5.23 | 0 (0) | 7.13 | 0 (0) | 7.4 | 0 (0) | 7.62 |
| **HoP + Nisin A** | 6.37 (0.29) | 0 (0) | 6.68 | 0 (0) | 7.13 | 0 (0) | 7.4 | 0 (0) | 7.62 |
| **Microfiltration** | 6.37 (0.29) | 0 (0) | 6.68 | 1.1 (1.56) | 6.03 | 1.05 (1.48) | 6.35 | 0 (0) | 7.62 |
| **Lactose Oxidase** | 6.37 (0.29) | 5.77 (0.34) | 0.91 | 5.8 (0.22) | 1.33 | 6.24 (0.59) | 1.16 | 5.9 (0.52) | 1.72 |
| **Nisin A** | 6.37 (0.29) | 6.77 (0.17) | - | 4.86 (2.82) | 2.27 | 5.05 (3.2) | 2.35 | 5.36 (3.07) | 2.26 |

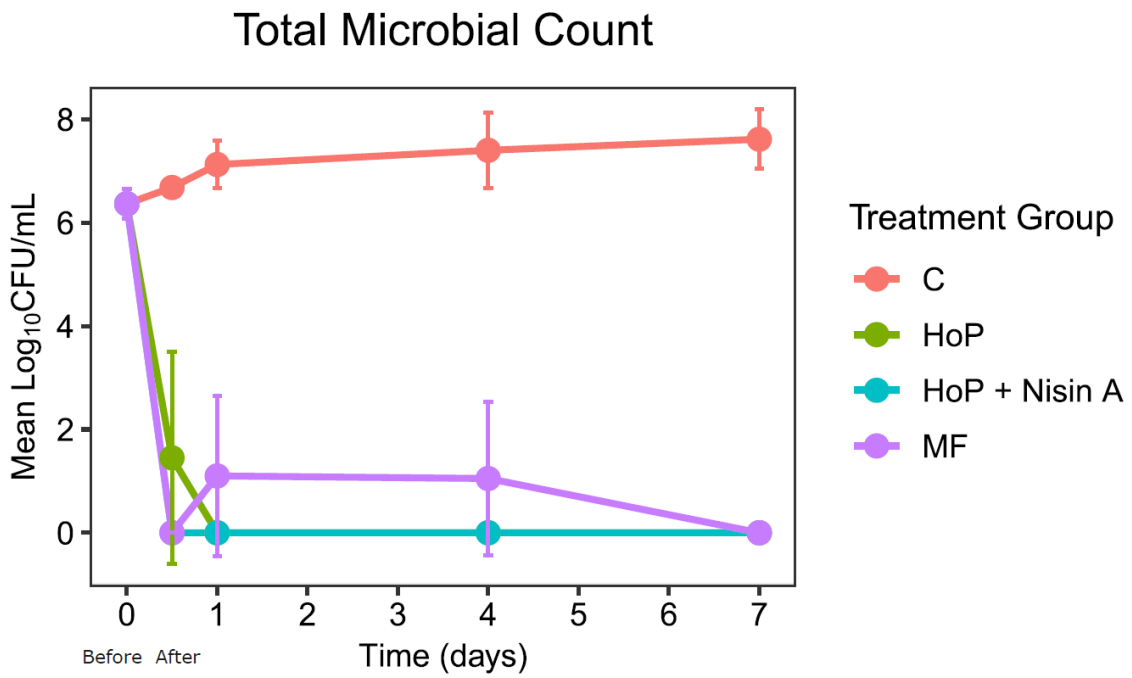



Figure 2: The total microbial count of DHM following novel processing techniques for up to 7 days of storage at 4 °C. Treatment groups: C = control, HoP = Holder pasteurized, HoP + Nisin A = Holder pasteurized and treated with Nisin A, MF = membrane filtered

Table 2: Monitoring of bacterial growth during incubation B (10°C for 7 days) of pooled milk pre-treatment for both trials. All values presented as $\log_{10}$ CFU $mL^{-1}$.

| | Media | Day 0 | Day 4 |
|---|---|---|---|
| Trial 1 | Columbia blood agar | 3.01 | 3.69 |
| | Baird Parker | 2.84 | 1.65 |
| | MacConkey Agar | 2.93 | 3.02 |
| | Edwards modified medium | 1.00 | 2.87 |
| | *Pseudomonas* isolation agar | 1.81 | 3.40 |
| Trial 2 | Columbia blood agar | 2.28 | 3.18 |
| | Baird Parker | 1.54 | 0 |
| | MacConkey Agar | 1.18 | 3.13 |
| | Edwards Modified Medium | 1.88 | 2.06 |
| | *Pseudomonas* Isolation Agar | 1.88 | 3.22 |

### *3.2.3 Pseudomonas*

Quantification of *Pseudomonas* growth by culturing pooled human milk on Pseudomonas Isolation Agar revealed a variable effect of treatments. A substantial reduction in *Pseudomonas* was observed in milk following treatment with HoP, HoP + Nisin A and Microfiltration with a log reduction of 6.24, 5.82 and 6.53, respectively. Bacterial counts remained low in all three groups at T2 and T3 (0-1.4 $\log_{10}$ CFU $mL^{-1}$) (Figure 3A), with undetectable levels by the final time-point (T4). In contrast, a lower reduction of 3.2-log were observed in lactose oxidase treated milk at T1, with residual bacterial levels of 4.43 $\log_{10}$ CFU $mL^{-1}$. Despite an initial reduction in lactose oxidase treated milk, after 24 hours, pseudomonas counts increased to 6.41 $\log_{10}$ CFU $mL^{-1}$. A similar level of *Pseudomonas* was observed at T3 and T4 of 6.45 $\log_{10}$ CFU $mL^{-1}$ and 6.28 $\log_{10}$ CFU $mL^{-1}$, respectively. The highest levels of bacteria following treatment were observed in the Nisin A treated milk (6.47 $\log_{10}$ CFU $mL^{-1}$). Indeed, at all subsequent time-points, a > 0.5-log reduction was observed when compared with the untreated control (Table 3).

Table 3: Mean *Pseudomonas* counts ($log_{10}$ CFU $mL^{-1}$) of pooled human milk following novel processing treatments across 7 days of storage at 4 °C. Averaged data is presented as mean $log_{10}$ CFU $mL^{-1}$ (standard deviation). Log reduction and percentage reduction presented in brackets are based on the treatment group when compared with the untreated control at the same time-point. T0 = before treatment, T1 =after treatment, T2 = after 24 hours, T3 = after 4 days, T4 = after 7 days.

| Treatment group | T0 | T1 | | T2 | | T3 | | T4 | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction |
| **Control** | 7.58 (1.37) | 7.63 (1.39) | - | 7.13 (0.62) | - | 7.5 (0.54) | - | 7.63 (0.59) | - |
| **HoP** | 7.58 (1.37) | 1.39 (1.96) | 6.24 | 0 (0) | 7.13 | 0.77 (1.08) | 6.73 | 0 (0) | 7.63 |
| **HoP + Nisin A** | 7.58 (1.37) | 1.81 (1.56) | 5.82 | 0.93 (1.31) | 6.2 | 1.29 (1.82) | 6.21 | 0 (0) | 7.63 |
| **Microfiltration** | 7.58 (1.37) | 1.1 (1.56) | 6.53 | 0 (0) | 7.13 | 1.41 (1.99) | 6.09 | 0 (0) | 7.63 |
| **Lactose Oxidase** | 7.58 (1.37) | 4.43 (2.31) | 3.2 | 6.41 (0.65) | 0.72 | 6.45 (0.86) | 1.05 | 6.28 (0.60) | 1.35 |
| **Nisin A** | 7.58 (1.37) | 6.47 (0.26) | 1.16 | 7.1 (0.57) | 0.03 | 7.11 (0.32) | 0.39 | 7.3 (0.38) | 0.33 |

### *3.2.4 Coliforms*

In this study, the untreated human milk had high levels of coliforms at T1 of 7.66 $log_{10}$ CFU $mL^{-1}$ while a greater than 6-log reduction was observed in HoP, HoP + Nisin A and microfiltered milk, with bacterial counts of 1.46 $log_{10}$ CFU $mL^{-1}$, 1.38 $log_{10}$ CFU $mL^{-1}$ and 1.30 $log_{10}$ CFU $mL^{-1}$, respectively (Table 4). Further reduction in coliforms was observed at subsequent time-points with undetectable levels after 24 hours (T2), 4 days (T3) and 7 days (T4) (Figure 3B). In contrast, high levels of coliform counts persisted in the control group, with 6.86 $log_{10}$ CFU $mL^{-1}$ after 7 days of storage at 4 °C. Milk treated with Nisin A failed to suppress coliforms , with 6.49 $log_{10}$ CFU $mL^{-1}$ at T1, and a minimal reduction in coliform counts over the 7 days of storage, representing less than 1-log reduction at each time-point. Following lactose oxidase treatment (T1), coliform levels of 5.73 $log_{10}$ CFU $mL^{-1}$ remained. However, after 24 hours, a greater than 5-log reduction in coliform counts was

observed (1.44 $log_{10}$ CFU $mL^{-1}$). Despite the observed reduction after 24 hours, coliform levels increased at T3 and T4 with 5.13 $log_{10}$ CFU $mL^{-1}$ and 3.07 $log_{10}$ CFU $mL^{-1}$, respectively (Table 4).

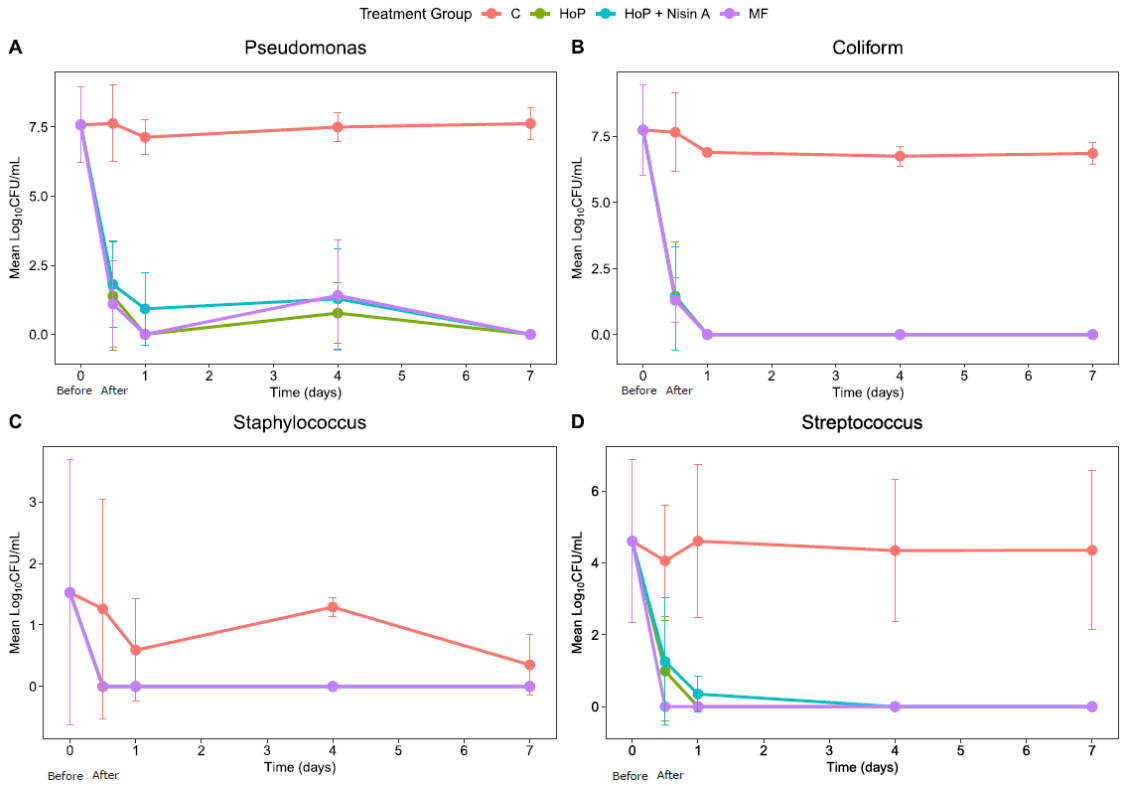



Figure 3: Bacterial growth of pooled human milk following novel processing techniques and storage for 7 days at 4°C. (**A**) *Pseudomonas* (**B**) Coliforms (**C**) *Staphylococcus* (**D**) *Streptococcus*. Treatment groups: C = control, HoP = Holder pasteurized, HoP + Nisin A = Holder pasteurized and treated with Nisin A, MF = membrane filtered

### *3.2.5 Staphylococcus*

At T1, low levels of *Staphylococcus* were observed in the untreated control group (1.26 $log_{10}$ CFU $mL^{-1}$). *Staphylococcus* counts remained low in the control group at all time-points (Table 5). No detectable levels of *Staphylococcus* were observed in HoP, HoP + Nisin A and the microfiltration treated milk at T1, T2, T3 and T4 ( Figure 3C). Despite low starting levels, *Staphylococcus* populations were detectable in Lactose oxidase and Nisin A treated milk following treatment, with counts of 0.80 $log_{10}$ CFU $mL^{-1}$ and 0.89 $log_{10}$ CFU $mL^{-1}$, respectively. The low initial levels of

*Staphylococcus* in the untreated milk do not allow a true effect of treatment on *Staphylococcus* to be determined.

Table 4: Mean coliform counts following treatment at each time-point as per growth on the selective media, MacConkey's agar, for the enumeration of coliforms. Averaged data is presented as mean $\log_{10}$ CFU $mL^{-1}$ (standard deviation). Log reduction and percentage reduction presented in brackets are based on the treatment group when compared with the untreated control at the same time-point.

| Treatment group | T0 | T1 | | T2 | | T3 | | T4 | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction |
| **Control** | 7.74 (1.7) | 7.66 (1.49) | - | 6.9 (0.10) | - | 6.75 (0.38) | - | 6.86 (0.42) | - |
| **HoP** | 7.74 (1.7) | 1.46 (2.06) | 6.2 | 0 (0) | 6.9 | 0 (0) | 6.75 | 0 (0) | 6.86 |
| **HoP + Nisin A** | 7.74 (1.7) | 1.38 (1.95) | 6.28 | 0 (0) | 6.9 | 0 (0) | 6.75 | 0 (0) | 6.86 |
| **Microfiltration** | 7.74 (1.7) | 1.3 (0.85) | 6.36 | 0 (0) | 6.9 | 0 (0) | 6.75 | 0 (0) | 6.86 |
| **Lactose Oxidase** | 7.74 (1.7) | 5.73 (0.16) | 1.93 | 1.44 (2.03) | 5.46 | 5.13 (0.87) | 1.62 | 3.07 (4.33) | 3.68 |
| **Nisin A** | 7.74 (1.7) | 6.49 (0.16) | 1.17 | 5.91 (1.29) | 0.99 | 6.25 (1.53) | 0.5 | 6.28 (1.85) | 0.47 |

Table 5: Mean bacterial counts of *Staphylococcus* following treatment at each time-point as per growth on selective media Baird Parker. Averaged data is presented as mean $\log_{10}$ CFU $mL^{-1}$ (standard deviation). Log reduction and percentage reduction presented in brackets are based on the treatment group when compared with the untreated control at the same time-point. T0 = before treatment, T1 =after treatment, T2 = after 24 hours, T3 = after 4 days, T4 = after 7 days.

| Treatment group | T0 | T1 | | T2 | | T3 | | T4 | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction |
| **Control** | 1.53 (2.16) | 1.26 (1.78) | - | 0.59 (0.83) | - | 1.29 (0.16) | - | 0.35 (0.49) | - |
| **HoP** | 1.53 (2.16) | 0 (0) | 1.26 | 0 (0) | 0.59 | 0 (0) | 1.29 | 0 (0) | 0.35 |
| **HoP + Nisin A** | 1.53 (2.16) | 0 (0) | 1.26 | 0 (0) | 0.59 | 0 (0) | 1.29 | 0 (0) | 0.35 |
| **Microfiltration** | 1.53 (2.16) | 0 (0) | 1.26 | 0 (0) | 0.59 | 0 (0) | 1.29 | 0 (0) | 0.35 |
| **Lactose Oxidase** | 1.53 (2.16) | 0.8 (1.13) | 0.46 | 0.5 (0.71) | 0.09 | 0.65 (0.92) | 0.64 | 0 (0) | 0.35 |
| **Nisin A** | 1.53 (2.16) | 0.89 (1.13) | 0.37 | 0 (0) | 0.59 | 0.85 (0.21) | 0.44 | 0 (0) | 0.35 |

### *3.2.6 Streptococcus*

Samples subjected to microfiltration had the highest reduction in *Streptococcus* growth with no detectable growth immediately after treatment or for the 7 days of refrigerated storage (Table 6). Microfiltration resulted in the maximum possible log reduction at all time-points, with the highest log reduction of 4.61 observed 24 hours after treatment. Milk subjected to HoP or HoP + Nisin A treatment, resulted in an immediate log reduction of 3.07 and 2.8, respectively. Furthermore, after 24 hours of refrigerated storage, *Streptococcus* was undetectable in HoP treated milk, which remained until T4 (Figure 3D). At T2, low bacterial levels were detected in the HoP + Nisin A treated milk, measured at 0.35 $\log_{10}$ CFU $mL^{-1}$. No *Streptococcus* growth was evident at T3 and T4. On the other hand, minimal reductions in bacterial

levels was observed in Nisin A and lactose oxidase treated milk with an initial *Streptococcus* count of 4.19 $log_{10}$ CFU $mL^{-1}$ and 3.48 $log_{10}$ CFU $mL^{-1}$, respectively. Indeed, *Streptococcus* persisted at subsequent time-points, with a log reduction of less than 1.5 observed in both groups across the 7 days.

Table 6: Mean bacterial counts of *Streptococcus* following treatment at each time-point as per growth on Edwards modified media. Averaged data is presented as mean $log_{10}$ CFU $mL^{-1}$ (standard deviation). Log reduction and percentage reduction presented in brackets are based on the treatment group when compared with the untreated control at the same time-point. T0 = before treatment, T1 =after treatment, T2 = after 24 hours, T3 = after 4 days, T4 = after 7 days.

| Treatment group | T0 | T1 | | T2 | | T3 | | T4 | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction | Mean (SD) | Log reduction |
| **Control** | 4.6 (2.26) | 4.06 (1.55) | - | 4.61 (2.13) | - | 4.34 (1.98) | - | 4.35 (2.21) | - |
| **HoP** | 4.6 (2.26) | 0.99 (1.40) | 3.07 | 0 (0) | 4.61 | 0 (0) | 4.34 | 0 (0) | 4.35 |
| **HoP + Nisin A** | 4.6 (2.26) | 1.26 (1.77) | 2.8 | 0.35 (0.49) | 4.26 | 0 (0) | 4.34 | 0 (0) | 4.35 |
| **Microfiltration** | 4.6 (2.26) | 0 (0) | 4.06 | 0 (0) | 4.61 | 0 (0) | 4.34 | 0 (0) | 4.35 |
| **Lactose Oxidase** | 4.6 (2.26) | 3.48 (1.87) | 0.58 | 3.88 (1.70) | 0.73 | 2.86 (0.96) | 1.48 | 3.1 (0.78) | 1.25 |
| **Nisin A** | 4.6 (2.26) | 4.19 (0.95) | - | 3.11 (0.30) | 1.5 | 4.28 (0.82) | 0.06 | 3.32 (0.23) | 1.03 |

### 3.3 The nutritional composition and pH of milk

Following incubation for 7 days at 10 °C, untreated human milk had an average fat, carbohydrate and protein level of 2.85 (0.42) %, 7.15 (0.07) % and 1.13 (0.18) %, respectively. The macronutrient levels in the untreated control sample remained stable during the 7 days of refrigerated storage with levels of 2.7 (0.42) %, 7.15 (0.15) % and 1.05 (0.21) %, in fat, carbohydrate and protein, respectively. Following treatment with HoP, lactose oxidase, nisin A and HoP + Nisin A, the macronutrient levels of milk remained stable, with a range of 2.85-2.95 % in fat, 6.98-7.13 % in

carbohydrate and 1-1.1 % in protein. In contrast, low levels of fat and protein retention were observed in the membrane filtered skimmed milk, 0.2 (0) % and 0.85 (0.21) %, respectively. However, carbohydrate retention remained high, with a value of 7.33 (0.18) %. The macronutrient composition of human milk following processing over 7 days of refrigerated storage is shown in Table 7.

The pH of untreated human milk, following 7 days of incubation at 10 °C was 6.11 (0.13). Immediately following treatments, the pH level of milk remained within a range of 6.05-6.32. By day 7 of refrigerated storage, the pH level of milk in the untreated control, showed a slight reduction that was similar to that observed in the treatment groups, except for lactose oxidase treated milk (Figure 4). The lactose oxidase treated milk had a pH of 3.7 (0.04) following 7 days of storage at 4 °C, indicating spoilage.

Table 7: The macronutrient composition of DHM. Day 0 (following incubation B and immediately following treatment). Day 7 (following treatment and subsequent storage at 4 °C for 7 days). Data represented as mean (SD).

| | Fat (%) | | Total protein (%) | | Carbohydrate (%) | | Energy (kcal dL-1) | |
|---|---|---|---|---|---|---|---|---|
| | Day 0 | Day 7 | Day 0 | Day 7 | Day 0 | Day 7 | Day 0 | Day 7 |
| **Control** | 2.85 (0.42) | 2.7 (0.42) | 1.13 (0.18) | 1.05 (0.21) | 7.15 (0.07) | 7.15 (0.07) | 59.75 (4.6) | 58 (5.66) |
| **HoP** | 2.93 (0.53) | 3.08 (0.81) | 1.1 (0.14) | 1.08 (0.11) | 7.08 (0.04) | 7.03 (0.07) | 60 (5.66) | 61.25 (7.42) |
| **Membrane filtration** | 0.2 (0) | 0.2 (0) | 0.85 (0.21) | 0.9 (0.14) | 7.33 (0.18) | 7.48 (0.04) | 34.75 (1.77) | 35.5 (0.71) |
| **Lactose oxidase** | 2.85 (0.5) | 3.63 (0.88) | 1 (0.14) | 0.85 (0.21) | 7.13 (0.04) | 6.7 (0.14) | 59.5 (4.95) | 64 (8.49) |
| **HoP + Nisin A** | 2.95 (0.5) | 3 (0.64) | 1.1 (0.14) | 1.05 (0.07) | 7.05 (0.07) | 7.05 (0.07) | 60.75 (6.01) | 60.5 (5.66) |
| **Nisin A** | 2.85 (0.42) | 2.58 (0.39) | 1.1 (0.14) | 1.1 (0.14) | 6.98 (0.04) | 6.98 (0.25) | 59 (4.24) | 56.25 (3.18) |

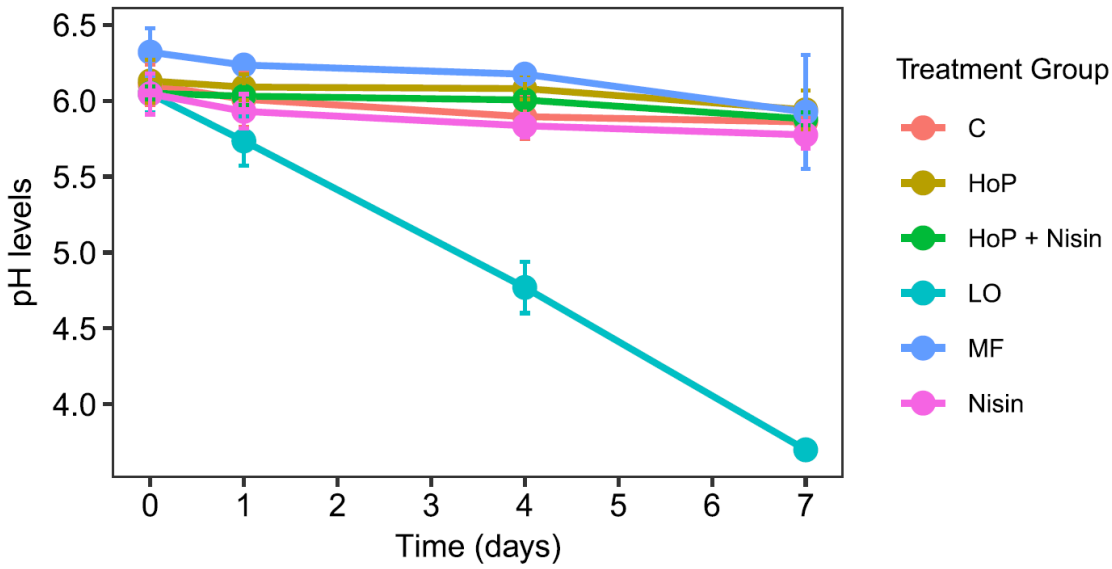



Figure 4: The pH levels of human milk following treatment and subsequent storage at 4 °C for 7 days. Treatment groups: C = control, HoP = Holder pasteurized, HoP + Nisin A = Holder pasteurized and treated with Nisin A, MF = membrane filtered

## 4 Discussion

To date, the potential of bio-preservation or microfiltration for the processing of DHM remains unknown. In this study, we assess the efficacy of a bacteriocin, nisin A, and an enzyme, lactose oxidase, when applied to pooled human milk, as a method of achieving microbial safety. In addition, the mechanical processing of skimmed human milk was performed through microfiltration. These three methods have previously been assessed in food stuffs (22, 34, 43), however, to the best of our knowledge no other studies have looked at their application in human milk. Thus, we carried out bacterial evaluation following the use of these novel processing techniques and present findings herein demonstrating their potential and the need for further evaluation and development of protocols to optimize their efficiency.

Firstly, to increase the natural microbial population of human milk in this study, pooled milk samples were subjected to a pre-treatment incubation period of 7 days at 10 °C. Current recommendations for milk banks is to accept donated milk with a maximum total bacterial growth of $10^5$ CFU $mL^{-1}$ and $10^4$ CFU $mL^{-1}$ of *S. aureus* and

*Enterobacteriaceae*, respectively (44). In this study, milk samples prior to incubation had low levels of bacterial growth (1.44-2.65 $\log_{10}$ CFU $mL^{-1}$). In contrast, incubated storage resulted in high microbial starting counts of 4.60-7.74 $\log_{10}$ CFU $mL^{-1}$. Thus, incubation allowed a log reduction that would be required for milk bank setting to be tested, enabling a true effect of treatment to be determined. However, it is important to note that *Staphylococcus* growth, determined by growth on Baird parker media, was not well conserved when exposed to the incubation conditions (1.53 $\log_{10}$ CFU $mL^{-1}$). Therefore, insights into the effect of the applied treatments on *Staphylococcus* growth were limited. The cause of *Staphylococcus* suppression during incubation at 10 °C is unclear but may be due to a number of influential factors. For instance, pH is known to influence growth, with an optimum pH of 6-7 for *Staphylococcus* growth, and a survival range of 4-10 (45). However, as the pH of milk used in this study was an average of 6.11 post-incubation it is unlikely to be the limiting factor. Similarly, the fat content of the food matrix can impact growth, with poor growth observed in high milk fat products including cream (46). As the fat content of untreated milk used in this study was approximately 2.9%, the fat content should not prevent growth. A critical environmental factor for *Staphylococcus* growth is temperature, with an optimum temperature of 37 °C, although *Staphylococcus* survival has been shown at 7-48 °C (45). Importantly, the robustness of *Staphylococcus* to environmental temperature is strain dependent. For instance, *S. aureus* growth occurred at 7-12 °C (47); elsewhere, however, *S. aureus* did not grow when incubated at 8 °C (48). Finally, the presence and ratio of competing microbes can limit *Staphylococcus* proliferation and survival (45). The ability of *Staphylococcus* present in our samples to tolerate sub-optimal environmental conditions may have been exacerbated by the presence of other sub-optimal conditions, resulting in the low levels of growth. We suggest that both the temperature and the presence of competing microbes are likely contributors to the low levels of *Staphylococcus* survival observed following incubation at 10 °C for 7 days.

Bacterial counts following HoP further demonstrate its efficacy at microbial elimination for the processing of human milk. Indeed, a > 5-log reduction occurred

in total microbial growth immediately following treatment, with a > 7-log reduction observed at all subsequent time-points. Moreover, a > 6-log reduction in *Pseudomonas* and coliform growth was achieved. Similar reductions of *S. aureus* and *E. coli* were recorded previously following the artificial inoculation of donor human milk (49, 50). As can be expected due to low initial counts, *Staphylococcus* was undetectable at all time-points following treatment. However, *Streptococcus* growth remained (0.99 $\log_{10}$ CFU $mL^{-1}$), suggesting *Streptococcus* was more resistant to HoP treatment in this study. HoP treatment has previously resulted in undetectable levels of *Streptococcus* following processing (51).

Previous studies have shown the anti-microbial effect of nisin A in a variety of dairy based food models. For instance, nisin exhibited anti-listerial effects in cottage cheese (52, 53), inhibited yeasts and extended the shelf life of fresh galytori cheese (54) and demonstrated bacteriostatic capacity against *S. aureus* growth in milk and minas frescal cheese (55). Furthermore, the addition of nisin in combination with a variety of anti-microbial compounds has proven effective. A mixture of lysozyme and nisin worked synergistically in pasteurised bovine milk, inhibiting total bacteria, aerobic spore formers and psychotropic growth, extending the shelf life of milk up to 15 days (10). Additionally, in bovine milk, a synergistic anti-microbial effect of nisin when combined with garlic root juice (56), phenolic compounds (57) and reuterin (58) has been demonstrated. A similar approach is the combination of nisin with various processing technologies. The use of nisin in addition to other methods of processing, enables the damage of gram-negative bacteria's cell wall, allowing nisin to permeate and make direct contact with the cytoplasmic membrane, resulting in greater efficacy and increased sensitivity of the microorganism (20). Indeed, when nisin was combined with thermoultrasonication, an enhanced bactericidal effect on total bacteria and the complete eradication of *E. coli* was observed in orange juice (59). Similarly, a combination of nisin A and high-pressure processing at 500 MPa was effective against *Bacillus spp.* spores (60). Studies to date demonstrate the capacity of nisin via direct application, as part of an anti-microbial formulation or as a post-processing tool to achieve the microbial safety and shelf-life stability of foods.

In this study, when HoP was combined with nisin A, an even greater reduction in the total microbial growth occurred (6.68-log reduction) and was sustained for the 7 days of refrigerated storage. For coliforms, *Staphylococcus* and *Streptococcus*, bacterial growth was reduced in line with HoP treatment alone. In contrast, *Pseudomonas* growth was better reduced by HoP alone, although the necessary > 5-log reduction was still achieved with HoP + Nisin A. Overall, on non-selective agar a greater reduction in microbial growth occurred when HoP was combined with Nisin A, however, on selective media, no significant advantage was observed. On the contrary, the direct inclusion of nisin A proved inadequate for the treatment of human milk. Indeed, total bacterial growth was only reduced 24 hours after application by ~2 log, which is not sufficient for milk banks. Furthermore, nisin A was the least effective treatment against *Pseudomonas* and coliforms with an initial ~1 log reduction that was not maintained with cold storage of 1-7 days.

To date, although nisin A has been effective in combating gram positive bacteria (10), a significant limitation commonly associated with nisin A application is its inability to suppress gram-negative bacterial growth, as previously demonstrated in bovine milk (58). However, a recent study demonstrated the inhibitory capacity of Nisin A (0.2065 - 423 μg $mL^{-1}$) against a diverse range of gram negative bacterial strains from 17 different genera, including *Pseudomonas* and *Enterococcus* (9). Despite previous findings, in this study, the direct application of nisin had poor anti-microbial effects within the human milk matrix, regardless of gram-positive or gram-negative bacteria. Indeed, reductions in gram-positive bacteria were low (0.5 - 1.5-log reduction). Interestingly, a similar trend was observed in *Staphylococcus* and *Streptococcus* growth, after 24 hours a reduction in bacterial growth occurred; however, by day 4, growth returned, before levels were undetectable at day 7. These findings indicate a failure to fully eradicate gram-positive bacteria, instead causing sublethal damage that allows for their subsequent recovery. The undetectable levels at day 7 is likely due to cold storage. Previous findings suggest that the inhibitory action of nisin is influenced by the food matrix, in particular the fat content (55). For example, the anti-microbial action of nisin is higher in skimmed and low-fat cow's milk when compared with whole milk (56). The direct application of nisin

A does not exhibit strong anti-microbial effects within the human milk matrix, however, these findings-support the complementary use of nisin with HoP. Nisin remains a promising therapeutic agent for the milk bank industry.

Although not screened for in this study, the outgrowth of bacterial spores in DHM following HoP is a persistent challenge facing milk banks, most commonly *Bacillus* spores (61-64). Importantly, unlike HoP, nisin A has demonstrated bactericidal capacity against spore-forming bacteria (10). Furthermore, when tested against *Bacillus* and *Paenibacillus* spores in a milk-based pudding, nisin A inhibited spore outgrowth (43). The potential for anti-microbial peptides to prevent spore germination is a compelling advantage to its inclusion in donor human milk processing steps. The control of bacterial spores in donor human milk is essential to maintaining milk safety and ensuring shelf-life stability. Future studies should explore the use of nisin A in combination with processing technologies or as part of an anti-microbial formulation. In addition, further investigation into the sustained shelf-life of pasteurized DHM when cold-chain storage facilities are limited may help provide safer DHM in these contexts.

Existing findings demonstrate the ability of lactose oxidase to activate the LPO system of bovine milk, resulting in microbial inhibition in dairy foods including yogurt (30), milk (65) and cheese (31) at concentrations of 1.2 g $L^{-1}$, 12 g $L^{-1}$ or 1.2 g $L^{-1}$ and 0.6 g $L^{-1}$, respectively. The inhibition of bacterial strains by lactose oxidase was demonstrated in targeted overlay assays against a number of bacterial strains including *Escherichia coli*, *L. monocytogenes*, *S. aureus* and *Pseudomonas fragi* (29). The same group further demonstrated the capacity of lactose oxidase at concentrations of 0.12 g $L^{-1}$ to reduce *P. fragi* growth in skimmed milk by 2.5-log within 24 hours (22). Furthermore, when lactose oxidase was applied at a higher concentration of 1.2 g $L^{-1}$, *P. fragi* growth was reduced below the limit of detection and this was maintained for 7 days of cold storage. Despite the ability of lactose oxidase to activate the LPO system in bovine milk, lactose oxidase inclusion in this study did not cause sufficient microbial inhibition in human milk. We observed a minimal reduction in total bacterial growth after treatment, with a 1.33-log reduction following 24 hours. A greater immediate reduction was observed for

*Pseudomonas* of 3.2-log. However, after 24 hours *Pseudomonas* growth returned, despite no increase in growth observed in the control sample.

An opposite effect was seen for coliform growth, the initial reduction in growth was low (1.93-log) but increased after 24 hours (5.46-log), before growth returned at T3 and T4. In addition, lactose oxidase was inadequate at eradicating *Staphylococcus* and *Streptococcus* growth. Overall, lactose oxidase at a concentration of 1.2 g $L^{-1}$ was ineffective at eliminating the microbial load of human milk used in this study. One potential reason for the poor microbial inhibition observed may be due to the high starting bacterial counts. Indeed, FAO/WHO noted, that at high levels of bacterial cell density, the activity of the LPO system is low, whereas, at moderate and low levels of bacterial contamination, the activity of the LPO system is moderate or high, respectively (66). As such, the high levels and diversity of bacteria present in our samples post-incubation, may have inhibited the activity of the LPO system. Furthermore, previous findings in bovine milk noted significant differences in the bacteriostatic effect of lactose oxidase in raw and pasteurized milk, likely due to lower available levels of LPO in milk post-pasteurization (22, 67). In our own study, although samples were not pasteurized, human milk is known to have lower levels of LPO when compared with bovine milk (24). Thus, the increased presence of hydrogen peroxide alone may not be adequate to activate the LPO system due to low LPO levels. It appears lactose oxidase alone is unlikely to be sufficient for the suppression of bacterial growth. However, the potential for lactose oxidase as a component of an anti-microbial formulation to maintain microbial safety, in particular where cold chain storage is limit, could be explored further. Indeed, Aouadhi et al. (68) combined nisin and activation of the LPO system through the addition of thiocyanate and hydrogen peroxide, resulting in a 4-6 log reduction in microbial growth in raw milk. A further consideration for the application of lactose oxidase, is its effects at various concentrations on the physiochemical and organoleptic properties of milk. Lactose oxidase was previously shown to decrease the pH of milk and cheese during refrigerated storage (22, 31). Indeed, the pH of lactose oxidase-treated milk in this study was drastically reduced when compared with the untreated control. Future studies will need to identify the optimal

parameters for lactose oxidase activity within the human milk matrix, the potential for sensorial consequences, and the necessary concentration to achieve microbial suppression. We suggest there is greater potential for lactose oxidase inclusion in DHM as part of an anti-microbial formulation, enabling synergistic inactivation of microbes. It appears, that when applied in human milk at the concentration used in this study, lactose oxidase preserves the initial microbial quality of milk but does not improve its microbial safety.

Of all the treatments tested, microfiltration was the most successful at eliminating the microbial contamination of DHM immediately following treatment (> 6-log). Previous studies have demonstrated the pasteurization of skimmed bovine milk by microfiltration with varying outcomes. Following microfiltration with a membrane pore size of 1.4 µm, a 3.4-3.8 log reduction in total bacteria was achieved in skimmed bovine milk (34, 69). However, a lower reduction of 1-2 log was reported elsewhere (35). The choice of membrane pore size appears to be a key factor in attaining microbial safety. For example, with a membrane pore size of 1.4 µm a > 4-log reduction in total bacteria was achieved, although *Bacillus subtilis*, *Listeria innocua* and *E. coli* remained above the level of detection (70). However, when microfiltration with a smaller pore size of 0.8 µm was applied, a greater reduction in total bacteria occurred (<10 CFU $mL^{-1}$) and *B. subtilis*, *L. innocua* and *E. coli* levels were undetectable (70). Although a separate study noted similar reductions in bacterial growth following microfiltration with either a 1.4 µm and 0.8 µm pore size (32).

In this study, despite undetectable levels of bacteria immediately after treatment, bacterial growth was observed at T2 (24 hrs) and T3 (4 days). The same was observed in *Pseudomonas* growth at T3. Despite the re-occurrence of low levels of bacterial growth, a > 6-log reduction was still achieved. However, the low levels of bacterial re-growth suggests failure of microfiltration to fully prevent the passage of all bacterial cells. A similar finding was noted in microfiltered bovine milk, despite initial reductions in bacterial growth, re-growth of 4-log was observed after 6 days of refrigerated storage (35). Those authors suggested that bacterial re-growth could be a result of spore outgrowth, environmental contamination or biofouling. In this

study, a pore size of 0.45 µm was applied and should prevent the passage of bacterial cells. For instance, the approximate cell size of *Pseudomonas aeruginosa* and *B. cereus* is 0.8 µm x 3.0 µm and 1.0 µm x 4.0 µm, respectively (33). However, the small pore size, combined with the conventional flow of lab scale microfiltration, may have contributed to bacterial adhesion and membrane fouling, effecting filtration efficacy. The majority of studies to date on microfiltered bovine milk have successfully used cross-flow ceramic membrane filtration to minimize risk of membrane fouling. Future studies assessing the microfiltration of human milk should implement this approach. Alternatively, microfiltration has also been combined with other pasteurization techniques including high-pressure and high-temperature short-time processing and demonstrated even greater reductions in bacterial growth (69, 70). A combination of microfiltration with other processing techniques would enable the use of larger pore sizes, minimizing the risk of membrane fouling. Importantly, high levels of bioactive protein and enzyme retention have been reported following microfiltration of bovine milk (32, 70). Indeed, a 100% and 86-100% retention of IgG, IgA, IgM, LPO and lactoferrin was demonstrated following microfiltration with 1.4 µm and 0.8 µm, respectively (32). In addition, when sensory analysis was performed on bovine milk following microfiltration treatment, no difference was reported when compared with the skimmed milk control (69). The influence of microfiltration on the bioactive constituents and sensorial properties of human milk still needs to be established. Any future processing technology, including microfiltration, would need to ensure high levels of bioactive retention.

There are a number of limitations in this study. Although the incubation step provided high starting counts, the implementation of this step as the method to increase pre-treatment bacterial load is not recommended for future experiments. Due to the fact that human milk is not a standardized product and has variable range of starting microbial counts (2.6 – 5.2 $\log_{10}$ CFU $mL^{-1}$) (61, 64), the incubation step does not guarantee high levels of microbial growth. As such, incubation did not always result in high starting counts, indeed 3 further attempts at incubation were performed but resulted in low levels of bacterial growth, preventing their use in this

study (data not shown). Furthermore, the bacteria observed in our samples was limited to those with capacity to grow at 10 °C, limiting our insight into *Staphylococcus*. Also, incubation at 10 °C for 7 days may facilitate biofilm formation, as such, the post-incubation milk analysed may not accurately reflect the microbial profile of milk or the true effect of treatments tested.

In addition, although CBA is commonly used for aerobic bacterial cultivation, in this study, it appeared to underestimate total viable counts, as evidenced by higher recoveries on selective media for *Pseudomonas* and coliform growth. This suggests that CBA does not sufficiently support the full aerobic population present in human milk. Accordingly, future studies are necessary to determine the optimum non-selective medium for total bacterial screening of human milk to more accurately quantify total bacterial loads.

As a starting point, this study does provide insight into novel treatments not previously examined and their interaction with high microbial loads within milk matrix. However, we recommend the artificial inoculation of human milk with target microorganisms to examine effect of treatments going forward (i.e., spiking). If the treatments prove effective in spiking studies, subsequent studies on endogenous bacterial growth at lower levels reflecting conditions of milk donation should be performed. In addition, incubation does influence physiochemical and nutritional properties, therefore, when investigating anti-microbial peptides, the higher levels of psychotropic bacteria and enzymatic reactions may influence the anti-microbial activity observed. In addition, we did not perform spore analysis. Future studies should implement the use of spore assay to determine the efficacy of treatments against *Bacillus* spores. Spores remain a key challenge for the milk bank sector and any future treatment would be advantageous if spore suppression was achieved.

## 5 Conclusions

This study provides insights into the unexplored area of anti-microbials as a non-thermal alternative for the processing of donor human milk. The inhibitory potential of nisin A and lactose oxidase was not well supported in this study, however, future studies may achieve higher levels of microbial inhibition if these anti-microbial peptides are used as part of formulation or in combination with existing processing technologies. In particular, nisin A as a synergistic component to milk processing with HoP could aid in preventing spore germination and maintain shelf-life stability. Both microfiltration or HoP combined with Nisin A proved effective in reducing the microbial load of DHM, supporting the need for further evaluation and optimization of these treatments for DHM processing. This study opens promising avenues for alternatives to conventional processing methods, however, additional research is necessary to support their practical implementation.

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## 7 Contributions

The parallel nutritional analysis referred to in this study was performed by Dr. Fanyu Meng as part of a collaboration with the School of Food and Nutritional Sciences. Dr. Fanyu Meng also participated in the running of treatment trials outlined in this study.

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# Chapter 7

General Discussion

# 1 Discussion

Milk is defined as *"a whitish fluid, rich in fat and protein, secreted by the mammary glands of female mammals (including humans) for the nourishment of their young, and taken from cows, sheep, etc., as an article of the human diet"* (1). Milk is uniquely designed to provide tailored species-specific nutrition to the newborn, fulfilling the complete nutritional requirements needed to sustain life during the initial critical window of development (2-4). Milk in its most natural form is designed to be delivered via direct feeding, from the female mammal to their offspring. However, in today's world, the provision of milk increasingly necessitates human intervention, this is particularly true when milk is not for direct consumption by the offspring. Milk now holds significant commercial value as an established component of the human diet. As the potential applications of milk grows, so too does the demand for and expansion of milk production systems.

Globally, bovine milk accounts for more than 80 % of total milk production (5). In Ireland alone, 8.3 billion litres of raw milk are produced annually. This has led to the intensification of dairy production systems and the diversification of dairy manufacturing processes. Within dairy farming, milk management strategies, rooted in good farming practice, begin even before calving or the onset of lactation and continue through to feeding calves or preparing milk for export and commercialization (6, 7). Subsequently, milk undergoes pasteurization and technological processing, depending on the desired end product.

In humans, where exclusive breast feeding is not possible, whether due to inability or personal choice, the use of breast pumps and manual expression has emerged. These methods support sustained mother's milk supply to her infant, facilitate the return to work of the mother and enable storage (8-11). These resources are also essential to the functioning of milk banks, enabling the collection and donation of donor human milk (DHM). Once donated, DHM undergoes a series of collection, processing and storage procedures to ensure safety and quality prior to distribution (12, 13).

In both the farming and human milk banking sectors, each step in the milk handling process plays a critical role. These steps can either protect and enhance the natural properties of milk, ensuring optimal nutrition, immune value and stability, or, conversely, introduce the risk of contamination, degradation and safety hazards, the consequences of which ultimately affect the consumer. Moreover, the widespread consumption of milk has opened the door for its development as a functional food. Furthermore, as milk harbours an array of bioactive metabolites, milk and its constituents also present promising potential for biotherapeutic or biopreservative development. Whether the production of milk is for human or animal use, food or health applications, the steps involved in milk production, development and distribution are diverse and can influence end-product quality and consumer well-being. Milk management demands careful consideration and implementation, as improper handling may lead to fallout in terms of animal welfare, economic losses and environmental damage. This thesis addresses the optimization of milk quality and safety with milk management practices from a microbial perspective, through the exploration of three key areas (i) dairy farm management practices during dry cow therapy (DCT) (ii) harnessing milk properties for targeted product development (iii) human milk bank processing for improved DHM profile for the neonate.

Chapter 1 provides a comprehensive overview of the nutritional and microbial profile of DHM as influenced by both current and emerging processing techniques. The review highlights the potential degradation of DHMs bioactive properties following thermal exposure and provides insights into the growing area of proposed alternatives. However, the consolidation and interpretation of available data is limited by the absence of a standardized approach in human milk studies to date including, inconsistencies in study design, unknown sample history and the use of varying analytical techniques. These limitations could be addressed through the development and implementation of standardized workflows, enabling a direct comparison of processing methods and ultimately, accelerate the development of the most effective processing technologies for optimum infant nutrition. Indeed, a basic workflow template has previously been proposed by the European Milk

Banking Association, suggesting the inclusion of three study groups (raw human milk, holder pasteurized and the proposed pasteurisation technique) (14). Recommended evaluation parameters include biochemical quality markers (secretory Immunoglobulin A (sIgA), bile salt stimulated lipase and lysozyme) as well as indicators for the microbial quality (*Listeria monocytogenes*, *Staphylococcus aureus*, *Chronobacter sakazakii*, cytomegalovirus and human milk virome) (14). To facilitate meaningful comparisons, this template should be expanded upon to include details on sample handling, storage history and analytical techniques.

In Chapters 2, 3 and 4, the studies are centred around bovine colostrum and milk from both a farm management standpoint and also, the potential opportunities to harness milks' natural properties for potential health and commercial applications.

Milk is frequently subjected to a variety of manufacturing and process technologies, depending on the desired end product and target consumer. These steps offer opportunities for modification and optimization. In Chapter 2, the potential of dairy based functional foods, with a particular focus on increasing the carotenoid content was explored, for improved nutritional and marketable value. In addition, farm and production management strategies, and solutions to support functional food development are discussed. This chapter also outlines key challenges associated with functional food development, including, bioaccessibility, bioavailability, stability and scalability. These are universal considerations that need to be addressed when undertaking the development and delivery of milk, its by-products and milk-based foods for industrial and commercial applications.

Chapter 3 investigated the impact of DCT with or without antibiotics on the microbial composition of Irish bovine colostrum in a cohort of healthy cows (n=90). Colostrum samples were collected from 5 farms, employing different methods of DCT. Two farms administered Cephagaurd (CEF), two farms administered UBRORED, and one farm used no antibiotic during the drying off period. 16S rRNA analysis revealed the predominant phyla (Firmicutes, Actinobacteriota, Bacteroidota and Proteobacteria) and genera (*Acinetobacter, Corynebacterium, Facklamia, Jeotgalicoccus, Lactococcus, Leuconostoc, Psychrobacter* and

*Staphylococcus)* in bovine colostrum, regardless of DCT treatment. A significant effect of antibiotic administration during DCT on the microbial composition and diversity of bovine colostrum was observed. Colostrum from cows that underwent natural DCT (the 'NOAB' group) had the highest alpha-diversity according to Shannon index ($p < 0.05$). Furthermore, beta-diversity analysis revealed distinct microbial clustering when compared with the antibiotic treated groups ($p < 0.001$). An effect of DCT was also evident between colostrum from the two antibiotic treated groups, with significant differences in both alpha-diversity ($p < 0.01$) and beta-diversity ($p < 0.001$), depending on the choice of antibiotic used. Critically, differential abundance analysis did not reveal an increase in genera commonly associated with mastitis in the NOAB group. However, a significantly higher abundance of *Pseudomonas*, *Staphylococcus* and *Enterococcus* were observed in the CEF group. Similarly, increased abundance of *Corynebacterium* and *Staphylococcus* were demonstrated in the UBRORED treated group. These findings indicate that the microbial profile of bovine colostrum is influenced by antibiotic treatment administered during DCT. These data support the potential for non-antibiotic based DCT as a viable alternative to managing udder health. However, the findings were subject to two key limitations. Firstly, sampling was only conducted at a single time point, restricting the ability to assess temporal changes. Secondly, the use of 16S rRNA sequencing, while effective for broad community profiling, lacked the sequencing dept needed to assess influence of antibiotic usage at a species level. Recent advances in next-generation sequencing technologies alongside reduced costs, offer high-power tools for deeper genomic research. Shotgun metagenomics or whole-genome sequencing (WGS) could provide more detailed insights into the influence of DCT. Indeed, a previous longitudinal study conducted by our group on a subset of the same sample population used in this study (n=24), examined the influence of antibiotic treatment on bovine colostrum using WGS (15). The study demonstrated the long-lasting effects of antibiotic exposure, with microbial and genomic changes still evident in milk collected at 6 months. Furthermore, the genetic analysis revealed the presence of antibiotic-resistant genes (ARGs) across all three cohorts, with the highest abundance observed in the antibiotic treated groups. Based on the findings of both studies, we

suggest a future longitudinal study (0-6 months), with a similar sample size to that of our current study (n=90) using shotgun sequencing. This approach would strengthen the data on the influence of on-farm antibiotic usage and provide more insight into the potential resistome of bovine colostrum and milk. Identifying the source of ARGs on the dairy farm is critical to mitigate the global spread of antibiotic resistance. Further investigations is needed to determine the origin and mechanism of transmission, including horizontal and vertical gene transfer. To this end, the inclusion of both environmental (bedding, water, soil) and faecal samples may provide greater insights into resistome dynamics on farm. The emergence and spread of antibiotic-resistant microbes and ARGs throughout the environment poses a major threat to animal, human and environmental health. In recognition of this, a reduction in on-farm use is already underway as a result of recent EU regulatory policies (16). However, the role of the dairy farm in dissemination of ARGs remains. Strict adherence to good farming practice, the development of alternative therapies and farmer outreach and education is critical in mitigating the spread and reducing usage of antibiotics. Furthermore, Immunoglobulin(Ig)G levels and somatic cell count (SCC) were not measured, instead, we relied on visual inspection by the farmers to assess the infection status of the cows. To strengthen future investigations, the inclusion of IgG and SCC testing is recommended, as they are key indicators used for colostrum quality and udder health. IgG testing can be performed on-farm using a Brix refractometer, however for improved accuracy, laboratory-based methods such as radial immunodiffusion assay or ELISA can be performed (17, 18). For SCC testing, several on farm techniques are available, including the California Mastitis Test, the Porta SCC test and the Delaval Cell Counter (19). In addition, an automated fluorescence-based cell counter, the Fossomatic, is widely used and provides high-throughput, accurate results. Additionally, when colostrum samples were evaluated for inter-farm variation, significant differences were observed in the microbial composition even when the same DCT protocols were undertaken. This suggests the presence of additional influencing factors most likely, feed and the surrounding environment. However, due to the lack of metadata and supporting samples, the degree to which these factors influence the microbial composition of colostrum remains unknown.

In Chapter 4, we examined bovine colostrum-derived microbes at both the phenotypic and genomic levels, highlighting the potential of bovine colostrum as a reservoir of lactic acid bacteria (LAB) with health-promoting and protective attributes. Bacterial isolates (n=166) from pooled bovine colostrum emanating from 10 dairy farms in Ireland underwent a series of identification and differentiation, including RAPD PCR, genomic extraction and 16S rRNA Sanger sequencing, to identify 30 LAB strains. Following identification, LAB strains underwent phenotypic characterization, resulting in 4 (13 %), 26 (87 %) and 5 (17 %) strains with potential bacteriocin, exopolysaccharide (EPS) and bile salt hydrolase (BSH) producing capacity, respectively. To enable genomic level analysis, 10 strains belonging to the genera *Loigolactobacillus*, *Levilactobacillus*, *Limsoilactobacillus*, *Lactiplantibacillus*, *Lacticaseibacillus*, *Lentilactobacillus* and *Lactococcus* were selected for WGS. A putative plasmid-borne bacteriocin gene cluster encoding the bacteriocins Acidocin 8912 and BacSJ was identified in a *Lacticaseibacillus paracasei* strain with demonstrated inhibition against *Enterococcus thailandicus*. Furthermore, genome mining identified a second cluster encoding the core peptide LSEI_2386. Similarly, a putative bacteriocin gene clusters encoding LSEI_2386 were also identified in two further *Lc. paracasei* strains. However, for both strains, the well diffusion assays against *Lactobacillus delbrueckii subsp. bulgaricus* resulted in zones of inhibition with diffuse boundaries, as such their anti-microbial potential remains speculative. In addition, genome analysis of a further two strains with a positive EPS phenotype, identified a potential γ-Aminobutyric acid (GABA)-producing *Levilactobacillus brevis* strain and a BSH-producing *Limsoilactobacillus mucosae* strain. Importantly, despite the routine use of antibiotics in the farm environment, ARGs were not prevalent amongst the LAB strains of interest in this study, supporting the safety of these strains for future applications.

While our findings are promising, they remain putative and warrant further investigation. In particular, additional *in vitro* studies are needed to better characterize the inhibition spectrum of the identified bacteriocin producing strains. Expanding the range of indicator strains used may also help uncover missed bacteriocin producers within our culture bank. The anti-microbial potential of

bovine colostrum-derived isolates is especially relevant to the ongoing fight against antibiotic resistance. Indeed, the detection of bacteriocin producers in colostrum suggests that potential natural anti-microbial solutions to resistance may already exist within milk itself. Given that mastitis is a common source of infection on dairy farms and a major cause of antibiotic usage, future inhibition assays should include common bovine mastitis associated pathogens as indicator strains. Furthermore, to fully understand the anti-microbial potential, determining the mechanism of action and operon characterization of putative bacteriocin gene clusters is required. The design and use of plasmid constructs could facilitate functional validation by helping to determine the role and necessity of individual genes present within the cluster. Furthermore, genomic analysis would identify the presence of BSH and EPS biosynthetic gene clusters. To fully confirm the production capacity of colostrum isolates, these genomic investigations should be undertaken in future studies. Finally, bacteria exhibiting desirable functional traits have potential applications across a wide range of sectors, including, human health, veterinary medicine and food processing. However, for any candidate strain or its purified bioactive by-product to be considered, its potential for commercial development must be established. The challenges outlined in Chapter 2 regarding functional food development also apply here. Indeed, the bioavailability, stability, scalability and safety of any proposed strain must be attainable. Moreover, the potential of strains remains speculative until validated by *in vivo* studies, confirming their efficacy and functionality within the host environment.

In Chapter 5, we evaluated the efficacy of Holder pasteurisation (HoP) and high-pressure processing (HPP) in reducing microbial populations in DHM and maintaining microbial safety during prolonged refrigerated storage. Bacterial screening on non-selective and selective media was performed on pooled human milk following HoP or HPP at 200, 400 and 600 MPa for 10 min, to determine the microbial safety of DHM when stored at 4 °C for 7 days. Additionally, a parallel study monitoring the macronutrient and enzymatic (lactoperoxidase, lysozyme) content of milk was performed. The data contributes to the growing body of evidence supporting HPP as a promising non-thermal technique for the preservation of DHM.

In this study, total microbial counts confirmed that HoP-treated milk remained within acceptable growth limits (<10 CFU $mL^{-1}$) when stored at 4 °C for 7 days. Similarly, HPP at 600 MPa resulted in undetectable microbial counts which persisted for the 7 days of cold storage. Furthermore, milk treated with HPP at 400 mPa resulted in an immediate reduction in total microbial counts (3.45-log) and bacterial growth was undetectable at subsequent time-points. In addition, HPP at 400 MPa demonstrated an immediate and complete reduction in *Staphylococcus*, *Pseudomonas* and *Streptococcus* counts.

These results demonstrate that processing DHM at 600 MPa for 10 min ensures microbial safety but this comes with notable reductions in the levels of lysozyme and lactoferrin. This aligns with a previous report of the diminished Ig profile of milk when processed at 600 MPa, although the damage was mitigated with a reduced exposure time (20). Conversely, in our study, HPP treatment at 400 MPa for 10 min, resulted in greater conservation of the examined enzymatic constituents, while immediate microbial inhibition was less robust (~3.5 $log_{10}$ CFU $mL^{-1}$), although at subsequent time-points bacterial growth was undetectable. Although our findings are promising, as noted in Chapter 1, and reaffirmed with this study, the specific parameters applied during HPP are critical in determining the end-product safety and nutritional value. Evidently, the need for further research to determine optimum HPP parameters remains. As such, future studies should evaluate a broader range of holding times to elucidate the minimum time required to achieve both microbial safety and nutritional retention. Specifically, exposure times under 10 min should be examined for 600 MPa, while extending holding time to ~15 min may improve outcomes at 400 MPa. Notably, we did not assess the Ig profile of DHM following processing, this should be a priority in future work. This can be achieved through ELISAs or radial immunodiffusion assays, as demonstrated elsewhere (21, 22). Additionally, a limitation of this study was the number of replicates performed. In this study, HPP was performed in duplicate, while further replicates would have allowed for robust statistical analysis and assessment of significance between the treatment groups.

Finally, a practical consideration when evaluating the potential integration of HPP into the milk bank sector, is the feasibility of implementing this method of processing. As noted in a previous study, feasibility assessments are warranted when evaluating alternative processing techniques (23). A previous cost-analysis study noted that pascalization (HPP) is nearly seven times more expensive than pasteurization (24). Thus, a longitudinal cost-benefit evaluation is necessary to determine whether such an investment is sustainable. This chapter also demonstrated the potential for the sustained microbial safety of DHM under refrigeration conditions for one week. These finding suggests a simple and practical handling modification that could help conserve DHM supply and reduce unnecessary waste. In this study, DHM was removed from refrigeration conditions at four timepoints for brief periods to allow for sampling. However, future research to determine how many times, and for how long, a sample can be removed from refrigeration without compromising safety is warranted. This information is needed for the realistic implementation and acceptance of any revised protocol within milk banks, where handling and storage procedures are rigorously regulated and require detailed protocols. Clear, evidence-based guidelines will ensure the quality and safety of DHM is maintained.

In Chapter 6, we undertook previously untested methods for the treatment of DHM, to evaluate their ability to produce DHM within acceptable microbial safety limits, with varying outcomes. In this study, pooled human milk was first subjected to an incubation period of 7 days at 10 °C to increase starting microbial counts and ensure a true effect of treatment could be seen. Following incubation, human milk was subjected to a series of processing methods, including the direct inclusion of nisin A and lactose oxidase. In addition, a hybrid technique of nisin A application directly following HoP was performed. Also, de-fatted human milk was subjected to microfiltration. The microbial safety of pooled milk samples were monitored immediately following treatments and for a further 7 days of refrigerated storage. Immediately following treatment, a complete reduction in total microbial growth (> 6-log) was observed following microfiltration or nisin A combined with HoP. Similarly, a > 6-log reduction in *Pseudomonas* and Coliform counts was observed

in both treatment groups. In contrast, low levels of microbial inhibition were achieved with the direct inclusion of either nisin A or lactose oxidase across all media and time-points. These findings indicate that under the applied experimental conditions, the direct inclusion of either nisin A or lactose oxidase is not suitable for the preservation of DHM, despite previous successes in raw bovine milk (25, 26). Both techniques failed to produce milk within acceptable microbial safety standards. However, this study highlights the potential of hybrid processing techniques, demonstrating effective microbial safety when nisin A was combined with HoP. The potential of hybrid processing is vast, offering the ability to combine a multitude of techniques with the ultimate aim of reducing the severity of conventional processing conditions. However, since nisin A was only tested alongside standard HoP parameters in this study, it remains to be determined whether hybridization could allow for reduced thermal exposure (lower temperature or shorter duration). In addition, future studies should also consider the optimum antimicrobial concentration, balancing efficacy with consumer safety and perspective.

A limitation in this study was the experimental design. The inclusion of a pre-treatment incubation step to elevate the starting microbial load is not recommended for future studies as it introduced variability that was difficult to control, limiting reproducibility. Indeed, given that milk is not a standardized product, microbial counts post-incubation were unpredictable and dependent on the starting microbial population. This approach risked the over representation of some species and the unintentional exclusion of others. For example, in this study, *Staphylococcus* growth was poorly supported at 10 °C, limiting insights into the effects of applied treatments. *Staphylococcus* is a key contaminant of concern in DHM (13, 27), frequently associated with mastitis (28-30), heat-resistant enterotoxins (31) and ARGs, notably the *mecA* gene linked to methicillin resistance (32, 33). A more reliable and controlled approach for assessment of anti-microbials or microfiltration would be the evaluation of targeted pathogenic growth through artificial inoculation. Once a treatment demonstrates potential with targeted

assessment, it can then be further validated using the natural unaltered population of microbes in milk.

Regarding microfiltration, the use of a lab-scale, conventional flow set up provided pre-liminary insights into microbial suppression. However, processing was limited by the use of a small pore size, intended to exclude bacteria, and may have resulted in bacterial adhesion and biofouling, potentially allowing the re-growth of sub lethally damaged cells. In contrast, cross flow, ceramic membrane filtration using a larger pore size (0.8-14 μm) has shown promise in food-based studies. Again, to enhance performance outcomes and potentially increase the required pore size, a hybrid strategy could be considered, as demonstrated previously in bovine colostrum when complemented with HPP (34). Future work should expand to include the reintroduction of the lipid fraction, a vital energy source for the neonate.

A shared limitation in both studies presented in Chapters 5 and 6 was the failure to screen for bacterial spores. This represents a critical oversight, as bacterial spores remain a major limiting factor associated with HoP and a persistent challenge within the milk banking sector. This concern is emphasized by previous findings that demonstrate an increase in spore presence following exposure to processing conditions (27, 35). For novel methods, HPP, or the extension of refrigerated storage, their efficacy against bacterial spores, mainly *Bacillus cereus,* must be established. Previous findings noted the ability of nisin A to suppress bacterial sporulation (36, 37). Similarly, hybrid processing strategies with the integration of an anti-microbial formulation may offer a viable solution to control bacterial spores during DHM processing. Future studies should explore the application of nisin A during pre-processing, to proactively prevent sporulation. The inclusion of spore screening in this study would strengthen the data and should be included in future studies.

Additionally, although beyond the scope of these studies, the potential reintroduction of mother's own milk microbiota and the fortification of DHM are emerging areas of research, providing targeted nutrition to the infant (38-42). Future analysis of DHM processing technologies would benefit from considering these factors. Critical questions include: What is the optimal point in the processing cycle

to introduce these components? Does their addition increase risk of contamination? Does the post-processed milk matrix remain suitable for fortification or reinoculation? Furthermore, this work focused on the processing of DHM in line with techniques and facilities used and studied in developed regions, however, the need for optimal nutrition in under resourced setting is also under examination (43, 44). Although we did not investigate this context directly, the development and inclusion of anti-microbial options suitable for areas where refrigeration and storage capacity are limited warrants serious exploration.

In conclusion, in this thesis a series of studies were conducted that demonstrated the value of both culture-dependent and culture-independent techniques in the evaluation and potential optimization of mammalian milk. The research presented spans multiple areas and highlights the diverse and complex nature of milk and its constituents. Milk contains an array of components with complex and differing interactions, within the milk matrix, with their host environment and the consumer. While the existence of a milk microbiome remains speculative and is disputed (45), the value and influence of the microbial composition of milk is without question.

The expansion, diversification and upscaling of both the dairy industry and human milk bank sector as a result of increasing demand, presents a multitude of challenges which can influence the quality of milk retained. This thesis not only highlights the valuable properties of milk to the neonate and broader consumer but emphasizes that the value of milk, nutritionally, commercially and environmentally, is highly dependent on the implemented management practices. The studies on factors influencing milk quality and safety highlight how targeted milk management practices can help preserve and enhance milks beneficial properties. As our knowledge of individual milk components, their interaction with the host and the needs of the target consumer continues to grow and evolve, so too must our approach to milk handling and processing. Ongoing surveillance of safety hazards, development of innovative processing solutions and adherence to best practices are essential in conserving the beneficial properties of milks for optimum nutrition.

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