



**THE DETECTION AND ENUMERATION OF PROPIONIBACTERIUM SPP
ISOLATED FROM A KEFIR BEVERAGE USING PCR AND MICROBIOLOGICAL
METHODS**

By

Lynette Cloete

Thesis submitted in fulfilment of the requirements for the degree

Master of Food Science and Technology

In the Faculty of Applied Science

At the Cape Peninsula University of Technology

Supervisor: Dr Z Keyser

Co-Supervisor: Prof. VA Jideani

Bellville Campus

19 January 2026

CPUT copyright information

The thesis may not be published either in part (in scholarly, scientific or technical journals), or as a whole (as a monograph), unless permission has been obtained from the University

DECLARATION

I, Lynette Cloete declare that the contents of this thesis represent my own unaided work, and that the thesis/dissertation has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed  _____

Date 12/12/2025

ABSTRACT

Malnutrition and vitamin deficiencies remain major global food challenges. To overcome these issues, naturally healthy products are often fortified with beneficial ingredients or probiotics to further enhance their nutritional profile. Kefir is one such example, as it has successfully been fortified with *Propionibacterium freudenreichii*. This probiotic provides multiple gut health benefits while also producing important micronutrients such as vitamin B12 and folate. Kefir itself is a probiotic-rich beverage that naturally contains a diverse matrix of symbiotic lactic acid bacteria, yeasts, and other beneficial microorganisms. However, secondary yeast fermentation during storage may compromise product stability, potentially leading to nutrient loss and other shelf-life defects. Ensuring that fortified dairy products reach the consumer with their nutrients intact is therefore essential. This remains challenging due to the inherently short shelf life of such products and the time-consuming nature of many conventional microbiological tests. Furthermore, detection of *propionibacteria* in complex food matrices can be cumbersome and unreliable. The present study aimed to optimise a method to detect *P. freudenreichii* in kefir during refrigerated, frozen, and freeze-dried storage for 28 days. Conventional enumeration was compared with the polymerase chain reaction (PCR) method. For culture-based analysis, serial dilutions were prepared and plated on YEL, PCA, and Pal-Propiobac. DNA was extracted using the Power Food Microbial DNA Isolation Kit. Because DNA from dead cells may remain intact, leading to false positives, samples were pretreated with propidium monoazide (PMA) prior to extraction. This intercalating dye penetrates non-viable cells, binds to their DNA, and prevents its amplification, ensuring that only viable cells with intact membranes are detected. The PCR protocol was optimised specifically for *P. freudenreichii*, with amplification of the V3 variable region within the 16S rRNA gene carried out using species-specific primers Prop1 (5'-GATACGGGTGACTTGAGG-3') and Prop2 (5'-GCGTTGCTGATCTGCGATTAC-3'). Viable *P. freudenreichii* could be detected in kefir across all storage treatments for up to 28 days with PCR with PMA treatment. Selective enumeration was difficult using PCA and YEL agar due to the complex, diverse microbial flora naturally present in kefir and to exposure to low temperatures. In contrast, Pal-propionibac successfully suppressed competing microbial species, enabling more accurate detection of propionibacteria. Overall, this study highlights that optimised PCR, combined with selective pretreatment and culture-based confirmation, offers a reliable and efficient method for monitoring *P. freudenreichii* in fortified kefir during storage, thereby ensuring both probiotic integrity and product quality.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people and institutions:

- Dr Z Keyser, my supervisor, at the Cape Peninsula University of Technology (CPUT), for his guidance and assistance during my research.
- Prof VA Jideani, my co-supervisor, Professor and head of the Cereals and Legumes Biopolymer Research for Food Security at CPUT, for her guidance, motivation and expertise.
- Prof J van Wyk, Associate Professor at CPUT, for introducing me to the wonderful world of kefir.
- Fermentation friends, Bongisiwe Zozo, Nontsikelelo Tafu, Letlotlo Nyenye, Zaa-id Rinquest, Ryan Morkel for your assistance and unending motivation.
- I am truly grateful to the DFST Technical Team at CPUT for their expertise, creativity, teamwork, technical assistance, and unwavering support.
- Foodbev Seta, for financial assistance for the execution of the experimental process for the fulfilment of my thesis.

DEDICATION

This thesis is dedicated to my loving husband, Fernando, and our beautiful children, Hayley, Amy and Chadley. Thank you for your love, patience, understanding and endless sacrifices during the fulfilment of my thesis. You are and will always be my inspiration.

TABLE OF CONTENTS

Declaration	i
Abstract	ii
Acknowledgements	iii
Dedication	iv
Abbreviations/Acronyms/Glossary	viii

CHAPTER 1: MOTIVATION AND THE DESIGN

1.1	Introduction	1
1.2	Statement of the research problem	2
1.3	Objectives of the research	2
1.3.1	Broad objectives	2
1.3.2	Specific objectives	3
1.4	Hypotheses	3
1.5	Delineation of the research	3
1.6	Significance of the research	3
1.7	Expected outcomes, results and contribution of the research	4
1.8	Ethical considerations	4
1.8	Overview of the study	4
	References	6

CHAPTER 2: LITERATURE REVIEW

2.1	Introduction	8
2.2	Fortification and fermentation of food	9
2.3	Lactic acid bacteria fermentation	12
2.4	Milk	14
2.5	Kefir	16
2.5.1	History of kefir	17
2.5.2	Microbiological characteristics of kefir grains	18
2.5.3	The production of kefir	19
2.5.4	Properties of kefir	20
2.6	Probiotics	22
2.6.1	Characteristics of probiotics	23
2.6.2	Benefits of probiotics	24
2.7	Propionic acid bacteria (PAB)	26
2.7.1	Nutritional characteristics of PAB	27
2.7.2	Metabolic pathways of PAB	27
2.7.3	Commercial importance of PAB	28
2.7.4	Potential application of PAB in kefir	29
2.7.5	Methods of PAB enumeration and detection	30
	References	32

CHAPTER 3: DETECTION OF *PROPIONIBACTERIUM SPP.* USING PCR AND MICROBIOLOGICAL METHODS

	Abstract	48
3.1	Introduction	48
3.2	Materials and methods	50
3.2.1	PAB strains	50
3.2.2	Preparation of YEL agar	50
3.2.3	Preparation of Pal propiobac	51
3.2.4	Preparation of PCA	51
3.3	Streaking of PAB onto conventional agar	51
3.4	Preparation of PCR reagents	51
3.4.1	Preparation of Tris/Borate/EDTA (TBE) buffer	51
3.4.2	Preparation of 1% Agarose gel with wells	51
3.4.3	Preparation of primers	52
3.5	Treatment of pure PAB samples with PMA reagent	52
3.5.1	Procedure 1: Preparation of control PAB samples	50
3.5.2	Procedure 2: Treatment of PAB pure culture control samples with PMA reagent	52
3.5.3	Isolation of DNA from PMA-treated PAB	54
3.6	Optimisation of the PCR protocol	54
3.6.1	PCR protocol for the detection of <i>P. freudenreichii</i>	54
3.6.2	Loading of agarose gel	56
3.6.3	Electrophoresis	56
3.7	Results and discussion	56
3.7.1	<i>Propionibacterium freudenreichii</i> growth on conventional media	56
3.7.2	Optimised Polymerase chain reaction protocol to detect <i>P. freudenreichii</i>	59
3.7.3	Treatment of viable and non-viable cell DNA using PMA	61
3.8	Conclusion	63
	References	64

CHAPTER 4: THE DETECTION OF *PROPIONIBACTERIUM SPP.* IN KEFIR USING PCR AND MICROBIOLOGICAL METHODS UNDER VARIOUS STORAGE CONDITIONS

	Abstract	69
4.1	Introduction	69
4.2	Materials and methods	71
4.2.1	Source of materials	71
4.3	Activation of kefir grains and PAB	71
4.4	Production of kefir control (KC) and kefir inoculated with PAB	71
4.5	Storage of kefir samples	72
4.6	Preparation of growth media and reagents	72
4.6.1	Preparation of YEL agar	73

4.6.2	Preparation of Plate Count Agar	73
4.6.3	Preparation of Pal propiobac	73
4.6.4	Preparation of Quarter-strength ringer's solution	73
4.7	Preparation of PCR reagents	74
4.7.1	Tris/Borate/EDTA (TBE) buffer	74
4.7.2	Preparation of 1% Agarose gel tray with wells	74
4.7.3	Preparation of primers	74
4.8	Treatment of kefir samples with PMA reagent	74
4.9	Isolation of the DNA of PMA-treated kefir samples	75
4.10	PCR analysis of kefir	75
4.10.1	PCR	75
4.10.2	Inoculation of wells	76
4.10.3	Electrophoresis	76
4.10.4	Enumeration of kefir samples	76
4.11	Data analysis	76
4.12	Results and discussion	77
4.12.1	Enumeration of kefir on conventional media	77
4.12.2	Effect of PAB type, storage type and storage time on the kefir microbial load	80
4.12.3	Effect of storage time on the kefir microbial load based on PAB type	81
4.13	Results for PCR analysis of kefir	86
4.14	Conclusion	88
	References	88

CHAPTER 5: SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1	Summary of results and discussion	92
5.2	Conclusion	93
5.3	Recommendations	94

LIST OF FIGURES

Figure 1.1	Overview of the study	5
Figure 2.1	Major developments in food fermentation technology through the years	13
Figure 2.2	Kefir grains used in the production of the kefir	17
Figure 2.3	Tibetan kefir grains as observed by Lu et al., (2014:4) using an electron microscope: a single grain unit as indicated by the circle (A), view from the side (B), from the top (C), and from the bottom (D)	18
Figure 2.4	The beneficial effects of probiotics on human health	25
Figure 2.5	Wood-Werkman metabolic pathway	29
Figure 2.6	Schematic diagram on the intercalation of non-viable DNA with Propidium mono-azide	32

Figure 3.1	Schematic diagram of sample treatment with PMA reagent	53
Figure 3.2	Isolation of DNA of lyophilised pure PAB cultures using the Powerfood Microbial DNA isolation kit	55
Figure 3.3	Propioni bacteria streaked on Pal propiobac at various incubation periods	58
Figure 3.4	Agarose gel with overloaded marker	60
Figure 3.5	Agarose gel using ethidium bromide as a stain following the PCR protocol with an annealing temperature of 52°C	60
Figure 3.6	Validation of the PCR-based method based on a response from target (PAB) and non-target (E. coli) DNA using pure bacterial strains	61
Figure 3.7	DNA treated with PMA	62
Figure 3.8	PMA treated DNA of PAB exposed to fluorescent light	62
Figure 4.1	Schematic diagram of the production of kefir	72
Figure 4.2a	PAB in kefir on Pal propiobac	78
Figure 4.2b	High number of PAB in Pal propiobac	78
Figure 4.3a	Kefir control in PCA	79
Figure 4.3b	Kefir with PAB in PCA	79
Figure 4.3c	Kefir in PCA (Dilution 1×10^{-7})	79
Figure 4.3d	Kefir in PCA (Dilution 1×10^{-6})	79
Figure 4.4a	Kefir in YEL agar	79
Figure 4.4b	Kefir in YEL agar	80
Figure 4.4c	Kefir in YEL agar	80
Figure 4.5	The values are mean $\pm$ standard deviation of three independent trials showing the succession microbial counts (Log cfu/mL) over the storage time of 28 days for: (a) KC = Kefir control, kefir not inoculated with PAB. (b) KJ15 = Kefir inoculated with J15h PAB. (c) KJ17 = Kefir inoculated with J17 PAB. TPC = Total plate count, YEL=Yeast extract lactate, PAL = Pal propiobac. There is no significant ($p > 0.05$) difference for the values with the same letter in the same column	82
Figure 4.6	Mean microbial succession counts (cfu/ mL) showing the effect of the interaction of: (a) Kefir control, (b) KJ15, (c) KJ17 with treatment refrigerated, frozen and freeze-dried. TPC = Total plate count, YEL=Yeast extract lactate, PAL = Pal propiobac. There is no significant ($p > 0.05$) difference for the values with the same letter in the same column	84
Figure 4.7	PCR images for kefir under various storage conditions: (A) Day 0, (B) day 14, (C) day 28	87

LIST OF TABLES

Table 2.1	Fortified dairy products and dietary nutritional recommendations	10
Table 2.2	The chemical composition of cow's milk	16
Table 4.1	Main effect of PAB inoculation on the microbial load	81

ABBREVIATIONS AND ACRONYMS

PAB	<i>Propionibacteria freudenreichii</i>
DNA	Deoxyribonucleic acid
UHT	Ultra-high temperature
PCR	Polymerase chain reaction
PCA	Plate count agar
YEL	Yeast extract, lactate agar
PMA	Propidium monoazide

GLOSSARY

Terms	Definition/Explanation
<i>Kefir</i>	Is a nutritional fermented milk drink with a creamy texture, sour taste and subtle effervescence. It is a probiotic product which contains lactic acid bacteria and yeasts.
Kefir grains	The grains appear as smooth, shiny, globular double walled structures, resembling clumps of cauliflower.
Fermentation	Is a process that helps break down large organic molecules into simpler ones via the action of microorganisms. Alcohols, carbon dioxide, and/or organic acids are produced by microorganisms, primarily from sugars and under mostly anaerobic conditions, for production of energy.
Fortification	Is the process whereby nutrients that are not naturally present in the food is deliberately added to improve or maintain its quality
Freeze	Is the process where water is removed by sublimation after food is frozen at ultra-low temperatures.
PMA	Propidium monoazide is an intercalating dye used to bind with DNA of compromised or dead cells, thus preventing them from amplification during PCR
Probiotic	Is a bacteria-based product which is consumed to benefit the host.
PCR	The polymerase chain reaction is an analytical method used to rapidly amplify DNA by making millions to billions of copies of a specific DNA sample using primers.
DNA	is a double helix polymer composed of two polynucleotide chains coiled around each other which carries genetic instructions responsible for the development, functioning, growth and reproduction of all known organisms.

CHAPTER 1

MOTIVATION AND DESIGN OF THE STUDY

1.1 Introduction

Over the years, the need for food with enhanced nutritional value has increased, especially in low to medium socio-economic communities where disease and malnutrition are common (Van Wyk et al., 2011:69). Not all foods are nutritionally complete, hence the need to fortify certain foods with vitamins and mineral complexes. Vitamins play an important role in the normal functioning of the human body. Vitamin B₁₂ deficiency, for example, can lead to fatigue, nausea, constipation, flatulence, loss of appetite, weight loss, neurological changes or permanent damage to the nervous system, fermenter's disease, and anaemia. It can also lead to cardiovascular disease and cancer (Fenech, 2012:236; Yildirim, 2015:344). As humans do not naturally synthesise vitamin B₁₂, it must be obtained from other sources like microbial synthesis (Kozyraki & Cases, 2013:1003; Watanabe & Bito, 2018:155). To combat this problem, functional foods are being developed by fortifying foodstuffs with vitamins and probiotics (Kalfirtova & Sovjac, 2005:22). *Propionibacterium* spp. (PAB) plays an important role in the development of functional foods due to their unique probiotic qualities, including the production of vitamin B₁₂. Specific human populations with health problems (like intolerance, allergy, inflammation and cancer) linked to certain lifestyles will require specialised diets. PAB can be crucial in this regard by modulating important health factors (Rabah et al., 2020:2).

PAB has been used for ages to provide Swiss cheese with its distinctive flavour and "eyes." The ability of these organisms to efficiently biosynthesise a wide range of useful macromolecules, such as vitamins, antimicrobials, conjugated linoleic acid, and trehalose, is what has reignited interest in them as cell factories (Piwowarek et al., 2018:515). PAB is widely used for its probiotic and cancer-prevention properties, as well as its production of vitamin B₁₂ (Thierry et al., 2011:19; Morkel, 2016:96). PAB forms part of the phylum *Actinobacteria*, and is divided into two classes, generally known as dairy propionibacteria and cutaneous propionibacteria, which are found on the human skin. PAB are Gram-positive rod-shaped, catalase variable organisms. They are also non-motile, non-spore forming facultative anaerobes (Dasen et al., 1998:251; Jeantet & Jan, 2021:3486). *P. freudenreichii* was first described by Orla-Jensen and von Freudenreich (1906) when they investigated the production of propionic acid and the formation of round eyes in cheese (Bücher et al., 2021:4312). Later, it was used as a ripening culture in Swiss cheese and as a starter culture for other dairy products (Theirry et al., 2011:19). PAB converts the lactate produced by lactic acid bacteria into propionate, acetate, and carbon dioxide. Propionic fermentation mainly occurs in warm environments and is markedly slowed at colder temperatures. However, *P. freudenreichii* remains active during the storage of cheese at 4°C, as in the ripening of Emmental cheese

(Cousin et al., 2012:135). Van Wyk et al. (2011:73) proved that fortifying dairy products with PAB can significantly increase vitamin B₁₂ in products like kefir.

Kefir is a fermented, smooth-textured, acidic, low alcohol milk beverage with a pleasant taste and is prepared using milk of various origins and inoculation with kefir grains (Xie et al., 2012:395). It can be prepared from milk of animal or plant origin, i.e., cow, goat, sheep, rice, coconut, and soy (Altay et al., 2013:44). Kefir grains consist of a mixture of various yeast and bacteria spp. which co-exist in a symbiotic relationship (Leite et al., 2013:215; Xie, 2012:395; Nalbantoglu, 2014:43). Among the many beneficial probiotic effects of kefir grains is antimicrobial and anti-inflammatory properties (Altay et al., 2013:50; Tingirikari et al., 2024:12). Isolation of PAB from kefir can be performed using various microbiological techniques, including the use of lactate-rich medium such as Yeast Extract Lactate (YEL) agar and Pal propiobac medium (Cat no AO6001, Standa Laboratories, France). However, the PAB colonies obtained with lactate-rich media are not easily distinguishable from other types of bacteria, and the patented Pal propiobac medium is not readily accessible in South Africa and is very costly. There is a need for reliable and cost-effective culturing and Polymerase Chain Reaction (PCR) methods to detect PAB strains. Dasen et al. (1998:258) developed a faster method to differentiate PAB from other genera by creating a multiplex-PCR using the 16S rRNA motif, which is unique to PAB. Thus, an organism could be successfully identified in less than 2 days based on the similarity of its 16S rRNA motif with other organisms in a known database. However, distinguishing between metabolically active or viable and non-viable microbes in a community remains cumbersome (Wang et al., 2020:3).

1.2 Statement of the Research Problem

P. freudenreichii subsp. *shermanii* is a probiotic bacterium that increases the levels of Vitamin B₁₂ in complex probiotic matrixes such as kefir. Detection and enumeration of this bacterium on laboratory level during processing, freeze-drying and storage are essential. However, microbiological isolation and enumeration methods are often expensive and very cumbersome. Therefore, it is important to investigate reliable detection and enumeration methods for this organism in kefir.

1.3 Objectives of the Research

1.3.1 Broad objectives

The research study aimed to detect viable *P. freudenreichii* subsp. *shermanii* in a kefir matrix during storage (refrigeration, frozen storage and freeze-dried) by using conventional culturing methods and a PCR protocol.

1.3.2 Specific Objectives

The following objectives in this research study were achieved:

- (i) Establishment of an optimum growth medium for the cultivation and detection of *P. freudenreichii* subsp. *shermanii*.
- (ii) Enumerate the total bacterial count of kefir stored under different storage conditions via conventional microbiological techniques.
- (iii) Design of an effective PCR protocol for optimum detection of viable *P. freudenreichii* subsp. *shermanii* isolated from kefir stored under different conditions.

1.4 Hypotheses

The following hypotheses were tested:

- (i) Pal propiobac was a better medium for the cultivation of *P. freudenreichii* than the YEL and PCA.
- (ii) *P. freudenreichii* subsp. *shermanii* in kefir beverage was enumerated using conventional culturing methods over 28 days under various storage conditions.
- (iii) Viable *P. freudenreichii* subsp. *shermanii* was detected using a PCR protocol in kefir.

1.5 Delineation of the Research

Two strains of *P. freudenreichii* subsp. *shermanii* were used to inoculate the kefir beverage. Kefir grains from one supplier were used in this study. Ultra-high temperature (UHT) full cream milk was used to produce kefir. This study focused on the qualitative detection of the target gene of *P. freudenreichii* subsp. *shermanii* limited to conventional PCR, instead of the quantitative real-time PCR (qPCR). A primer sequence from a previous study was used to detect the presence or absence of the target 16S ribosomal DNA fragment using the Eppendorf conventional PCR amplifier.

1.6 Significance of the Research

Previous research has shown that fortifying food with vitamin B₁₂, producing PAB, enhances the nutritional properties of certain food products by increasing the vitamin B₁₂ levels. Vitamin B₁₂ is not normally synthesised by the human body and by successfully fortifying food with vitamin B₁₂ producing PAB, various illnesses and neurological diseases can be combated. Enumeration of PAB during different storage conditions is valuable to the food industry, the public and especially low socio-economic communities. The industry will benefit from the new knowledge of analysis as current techniques are time-consuming and cumbersome. Rapid methods of detection are less expensive, leading to the less expensive overhead of products. This will enable the community to access healthier, more sustainable, and more affordable food, thus eradicating deficiency of vitamins and minerals. Conventional culturing of PAB may

take up to 14 days to obtain the total viable count, whereas detection of the organism using PCR will take only four days (Bossard et al., 2016:3043).

1.7 Expected Outcomes, Results, and Contributions of the Research

The ability to detect viable from non-viable *P. freudenreichii* subsp. *shermanii* cells in a complex probiotic matrix such as kefir using PCR, without depending on conventional microbiological culturing methods with its many limitations, will significantly value the probiotic manufacturing industry. It is expected that viable *P. freudenreichii* subsp. *shermanii* will be detected from kefir following processing, freeze-drying and storage. The study will make a positive contribution to future research as well as economic factors in the probiotic manufacturing industry if it is proven that *P. freudenreichii* subsp. *shermanii* in kefir significantly survives all these processes. At least one manuscript will be published in a DHET-accredited scientific journal, and the research output will be presented at one national conference. After completing the study, the researcher will obtain a Master's degree in Food Science and Technology.

1.8 Ethical Considerations

The research does not involve human, animal or sensitive data. Kefir and microbiological waste were discarded and collected as per the agreement with BCL medical waste. Formal ethical clearance was not required by Faculty Research Ethics Committee of the Faculty of Applied Sciences

1.9 Overview of the Study

This thesis overview as shown in figure 1.1, consists of five chapters, where each chapter is written in a journal manuscript format. A list of references is added at the end of each chapter. Chapter one outlines an introduction, statement of the research problem, background, objectives, hypothesis, delineations, significance and the expected outcomes of the study. Chapter two presents a comprehensive literature review, highlighting the background and key sub-topics relevant to the research topic. Chapter three, the first research chapter, establishes the growth of *Propionibacterium freudenreichii* ssp *shermanii* (PAB) using traditional culturing methods using PCA, YEL agar and Pal propiobac. This chapter also includes the optimisation of a conventional PCR method utilising Propidium monoazide (PMA) to detect PAB from a pure culture. Chapter 4 explores the detection and survival of PAB in kefir fortified with PAB during refrigeration, frozen and freeze-dried storage over 28 days using conventional methods and the optimised PCR method as described in chapter 3. The summary is contained in chapter 5, as well the conclusion and further recommendations.

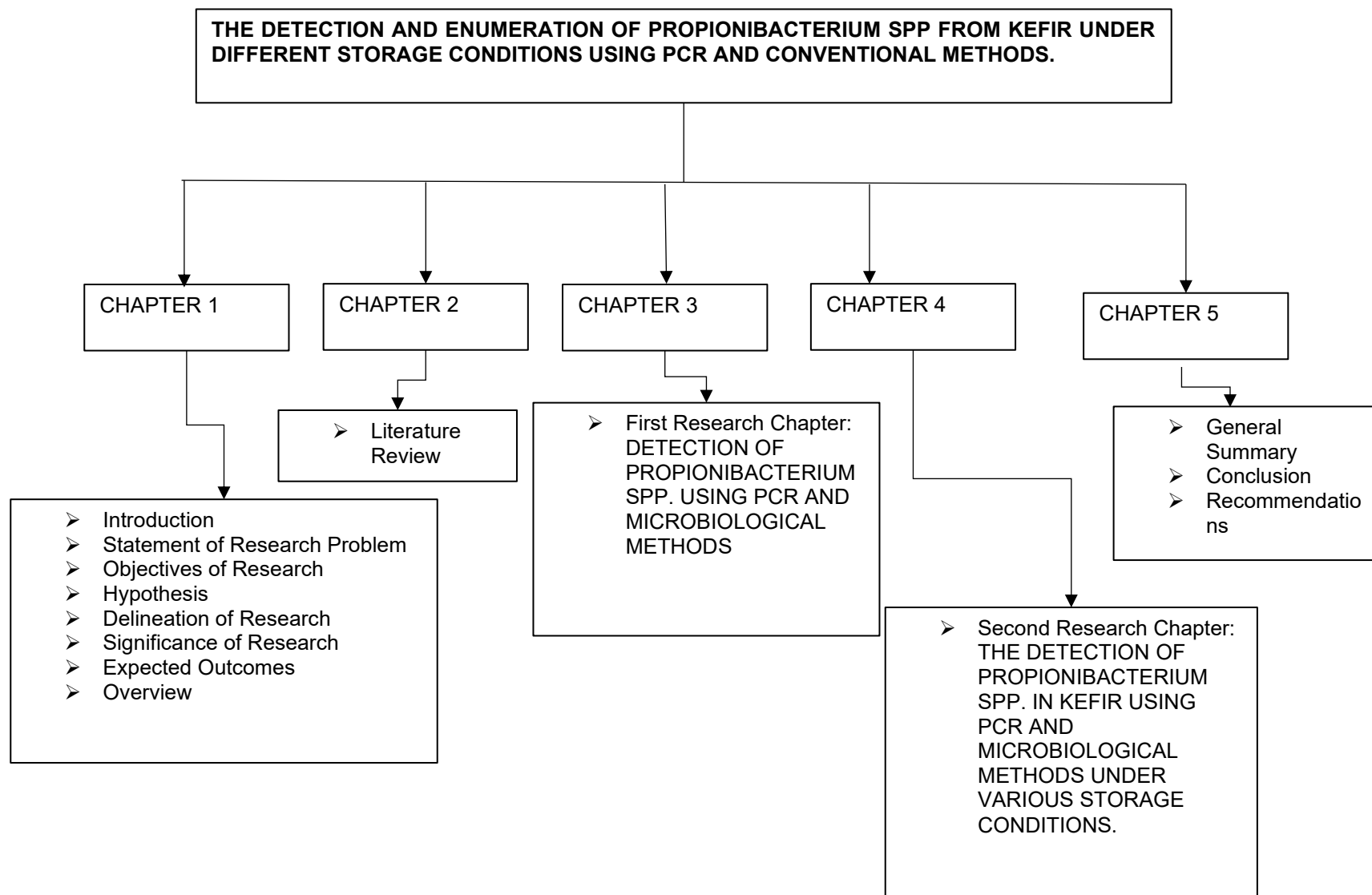


Figure 1.1 Overview of the study

References

- Altay, F., Karbancioglu-Güler, F., Daskaya-Dikmen, C. & Heperkan, D. 2013. A review on traditional Turkish fermented non-alcoholic beverages: Microbiota, fermentation process and quality characteristics. *International Journal of Food Microbiology*, 167(1): 44-56.
- Bossard, D.A., Ledergerber, B., Zingg, P.O., Gerber, C., Zinkernagel, A.S., Zbinden, R. & Achermann, Y. 2016. Optimal length of cultivation time for isolation of *Propionibacteria* acnes in suspected bone and joint infections is more than 7 days. *Journal of Clinical Microbiology*, 54(12): 3043-3049.
- Bücher, C., Burtscher, J. & Domig, K.J. 2021. Propionic acid bacteria in the food industry: An update on essential traits and detection methods. *Comprehensive Reviews in Food Science and Food Safety*, 20(5): 4299-4323.
- Cousin, F.J., Louesdon, S., Maillard, M., Parayre, S., Falentin, H., Deutsch, S., Boudry, G. & Jan, G. 2012. The first dairy product exclusively fermented by *Propionibacterium freudenreichii*: A new vector to study probiotic potentialities *in vivo*. *Food Microbiology*, 32(1): 135-146.
- Dasen, G., Smutny, J., Teuber, M. & Meile, L. 1998. Classification and identification of *Propionibacteria* based on ribosomal RNA genes and PCR. *Systematic and Applied Microbiology*, 21(2):251-259.
- Fenech, M. 2012. Folate (vitamin B9) and vitamin B12 and their function in the maintenance of nuclear and mitochondrial genome integrity. *Mutation Research*, 733(1-2): 21-33.
- Jeantet, R. & Jan, G. 2021. Improving the drying of *Propionibacterium freudenreichii* starter cultures. *Applied Microbiology and Biotechnology*, 105:3485-3494. <https://doi.org/10.1007/s00253-021-11273-3>
- Kalfirtova, P. & Sovajak, R. 2005. Improve the nutritional value of fermented dairy products by nutraceutical-producing food-grade microorganisms. *Agricultura Tropica et Subtropica*, 38:22-24.
- Kozyraki, R. & Cases, O. 2013. Vitamin B₁₂ absorption: Mammalian physiology and acquired and inherited disorders. *Biochimie*, 95(5): 1002-1007.
- Leite, A.M.D.O., Miguel, M.A.L., Peixoto, R.S., Rosado, A.S., Silva, J.T. & Paschoalin, V.M.F. 2013. Microbiological, technological and therapeutic properties of kefir: a natural probiotic beverage. *Brazilian journal of microbiology*, 44: 341-349.

- Morkel, R.A. 2016. Vitamin B12 and folate enrichment of kefir by *Propionibacterium freudenreichii* and *Streptococcus thermophilus* strains. Unpublished Masters' dissertation, Cape Peninsula University of Technology, Bellville.
- Nalbantoglu, U., Cakar, A., Dogan, H., Abaci, N. & Ustek, D. 2014. Metagenomic analysis of the microbial community in kefir grains. *Food Microbiology*, 41: 42-51.
- Piwożarek, K., Lipińska, E., Hać-Szymańczuk, E., Kieliszek, M. & Ścibisz, I. 2018. *Propionibacterium spp.*—source of propionic acid, vitamin B12, and other metabolites important for the industry. *Applied Microbiology and Biotechnology*, 102(2): 515-538.
- Rabah, H., Do Carmo, F.L.R., Carvalho, R.D.D.O., Cordeiro, B.F., Da Silva, S.H., Oliveira, E.R., Lemos, L., Cara, D.C., Faria, A.M.C., Garric, G. & Harel-Oger, M. 2020. Beneficial propionibacteria within a probiotic emmental cheese: impact on dextran sodium sulphate-induced colitis in mice. *Microorganisms*, 8(3): 1-20.
- Thierry, A., Deutch, S., Falentin, H., Dalmaso, M., Cousin, F.J. & Jan, G. 2011. New insights into physiology and metabolism of *Propionibacterium freudenreichii*. *International Journal of Food Microbiology*, 149(1): 19-27.
- Tingirikari, J.M.R., Sharma, A. & Lee, H.J. 2024. Kefir: a fermented plethora of symbiotic microbiome and health. *Journal of Ethnic Foods*, 11(1): 1-15.
- Van Wyk, J., Witthunn, C.R. & Britz, T.J. 2011. Optimisation of vitamin B₁₂ and folate production by *Propionibacterium freudenreichii* strains in kefir. *International Dairy Journal*, 21(2): 69-79.
- Wang, Y., Yan, Y., Thompson, K.N., Bae, S., Accorsi, E.K., Zhang, Y., Shen, J., Hartman, E.M., Huttenhower, C. & Chan, H.T.H. 2020. Whole microbial community viability is not quantitatively reflected by propidium monoazide sequencing approach: 1-22. <https://doi.org/10.21203/rs.3.rs-59563/v1>
- Watanabe, F. & Bito, T. 2018. Vitamin B12 sources and microbial interaction. *Experimental Biology and medicine*, 243(2): 148-158. [Doi:10.1177/1535370217746612](https://doi.org/10.1177/1535370217746612)
- Xie, N., Zhou, T. & Li, B., 2012. Kefir yeast enhance probiotic potentials of *Lactobacillus paracasei* H9: The positive effects of coaggregation between the two strains. *Food Research International*, 45(1): 394-401.
- Yildirim, T., Yalcin, A., Atmis, V., Cengiz, O., K., Aras, S., Varlı, M., & Atli, T. 2015. The prevalence of anemia, iron, vitamin B₁₂, and folic acid deficiencies in community dwelling elderly in Ankara, Turkey. *Archives of gerontology and geriatrics*, 60(2): 344-348.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The human body requires at least thirty types of vitamins ranging from water-soluble to fat-soluble, minerals and dietary components to promote bio-functionality. These nutrients convert food into energy and play a fundamental role in cell damage repair. Minerals are easily absorbed by the human body from plants, fish and fluids consumed in the human diet. However, vitamins are not absorbed easily into the body from food and other sources. The beneficial effects of vitamins are also easily denatured by processes, namely, cooking and storage or exposure to oxygen (Ofoedu et al., 2021:2).

Vitamins are categorised as a family of micronutrients consisting of 13 groups of organic compounds with a recommended daily intake ranging from micrograms to milligrams. The 13 groups of vitamins consist of four fat-soluble and nine water-soluble compounds (Shiozawa, 2022:8). Micronutrient deficiency, defined as the chronic lack of vitamins and minerals, may have severe health consequences, particularly in young children and pregnant women. This will also influence the future health and productivity potential of these individuals. It has been proven that children in low socio-economic areas have a high prevalence of micronutrient deficiency as they do not always have access to medical care and adequate nutrition (Wieser et al., 2013:2). In recent years, research has shown that nearly more than half of children under the age of five are severely affected in impoverished countries due to malnutrition, which remains a critical public health challenge (Tamir et al., 2025:1). Scurvy, blindness, dermatitis, peripheral neuropathy, rickets, bone, teeth, and gum diseases result from vitamin deficiency in the human diet. Other types of nutrient deficiencies may occur at various stages in life, from infancy to the elderly, including birth defects, childhood anaemia, cardiovascular diseases, and neural tube defects (Shiozawa, 2022:8). Moreover, the deficiency in vitamin B₁₂ can lead to fatigue, nausea, constipation, flatulence, loss of appetite, weight loss, neurological changes or permanent damage to the nervous system, fermenter's disease, and anaemia (Kalfirtova & Sovajak, 2005:23). It can also lead to cancer, cardiovascular disease, developmental and neural abnormalities (Fenech, 2012:22). Furthermore, the deficiency in vitamin B₁₂ in the elderly can be associated with cognitive impairment, dementia, which includes Alzheimer's disease. Other disorders associated with the deficiency of this micronutrient are vascular dementia, Parkinson's disease, multiple sclerosis and megaloblastic anaemia, which can be fatal. The leading cause of these diseases is the insufficient intake of vitamin B₁₂ due to poor dietary habits (McCaddon, 2013:3). To address this, certain foods must be fortified with vitamin and mineral complexes to enhance their nutritional value. Vitamin B₁₂ must be obtained from bacterial synthesis or dietary intake of these products as it is not naturally synthesised by humans. The pancreas, gut, and stomach

are all involved in the multi-step process of vitamin B₁₂ absorption (Kozyraki & Cases, 2013:1003; Bohrer, 2017:108). Thus, the need for food with enhanced nutritional value has increased, especially in low socio-economic communities where disease and malnutrition are common (Van Wyk et al., 2011:69). There are numerous low-cost agricultural processing areas and modern food applications that may mitigate these deficiencies. Recent trends and developments have shown that fermented beverages contain probiotics that are directly incorporated into the beverages and are therefore regarded as bio-functional food products (El-Sohaimy & Hussain, 2023:1). This, together with novel and aseptic packaging, will further increase shelf life and maintain the products' organoleptic properties. Direct liquid inoculation systems have aided in microorganisms being added directly to the products without the challenges associated with contamination or survival during processing. It is believed that this innovation will significantly create a vehicle for probiotics to increase the product's health benefits (Prado et al., 2008:120). Food fortification and enrichment programs have been introduced on a global platform to enhance vitamin and mineral intake via various food products, such as milk supplements using nano-emulsion technology. Hence, companies are fortifying foods such as yoghurt, bread and milk with vitamins and probiotics (Kalfirtova & Sovjac, 2005:22). Probiotic foods are short shelflife products. During fermentation and subsequent storage, the products changes in terms of its various attributes. One of the factors which is of high importance in fermented foods is to ensure that the probiotic flora remains viable, especially in the presence of other symbiotic cultures like lactic acid bacteria (Mani-Lopez et al., 2014:2579). The objective of this study is to establish an effective protocol to enumerate and detect viable PAB in kefir under different storage conditions over 28 days.

2.2. Fortification and fermentation of food

Food fortification, the practice of enriching commonly consumed foods with vitamins and minerals, was established in the 1920's. The main aim of food fortification was to increase the nutritional value of foods safely and cost-effectively with a view to improve diets and to prevent and control micronutrient deficiencies. Commonly fortified food includes staple products such as salt, maize flour, sugar, vegetable oil and rice (Olson et al., 2021:3). According to the World Health Organisation (WHO), iron and vitamin A deficiencies are the main contributors to mortality rate each year (Mkambula et al., 2020:1). A recent study has proven that fortification of foods with iodine, folic acid, vitamin A, and iron has dramatically reduced serious diseases (Olson, 2021:4). Hence, food fortification can be regarded as one of the most important processes used to improve the quality and quantity of nutrients in food. This process, however, is very expensive. Table 2.1 summarises the dietary nutritional recommendations of fortified dairy products. Products such as yoghurt are produced by fermentation processes where enzymes break down organic substances into more minor compounds and products that are more digestible, stable, flavoured, and nutritionally enhanced. Due to the high consumption

rate of yoghurt, it is one of the best ways to improve the overall nutrient intake with minimal processing (Gahruie, 2015:1).

Table 2.1. Fortified dairy products and dietary nutritional recommendations (Santillán-Urquiza et al., 2016:300)

Vitamins/Minerals	Daily dietary recommendation (Men, mg.day⁻¹)	Daily dietary recommendation (Women, mg.day⁻¹)	Fortified products	Amount added (mg.100g⁻¹)
Vitamin A	0.1	0.8	Milk, yogurt, and powder milk	0.12–0.90
Vitamin D	0.01	0.01	Milk, yogurt, powder milk, and cheese	0.00008–0.008
Vitamin E	12	12	Milk, yogurt, and powder milk	1.5–4.0
Vitamin B6	1.6	1.8	Milk, fermented milk, and yogurt	0.4–0.8
Vitamin C	60	60	Milk	50
Calcium	1000	1000	Milk, yogurt, and cheese	160–480
Iron	12	10	Milk	2.2
Magnesium	350	330	Milk and yogurt	45–68
Phosphorus	700	700	Milk	350

Fermentation was originally used to produce food with extended shelf life before canning, pasteurisation and refrigeration were developed (Chavan & Kadam, 1989:352). Figure 2.1 shows major developments in food fermentation over the years. Fermentation methods date to 6000 BC, when milk, vegetables and meats were used as substrates, but the process was mainly traditional. The benefits of fermentation also include reducing the volume of food during transportation, destroying undesirable microorganisms, and enhancing the nutritive and organoleptic qualities of foods. Fermentation reduces the amount of energy required during production and aids in producing a safer product (Blandino et al., 2003:528). Fermented foods also have health beneficiation properties due to their antioxidant, antimicrobial, antifungal, anti-inflammatory, antidiabetic, and anti-atherosclerotic activities (Tzavaras et al., 2022:1).

The process of enhancing food by fermentation is further beneficial to the consumer as it improves safety, digestibility, nutritional value and the methods involved are relatively inexpensive. Fermentative activities are defined as the products that have been subordinate to the effect of microorganisms or enzymes to cause desirable biochemical changes in food products (Marco et al., 2017:94). The organoleptic properties and the general acceptability of food are also improved significantly. The microflora responsible for the fermentative activities can be inherently present or added as a starter culture during later stages of production. A

group of bacteria known as the lactic acid bacteria (LAB) is typically found in dairy-based fermented foods and includes genera such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Streptococcus*. A long and significant history of culture and cuisine surrounds fermented foods and beverages (Marco et al., 2021:197). For thousands of years, staples of human diets have included fermented foods, such as milk, meat, and plant-based meals. The microorganisms involved in fermentation have been associated with complex metabolic pathways and classified as probiotics due to their actions in the digestive tract of the consumer (Voidarou et al., 2021:19). When fresh food was in short supply, fermented foods made a reliable food source. Additionally, consumers would have been drawn to fermented foods and beverages because of their distinctive sensory qualities. Improved utility, such as transforming common cereal grains into beer or bread, would have also increased public acceptance of fermented foods (Siddiqui 2023:1).

It is common practice to add probiotic organisms such as *Lactobacillus* and *Bifidobacterium* spp. to fermented foods to create more stable, therapeutic and nutritious foods, thus becoming a delivery vehicle for probiotics (Turkmen, 2019:65; Majuwana, 2021:2467). Various studies have shown the ability of fermented food to act as carrier of probiotics and deliver the probiotic to the host efficiently and simultaneously providing a health benefit to the modified food (Van Wyk et al., 2011:73; Leeuwendaal, 2022:7; Nithya et al., 2023:2). Probiotics involved in spontaneous fermentation, which is intended for human consumption is poorly defined. Additionally, there will be variations in the microbes found in kimchi, sauerkraut, miso, and other spontaneous food fermentations from batch to batch as well as between makers and regions. It is impossible to establish their probiotic properties if the selected strains were not isolated from each fermentation. This indicates that not all fermented foods will be classified as probiotics as they do not meet the criteria to be regarded as probiotics. The minimum criteria of food to qualify as a probiotic include attributes such as being safe to consume, tolerate bile and acids and colonise the intestinal tract (Zielińska, 2018:2). However, when a probiotic is added to a fermented food, the fermented food can be rendered a probiotic. It was determined that yoghurt could improve digestion of lactose among lactose intolerant individuals due to the yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) that are present. Thus, the European Food Safety Authority (EFSA Panel on Dietetic Products 2015) has approved a health claim for yoghurt. Furthermore, the numerous beneficial health effects of consuming yoghurt rich diets and other fermented foods such as the reduced risk of metabolic syndrome and weight management over the long term, have been proven by numerous studies (Mozaffarian et al., 2011:2871; Savaiano & Hutkins, 2021:610; Britos et al., 2024:2). Similarly, high consumption of kimchi and other fermented food resulted in a reduced prevalence of atopic dermatitis (Park & Bae, 2016:125).

2.3. Lactic acid bacteria Fermentation

Numerous studies have been documented about the advantages of fermented foods for health beneficiation. The microorganisms found in fermented foods are typically classified as generally regarded as safe (GRAS) by the United States Food and Drug Administration (US FDA). They can produce a variety of beneficial by-products and metabolites, including ethanol, alkali, acetic acid, organic acids, fatty acids, carbon dioxide, and antimicrobial peptides (bacteriocins) as well as lactic acid fermentation products (Sanlier et al., 2017:2). Lactic acid fermentation in food processing, involves the conversion of fermentable sugars present in the substrate to lactic acid. LAB also earned GRAS status (Mokoena et al., 2016:2). Microorganisms like LAB and yeasts use the chemicals in the raw substrate during fermentations (Sharma et al., 2020:1).

During the industrial revolution in the mid-19th century, populations increased in towns and cities, necessitating the production of food on a larger scale to supply local communities (Ross et al., 2002:4) LAB were mostly used in the fermentation processes of a variety of food commodities, namely, meat, dairy and vegetable products, e.g., cabbage used in the production of sauerkraut. Figure 2.1 shows a timeline of how the science of microbes, food processing-, preservation techniques and fermentation has evolved since 4000BC until 1988AD. The industrial revolution also led to the large-scale development of fermentation processes for the commercial production of alcoholic beverages using yeast and other microorganisms in the production of beer, wine, and spirits (Ross et al., 2002:4). Fermentation methods utilising milk, meat and vegetables have reported with the earliest records dating back to 6000BC. However, the role of microorganisms was not recognised. In 1850, the microbiology of fermentation as a science and the role of bacteria and yeasts were understood for the first time, resulting in more control and efficiency of the process (Caplice & Fitzgerald, 1999:132). Research has shown that among emerging functional foods, probiotics-based foods and beverages, including fermented dairy products are a growing part of the functional food market and are recognised as the future foods that are more prominent with wider acceptability among consumers (Ozer & Kirmaci, 2010:1; Koirala & Anal, 2021:1). They inhibit undesirable microbes by cross-feeding other beneficial gut microbes, thus playing an important role in creating a healthier gut environment (Sanders et al., 2018:213). A relatively frequent medical disorder known as lactase (galactosidase) insufficiency is the inability to properly hydrolyse lactose into two absorbable monosaccharides, glucose and galactose.

Lactose is a carbohydrate sugar found in milk and is broken down by the enzyme lactase. In cases where the small intestine is incapable of producing enough lactase to break

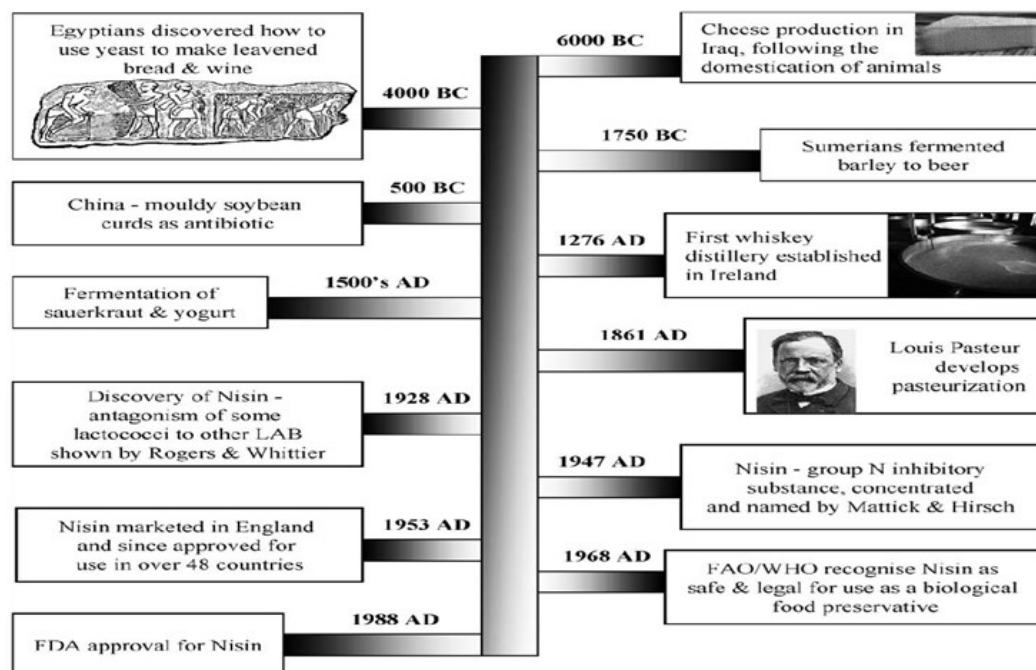


Figure 2.1. Major developments in food fermentation technology through the years (Ross et al., 2002:4)

down or digest lactose found in milk products, it results in lactose intolerance (Facioni et al., 2020:1). The lack of lactose in the diet by avoiding lactose-containing products can lead to a deficiency in essential carbohydrates as well as some important nutrients like vitamin B12 and calcium. Lactose also shows beneficial effects in the microbiota in the gut. (Romero-Velarde et al., 2019:1). Due to the quick colonic fermentation of the undigested lactose, this enzyme shortage may cause symptoms of lactose malabsorption, such as bloating, gas, changes in bowel habits, and abdominal cramps. After consuming dietary products containing lactose, symptoms of lactose malabsorption typically manifest 30 minutes to 2 hours later (Windey et al., 2015:7506). Almost complete abstinence from dairy products is the most popular strategy for treating lactose intolerance. However, this can have an adverse health effect (Ibrahim et al., 2021:357). Due to a decrease in calcium, phosphorus, and vitamin consumption, this strategy may cause a dangerous drop in bone density in some people. Lactase supplements or lactose and its derivatives may benefit gut health. If lactose is distributed over meals or prior to the consumption of dairy products, it may act as a prebiotic and may restore adequate levels. However, the alternative of adding over-the-counter lactase-containing pills to the meal is limited by their high cost and the side effect of sweetening the taste of the food. Lactose digestion may be aided by lactase activity (Robles & Priefer, 2020:8). According to studies, people who are lactose intolerant can tolerate lactose in yoghurt better than the same quantity of lactose in milk or other fermented dairy products to meet the recommended daily nutritional requirements (Adolfsson et al., 2004:249). The presence of lactase-producing bacteria, particularly *Lactobacillus* (L.) *acidophilus*, is thought to have contributed to the digestion and absorption of lactose in yoghurt. Other species that produce lactase include *L. bulgaricus* and

Streptococcus (S.) thermophilus, which may be helpful for lactose-intolerant people (Ranasinghe & Perera, 2016:99).

A study by Huang et al. (2013:2824) was conducted where *Lactobacillus plantarum* (isolate Lp27) was isolated from Tibetan kefir grains and fed to hypercholesterolemic rats at a dose of 109 colony forming units (cfu) per day for 4 weeks. It is known that Lp27 usually develops in 0.3% ox gall and survives 3 hours of incubation at pH 2.0. According to tests for antibiotic susceptibility, Lp27 was susceptible to gentamicin, tetracycline, erythromycin, and chloramphenicol but resistant to vancomycin, with a minimum inhibitory concentration of 23 g.L⁻¹. It was demonstrated that Lp27 reduced cholesterol absorption of Niemann-Pick C1-like 1 (NPC1L1) protein in rats. NPC1L1 is a critical protein for intestinal cholesterol absorption and is highly expressed in the small intestine, especially at the surface of enterocytes (Ge et al., 2008:517). By reducing NPC1L1 expression in Caco-2 cells, Lp27 reduced cholesterol absorption. The *in vitro* uptake of cholesterol and the *in vivo* effects of Lp27 on serum cholesterol levels in rats were also determined. The Lp27 feeding of rats significantly lowered serum total cholesterol, low-density lipoprotein cholesterol, and triglycerides concentrations. The study aimed to develop a new LAB strain that can serve as a probiotic to lower cholesterol levels in humans. The outcome indicated that down regulation of NPC1L1 might be an activity of most *Lactobacillus* sp. strains and may provide a possible molecular mechanism for the modulation of cholesterol concentrations, thus exhibiting efficient cholesterol-reducing ability *in vitro* and *in vivo*. Moreover, Lp27 reduced the concentrations of rats fed a high-cholesterol diet, as reported earlier for other *L. plantarum* strains. The results of this study indicated the probiotic potential of Lp27 for the control of cholesterol (Huang et al., 2013:2822).

2.4. Milk

Milk is a clear, white fluid taken from the mammary glands of female mammals. It is rich in nutrients such as caseins, lipids, and the carbohydrate lactose, which consists mainly of glucose and galactose. It is taken out during or after pregnancy (Sadovnikova et al., 2021:8). More than 85% of the milk produced and consumed globally is from cows; however, it can be derived from any female mammal. Since approximately 8000 years ago, humans have consumed milk from other species, and a sizable business has developed around milk preparation for human consumption. Milk processing is a common activity worldwide, particularly in Europe and North America. About 1000 different types of cheeses are produced which uses certain physicochemical characteristics of milk. Degradation of milk proteins into peptides and free amino acids by bacteria results in flavours and aromatic compounds formed through numerous further catabolic reactions (Sabikhi, 2007:14; Priyashantha and Lundh, 2021:12183). Due to the presence of casein and triglycerides, which are present as colloidal sol and emulsion, respectively, along with carbohydrates, serum proteins, and minerals, milk has a turbid look as one of its physical characteristics. Table 2.2 presents the general chemical

composition of cow's milk. The density of milk ranges from 1.028 to 1.034 kg.L⁻¹ depending on the chemical composition. The pH of milk is between 6.4 and 6.8 (Kandeel, 2019:1422). Milk is a nutritious and complete food that provides carbohydrates, lipids, proteins, and minerals to a regular diet (Górska-Warsewicz et al., 2019:2)

Globally milk is an important dairy product with a long history of production and consumption. The production of milk has greatly increased, reaching the highest levels in recent decades (0.9 billion tons) with the majority (0.8 billion tons) produced by cattle, followed by buffalo (0.12 billion tons) and goats (20.7 million tons). The significant increase in milk production can be attributed to its high nutritional and health value (Elamshity & Alhamdan, 2024:2). Dairy products unquestionably have the highest nutritional value in the world. Milk contains most of the compounds vital for human life and is an excellent source of most vitamins and minerals as well as protein and calcium (Kalfirtova & Sovjac, 2005:22; Górska-Warsewicz, 2019:1). Cow's milk is a good source of water-soluble vitamins except ascorbic acid and vitamin B₁₂. Milk is used in many fermented applications, such as cheese and yoghurt production, which is used to improve digestion and reduce intestinal infections and certain cancers. Milk is a physiological liquid containing beneficial bioactive and nutritional compounds that include bioactive peptides, organic acids, and oligosaccharides (Robinson, 2019:3). Since milk has been described as a vitamin source and the ability of some starters to synthesise vitamin B, fermented milk will have different vitamin content depending on the starter culture used. However, evaluation of vitamins is challenging due to processes like heat treatment, incubation time, temperature and storage conditions, which will change the vitamin content in the product (Gahruie et al., 2015:3).

Buffalo is the second most popular source of milk production (15.43%) originating from animals followed by goats, sheep, while camel milk recording the lowest production (0.44%) (Rashid et al., 2024). It has higher nutritional value than the other milk sources, because of its 7% to 9% fat content, high protein and mineral content, and somewhat low cholesterol content. It also has more dry matter, fat, and calories than cow milk, as well as more minerals, vitamins, and microelements (Abdel-Hamid, 2023:1). It is suggested that buffalo milk rather than cows' milk should be used in the commercial production of kefir due to its sensory characteristics. Since the texture score of buffalo milk is not as favourable as cow's milk, it is recommended that buffalo milk should be used for industrial kefir production due to its lower serum separation which is a problem for kefir during storage (Gul et al., 2015:1518). The long tradition of kefir production started with the fermentation of milk in skin bags as a preservation method. It is believed that the first fermented foods were started by accident (Bintsis, 2022:1). Fermentation of foods has proven to be beneficial to the consumer due to its health-promoting properties and enhancement of the product's organoleptic attributes. These processes also enhance mineral bioavailability and assist with the digestion of proteins and carbohydrates (Valentino et al., 2024:1; Erem & Kilic-Akyilmaz, 2024:2).

Table 2.2. The chemical composition of cow's milk (Santillán-Urquiza et al., 2017:298)

Component	Content per 100 mL
Protein (g)	3.5
Casein (g)	2.8
Whey proteins (α -lactalbumin and β -lactoglobulin; g)	0.7 (0.4 and 0.3)
Fat (g)	3.7
Lactose (g)	4.8
Minerals (g)	0.72
Calcium (mg)	125
Phosphorus (mg)	103
Magnesium (mg)	12
Potassium (mg)	138
Sodium (mg)	58
Iron (mg)	0.1
Vitamin A (I.U.)	158
Vitamin D (I.U.)	2
Thiamine (mg)	0.04
Riboflavin (mg)	0.18
Vitamin B6 (mg)	0.035
Folic acid (mcg)	2
Vitamin B12 (mcg)	0.5
Vitamin C (mg)	2

Research has proven that by fortification of food with *Propionibacterium freudenreichii* (PAB), it is possible to enhance the nutritional properties of specified food products by increasing vitamin B₁₂ levels (Chamlagain et al., 2021:499). Further research is important to investigate the organism's survival during the process of kefir production using shorter fermentation time and subsequent storage. Such findings will contribute significantly towards future product development in the industry (Van Wyk et al., 2011:73). Kefir is also an example of such a naturally fermented milk beverage (Dahiya & Nigam, 2023:2). Kefir is a milk product produced by the fermentation of various types of milk with kefir grains that have historically been used to promote and maintain good health. It is regarded as the best source of natural probiotics and it is easily digestible (Satir & Guzel-Seydim, 2016:1).

2.5. Kefir

Kefir is a smooth, textured, acidic, low alcohol milk beverage with a clean, pleasant taste. It is fermented by using milk of various origins and inoculating it with kefir grains with unique properties (Ganatsios et al., 2021:2). It can be prepared from milk of plant or animal origin, including cow, goat, sheep, rice, coconut, and soy (Altay et al., 2013:50; La Torre et al., 2024:2). The use of specific milk and bacterial cultures during production leads to comparable chemical and microbiological characteristics (Dimitrellou et al., 2019:3). The flavour, aroma, and characteristics of kefir are dependent on the yeast and microbial community naturally

present in kefir grains and the milk type used (Ding et al., 2022:343). Kefir, made from cow's milk, proved to have better sensory and organoleptic properties than goat's milk. Fresh goat's milk is therapeutic and has better digestibility than cow's milk, but consumers usually dislike the flavours and aromas. However, kefir produced from goat's milk does not have these adverse attributes, making fermentation of goat's milk with kefir grains ideal (Tratnik et al., 2006:45). Arslan-Tontul (2018:4779) proved that kefir could be a suitable yoghurt replacement in tarhana production as it successfully increased the LAB cell count without affecting yeast and other mesophilic bacterial counts. Yeast counts were significantly increased, while LAB was confirmed to be a major producer of organic acid in the tarhana samples. Microbiological counts revealed that adding kefir increased the LAB cell count but not the yeasts and mould.

2.5.1. History of kefir

The name “kefir” reportedly originates from the Turkish word “keyif” as it means “good feeling” resulting from the feelings experienced after drinking it (Mei et al., 2016:1). Kefir grains have their origin in the Caucasus mountains and Anatolia Peninsula and have existed since 1884 in modern Russia. (Aladeboyeje & Sanli, 2021:550). It is gaining popularity globally due to its nutritional attributes (Azizi et al., 2021:1). The grains resemble elastic pieces or clumps of cauliflower or coral (Figure 2.2). When viewed under a stereomicroscope (Figure 2.3), the grains consist of numerous small units between 2.0 and 9.0 mm in diameter. Each of these units is hollow globular structure with a polyhedral-like cell wall of approximately 200 µm and



Figure 2.2 Kefir grains used in the production of the kefir

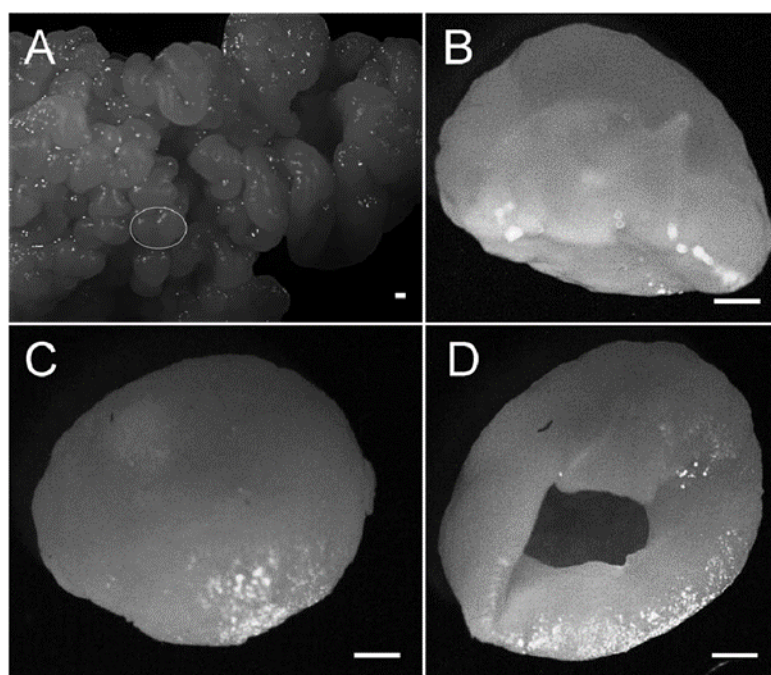


Figure 2.3 Tibetan kefir grains as observed by Lu et al. (2014:4) using an electron microscope: a single grain unit as indicated by the circle (A), view from the side (B), from the top (C), and from the bottom (D). Scale bar =1.2mm.

It consisted mainly of bacteria (Lu et al., 2014:4). Kefir grains appear to have a smooth and shiny surface, but scanning electron microscopic examinations prove they have a slimy but firm and rugged texture (Mei et al., 2016:1; Gao & Li, 2016:2). Kefir grains can last for years if the grains are maintained under suitable conditions (Alraddadi et al., 2023:2).

2.5.2. Microbiological characteristics of kefir grains

Kefir grains consist of a population of various yeasts and bacteria which include LAB, (*Lactococcus* and *Lactobacillus* spp.), acetic acid bacteria and fungi which are embedded in a matrix with a chemical composition of 890 to 900 g.kg⁻¹ water, 2 g.kg⁻¹ lipid, 30 g.kg⁻¹ protein, 60 g.kg⁻¹ sugars and 7 g.kg⁻¹ ash. LAB, which included *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Acetobacter*, *Streptococcus* and yeasts, which included *Kluyveromyces*, *Torula*, *Candida* and *Saccharomyces* spp., were among the symbiont mixture of microbes, isolated in various studies (Xie, 2012:394; Nalbantoglu, 2014:45). Ding et al. (2022:290) found that the outer layer of the kefir grain contained almost no yeast when kefir grains from different regions globally was investigated. This study proved that lactobacilli, lactococci and *Leuconostoc* were amongst the most prominent flora. These bacteria exist in a symbiotic relationship in various fermentation processes, including bread production as baker's yeast, polysaccharide production, and novel fermented beverages (Gorsek & Tramsek, 2008:153). The findings by Dertli & Con (2017:152) revealed that *L. kefirianofaciens* and LAB were the predominant bacteria present in all the kefir grains, while *Kazchstania unispora* was only present in one of

the samples tested. The yeast microflora, as determined with Next Generation Sequencing (NGS), were dominated by species relating to the *Dipodascaceae* family. *Saccharomyces cerevisiae* was present in low numbers. It was also found that several undesirable bacteria were detected amongst the beneficial microflora present in kefir grains, including *Enterobacteriaceae*, *Pseudomonadaceae* and *Moraxellaceae*. The polysaccharide matrix surrounding the microbial complement in kefir, is called kefiran (Rodrigues et al., 2005:404; Zajšek et al., 2013:970). Kefiran is described as a glucogalactan which is water-soluble which is produced by micro-organisms like *Lactobacillus kefiranofaciens* and *Lactococcus plantarum* (Gao & Li 2016:2). The favourable health properties of kefir may be attributed, but not restricted to kefiran (Santos et al., 2003:437). Kefiran also described as an appealing flexible biopolymer that produces polysaccharides) (Ellatif et al., 2022:2). According to Garbers et al. (2004:687), obtaining a constant starter culture required for a good quality kefir product is difficult due to its complexed microbial composition. However, this spectrum of microbial species combined with the fermentation process is responsible for the characteristic qualities of kefir. Kefir is obtained by the fermentation of the milk with kefir grains. The kefir grains recovered after the fermentation process are reusable as a starter culture for future fermentations. Reusing the kefir will also increase the biomass (Alraddadi et al., 2023:2). According to Mei et al. (2016:1), kefir grains can retain their activity for years if preserved and incubated under appropriate culture and physiological conditions. It is of the utmost importance to produce kefir under controlled conditions to fully benefit from its health promoting properties and control contamination levels as undesirable bacteria such as *P. acnes* have previously been isolated (Dertli & Con, 2017:153).

2.5.3. The production of kefir

Kefir grains are natural starter cultures in the process of kefir production. Historically, continuous fermentation in sheep skin bags produced kefir under uncontrolled conditions by the removal of fermented milk, followed by the addition of fresh milk (Guzel-Sydim et al., 2010:144). In current practice, kefir is produced by inoculating kefir grains into milk, as the kefir grains contain a diverse microbial community formed by a matrix of bacteria, yeasts, and fungi. This process is followed by incubation at room temperature for approximately 24 hours. The resultant product will be a unique self-carbonated beverage which resembles and tastes like yoghurt but with exotic sour properties (Dertli & Con, 2017:151). The microorganisms in kefir are responsible for the fermentation of the milk and the grains can be recovered to be reused at the end of the process (Altay et al., 2013:50; Leite et al., 2013:345). The quality and microflora of the kefir grains can be drastically affected by the original microbial distribution in grains, the grain to milk ratio, incubation and cold storage conditions, and aseptic handling during separation and washing. Differences in common microorganisms were obtained between kefir grains from different regions. It is also challenging to obtain a constant and

defined starter culture that can be used for industrial kefir production due to the vast differences and complexity in the microbial composition (Mei et al., 2016:1). The unique taste and texture of kefir are largely due to the interactions and fermentation processes of the yeasts and other microflora present in the kefir that also produce a variety of bioactive ingredients and peptides. A variety of bacteria have exhibited proteinase activity. The action of proteinase and peptidase enzymes on milk proteins can theoretically result in the formation of many possible peptides. Kefir consumption has many beneficial effects, including improved lactose digestion and enhanced metabolic and immune functions in humans (Farnworth, 2005:12). Dehydrated and hydrated kefir grains are inoculated into milk to produce many novel products. Bacteria and yeasts isolated from kefir grains were used in the production of malted beer and beer-like fermented beverages (Mikyska et al., 2015:311). During unfiltered and unpasteurised kefir beer production, dried starter grains were thawed and repeatedly cultured in 50 g.L⁻¹ molasses until a concentration of 30 g.L⁻¹ was obtained for mash fermentation. The grains were allowed to grow in a nutrient medium, washed in distilled water and settled in a fresh nutrient medium. The process involved the polyphenol content of barley malt, along with some of the probiotic and prebiotic qualities that are associated with kefir (Rodrigues et al., 2016:59).

2.5.4. Properties of kefir

Traditional home production, combined with commercial kefir production, has contributed to increased consumption. According to Leite et al. (2012:341), there is growing evidence that kefir is increasingly consumed in many countries due to its beneficial effects, the belief that it may be helpful in many diseases and infectious conditions, and its perceived benefits for general health. Kefir was described as a functional food and a natural probiotic beverage (Ganatsios et al., 2021:9; Apalawo et al., 2024:2). Kefir is safe, inexpensive and a good source of magnesium, calcium and Phosphorus. Kefir can easily be prepared at home (La Torre et al., 2024:2).

Kefir grains exhibit antimicrobial activity against many bacteria, including *Salmonella typhi*, *Shigella sonnei*, *Staphylococcus aureus*, *Candida albicans*, *Streptococcus enteritidis*, and *Escherichia coli*. Fermented milk also had an inhibitory effect against *Enterobacter faecalis*, *Bacillus subtilis*, *S. enteritidis*, but exhibited no effect against *Pseudomonas aeruginosa* and *Candida albicans* (González-Orozco, 2022:3709). The growth of many Gram-positive and Gram-negative bacteria like *S. aureus*, *E. faecalis*, *S. enteritidis*, *Bacillus cereus*, *E. coli*, and *Clostridium tyrobutyricum* strains was prevented during *in vitro* studies using kefir extracts. However, the inhibitory properties varied with different grain types and kefir fermentation times (Kim et al., 2016:787). Kefir also has anti-inflammatory properties and has acted against pathogenic genera, including *Salmonella*, *Helicobacter*, *Shigella* and *Staphylococcus spp.* and *E. coli*. Kefir aids in wound healing. Kefir was effective as a protective dietary supplement against COVID-19 during the pandemic. Lactic acid bacteria in milk

fermentation, produced nisin, which is a food-grade antimicrobial peptide, was studied for the control of viral infections (Dahiya & Nigam, 2023:3). The polysaccharide matrix surrounding the bacteria and yeast, called kefiran, is a water-soluble glucogalactan with reportedly antimycotic, antibacterial and antitumor activities (Simonelli et al., 2022:101). Strains of *Lactobacillus* spp. were tested *in vitro* for their ability to adhere to human enterocyte-like Caco-2 cells, resistance to acidic pH, bile acid and antimicrobial activity, which are among the attributes of the best probiotics. *L. acidophilus* and *L. kefiranofaciens*, which are also present in the complex microbiota of kefir grains, exhibited good adhesion, resistance to acidic pH values and bile acids, as well as inhibition of some pathogenic bacteria. *L. kefiranofaciens* produced a kefiran-related exopolysaccharide. In vivo, oral administration of kefir grain kefiran significantly slowed tumour growth. The exopolysaccharide produced by *L. kefiranofaciens* had a better emulsifying capability than xanthan gum, guar gum, and locust gum. It has thus been proven that *L. kefiranofaciens* in kefir grains is of significant industrial interest due to these probiotic characteristics as well as possible further therapeutic applications due to its unique ability to produce kefiran-related polysaccharides (Altay et al., 2013:51).

Epidemiological studies have shown a negative correlation between cancer development and the consumption of fermented dairy products, such as kefir. Metabolic activity of bacteria can alter the nutrients and release of bioactive compounds during the fermentation process, resulting in its health-promoting properties (Savaiano, 2021:611). During a study where experimental mice were subjected to subcutaneous transplants of Ehrlich's carcinoma cells and then orally administered with a water-soluble polysaccharide isolated from kefir grains, the carcinoma cells were inhibited. It was proven in a study where rats were fed with natural kefir under normal metabolic conditions that the kefir had the ability to protect colonic crypt cells against radiation-induced apoptosis, which was most pronounced in the stem cells (Hlastan-Ribič, 2005:51). Sabir et al. (2010:570) evaluated the probiotic characteristics of LAB isolated from kefir which included *L. helveticus*, *L. casei*, *Pediococcus dextrinicus*, *P. pentosaceus*, *L. cremoris* and *L. lactis*. It has been established that these species were able to survive acid conditions and various bile salt concentrations. They were also able to auto-aggregate and co-aggregate *E. coli*. As previously discussed, the probiotic characteristics of *L. acidophilus* and *L. kefiranofaciens* proved to be the best in the spectrum of LAB tested, which included *L. kefir*, *L. brevis*, *L. paracasei* and *L. plantarum* (Santos et al., 2003:437). It is, therefore, possible to select high-quality kefir starter cultures according to their antimicrobial and probiotic properties; the symbiotic relationship of microbes with the kefir matrix is still poorly understood (Tingirikari et al., 2024:12).

In a study by Mikyška et al. (2015:315), various cereals and grains or combinations thereof were fermented by yeasts and bacteria isolated from kefir grains. Yeast strains RIBM163 and RIBM164 (*Saccharomyces cerevisiae*) and LAB strains RIBM2-107 and RIBM2-108 (*Lactobacillus casei* subsp. *paracasei*) were used to inoculate wort of malted wheat, oats

and barley successfully. Kefir, like other fermented dairy products, has been linked to several health advantages, including cholesterol metabolism, antibacterial activity, tumour suppression, and accelerated wound healing. Rodriques et al. (2016:61) produced a craft beer using kefir grains. Male Wistar rats were divided into groups and fed kefir beer, control beer, kefir-fermented molasses, water, and 4% ethanol for 30 days. The rats were weighed every 5 days; rats fed with both beer samples and kefir suspension resulted in 4.82% reduction in animal body weight. The rats were also subjected to carrageenan-induced paw oedema. An average decrease in inflammation after 7 days of treatment with the kefir beer was observed, which was more pronounced than that observed with the control beer.

2.6. Probiotics

The joint World Health Organisation and Food and Agriculture Organisation of the United Nations (WHO/FAO) defined probiotics as “live microorganisms, which when administered in adequate amounts, confer a health benefit to the host”. In the EU, these non-pathogenic microorganisms must be present in doses of at least 10^6 cfu.mL⁻¹ to be effective and exert a positive physiological influence and health benefit to the consumer (Hill et al., 2014:511; Peruzzolo et al., 2025:2). The safety criteria must be crucially evaluated as it is consumed by a diverse population including infants, elderly and immunocompromised individuals. Identification, purity, and viability, including dosage, are important for the characterisation of probiotics. Its genetic and phenotypic characteristics, including pathogenicity, must also be investigated (Sarita et al., 2025:3). Guidelines for the evaluation of probiotics in food were released in 2002 by the joint WHO/FAO Working Group. According to these guidelines; the minimum requirements needed for probiotic status include, (i) well-defined strain identification which must be assessed to genus, species and strain level, (ii) screening of potential probiotics *in vitro* for attributes such as resistance to gastric acidity, bile acidity, digestive enzymes, antimicrobial activity against potentially pathogenic bacteria, (iii) safety assessment, and (iv) *in vivo* studies for substantiation of the health effects in the target host (Binda et al. 2020:1). It is difficult for the consumer to track the product research if the label does not state the strain of the organism. Sometimes, no live bacteria are present in the products. This includes skin- and haircare, cosmetics and deodorants (Puebla-Barragan et al., 2021:2). There are many overemphasised probiotic labels on the market, such as for pizza, coffee, shampoo and toothpaste. For this reason, scientific evidence of the health effects of a defined application is necessary to claim the functional property. Consumers are becoming increasingly aware of the side effects of medicine, thus consuming probiotic food is more acceptable to receiving the daily dose of probiotics (Latif et al., 2023:8).

2.6.1. Characteristics of probiotics

The International Association of Prebiotics and Probiotics (ISAPP) stipulates that for a microbe to gain probiotic status, it must be alive, administered at an effective dose, and confer health benefits (Sanders et al., 2018:212). According to the ISAPP consensus panel, the definition of a probiotic implies that it must be safe for its intended use and be a well-defined mixture to allow appropriate identification of the strain level. It is not necessary for probiotics to demonstrate traits in pre-clinical tests such as colonisation, intestinal transit survival, adherence, anti-pathogenic activities, and host microbiota balance. Furthermore, the term does not specify whether the probiotic strain is derived from a human source. These characteristics might be beneficial for probiotics made for certain uses but may not be necessary for all applications. For instance, a probiotic used for dental health might not need to survive intestinal transit. Any probiotic's safety must be firmly demonstrated for its intended use, despite the outstanding safety record of probiotics that are widely marketed (Sanders et al., 2016:213; Merenstein et al., 2023:10). Probiotics tailored for vulnerable patient populations may raise extra concerns (Sanders et al., 2016:683; Spacova et al., 2023:7).

Probiotics are not consumed as a cure or treatment or disease, but merely as nutritional attributes to health (Garg et al., 2024:2). The use of probiotics in vulnerable populations, especially pregnant women and the elderly, must be prescribed by specifically educated health officials as safety trials are scarce. Safety concerns may relate to various aspects, such as product quality, suitability, strain type, posology, and intended use (Fernández et al., 2024:10).

Since probiotics are live microorganisms, good manufacturing practices must be met, and special care must be taken to prevent undesirable contamination of the product. Labelling of probiotic products must include the current name of the genus, species, and strain classification for all microbes (FAO & WHO, 2002). Information on product labels should also contain information on the potential of the product, typically expressed as colony-forming unit (CFU). CFU refers to the enumeration of viable organisms interpreted from a unit that was able to form a colony on an agar plate (Chiron et al., 2018:573; Sanders et al., 2018:20). Probiotics can be found in three primary categories on the international market: foods, dietary supplements with probiotics for human use, and medications. Some more recent products that contain probiotics are not classified in a regulatory category. Numerous regulatory systems rigorously control the number of live microorganisms in pharmaceuticals and cosmetics, including probiotics for human use (Zommiti et al., 2020:9). Probiotic cosmetics are now available and accessible. For safety concerns, there are many regulatory frameworks that strictly regulate the amount of living microorganisms in cosmetic goods. However, for a product to be deemed a "probiotic", it must deliver adequate quantities of live microbes. Another concern is whether cosmetic advantages like teeth whitening qualify as "health benefits" under the probiotic definition. Products like chewing gum, throat sprays, and probiotic lozenges do

not clearly fit into the food or supplement categories. Additionally, probiotics not only benefit humans but can also be used in pet and animal husbandry settings (Sanders et al., 2018:213).

2.6.2. Benefits of probiotics

Propionibacteria in dairy has been proven to have probiotic properties as the intestinal population of bifidobacteria is enhanced due to the ingestion thereof (Rossi et al., 2025:24). Probiotics can directly or indirectly influence intestinal physiology through the regulation of the indigenous flora and help to inhibit pathogens (Kıvanç & Yapıcı, 2018:225; Chandrasekaran, 2024:1). Figure 2.4 shows how probiotics can be beneficial to the human body. In recent years, lactose intolerance and the number of vegan individuals has increased. This sparked interest in alternative probiotic sources that can be consumed by affected individuals (Jang et al., 2024:257). Sugary kefir is a substitute source of probiotics for people with lactose sensitivity and vegans. According to research, sugary kefir contains probiotic qualities that allow pathogens to colonise the mucosa and adhere to it by penetrating its lumen (Açik et al., 2020:527).

Various lactic acid bacteria (LAB), more specifically *Lactobacillus plantarum*, was evaluated for their therapeutic potential in food as probiotics. LAB is also used in conjunction with other bacteria in different types of food (Zago et al., 2011:1033). LAB isolated from various fermented foods and beverages traditionally of African origin, have emerged to be a good source of antimicrobial and therapeutic agents, apart from being fermented for their numerous nutritional benefits (Obafemi et al., 2022:2). LAB has shown antioxidant and anti-inflammatory activity and are effective against necrotic cancer cells which also highlighted its potential as a nutraceutical product and probiotic strain. Some strains of LAB isolated from kefir grains possess antioxidant properties and exhibit antimicrobial effects against pathogens such as *Listeria monocytogenes*, *E. coli* and *P. aeruginosa* (Elhalik et al., 2024:2).

Fermented dairy products contain a complex mixture of lactic and propionic acid bacteria, which protect propionibacteria against digestive stress (Cousin et al., 2012:135). Probiotic starter cultures are very useful in improving the quality and preservation of fermented meat and fish as they may inhibit the growth of pathogenic bacteria. Probiotics also improved gut microflora composition, immune response, improve product quality and animal growth as well as sensory properties (Al-Shawi et al., 2020:14). Animal feed fortified with probiotic cultures proved significant in *in vivo* experiments. Fish fed with probiotics presented significantly higher growth rates than their counterparts which were fed with control diets. Supplementing with probiotics resulted in a higher survival rate, enhanced growth and immunity (Amin et al., 2025:2).

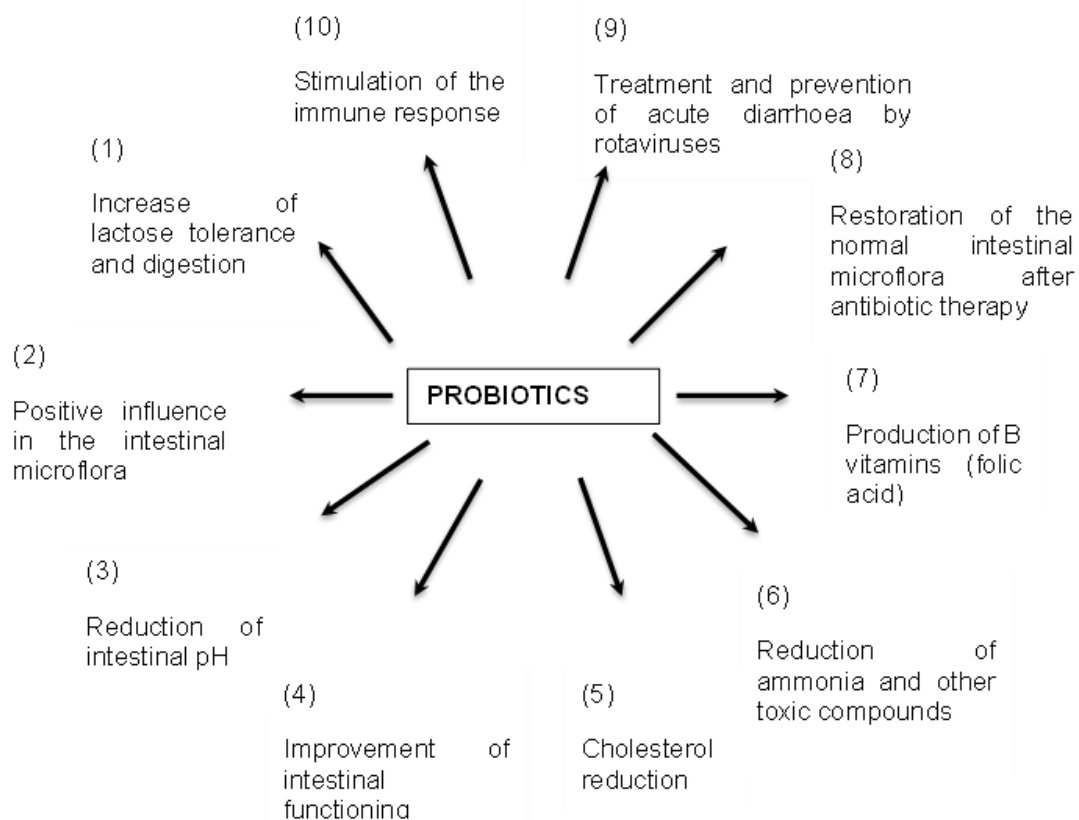


Figure 2.4 The beneficial effects of probiotics on human health (Prado et al., 2008:112).

Supplement feeding the avian industry avoided the undesirable effects of lectins on animal health by blocking their binding to mucosal receptors. Probiotic bacteria were administered to broilers to control the adverse effects of these proteins on digestive enzymes (Babot et al., 2021:30). Similarly, chickens challenged with various *Salmonella* spp. were fed with LAB probiotics which included *Bifidobacterium bifidum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Aspergillus oryzae* and *Torulopsis* species. The recovery of *Salmonella* including coliforms and campylobacter was significantly reduced compared to the chickens fed with the control diet. Chickens fed with the probiotic diet showed increased moisture, protein and ash content and lower fat content in the leg and breast meat (Khaksefidi & Rahimi, 2005:1153). Probiotics like LAB and *Bifidobacterium* ingested from dairy, tablets, lozenges and chewing gum in various dose regimes may have beneficial effects in the prevention and treatment of dental caries due to their inhibiting effect on cariogenic bacteria (Li et al., 2024:13). Although randomised clinical study results look promising, large-scale studies should be performed to evaluate orally derived anti-caries bacteria. Previous studies have also proven that plasma glucose levels in mice were significantly reduced following probiotics probiotic treatment, which proved significant in controlling insulin-independent diabetes. Host wellbeing, in terms of obesity, is positively affected by nutrients containing prebiotics and probiotics. Bacterial metabolism in the gut influences the release of bioactive compounds interacting with host cellular targets to control energy metabolism and immunity. This, in turn, affects energy

metabolism and immunity, ultimately resulting in less fat mass development, diabetes and low levels of inflammation associated with obesity (Mokoena et al., 2016:5).

2.7. Propionibacteria (PAB)

Propionibacterium freudenreichii forms part of the phylum *Actinobacter*. *P. freudenreichii* is divided into two classes, generally known as classical or dairy and cutaneous PAB, which is of clinical importance. When propionibacteria are associated with food products, they are classified as *Propionibacterium*. Currently, food related propionibacteria are *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici*, *A. jensenii* and *A. thoenii* (Rossi et al., 2025:2). Both classes are of equal importance to humans. Classical PAB, also known as dairy propionibacteria (PAB), can play a role in the dairy industry as a starter culture or in food spoilage as a potential pathogen. Cutaneous PAB is pathogenic and mainly causes acne and food spoilage (Ahmadi et al., 2017:68). Theiry et al. (2011:20) described PAB as Gram-positive pleomorphic rods with a high G+C content, requiring low levels of nutrition and capable of surviving in various environments. They are also non-motile, non-spore-forming, catalase variable, and facultative anaerobes. PAB grows optimally at 30-37°C and pH 6-7. The growth is inhibited at pH 4.5 and no propionic acid is produced (Ahmadi et al., 2017:69). PAB forms part of the genus *Actinobacter*. It is divided into two classes: dairy PAB and cutaneous PAB. Dairy PAB are good producers of vitamin B₁₂ and propionic acid. (Chamlagain et al., 2018:75; Zhang et al., 2025:8) They convert the lactate produced by lactic acid bacteria into propionate, acetate and CO₂ according to the formula: 3 lactate = 2 propionates + acetate + CO₂ + H₂O (Ahmadi et al., 2017:69). Cutaneous PAB is found on the skin and mostly causes acne and disease. The most important member of cutaneous PAB is *Propionibacterium acnes*, (*P. acnes*) forms biofilms and is responsible for severe acne and post-surgery infections. This class of bacteria causes damage and inflammation of the host cell tissues and can hydrolyse different proteins, gelatin and fibronectin (Platsidaki & Dessinioti, 2018:3). However, *P. acnes* were reclassified as *Cutibacterium acnes* due to its phylogenic and genomic analysis, which showed differences in the 16S RNA gene sequence among other differences (Mayslich et al., 2018:1; Platsidaki & Dessinioti, 2018:4; Corvec et al., 2019:27). *P. freudenreichii* was first described by Orla-Jensen and von Fredenreich in 1906 when they investigated the production of propionic acid and the formation of round holes in cheese (Theiry et al., 2011:20). Later, it was used as a ripening culture in Swiss cheese as well as a starter culture for other dairy products (Koskinen et al., 2015:1). Some dairy PAB have the potential to serve as food-protective cultures and can also synthesise antifungal and anti-yeast properties. PAB secretes bacteriocin and several antimicrobial organic acids acting in synergy, which includes acetic, propionic, succinic and lactic acids, 2-pyrrolidone-5-carboxylic acid, 3-phenyllactic acid, hydroxyphenyl lactic acid and 3-phenyllactic acid (Theiry et al., 2011:25). Propionic fermentation mainly occurs under warm conditions. However, *P. freudenreichii* is known to

remain active during the storage of cheese at 4°C as in the ripening of Emmental cheese (Cousin et al., 2012:140).

PAB is widely used for its probiotic- and protection properties as well as its nutraceutical properties (Rossi et al., 2025:8). They are good producers of vitamin B₁₂ and have successfully been used in the fortification of fermented dairy products. By incorporating propionibacteria into the kefir grains in the milk beverage, the vitamin B₁₂ levels can be increased (Van Wyk et al., 2011:72). A level of 5 % of *Propionibacterium freudenreichii ssp. shermanii* (PAB) was recommended for the fortification of fermented dairy products. Meat, fish, dairy and poultry products normally contain this essential vitamin which is water-soluble and unstable in light and high temperature.

2.7.1. Nutritional characteristics of PAB

PAB can grow anaerobically in media that contain only a few nutritional requirements, i.e., a carbon source, ammonium as the only nitrogen source, minerals, and two to four vitamins. PAB may utilise a range of carbon substrates, including sugars (lactose, galactose, D-glucose, and D-mannose), alcohols (erythritol, glycerol, and adonitol), and acids (lactic acid, gluconic acid). These carbon sources are catabolised by different pathways such as glycolysis, pentose phosphate, and Entner-Doudoroff pathways (Falentin et al., 2010:5). PAB can synthesise all the amino acids as they contain the necessary enzymes. They have low nitrogen requirements and can use ammonium sulphate as a special source for growth. PAB is dependent on vitamins such as biotin (vitamin H) and pantothenic acid (vitamin B₅) and minerals like sodium, phosphorus, and potassium for optimal growth. Magnesium, calcium, sulphur, and iron are required for rapid growth. PAB is inhibited by copper, while iron, manganese, cobalt, and magnesium have growth stimulatory effects.

2.7.2. Metabolic pathways of PAB

During PAB metabolism, several complex metabolic pathways can be interconnected and work simultaneously in multiple directions. Lactate produced by LAB can be catabolised in three main pathways namely the Fritz pathway, CO₂ fixing pathway and the aspartate deamination pathway. Pyruvate is one of the main nodes of PAB. Acetate and CO₂ synthesis via pyruvate dehydrogenase (PDH) and propionate production via the Wood-Werkman pathway are the main metabolic cycles utilised by PAB (Dalmaso et al., 2012:11).

Propionic acid is the major end-product of fermentation by PAB. Pyruvate production is aimed at three main pathways throughout the pyruvate intermediate branch. While some pyruvate is directed into the acetic acid pathway, most pyruvate is converted into propionic acid via the Wood-Werkman cycle (Figure 2.5), a propionate production mechanism. Oxaloacetate transcarboxylase catalyses the conversion of pyruvate to oxaloacetate and methylmalonyl Co-enzyme A (CoA) to propionyl CoA. Propionyl CoA is created when the

carboxyl group from methylmalonyl CoA is transferred to pyruvate. The release of oxaloacetate from this reaction does not occur, nor is there any evidence of an exchange between the propionyl CoA: succinyl CoA transferase in the enzyme that catalyses this linked reaction. A mole of pyruvate enters the Wood-Werkman cycle, producing a mole of propionate, a mole of NAD⁺, a mole of ATP and a mole of NAD⁺. In the Wood-Werkman cycle, propionic acid is created together with NAD⁺ regeneration for glycolysis. An important source of NAD⁺ produced in cell metabolism is the regeneration of glycolysis in the Wood-Werkman cycle. This linked reaction is catalysed by propionyl CoA: succinyl CoA transferase (Dalmasso:2012:10; Kim et al., 2023:5).

2.7.3. Commercial importance of PAB

Traditionally, PAB was used as a vitamin producer, a bio-preserver and widely used in the dairy industry as a starter culture for cheese ripening. *P. freudenreichii* is, however, currently emerging for its probiotic potential and is used commercially in probiotic preparations, available as tablets or capsules intended to improve intestinal transit and comfort (Rossi et al., 2025:8). The useful and beneficial probiotic properties, along with its industrial implication of PAB was granted European quality presumption of safety and the American GRAS (Generally Regarded as Safe) status (Coronas et al., 2023:2). PAB is widely used in the manufacturing of Swiss cheese in the dairy industry, propionic acid and Vitamin B₁₂.

Propionic acid (PA) is widely used in the synthesis of herbicide, cellulose plastics, fruit flavors, perfume industry, ester solvents and butyl rubber (Ahmadi et al., 2017:68). *P. freudenreichii*, can produce propionic acid during microbial fermentation. PA has commercially been produced by chemical synthesis from petroleum feedstock which includes oxidation of propanol or propenal and hydrolysis of esters using propane or propionaldehyde as raw material. However, it can also be produced by microbial or biotechnological methods by culturing microorganisms such as propionibacteria (Ranaei et al., 2020:116). Even though microbial biosynthesis of propionic acid is regarded as limited and time-consuming due to the fermentation proving to be fastidious, the end product inhibiting, and expensive downstream processing of recovery and concentration, the application of propionic acid is still considered an important natural preservative. Furthermore, the high price of oil and petrochemical products renders fermentative production attractive. By using inexpensive agro-industrial waste and renewable feedstock, microbial production of propionic acid can compete with the chemical processes (Ahmadi et al., 2017:69; Bezirci et al., 2023:2). The consumption of *P. freudenreichii* modulates the complex human intestinal microbiota by enhancing the bifidobacteria population at the expense of clostridia (Tarnaud et al., 2020:2). Therefore, PAB is rendered to be regarded as both prebiotics and probiotics. PAB also exhibits anti-inflammatory properties. Promising immunomodulatory properties was identified *in vitro* in animals and in humans (Le Marèchal, 2015:447; Rabah et al., 2017:2).

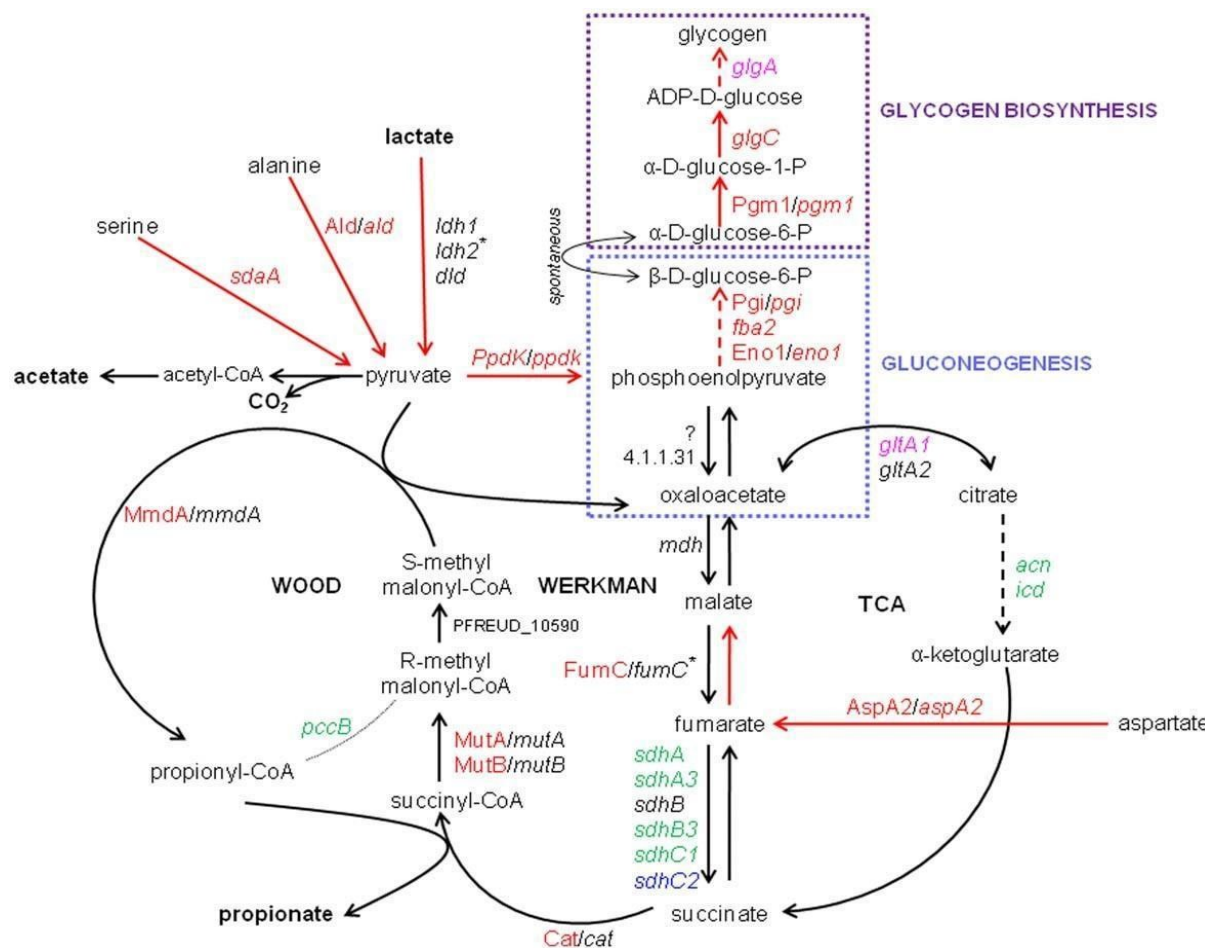


Figure 2.5 Wood-Werkman metabolic pathway (Dalmasso et al., 2012).

The consumption of *P. freudenreichii* modulates the complex human intestinal microbiota by enhancing the bifidobacteria population at the expense of clostridia (Tarnaud et al., 2020:2). Therefore, PAB is rendered to be regarded as both prebiotics and probiotics. PAB also exhibits anti-inflammatory properties. Promising immunomodulatory properties was identified *in vitro* in animals and in humans (Le Maréchal, 2015:447; Rabah et al., 2017:2). *P. freudenreichii* ssp. *shermanii*, is a complex probiotic that enhances the production of vitamin B₁₂ in kefir (Morkel, 2016:20). However, isolation and enumeration techniques for these bacteria are expensive and labour-intensive. Investigating methods for the accurate identification and enumeration of propionibacteria in kefir is therefore of interest (Nejati et al., 2020:2).

2.7.4. Potential application of PAB in kefir

Kefir is an ancient dairy-fermented product which dates far back to biblical times. It is reported that kefir was in use in the 2nd millennium BC, even though it was believed that it was first mentioned by Marco Polo in the 13th century (Yang et al., 2014:185). Kefir can be regarded as a functional food due to its high content of natural probiotics, microbial composition enriched with bioactive compounds, metabolites and peptides as well as being inexpensive (Apalowo et al., 2024:2). LAB are known to be dominant in dairy fermentation and have been investigated

intensively through the years (Ayivi et al., 2022:7008). The employment and survival of PAB in kefir under various storage conditions has not yet been sufficiently explored. Intricate research remains a priority to identify and establish mechanisms responsible for any health benefits which includes gut health (Sanders et al., 2018:213; McGovern et al., 2024:4274).

O'Brien et al. (2016) proved that the microbial load in frozen products prepared from traditional kefir would be high enough to be considered a beneficial supplement to the consumer. Moreover, distribution problems typically associated with packaging, storage, and transporting refrigerated products can be eliminated. Therefore, mass production and marketability of this nutritious food can be increased. PAB is known for naturally synthesising vitamin B₁₂. Enriching kefir with PAB during fermentation can facilitate natural fortification with vitamins to increase the nutritional value (Morkel, 2016:31). It was proven that PAB strains also possess probiotic qualities. It has been linked to a variety of possible health advantages, including the ability to increase immunity due to bioactive components produced during fermentation (Rocchetti et al., 2025:7). New knowledge was gained of how *P. freudenreichii* accumulated Carbon storage compounds to aid in its long-term survival. It is, therefore, important to investigate the maintenance of viable PAB populations during storage, particularly at low temperatures (Dalmaso et al., 2012:11).

2.7.5. Methods of PAB enumeration and detection

Over the years, it has been established that the ability to rapidly detect pathogens is of great importance for consumer safety and public health. Culture-based methods tend to be less expensive and can be very informative and may give insight into individual strains present. However, these methods do not always yield the total bacterial subset in the sample. Traditional culture-based detection techniques, which show microbial viability, are often labour-intensive, time-consuming, and slow to produce results (Adzitey, 2013:98; Foddai & Grant, 2020:4283).

In various studies, dairy PAB was cultured in a lactate-rich medium such as sodium lactate (SL) broth and Yeast extract lactate (YEL) agar and incubated under anaerobic conditions using an Anaerocult A system (Merck, Darmstadt, Germany) at 30 to 32°C for up to 10 days (Rossi, 1999:4241; Witthuhn et al., 2004:34; Savijoki et al., 2023:4). Phenotypic methods are still needed because molecular methods have not yet been successful in separating *P. freudenreichii* subspecies (de Freitas et al., 2015:467). The lack of phenotypic test standardisation may possibly have contributed to some inconsistencies between studies, especially where experimental conditions, including oxygen content, temperature, and incubation time, are factors (de Freitas et al., 2015:473). Pal propiobac (Standa-Industrie, Caen, France) was patented by Madec et al. (1994:316) for the selective enumeration of PAB species. It has been proven that viable cells can form colonies on Pal propiobac. Previous

studies used Pal propiobac plus to isolate PAB in cheese by exposing plates to 6 days incubation under anaerobic conditions at 30°C (Garofalo et al, 2024:6617; Busetta et al, 2026:2) Non-viable cells, viable but non-culturable cells, and cells that need special growth conditions cannot be detected as they will not grow on conventional culture media (Pazos-Rojas et al., 2023:1). Boyte et al. (2023:9) reported that in several studies enumeration results obtained via viability qPCR compares well is agreement with standard plate count method. However, polymerase chain reaction (PCR) and quantitative PCR can detect target DNA in a sample, this also includes the non-viable cells or DNA present in the environment leading to misrepresentation of the viable mass of cells (Foddai & Grant et al., 2019:4283; Jo et al., 2023:4). A novel intercalating agent has been employed in conjunction with PCR to distinguish between viable and non-viable cells. Propidium monoazide (PMA) is the foundation of this innovative approach (Husein et al., 2019:113). PMA is an intercalating dye which is highly selective in the penetration of viable or non-viable cells, being impermeable to viable cells. The DNA of dead cells are compromised, allowing the dye to enter the cell wall by diffusion. During photoactivation the dye irreversibly binds to the DNA causing the DNA to precipitate. The dye-bound DNA will not be able to amplify during the polymerase function of the PCR process. The DNA of live cells which were not affected by the azide group during photoactivation will amplify during the PCR process (Van Holm et al., 2021:1).

After treatment of the suspected PAB with PMA, the DNA was extracted using a Powerfood Microbial DNA isolation kit (Cat No 21000-50, Whitehead Scientific, South Africa). Figure 2.6 shows how PMA intercalates the DNA of non-viable cells, allowing only the DNA of viable cells to be amplified during the PCR. Molecular detection methods using conventional PCR or Real-Time PCR (RTP) can also be employed to evaluate the presence of certain organisms in food or environmental samples.

However, these methods rely heavily on the detection of species-specific nucleotide sequences, followed by amplification of the targeted nucleic acid. This is followed by visualisation on an agarose gel or in an amplification plot using computer software (Huq et al., 2012:3). It was proven that detection of foodborne pathogens can be achieved through a combination of the conventional and molecular-based techniques (Kabiraz et al., 2023:16). Most methods continue to involve various enrichment procedures, followed by plating onto selective agar or by plating directly onto selective agar without enrichment, and confirmation of presumptive bacteria colonies by biochemical tests (Adzitey et al., 2013:97).

The combination of PMA-PCR is a promising analytical tool to amplify DNA from live bacterial cells, however the limitations include variability of PMA intercalation and DNA extraction which can affect the results obtained. The effectiveness of PMA-PCR is compromised in complexed matrices which contain biofilms, type of sample, nature of certain cell types and viable, but not culturable cells (Jansriphibul et al., 2025:5).

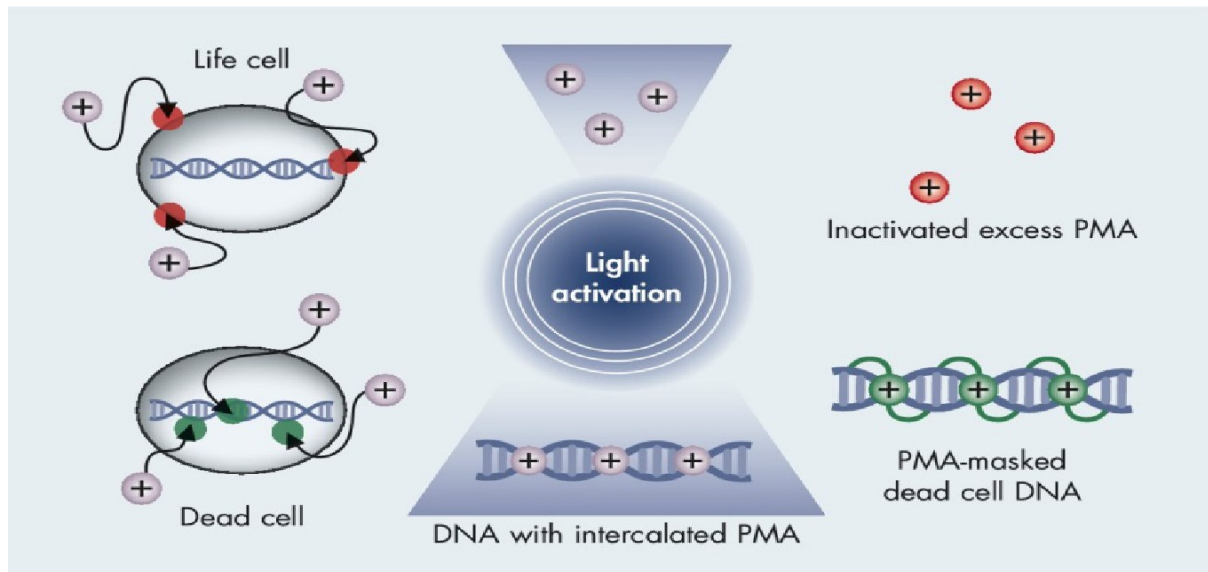


Figure 2.6 Schematic diagram on the intercalation of non-viable DNA with Propidium mono-azide (Qaigen, 2014:6, Figure 1).

References

- Abdel-Hamid, M., Huang, L., Huang, Z., Romeih, E., Yang, P., Zeng, Q., & Li, L. 2023. Effect of Buffalo Breed on the Detailed Milk Composition in Guangxi, China. *Foods*, 12(8): 1-10. <https://doi.org/10.3390/foods12081603>
- Acik, M., Çakiroğlu, F.P., Altan, M. & Baybo, T. 2020. Alternative source of probiotics for lactose intolerance and vegan individuals: sugary kefir. *Food Science and Technology*, 40: 523-531.
- Adolfsson, O., Meydani, S.N. & Russell, R.M. 2004. Yoghurt and gut function. *The American Journal of Clinical Nutrition*, 80(2): 245-256.
- Adzitey, F., Huda, N., & Ali, G.R.R. 2013. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. *Biotech*, 3(2): 97-107. <https://doi.org/10.1007/s13205-012-0074-4>
- Ahmadi, N., Khosravi-Darani, K. & Mortazavian, A.M. 2017. An overview of biotechnological production of propionic acid: from upstream to downstream processes. *Electronic Journal of Biotechnology*, 28: 67-75.
- Alraddadi, F.A.J., Ross, T. & Powell, S. M. 2023. Evaluation of the microbial communities in kefir grains and kefir over time. *International Dairy Journal*, 136:1-9. <https://doi.org/10.1016/j.idairyj.2022.105490>

- Al-Shawi, S.G., Dang, D.S., Yousif, A.Y., Al-Younis, Z.K., Najm, T.A. & Matarneh, S.K. 2020. The potential use of probiotics to improve animal health, efficiency and meat quality: A review. *Agriculture (Switzerland)*, 10(10): 1-14.
- Altay, F., Karbancioglu-Güler, F., Daskaya-Dikmen, C. & Heperkan, D. 2013. A review on traditional Turkish fermented non-alcoholic beverages: Microbiota, fermentation process and quality characteristics. *International Journal of Food Microbiology*, 167(1): 44-56.
- Amin, I., Can, E., Khan, T., Fatima, J. & Khalid, M. 2025. Synergistic effects of combined probiotic supplementation on growth performance, survival, and health status in *Labeo rohita*. *Aquaculture International*, 33(340): 1-11.
- Apalowo, O.E., Adegoye, G.A., Mbogori, T., Kandiah, J. & Obuotor, T.M. 2024. Nutritional Characteristics, Health Impact, and Applications of Kefir. *Foods*, 13(7): 1-33. Multidisciplinary Digital Publishing Institute (MDPI). <https://doi.org/10.3390/foods13071026>
- Arslan-Tontul, S., Mutlu, C., Candal, C., & Erbaş, M. 2018. Microbiological and chemical properties of wet tarhana produced by different dairy products. *Journal of Food Science and Technology*, 55(12): 4770-4781. <https://doi.org/10.1007/s13197-018-3410-9>
- Azizi, N.F., Kumar, M.R., Yeap, S.K., Abdullah, J.O., Khalid, M., Omar, A.R., Osman, M.A., Mortadza, S.A.S. & Alitheen, N.B. 2021. Kefir and its biological activities. *Foods*, 10: 1-26. MDPI AG.
- Babot, J.D., Argañaraz-Martínez, E., Quiroga, M., Grande, S.M., Apella, M.C. & Perez Chaia, A. 2021. Protection of the intestinal epithelium of poultry against deleterious effects of dietary lectins by a multi-strain bacterial supplement. *Research in Veterinary Science*, 135: 27-35.
- Bezirci, E., Taşpınar-Demir, H., Turanlı-Yıldız, B., Erdem, A., Alemdar, F. & Türker, M. 2023. Propionic acid production via two-step sequential repeated batch fermentations on whey and flour. *Biochemical Engineering Journal*, 192: 1-9.
- Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M.E., Tremblay, A. & Ouwehand, A.C. 2020. Criteria to Qualify Microorganisms as “Probiotic” in Foods and Dietary Supplements. *Frontiers in Microbiology*, 11: 1-9.
- Bintsis, T. & Papademas, P. 2022. The Evolution of Fermented Milks, from Artisanal to Industrial Products: A Critical Review. *Fermentation* 8(12): 1-21. Multidisciplinary Digital Publishing Institute (MDPI). <https://doi.org/10.3390/fermentation8120679>

- Blandino, A., Al-Alseeri, M.E., Pandiella, S.S., Cantero, D. & Webb, C. 2003. Cereal-based fermented foods and beverages. *Food Research International*, 36(6): 527-543.
- Bohrer, B. M. 2017. Review: Nutrient density and nutritional value of meat products and non-meat foods high in protein. *Trends in Food Science and Technology*, 65: 103-112. <https://doi.org/10.1016/j.tifs.2017.04.016>
- Boyte, M. E., Benkowski, A., Pane, M. & Shehata, H.R. 2023. Probiotic and postbiotic analytical methods: a perspective of available enumeration techniques. *In Frontiers in Microbiology*, 14: 1-15. Frontiers Media SA. <https://doi.org/10.3389/fmicb.2023.1304621>
- Britos, S., González, A.F., Flax Marcó, F., Katz, M., Schuldberg, J., Torresani, M.E. & Vinderola, G. 2024. Yogurt, in the context of a healthy diet, for the prevention and management of diabetes and obesity: a perspective from Argentina. *Frontiers in Nutrition*, 11: 1-6.
- Busetta, G., Garofalo, G., Taspinar, T., Guarcello, R., Franciosi, E., Sardina, M.T., Moschetti, G., Settanni, L., Erten, H. & Gaglio, R. 2026. Enhancing “Ewiss” cheese production using autochthonous lactic acid bacteria and *Propionisclava flava* as an alternative to commercial propionic acid bacteria. *International Dairy Journal*, 173: 1-11. <https://linkinghub.elsevier.com/retrieve/pii/S0958694625002924>
- Caplice, E. & Fitzgerald, G.F. 1999. Food fermentations: role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, 50: 131-149.
- Chamlagain, B., Peltonen, L., Edelmann, M., Ramos-Diaz, J.M., Kemppinen, A., Jouppila, K., Varmanen, P. & Piironen, V. 2021. Bioaccessibility of vitamin B12 synthesized by *Propionibacterium freudenreichii* and from products made with fermented wheat bran extract. *Current Research in Food Science*, 4: 499-502.
- Chamlagain, B., Sugito, T. A., Deptula, P., Edelmann, M., Kariluoto, S., Varmanen, P. & Piironen, V. 2018. In situ production of active vitamin B12 in cereal matrices using *Propionibacterium freudenreichii*. *Food Science and Nutrition*, 6(1): 67-76. <https://doi.org/10.1002/fsn3.528>
- Chandrasekaran, P., Weiskirchen, S., & Weiskirchen, R. 2024. Effects of Probiotics on Gut Microbiota: An Overview. *In International Journal of Molecular Sciences*, 25(11): 1-20. Multidisciplinary Digital Publishing Institute (MDPI). <https://doi.org/10.3390/ijms25116022>

- Chavan, J.K., Kadam, S.S. & Beuchat, L.R. 1989. Nutritional improvement of cereals by sprouting. *Critical reviews in food science & nutrition*, 28(5): 401-437.
- Chiron, C., Tompkins, T.A. & Burguière, P. 2018. Flow cytometry: a versatile technology for specific quantification and viability assessment of micro-organisms in multistrain probiotic products. *Journal of applied microbiology*, 124(2): 572-584.
- Coronas, R., Zara, G., Gallo, A., Rocchetti, G., Lapris, M., Petretto, G.L., Zara, S., Fancello, F. & Mannazzu, I. 2023. *Propionibacteria* as promising tools for the production of probiotic products: a proof-of-concept study. *Frontiers in Microbiology*, 14: 1-17.
- Corvec, S., Dagnelie, M.A., Khammari, A. & Dréno, B. 2019. Taxonomy and phylogeny of *Cutibacterium* (formerly *Propionibacterium*) *acnes* in inflammatory skin diseases. *Annales de Dermatologie et de Venereologie*, 146(1): 26-30.
- Cousin, F.J., Louesdon, S., Maillard, M.B., Parayre, S., Falentin, H., Deutsch, S.M., Boudry, G. & Jan, G. 2012. The first dairy product exclusively fermented by *Propionibacterium freudenreichii*: a new vector to study probiotic potentialities in vivo. *Food microbiology*, 32(1): 135-146.
- Dahiya, D. & Nigam, P. S. 2023. Therapeutic and dietary support for gastrointestinal tract using kefir as a nutraceutical beverage: Dairy-milk-based or plant-sourced kefir probiotic products for vegan and lactose-intolerant populations. *Fermentation*, 9(4): 1-13. MDPI. <https://doi.org/10.3390/fermentation9040388>
- Dalmasso, M., Aubert, J., Briard-Bion, V., Chuat, V., Deutsch, S.M., Even, S., Falentin, H., Jan, G., Jardin, J., Maillard, M.B., Parayre, S., Piot, M., Tanskanen, J. & Thierry, A. 2012. Temporal-omic study of *Propionibacterium freudenreichii* CIRM-BIA1. adaptation strategies in conditions mimicking cheese ripening in the cold. *Public Library of Science*, 7(4): 1-15.
- Dertli, E. & Con, A.H. 2017. Microbial diversity of traditional kefir grains and their role on kefir aroma. *LWT- Food Science and Technology*, 85, 151-157.
- Dimitrellou, D., Salamoura, C., Kontogianni, A., Katsipi, D., Kandylis, P., Zakynthinos, G. & Varzakas, T. 2019. Effect of milk type on the microbiological, physicochemical and sensory characteristics of probiotic fermented milk. *Microorganisms*, 7(9): 1-8. <https://doi.org/10.3390/microorganisms7090274>
- Ding, F., Stoyanova, L. G. & Netrusov, A. I. 2022. Microbiome and metabiotic properties of kefir grains and kefirs based on them. *Microbiology (Russian Federation)* 91(4): 339-355. Pleiades journals. <https://doi.org/10.1134/S0026261722100885>

- Elhalik, M. A., Mekky, A. E., Khedr, M. & Suleiman, W. B. 2024. Antineoplastic with DNA fragmentation assay and anti-oxidant, anti-inflammatory with gene expression activity of *Lactobacillus plantarum* isolated from local Egyptian milk products. *BMC Microbiology*, 24(1): 1-17. <https://doi.org/10.1186/s12866-024-03576-y>
- Ellatif, S.A., Abdel Razik, E.S., Abu-Serie, M.M., Mahfouz, A., Shater, A.F., Saleh, F.M., Hassan, M.M., Alsanie, W.F., Altalhi, A., Daigham, G.E. & Mahfouz, A.Y. 2022. Immunomodulatory efficacy-mediated anti-HCV and anti-HBV potential of kefir grains; unveiling the in vitro antibacterial, antifungal, and wound healing activities. *Molecules*, 27(6): 1-21.
- El-Sohaimy, S.A., & Hussain, M.A. 2023. Functional probiotic foods development: Trends, concepts, and products. *Fermentation* 9(3): 1-17. <https://doi.org/10.3390/fermentation9030249>
- Erem, E. & Kilic-Akyilmaz, M. 2024. The role of fermentation with lactic acid bacteria in quality and health effects of plant-based dairy analogues. *Comprehensive Reviews in Food Science and Food Safety*, 23(4): 1-3.
- Facioni, M.S., Raspiri, B., Pivari, F., Dogliotti, E. & Cena, H. 2020. Nutritional anagement of lactose intolerance: the importance of diet and food labelling. *Journal of Translational Medicine*, 18, (260): 1-9.
- Farnworth, E.R. 2005. Kefir – A Complex Probiotic. *Food Science and Technology Bulletin: Functional Foods* (edited by G.R. Gibson & R.G. Gibson), 1(2): 1-17. United Kingdom: IFIS Publishing.
- Fenech, M. 2012. Folate (vitamin B9) and vitamin B12 and their function in the maintenance of nuclear and mitochondrial genome integrity. *Mutation Research*, 733: 21-33
- Fernández, L., Orgaz, B. & Rodríguez, J. M. 2024. The safety of probiotics intended for use in pregnant and lactating women: From a desirable to a required task. *Foods*, 13(24): 1-17. Multidisciplinary Digital Publishing Institute (MDPI). <https://doi.org/10.3390/foods1324402>
- Foddai, A.C.G., & Grant, I.R. 2020. Methods for detection of viable foodborne pathogens: current state-of-art and future prospects. *Applied Microbiology and Biotechnology*, 104: 4281-4288.
- Gahruie, H.H., Eskandari, M.H., Mesbahi, G. & Hanifpour, M.A. 2015. Scientific and technical aspects of yogurt fortification. *Food Science and Human Wellness*, 4: 1-8.

- Ganatsios, V., Nigam, P., Plessas, S., & Terpou, A. 2021. Kefir as a functional beverage gaining momentum towards its health promoting attributes. *Beverages*, 7(3): 1-15. <https://doi.org/10.3390/beverages7030048>
- Gao, X., & Li, B. 2016. Chemical and microbiological characteristics of kefir grains and their fermented dairy products: A review. *Cogent Food and Agriculture*, 2(1): 1-10. Informa Healthcare. <https://doi.org/10.1080/23311932.2016.1272152>
- Garbers, I.M., Britz, T.J. & Witthuhn, R.C. 2004. PCR based denaturing gradient gel electrophoretic typification and identification of the microbial consortium present in kefir grains. *World Journal of Microbiology and Biotechnology*, 20: 687-693.
- Garg, V., Velumani, D., Lin, Y.C. & Haye, A. 2024. A comprehensive review of probiotic claims regulations: Updates from Asia-Pacific, United States, and Europe. *Pharma Nutrition*, 30: 1-13. Elsevier B.V. <https://doi.org/10.1016/j.phanu.2024.100423>
- Garofalo, G., Taspinar, T., Busetta, G., Mastrangelo, S., Portolano, B., Sardina, M.T., Gaglio, R., Erten, H. & Settanni, L. 2024. Description of Ewiss cheese, a new ewe milk cheese processed by Swiss cheese manufacturing techniques: Microbiological, physicochemical, and sensory aspects. *Journal of Dairy Science*, 107(9): 6614-6628.
- Ge, L., Wang J., Qi, W., Cao J., Qu, Y, Li, B. & Song, B. 2008. The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1. *Cell Metabolism*, 7: 508-519
- González-Orozco, B.D., García-Cano, I., Jiménez-Flores, R., & Álvarez, V.B. 2022. Invited review: Milk kefir microbiota—Direct and indirect antimicrobial effects. *Journal of Dairy Science*, 105(5): 3703–3715. <https://doi.org/10.3168/jds.2021-21382>
- Goršek, A., & Tramšek, M. 2008. Kefir grains production-An approach for volume optimization of two-stage bioreactor system. *Biochemical Engineering Journal*, 42(2): 153-158. <https://doi.org/10.1016/j.bej.2008.06.009>
- Górska-Warsewicz, H., Rejman, K., Laskowski, W. & Czeczotko, M. 2019. Milk and dairy products and their nutritional contribution to the average polish diet. *Nutrients*, 11(8): 1-19.
- Gul, O., Mortas, M., Atalar, I., Dervisoglu, M. & Kahyaoglu, T. 2015. Manufacture and characterization of kefir made from cow and buffalo milk, using kefir grain and starter culture. *Journal of dairy science*, 98(3): 1517-1525.

- Guzel-Seydim, Z., Kok-Tas, T.. & Greene, A.K. 2010. Kefir and Koumiss: Microbiology and technology. Development and manufacture of yogurt and functional dairy products. Boca Raton: Taylor and Francis Group, 143-154.
- Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S. & Calder, P.C. 2014. Expert consensus document: The International Scientific Association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature reviews Gastroenterology & hepatology*, 11: 506-514.
- Hlastan-Ribič, C., Cerar, A., Pokorn, D., Perše, M. & Zebič, A. 2005. Effects of kefir containing various levels of fat on chemically induced colorectal epithelial tumours in Wistar rats. *Nutrition Research*, 25: 55-63.
- Holm, W. Van, Ghesquière, J., Boon, N., Verspecht, T., Bernaerts, K., Zayed, N., Chatzigiannidou, I. & Teughels, W. 2021. A Viability Quantitative PCR Dilemma: Are Longer Amplicons Better? *Applied and Environmental Microbiology*, 87(5): 1-11. <https://doi.org/10.1128/AEM>.
- Huang, Y., Wu, F., Wang, X., Sui, Y., Yang, L. & Wang, J. 2013. Characterization of *Lactobacillus plantarum* Lp27 isolated from Tibetan kefir grains: A potential probiotic bacterium with cholesterol-lowering effects. *Journal of Dairy Science*, 96(5): 2816-2825.
- Huq, A., Haley, B.J., Taviani, E., Chen, A., Hasan, N.A. & Colwell, R.R. 2012. Detection, isolation, and identification of *Vibrio cholerae* from the environment. *Current Protocols in Microbiology*, 1(SUPPL.26): 1-58.
- Hussein, A.M., Salem, R.M., El-Diasty, EM. & Marwa, A.H., 2019. Real time PCR for identification of Viable But Non Culturable fungi isolated from mastitic cattle milk samples depending on propidium mono azide stain. *Journal of Current Veterinary Research*, 2: 112-118. <http://www.jcvtjournals.ekb.eg>
- Ibrahim, S.A., Gyawali, R., Awaisheh, S.S., Ayyivi, R.D., Silva, R.C., Subedi, K., Aljaloud, S.O., Siddiqui, S.A. & Krastanov, A. 2021. Fermented foods and probiotics: An approach to lactose intolerance. *Journal of Dairy Research*, 88, 357-365.
- Jang, H.J., Lee, N.K., & Paik, H.D. 2024. Overview of dairy-based products with probiotics: Fermented or non-fermented milk drink. *Food Science of Animal Resources*, 44(2): 255–268). <https://doi.org/10.5851/KOSFA.2023.E83>

- Jo, T.S. 2023. Utilizing the state of environmental DNA (eDNA) to incorporate time-scale information into eDNA analysis. *Proceedings of the Royal Society. Biological Sciences*, 209(1999): 1-11.
- Kabiraz, M.P., Majumdar, P.R., Mahmud, M.M.C., Bhowmik, S. & Ali, A. 2023. Conventional and advanced detection techniques of foodborne pathogens: A comprehensive review. *Heliyon*, 9(4): 1-24.
- Kalfirtova, P. & Sovajak, R. 2005. Improve the nutritional value of fermented dairy products by nutraceutical-producing food-grade microorganisms. *Agricultura Tropica et Subtropica*, 38: 22-24.
- Kim, S.K., Lee, M., Lee, Y.Q., Lee, H.J., Rho, M., Kim, Y., Seo, J.Y., Youn, S.H., Hwang, S.J., Kang, N.G., Lee, C.H., Park, S.Y. & Lee, D.Y. 2023. Genome-scale metabolic modeling and in silico analysis of opportunistic skin pathogen *Cutibacterium acnes*. *Frontiers in Cellular and Infection Microbiology*, 13:1 -13.
- Khaksefidi, A. & Rahim, S. 2005. Effect of probiotic inclusion in the diet of broiler chickens on performance feed efficiency and carcass quality. *Asian-Australian Journal of Animal Sciences*, 18(8): 1153-1156.
- Kivanc, M. & Yapici, E., 2018. Survival of *Escherichia coli* O157: H7 and *Staphylococcus aureus* during the fermentation and storage of kefir. *Food Science and Technology*, 39(1): 225-230.
- Kim, D.H., Jeong, D., Kim, H., Kang, I.B., Chon, J.W., Song, K.Y. & Seo, K.H. 2016. Antimicrobial activity of kefir against various food pathogens and spoilage bacteria. *Korean Journal for Food Science of Animal Resources*, 36(6): 787-7.
- Kim, S.K., Lee, M., Lee, Y.Q., Lee, H.J., Rho, M., Kim, Y., Seo, J.Y., Youn, S.H., Hwang, S.J., Kang, N.G., Lee, C.H., Park, S.Y. & Lee, D.Y. 2023. Genome-scale metabolic modeling and in silico analysis of opportunistic skin pathogen *Cutibacterium acnes*. *Frontiers in Cellular and Infection Microbiology*, 13: 1-13.
- Koirala, S. & Kumar, A. 2021. Probiotics-based foods and beverages as future foods and their overall safety and regulatory claims. *Future Foods*, 3: 1-12.
- Koskinen, P., Deptula, P., Smolander, O. P., Tamene, F., Kammonen, J., Savijoki, K., Paulin, L., Piironen, V., Auvinen, P. & Varmanen, P. 2015. Complete genome sequence of *Propionibacterium freudenreichii* DSM 20271T. *Standards in Genomic Sciences*, 10(83): 1-6. <https://doi.org/10.1186/s40793-015-0082-1>

- Kozyraki, R. & Cases, O. 2013. Biochimie Vitamin B12 absorption: Mammalian physiology and acquired and inherited disorders. *Biochimie*, 95(5):1002-1007.
<https://doi.org/10.1016/j.biochi.2012.11.004>
- Jansriphibul, K., Krohn, C. & Ball, A.S. 2025. Sources of variability for viability PCR using propidium monoazide. *Microbiological Research*, 298: 1-14.
- La Torre, C., Caputo, P., Cione, E. & Fazio, A. 2024. Comparing nutritional values and bioactivity of kefir from different types of animal milk. *Molecules*, 29: 1-22.
- Latif, A., Shehzad, A., Niazi, S., Zahid, A., Ashraf, W., Iqbal, M. W., Rehman, A., Riaz, T., Aadil, R. M., Khan, I. M., Özogul, F., Rocha, J. M., Esatbeyoglu, T. & Korma, S. A. 2023. Probiotics: mechanism of action, health benefits and their application in food industries. *Frontiers in Microbiology*, 14: 1-5.
Frontiers Media SA. <https://doi.org/10.3389/fmicb.2023.1216674>
- Le Maréchal, C., Peton, V., Plé, C., Vroland, C., Jardin, J., Briard-Bion, V., Durant, G., Chuat, V., Loux, V., Foligne, B., Deutch,., Falentin, H. & Jan, G. 2015. Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *Journal of Proteomics*, 112: 447-461.
- Leeuwendaal, N.K., Stanton, C., O'Toole, P.W. & Beresford, T.P. 2022. Fermented food, health and gut microbiome. *Nutrients*, 14(1527): 1-26.
- Leite, A. M., Miguel, .A.L.M, Peixoto, R.S., Ruas- Madiedo, P., Paschoalin, V.M.F. & Mayo, B., Delgado, S. 2015. Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains *Journal of Dairy Science*, 98: 3622-3632.
- Leite, A. M., Miguel, A.L.M., Peixoto, R.S., Rosado, A.S., Silva, J.T. & Pashoalin, V.M.F. 2013. Microbiological, technological and therapeutic properties of kefir: a natural probiotic beverage. *Brazilian Journal of Microbiology*, 44, (2): 341-349.
- Lu, M., Wang, X., Sun, G., Qin, B., Xiao, J., Yan, S., Pan, Y. & Wang, Y. 2014. Fine structure of Tibetan kefir grains and their yeasts distribution, diversity and shift. *PlosOne*, 9,(6): 1-10.
- Majuwana, K., Kariyawasam, G.M.M., Lee, N. & Paik, H. 2021. Fermented dairy products as delivery vehicles of novel probiotic strains isolated from traditional fermented Asian foods. *Journal of Food Science and Technology*, 58(7): 2467-2478.
- Mani-López, E., Palou, E. & López-Malo, A. 2014. Probiotic viability and storage stability of yogurts and fermented milks prepared with several mixtures of lactic acid bacteria. *Journal of Dairy Science*, 97(5): 2578-2590.

- Marco, M.L., Heeney, D., Binda, S., Cifelli, C.J., Cotter, P.D., Foligné, B., Gänzle, M., Kort, R., Pasin, G., Pihlanto, A. & Smid, E.J. 2017. Health benefits of fermented foods: microbiota and beyond. *Current Opinion in Biotechnology*, 44: 94-102.
- Marco, M.L., Sanders, M.E., Gänzel, M., Arrieta, M.C., Cotter, P.D., De Vuyst, L., Hill, C., Holzapfel, W., Sarah, L. & Merenstein, D. 2021. The international Scientific Association for Probiotics and Prebiotics (ISAAP) consensus statement on fermented foods. *Nature Reviews Gastroenterology and Hepatology*, 18(3): 196-208.
- Mayslich, C., Grange, P.A., Dupin, N. & Brüggemann, H. 2021. microorganisms *Cutibacterium acnes* as an Opportunistic Pathogen: An Update of Its Virulence-Associated Factors. *Microorganisms*, 9(303): 1-21. <https://doi.org/10.3390/microorganisms9020303>.
- McCaddon, A. 2013. Vitamin B12 in neurology and ageing; Clinical and genetic aspects. *Biochimie*, 95: 1-11.
- McGovern, C.J., González-Orozco, B.D. & Jiménez-Flores, R. 2024. Evaluation of kefir grain microbiota, grain viability, and bioactivity from fermenting dairy processing by-products. *Journal of Dairy Science*, 107(7): 4259-4276.
- Mei, J., Gao, X., Li, Y. 2016. Kefir grains and their fermented dairy products. *JSM Biotechnology and Biomedical Engineering*, 3(1): 1-7.
- Merenstein, D., Pot, B., Leyer, G., Ouwehand, A. C., Preidis, G. A., Elkins, C. A., Hill, C., Lewis, Z. T., Shane, A. L., Zmora, N., Petrova, M. I., Collado, M. C., Morelli, L., Montoya, G. A., Szajewska, H., Tancredi, D. J. & Sanders, M. E. 2023. Emerging issues in probiotic safety: 2023 perspectives. *Gut Microbes*, 15(1): 1-22. Taylor and Francis Ltd. <https://doi.org/10.1080/19490976.2023.2185034>
- Mikyška, A., Matoulková, D., Slabý, M., Kubizniaková, P. & Hartman, I. 2015. Characterization of the strains isolated from kefir grains and their use for the production of beer-based fermented beverages from nontraditional cereals. *Kvasny Prums*, 61: 311-319.
- Mkambula, P., Mbuya, M.N., Rowe, L.A., Sablah, M., Friesen, V.M., Chadha, M., Osei, A.K., Ringholz, C., Vasta, F.C. & Gorstein, J. 2020. The unfinished agenda for food fortification in low-and middle-income countries: quantifying progress, gaps and potential opportunities. *Nutrients*, 12(2): 1-21.
- Mokoena, M.P., Mutanda, T. & Olaniran, A.O. 2016. Perspectives on the probiotic potential of lactic acid bacteria from African traditional fermented foods and beverages. *Food & Nutrition Research*, 60: 1-12.

- Morkel, R.A. 2016. Vitamin B12 and folate enrichment of kefir by *Propionibacterium freudenreichii* and *Streptococcus thermophilus* strains. Unpublished Masters' dissertation, Cape Peninsula University of Technology, Bellville.
- Mozaffarian, D., Appel, L.J. and Van Horn, L. 2011. Components of a cardioprotective diet: new insights. *Circulation*, 123(24):2870-2891.
- Nalbantoglu, U., Cakar, A., Dogan, H., Abaci, N. & Ustek, D. 2014. Metagenomic analysis of the microbial community in kefir grains. *Food Microbiology*, 41: 42-51.
- Nejati, F., Junne, S., Kurreck, J. & Peter Neubauer, P. 2020. Quantification of major bacteria and yeast species in kefir consortia by multiplex TaqMan qPCR. *Frontiers in Microbiology*, 11: 1-13.
- Nithya, A., Misra, S., Panigrahi, C., Dalbhat, C.G. & Mishra, H.N. 2023. Probiotic potential of fermented foods and their role in non-communicable diseases management: An understanding through recent clinical evidences. *Food Chemistry Advances*, 3: 1-11.
- O'Brien, K.V., Aryana, K.J., Prinyawiwatkul, W., Ordonez, K.C. & Boeneke, C.A. 2016. The effects of frozen storage on the survival of probiotic microorganisms found in traditionally and commercially manufactured kefir. *Journal of Dairy Science*, 99(9): 7043-7048.
- Obafemi, Y. D., Oranusi, S. U., Ajanaku, K. O., Akinduti, P. A., Leech, J., & Cotter, P. D. 2022. African fermented foods: overview, emerging benefits, and novel approaches to microbiome profiling. *Science of Food*, 6(1): 1-9.
<https://doi.org/10.1038/s41538-022-00130-w>
- Ofoedu, C.E., Iwouno, J.O., Ofoedu, E.O., Ogueke, C.C., Igwe, V.C., Agunwah, I.M., Ofoedum, A.F., Chacha, 1,3, Onyinye, J.S., Muobike, P., Agunbiade, A.O., Njoku, N.E., Nwakaudu, Odimegwu, N.E., Ndukauba, O.E., Ogbonna, C.U., Naibaho, J., Korus, M., Odilichukwu, C. & Okpala, C.O.R. 2021. Revisiting food-sourced vitamins for consumer diet and health needs: a perspective review, from vitamin classification, metabolic functions, absorption, utilization, to balancing nutritional requirements. *PeerJ*: 1-45.
[9:e11940 DOI 10.7717/peerj.11940](https://doi.org/10.7717/peerj.11940)
- Olson, R., Gavin-Smith, B., Ferraboschi, C. & Kraemer, K. 2021. Food Fortification: The Advantages, Disadvantages and Lessons from Sight and Life Programs. *Nutrients*, 13(1118): 1-12.
- Ozer, B. & Kirmaci, H.A. 2010. Functional milks and dairy beverages. *International journal of Dairy Technology*, 63(1): 1-16.

- Park, S. & Bae, J.H. 2016. Fermented food intake is associated with a reduced likelihood of atopic dermatitis in an adult population (Korean National Health and Nutrition Examination Survey 2012-2013). *Nutrition Research*, 36(2): 125-133.
- Pazos-Rojas, L.A., Cuellar-Sánchez, A., Romero-Cerón, A.L., Rivera-Urbalejo, A., Van Dillewijn, P., Luna-Vital, D.A., Muñoz-Rojas, J., Morales-García, Y.E., & Bustillos-Cristales, M.R. 2023. *The Viable but Non-Culturable (VBNC) State, a Poorly-Explored Aspect of Beneficial Bacteria*: 1-25. <https://doi.org/10.20944/preprints202311.1369.v1>
- Peruzzolo, M., Ceni, G.C., Junges, A., Zeni, J., Cansian, R.L. & Backes, G.T. 2025. Probiotics: Health benefits, microencapsulation, and viability, combination with natural compounds, and applications in foods. *Food Bioscience*, 66: 1-17. Elsevier Ltd. <https://doi.org/10.1016/j.fbio.2025.106253>
- Piwowarek, K., Lipińska, E., Hać-Szymańczuk, E., Kieliszek, M. & Ścibisz, I. 2018. *Propionibacterium spp.*—source of propionic acid, vitamin B12, and other metabolites important for the industry. *Applied microbiology and biotechnology*, 102: 515-538.
- Platsidaki, E., & Dessinioti, C. 2018. Recent advances in understanding *Propionibacterium acnes* (*Cutibacterium acnes*) in acne. *F1000Research*, 7: 1-12. F1000 Research Ltd. <https://doi.org/10.12688/f1000research.15659.1>
- Prado, F.C., Parada, J.L., Pandey, A., Soccol, C.R. 2008. Trends in non-dairy probiotic beverages. *Food Research International*, 41: 111-123.
- Priyashantha, H., & Lundh, Å. 2021. Graduate Student Literature Review: Current understanding of the influence of on-farm factors on bovine raw milk and its suitability for cheesemaking. In *Journal of Dairy Science*, 104(11): 12173-12183. <https://doi.org/10.3168/jds.2021-20146>
- Puebla-Barragan, S., & Reid, G. 2021. Probiotics in cosmetic and personal care products: Trends and challenges. *Molecules*, 26(5): 1-12. <https://doi.org/10.3390/molecules26051249>
- Qiagen, 2014. *Blu-V Viability PMA Kit Handbook RevFeb2014*. Original Figure1:PMA intercalationschematic. QIAGEN. Available at: <https://www.qiagen.com/us/resources/resourcedetail?id=fa8ff67a-a8a5-43eb-b82c-5963ab23ee57> [Accessed 28 November 2025].
- Rabah, H., Do Carmo, F. L. R. & Jan, G. 2017. Dairy propionibacteria: Versatile probiotics. In *Microorganisms*, 5(2):1-17. MDPI AG. <https://doi.org/10.3390/microorganisms5020024>

- Ranasinghe, J.G.S. & Perera, W.T.R. 2016. Prevalence of *Lacticacid Bulgaricus* and *Streptococcus thermophilus* stability in commercially available yoghurts in Sri Lanka. *Asian Journal of Amiran Sciences*, 7,(5): 97-101.
- Ranaei, V., Pilevar, Z., Khaneghah, A.M. & Hosseini, H. 2020. Propionic acid: Method of production, current state and perspectives. *Food Technology and Biotechnology*, 58(2): 115-127.
- Rashid, M., Aboshady, H.M., Agamy, R. & Archimede, H. 2024. Milk production and composition in warm-climate regions: a systematic review and meta-analysis. *Tropical Animal Health and Production*, 56(8):1-21. Springer Science and Business Media B.V. <https://doi.org/10.1007/s11250-024-04214-5>
- Robinson, R.C. 2019. Structures and metabolic properties of bovine milk oligosaccharides and their potential in the development of novel therapeutics. *Frontiers in Nutrition*, 6: 1-8. Frontiers Media S.A. <https://doi.org/10.3389/fnut.2019.00050>
- Robles, L. & Priefer, R. 2020. Lactose intolerance: What your breath can tell you. *Diagnostics*, 10(412): 1-11.
- Rodrigues, K.L., Araújo, T.H., Schneedorf, J.M., de Souza Ferreira, C., Moraes, G.D.O.I., Coimbra, R.S. & Rodrigues, M.R. 2016. A novel beer fermented by kefir enhances anti-inflammatory and anti-ulcerogenic activities found isolated in its constituents. *Journal of Functional Foods*, 21: 58-69.
- Rodrigues, K.L., Caputo, L.R.G., Jose Carlos Tavares Carvalho, J.C.T., Evangelista, J. & Schneedorf J.M. 2005. Antimicrobial & healing activity of kefir and kefir extract. *International Journal of Antimicrobial Reagents*, 25: 404-408.
- Romero-Velarde, E., Delgado-Franco, D., García-Gutiérrez, M., Gurrola-Díaz, C., Larrosa-Haro, A., Montijo-Barrios, E., Frits A. J. Muskiet, F.A.J., Vargas-Guerrero, B. & Geurts, J. 2019. The Importance of lactose in the human diet: outcomes of a Mexican consensus meeting. *Nutrients*, 8: 1-8.
- Ross, R.P., Morgan, S. & Hill, C. 2003. Preservation and fermentation: past, present and future. *International Journal of Food Microbiology*, 79: 3-16.
- Rossi, F., Santonicola, S., Giaccone, V., Truant, A. & Colavita, G. 2025. Dairy Propionibacteria: Probiotic Properties and Their Molecular Bases. *Biomolecules*, 15(886): 1-35.
- Rossi, F., Torriani, S. & Dellaglio, F. 1999. Genus- and species-specific PCR-based detection of dairy Propionibacteria in environmental samples by using primers targeted to the

- genus encoding 16S rRNA. *Applied and Environmental Microbiology*, 65(9): 4241-4244.
- Sabikhi, L. 2007. Designer milk. *Advances in food and nutrition research*, 53: 161-198.
- Sabir, F., Beyatli, Y., Cokmus, C. & Onal-Darilmaz, D. 2010. Assessment of Potential Probiotic properties of *Lactobacillus spp.*, *Lactococcus spp.* and *Pediococcus spp.* Strains Isolated from Kefir. *Journal of Food Science*, 75(9): 568-573.
<https://doi.org/10.1111/j.1750-3841.2010.01855.x>
- Sadovnikova, I.S., Gureev, A.P., Ignatyeva, D.A., Gryaznova, M.V., Chernyshova, E.V., Krutskikh, E.P., Novikova, A.G. & Popov, V.N. 2021. Nrf2/ARE activators improve memory in aged mice via maintaining of mitochondrial quality control of brain and the modulation of gut microbiome. *Pharmaceuticals*, 14(7): 1-27.
- Sanders, M.E., Akkermans, L.M., Haller, D., Hammerman, C., Heimbach, J.T., Hörmannspurger, G. & Huys, G. 2010. Safety assessment of probiotics for human use. *Gut microbes*, 1(3): 164-185.
- Sanders, M.E., Benson, A., Lebeer, S., Merenstein, D.J. & Klaenhammer, T.R. 2018. Shared mechanisms among probiotic taxa: implications for general probiotic claims. *Current opinion in biotechnology*, 49: 207-216.
- Sanders, M.E., Merenstein, D.J., Ouwehand, A.C., Reid, G., Salminen, S., Cabana, M.D., Paraskevagos, G. & Leyer, G. 2016. Probiotic use in at-risk populations. *Journal of the American Pharmacists Association*, 56(6): 680-686.
- Şanlıer, N., Gökçen, B.B. & Sezgin, A.C. 2017. Health benefits of fermented foods. *Critical reviews in food science and nutrition*, 59(3): 506-527.
- Santos, A., San Mauro, M., Sanchez, A., Torres, J. M., & Marquina, D. 2003. The antimicrobial properties of different strains of *Lactobacillus spp.* Isolated from Kefir. In *System. Appl. Microbiol* 26: 434-437. <http://www.urbanfischer.de/journals/sam>
- Sarita, B., Samadhan, D., Hassan, M. Z., & Kovaleva, E. G. 2024. A comprehensive review of probiotics and human health-current prospective and applications. *Frontiers in Microbiology*, 15: 1-14.
- Savaiano, D.A. & Hutkins, R.W. 2021. Yoghurt cultured fermented milk, and health: A systematic review. *Nutrition reviews*, 79(5):599-614.

- Schrezenmeir, J. & de Vrese, M. 2001. Probiotics, prebiotics, and synbiotics-approaching a definition. *The American Journal of Clinical Nutrition*, 73(2): 361–364.
https://doi.org/10.1007/10_2008_097
- Sharma, R., Garg, P., Kumar, P., Bhatia, S.B. & Kulshrestha, S. 2020. Microbial fermentation and its role in quality improvement of fermented foods. *Fermentation*, 6: 1-20.
- Shiozawa, K., Agista, A.Z., Ohsaki, Y. & Shirakawa, H. 2022. Emergence of Novel Functions of Vitamins for the Prevention of Life-Style Related Diseases Emergence of Novel Functions of Vitamins for the Prevention of Life-Style Related Diseases (SY (T1) 8). *Journal of Nutritional Science and Vitaminology*, 68: 8-10.
- Siddiqui, S.A., Erol, Z., Rugji, J., Taşçı, F., Kahraman, H.A., Toppi, V., Musa, L., Giacinto, G., Bahmid, N., Mehdizadeh, M., Castro-Munoz, R. 2023. An overview of fermentation in the food industry - looking back from a new perspective. *Bioresources and Bioprocessing*, 10(85): 1-47.
- Simonelli, N., Gagliarini, N., Medrano, M., Piermaria, J.A. & Abraham, A.G. 2022. Kefiran. In *Polysaccharides of Microbial Origin: Biomedical Applications*. Springer International Publishing: 99-116.
- Spacova, I., Binda, S., Haar, J.A., Henoud, S., Legrain-Raspaud, S., Dekker, J., Espadaler-Mazo, J., Langella, P., Martín, R., Pane, M., & Ouwehand, A.C. 2023. Comparing technology and regulatory landscape of probiotics as food, dietary supplements and live biotherapeutics. *Frontiers in Microbiology*, 14: 1-14. Frontiers Media SA.
<https://doi.org/10.3389/fmicb.2023.1272754>.
- Tamir, T.T., Zegeye, A.F., Workneh, B.S., Ali, M. S., Gonete, A.T., Techane, M.A., Wassie, M., Kassie, A.T., Ahmed, M.A., Tsega, S.S., Wassie, Y.A., Tekeba, B. & Mekonen, E. G. 2025. Childhood wasting and associated factors in Africa: evidence from standard demographic and health surveys from 35 countries. *BMC Public Health*, 25(1): 1-13.
<https://doi.org/10.1186/s12889-025-21673-z>
- Tarnaud, F., Gaucher, F., do Carmo, F.L.R., Illikoud, N., Jardin, J., Briard-Bion, V., Guyomarc'h, F., Gagnaire, V. & Jan, G. 2020. Differential adaptation of *Propionibacterium freudenreichii* CIRM-BIA129 to cow's Milk versus soymilk environments modulates its stress tolerance and proteome. *Frontiers in Microbiology*, 11: 1-21.
- Thierry, A., Deutch, S., Falentin, H., Dalmaso, M., Cousin, F.J. & Jan, G. 2011. New insights into physiology and metabolism of *Propionibacterium freudenreichii*. *International Journal of Food Microbiology*, 149:19-27.

- Tratnik, L., Božanić, R., Herceg, Z. & Drgalić, I. 2006. The quality of plain and supplemented kefir from goat's and cow's milk. *International journal of dairy technology*, 59: 40-46.
- Turkmen, N., Akal, C., & Özer, B. 2019. Probiotic dairy-based beverages: A review. *Journal of Functional Foods*, 53: 62-75. <https://doi.org/10.1016/j.jff.2018.12.004>
- Tzavaras, D., Papadelli, M. & Ntaikou, I. 2022. From milk kefir to water kefir: Assessment of fermentation processes, microbial changes and evaluation of the produced beverages. *Fermentation*, 8(135): 1-16.
- Valentino, V., Magliulo, R., Farsi, D., Cotter, P.D., O'Sullivan, O., Ercolini, D. & De Filippis, F. 2024. Fermented foods, their microbiome and its potential in boosting human health. *Microbial Biotechnology*, 17(2): 1-30.
- Van Wyk, J., Witthunn, C.R. & Britz, T.J. 2011. Optimisation of vitamin B12 and folate production by *Propionibacterium freudenreichii* strains in kefir. *International Dairy Journal*, 21: 69-79.
- Voidarou, C., Antoniadou, M., Rozos, G., Tzora, A., Skoufos, I., Varzakas, T., Lagiou, A. & Bezirtzoglou, E. 2021. Fermentative Foods: Microbiology, biochemistry, potential human health benefits and public health issues. *Foods*, 10(69): 1-27.
- Wieser, S., Plessow, R., Eichler, K., Malek, O., Capanzana, M.V., Agdeppa, I. & Bruegger, U. 2013. Burden of micronutrient deficiencies by socio-economic strata in children aged 6 months to 5 years in the Philippines. *BMC Public Health*, 13: 2-15.
- Windey, K., Houben, E., Deroover, L. & Verbeke, K. 2015. Contribution of colonic fermentation and fecal water toxicity to the pathophysiology of lactose-intolerance. *Nutrients*, 15: 7505-7522.
- Witthuhn, R.C., Schoeman, T. & Britz, T.J. 2005. Characterisation of the microbial population at different stages of Kefir production and Kefir grain mass cultivation. *International Dairy Journal*, 15(4): 383-389. <https://doi.org/10.1016/j.idairyj.2004.07.016>
- Xie, N., Zhou, T. & Li, B. 2012. Kefir yeast enhance probiotic potentials of *Lactobacillus paracasei* H9: The positive effects of coaggregation between the two strains. *Food Research International*, 45: 394-401.
- Zago, M., Fornasari, M.E., Carminati, D., Burns, P., Suárez, Vinderola, G., Reinheimer, J. & Girraffa, G. 2011. Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiology*, 28: 1033-1040.

- Zajšek, K., Goršek, A. & Kolar, M. 2013. Cultivating conditions effects on kefir production by the mixed culture of lactic acid bacteria imbedded within kefir grains. *Food Chemistry*, 139: 970-977. <https://doi.org/10.1016/j.foodchem.2012.11.142>
- Zhang, R., Sha, Y., Chamlagain, B., Edelmann, M., Savijoki, K., Piironen, V., Deptula, P. & Varmanen, P. 2025. Oxygen determines the requirement for cobalamin but not riboflavin in the growth of *Propionibacterium freudenreichii*. *Scientific Reports*, 15(1): 1-12. <https://doi.org/10.1038/s41598-025-12983-8>
- Zielińska, D. & Kolożyn-Krajewska, D. 2018. Food-origin lactic acid bacteria may exhibit probiotic properties. *BioMed research international*, 2018(1): 1-15.
- Zommiti, M., Feuilloley, M.G & Connil, N. 2020. Update of probiotics in human world: a nonstop source of benefactions till the end of time. *Microorganisms*, 8: 1-31.

CHAPTER 3

DETECTION OF *PROPIONIBACTERIUM SPP.* USING PCR AND MICROBIOLOGICAL METHODS

Abstract

Propionibacterium freudenreichii subsp. *shermanii* has been used for centuries in dairy production where it plays a key role in developing flavour and other characteristics. The detection and isolation of propionibacteria (PAB) is challenging due to the absence of readily available and inexpensive selective media. Due to major interest and economic importance of PAB to the food industry, a faster reliable detection and identification process is necessary. The objective of this study was to evaluate the effectiveness of the streaking technique and the conventional polymerase chain reaction (PCR) for detecting viable *Propionibacterium freudenreichii* from freeze-dried cultures. Cells were pre-treated with propidium monoazide (PMA) for the selective intercalation of the genomic DNA of non-viable cells to reduce the possibility of false-positive results. The validated PMA treatment settings were a 2.5 mM PMA reagent concentration, followed by a 10-minute dark incubation, and 2 minutes of photoactivation using two 250 W halogen lamps. After treatment of the suspected PAB with PMA, the DNA was extracted using a DNeasy Powerfood Microbial kit and amplified using a conventional PCR master cycler, where the 16S rRNA gene was amplified and tailored for *P. freudenreichii* using primers Prop1 (5'GATACGGGTGACTTGAGG-3') and Prop2 (5'GCGTTGCTGATCTGCGATTAC-3'). The 50 µL final volume of the optimised PCR protocol was made up of 25 µL Ampliqon Master mix, 1 µL Prop1, 1 µL Prop2, 20 µL PCR grade water, and 3 µL DNA sample. The ideal PCR cycle required initial denaturation at 95°C for 5 minutes, followed by 98°C for 20 s, annealing at 52°C for 15 s, and chain elongation at 72°C for 1 minute. The PCR products of *P. freudenreichii* were electrophoresed and visualised in 1% agarose gel. Streaked plates were performed using a forty-eight-hour Yeast extract lactate (YEL) broth culture onto YEL agar, Plate count agar (PCA) and Pal propiobac and incubated anaerobically for six days at 30°C. Pale cream colonies were obtained on the PCA plates and grey-brown colonies were visible on the YEL plates. PAB was identified as grey colonies surrounded by a yellow halo on the Pal propiobac. The growth of *P. freudenreichii* was visible on all three media selected for the conventional method. However, the microbial load obtained on YEL and PCA required further confirmation tests. The microbiological culturing and PCR methods employed were successful in selectively identifying viable PAB in pure cultures.

3.1. Introduction

PAB are Gram-positive rod-shaped or coryneform, catalase variable organisms. They are non-motile, non-spore forming facultative anaerobes. PAB prefer microaerophilic to anaerobic

growth conditions and grow optimally at 30-37°C and pH 6-7. The growth is inhibited at pH 4.5, with the production of propionic acid (Ahmadi et al., 2017:70; Turgay et al., 2020:3).

P. freudenreichii was first described by Orla-Jensen and von Freudenreich in 1906 when they investigated the production of propionic acid and the formation of eyes in cheese. Later, it was used as a ripening culture in Swiss cheese and a starter culture for other dairy products (Faye et al., 2011:549; Danilova et al., 2012:49; Guggisberg et al., 2015:119). Classical PABs are primarily found in raw milk and dairy products such as Swiss cheese and buttermilk. They play an important role in the texture and flavour of dairy products by producing propionic and acetic acids. PAB convert lactate produced by lactic acid bacteria into propionate, acetate, and carbon dioxide. Propionic fermentation occurs mainly in warm environments and slows down in colder conditions. However, *P. freudenreichii* remains active during the storage of cheese at 4°C for up to 14 days, as observed in the ripening of Emmentaler cheese (Cousin et al., 2012:144). Dairy PAB is also known to be a strong producer of vitamin B₁₂ and propionic acid (Ahmadi et al., 2017:69). *P. freudenreichii* is regarded as a probiotic and is considered GRAS (Generally Recognised as Safe) by the United States Food and Drug Administration (US FDA) (Chamlagain et al., 2016:2).

In various studies, dairy PAB is cultured in a lactate-rich medium such as sodium lactate broth (SLB) and Yeast extract lactate agar (YEL) and incubated under anaerobic conditions using an Anaerocult A system at 30-32°C for 5 to 10 days (Witthuhn et al., 2005:384; Karimi, 2012:7; Coronas et al., 2023:3). Dairy PAB can synthesise all amino acids and can adapt to various environments. They can grow in media containing low to minimum nutrients, which contain ammonium as the sole nitrogen source, but elevated growth was observed in media containing amino acids (Wang et al., 2021:3). However, traditional culture-based methods for detecting bacteria are often time-consuming, labour-intensive, and costly, and phenotypic identification remains problematic. Alternative techniques to identify dairy PAB are therefore required. Polymerase chain reaction (PCR) has been identified for rapid and sensitive routine identification of the various species of dairy PAB (Riedel et al., 1998:420). This method is based on the amplification of the V3 variable region within the 16S rRNA gene and has been applied in various environmental settings and in the food industry, where rapid methods are often required.

Conventional PCR is an effective and rapid diagnostic tool but possesses limitations due to its high cost. To overcome these limitations, duplex and multiplex PCR assays that allow for simultaneous amplification and detection of more than one target sequence in a single reaction have been developed (Chen et al., 2017:40). A multiplex PCR assay was developed by Dasen et al. (1998:254), where genus-specific primers are targeted to the genes encoding the 16S rRNA, together with two universal primers. It has been reported that DNA in the environment is very stable, decays slowly and can persist intact for extended periods after cell death, resulting in positive amplification products (Josephson, 1993:3513; Bairoliya et al.,

2022:3). This is a huge obstacle for DNA-based profiling as it is important to obtain meaningful data by limiting the analysis to the viable bacterial population in the sample. To remedy the amplification of non-viable cell DNA, the treatment of cells with biological dyes such as propidium monoazide (PMA) and ethidium monoazide (EMA) has been used in conjunction with quantitative PCR (qPCR). The viability discrimination is based on the positive molecular charge of the dye. While viable bacterial cell membranes are negatively charged, PMA can enter bacteria with a compromised cell membrane. When this happens, the dye intercalates into nucleic acids and forms a covalent modification between the dye and the nucleic acid after exposure to bright visible light (Nocker et al., 2007:5112; Zi et al., 2018:2; Yuan et al., 2018:399). PMA has a light activatable azide group, which allows for cross-linking with the non-viable cell DNA, resulting in strong inhibition of PCR amplification. Performing the intercalating of the non-viable cell DNA by PMA before performing a PCR assay will amplify DNA of viable bacterial populations while inhibiting the compromised cell membrane reactions. It has been shown in various studies that PMA is more selective than EMA in inhibiting the amplification from non-viable cell DNA in qPCR applications (Chang et al., 2010:122; Chang, et al., 2017:95).

Gaucher et al. (2020:3145) described *P. freudenreichii* as a multipurpose starter culture and beneficial food grade, probiotic bacterium that has beneficial effects when consumed by humans. These benefits include increased intestinal bifidobacterial population and anti-inflammatory modulation of the gut mucosa. The efficacy of probiotics depends on the survival of the number of bacteria during digestion, as they need to be present in adequate amounts to confer a health benefit to the host (Le Maréchal et al., 2015:448). In this study, the effective enumeration and identification of *P. freudenreichii* was investigated using various growth media and conventional PCR utilising specific primers.

3.2 Materials and Methods

Source of Materials and Equipment

3.2.1 PAB strains

Freeze-dried PAB strains were obtained from the culture collection of the Cape Peninsula University of Technology (CPUT), Bellville, South Africa. The two strains, renamed J15 and J17, were originally isolated from Damlitter cheese and listed as ATCC 13637, respectively. The freeze-dried PAB strains were reconstituted by adding 1 mL of sterile Ringer's solution (Merck, Johannesburg) to the respective vials (Van Wyk et al., 2011:70).

Preparation of growth media

3.2.2 Preparation of YEL broth and agar

YEL broth, a lactate rich medium, was prepared as described by Van Wyk et al. (2011:70) with following composition: yeast extract (5 g.L⁻¹, Biolab, Merck, Johannesburg, South Africa),

peptone powder (2 g.L⁻¹, Biolab), KH₂PO₄ (10 g.L⁻¹, Merck), sodium lactate syrup 50% v/v (20 mL. L⁻¹ Sigma Aldrich, Germany), and Tween 80 (20 mL⁻¹, Saarchem, Roodepoort, South Africa). To prepare YEL agar, bacteriological agar (5 g.L⁻¹ Biolab) was added to the prepared YEL broth. The pH was adjusted to pH 7 using 1M NaOH. The media was dissolved in distilled water by boiling, autoclaved for 15 minutes at 121°C and cooled to 48°C.

3.2.3 Preparation of Pal propiobac

The Pal propiobac (Cat no AO6001, Standa Laboratoires, France) was provided as six bottles containing 80 mL solid base media and one freeze-dried vial of selective supplement. The base agar medium was placed in a boiling water bath (Mettler, Lasec, South Africa) until the agar had melted, then cooled to 48°C. The selective supplement was gently mixed with 7 mL of sterile distilled water and dispensed evenly into the six bottles of molten agar before use.

3.2.4 Preparation of PCA

PCA (Cat no C6, Biolab, Merck) was prepared by adding 23 g of dehydrated media to 1 L of distilled water, boiling until all solids were dissolved, autoclaving at 121°C for 15 minutes, and cooling to 48°C.

3.3 Streaking of PAB onto conventional agar

The reconstituted PAB strains (0.2 mL) were inoculated into 9 mL YEL broth and incubated at 32°C for 48 hours. The cultures were streaked onto PCA, YEL agar, and Pal propiobac. The plates were incubated for 6 days at 30°C anaerobically using Anaerocult A (Cat no 1.138929.0001 Merck). Any significant differences in the morphology of the mature colonies were documented.

3.4 Preparation of PCR reagents

3.4.1 Tris/Borate/EDTA (TBE) buffer

To prepare 0.5x concentration TBE buffer (Cat no 50843, Lonza, South Africa), 50 mL concentrated 10x TBE buffer was combined with 950 mL Milli-Q water in a Schott bottle and mixed by inverting the bottle several times.

3.4.2 1% Agarose gel with wells

Agarose gel (1%) was prepared by adding 1 g of agarose powder into 100 mL 0.5x TBE buffer and heated in a microwave for approximately 90 seconds on high power or until all the granules were dissolved. The agarose was cooled in a water bath (Mettler, Lasec, South Africa) set at 55°C. Five µL of ethidium bromide (Cat no BP 1302-10, Jansen Pharmaceuticals, Thermofisher, South Africa) was added to 100 mL agarose gel before casting into a gel mould

containing a comb. The gel was left undisturbed at room temperature to solidify. The combs were removed after the solidification of the gel.

3.4.3 Preparation of primers

Specific PCR primers (Prop 1 and Prop 2) based on aligned 16S rRNA gene sequence were designed for this study (Riedel et al., 1998:420). Primers were supplied in the form of a transparent film (Integrated DNA Technologies, Whitehead Scientific, South Africa) and resuspended in TE buffer (10 mM Tris pH 8; 1 mM EDTA) (Cat no 51235, Accugene, Lonza, Whitehead Scientific, South Africa).

3.5 Treatment of pure PAB samples with PMA reagent

3.5.1 Procedure 1: Preparation of control PAB samples

The Blu-V PMA viability kit (Cat no 296015, Qiagen, Whitehead Scientific, South Africa) was used according to the manufacturer's instructions (Figure 3.1). PMA reagent was prepared by adding 550 µL RNase-free water to each vial to obtain a 2.5 mM PMA solution and mixed gently by pipetting up and down several times, then centrifuged briefly. The reagent was stored in opaque test tubes to avoid light exposure, as the PMA is a photo-reactive agent. Four different sample matrices were prepared to be treated with PMA, namely non-viable cells with PMA treatment, non-viable cells without PMA, viable cells with PMA and viable cells without PMA. Non-viable PMA control samples were prepared by adding 1 mL PAB pure culture sample into two safe seal tubes. One tube was labelled "Dead plus PMA", and the other was labelled "Dead no PMA". The tubes containing the bacteria were placed in a water bath (Memmert, Lasec, South Africa) set at 80°C for 20 minutes to destroy the bacteria and then cooled to room temperature. The sample labelled "Dead plus PMA" was treated with PMA. In contrast, DNA of the sample labelled "Dead no PMA" was directly extracted using the Powerfood Microbial DNA isolation kit (Cat no 21000-50, Whitehead Scientific, South Africa). The following controls were included: a sample that was not exposed to heat and contained a mixture of viable and non-viable PAB cells labelled "Not heat-treated" plus PMA and without PMA, respectively.

3.5.2 Procedure 2: Treatment of PAB pure culture control samples with PMA reagent

One mL of each control PAB pure culture was transferred to a clean 2 mL safe seal microtube and centrifuged for 5 minutes at 13000 x g. The supernatant was carefully removed and discarded. The pellet was resuspended in 500 µL buffer EB, vortexed using a vortex mixer (Labsmart, United Scientific, South Africa) and 10 µL of the reconstituted PMA reagent added to the samples. PMA reagent was not added to the "No PMA" control sample, and the thermal lysis sample was labelled "non-viable no PMA". The tubes were mixed by vortexing for 5 to 6 seconds. The tubes were wrapped in aluminium foil and incubated for 10 minutes at room

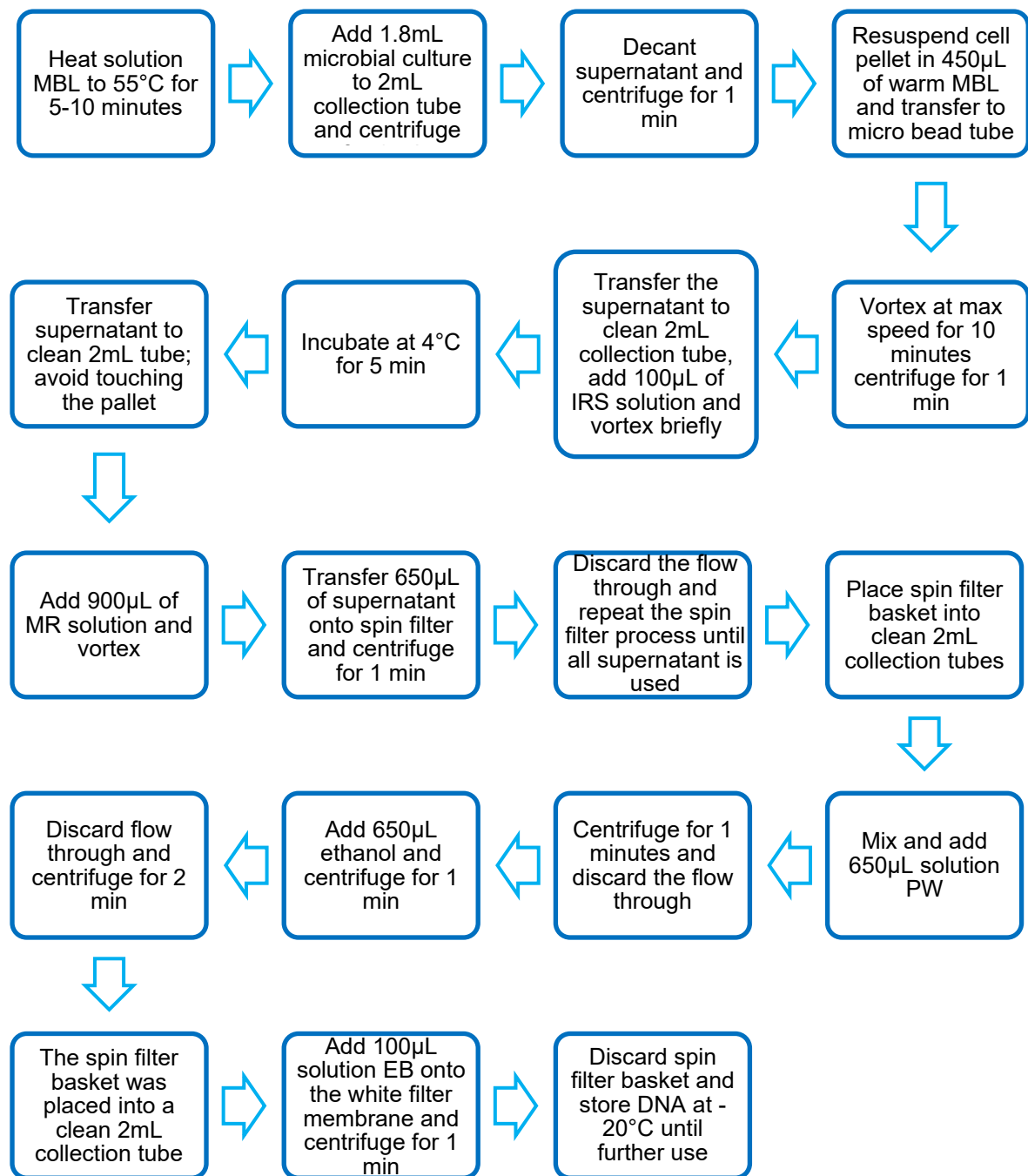


Figure 3.1 Schematic diagram of sample treatment with PMA reagent

temperature in the dark. After incubation, the samples were placed into an incubation box that contained two 230 V and 250 W halogen lamps for 10 minutes. The samples were removed, placed on ice and vortexed to allow for homogenous sample illumination. The samples were centrifuged for 5 minutes at 13000 x g and the supernatant was discarded (Yu, et al., 2017:7875).

3.5.3 Isolation of DNA from PMA-treated PAB

Isolation of the DNA was performed using the Powerfood Microbial DNA isolation kit (Cat No 21000-50, Whitehead Scientific, South Africa) (Figure 3.2). Solution MBL was heated to 55°C for 5 to 10 minutes until needed. The sample culture was homogenised in a lab stomacher (Model CLC-570, MRC, United Scientific, South Africa). A PAB culture of 1.8 mL was added to a 2 mL collection tube and centrifuged for 1 minutes at 13000 x g at room temperature. The supernatant was decanted and centrifuged for 1 minute at 13000 x g. The remaining media supernatant was removed with a pipette tip. The cell pellet was resuspended in 450 µL of solution MBL. The resuspended cells were transferred to the micro-bead tube. The micro-bead tube was secured horizontally to a vortex adapter and vortexed at maximum speed for 10 minutes. The tubes were centrifuged for 1 minute at 13000 x g at room temperature. The supernatant was transferred to clean 2 mL collection tubes, 100 µL of solution IRS was added and vortexed briefly to mix. The suspension was incubated at 4°C for 5 minutes. The tubes were centrifuged for 1 minute at 13 000 x g at room temperature. Without touching the pellet, the entire volume of the supernatant was transferred to a clean 2 mL collection tube and 900 µL of solution MR was added to the tube and vortexed to mix. Supernatant with a volume of 650 µL was transferred onto a spin filter and centrifuged for 1 minute at 13000 x g. The flow was discarded and repeated until all the supernatants had been loaded onto the spin filter. The spin filter basket was placed into a clean 2 mL collection tube. Solution PW was mixed thoroughly before adding 650 µL of this solution into the tube. This suspension was centrifuged for 1 minute at 13000 x g at room temperature. The flow was discarded and 650 µL of ethanol was added to the tube. This suspension was centrifuged for 1 minute at 13000 x g at room temperature. The flow-through was discarded and the pellet was centrifuged at 13000 x g for 2 minutes to remove the residual wash. The spin filter basket was placed into a clean 2 mL collection tube. Solution EB with a volume of 100 µL was placed into the centre of the white filter membrane and centrifuged for 1 minutes at 13000 x g. The spin filter basket was discarded. The DNA for each sample was stored at -20°C.

3.6 Optimisation of the PCR protocol

3.6.1 PCR protocol for the detection of *P. freudenreichii*

The PCR protocol was optimised for *P. freudenreichii*, and the amplification of the V3 variable region within the 16S rRNA gene was obtained using the species-specific primers Prop1 and Prop2. Primer Prop1 was resuspended in 349 µL TE buffer to obtain a 100 µM suspension, and primer Prop2 was resuspended in 390 µL TE buffer to obtain a 100 µM suspension (Whitehead Scientific, South Africa).

The primers were originally designed using specific reference strains of *P. freudenreichii* (Schoeman, 2001:113). All PCR amplifications were prepared in duplicate. The PCR reaction

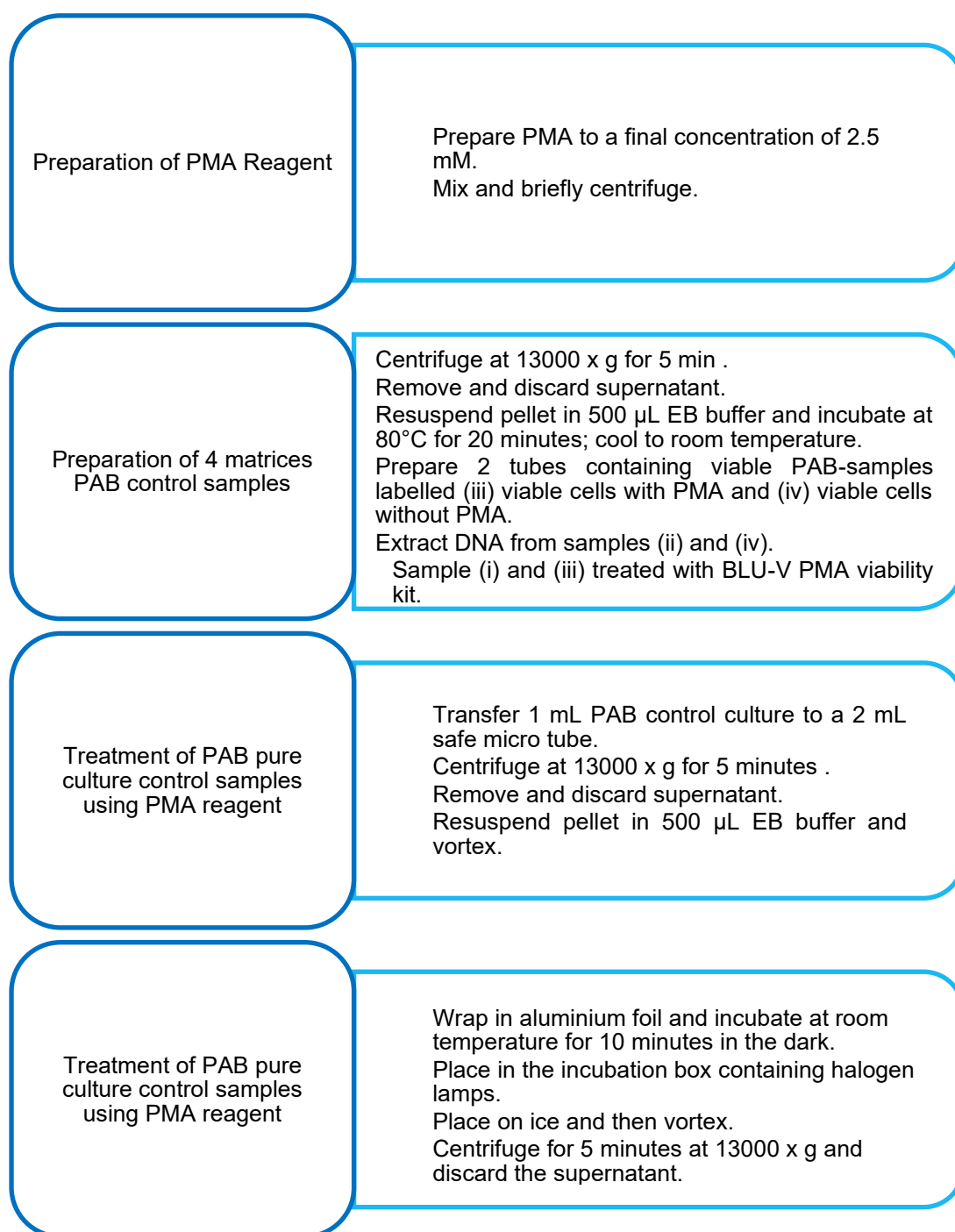


Figure 3.2 Isolation of DNA of lyophilised pure PAB cultures using the Powerfood Microbial DNA isolation kit (Cat No 21000-50, Whitehead Scientific, South Africa)

was prepared to a final volume of 50 µL, which contained 25 µL Ampliqon Master mix (Lasec), 1 µL Prop1, 1 µL Prop2, 20 µL PCR grade water and 3 µL DNA sample.

A negative control reaction was prepared with each PCR run, where the DNA template was substituted with PCR-grade water. The Taq DNA Polymerase master-mix red 1.5 mM MgCl₂ (Cat no 5200300-1250, Ampliqon, Lasec, South Africa) contained Taq DNA Polymerase, the NH₄⁺ buffer system, dNTPs and magnesium chloride. The master mix was a ready-to-use system. The PCR reaction was performed in 0.2 mL thin-walled tubes using an

Eppendorf Master cycler (Eppendorf, Merck, Germany). The optimised PCR amplification conditions consisted of initial denaturation at 95°C for 5 minutes, followed by 98°C for 20 s, annealing at 52°C for 15 s and chain elongation at 72°C for 1 minute to complete the cycle, followed by a chain elongation at 72°C for 5 minutes. The cycle was repeated 35 times and completed by a final chain elongation step at 72°C for 5 minutes and cooling the reaction products to 4°C.

3.6.2 Loading of the agarose gel

After the agarose gel had solidified, the comb was carefully removed. The gel was then removed from the gel mould bed plate and placed into the electrophoresis tray without removing the gel from the mould. The tray was filled with 0.5x TBE buffer until the gel was covered entirely. Five µL of ethidium bromide was added to the bottom of the tray before electrophoresis started. The first well of the gel was inoculated with a DNA marker, which consisted of 4 µL 1 kB DNA ladder (Ref no G571A, Promega, Anatech) and 1 µL bromophenol loading dye (Ref no G190A, Promega, Anatech). PAB bands must fluoresce in the region of 750 base pairs (BP) when comparing the fluorescent bands with the DNA ladder, as this is the size of the DNA fragment of interest. Bromophenol blue loading dye is useful in electrophoresis as it possesses a slightly negative charge and will migrate in the same direction as DNA. This characteristic allows the molecules to be monitored as they migrate through the agarose gel. Three µL of amplified DNA product was applied to each well. PCR products were electrophoresed in 1% agarose gel (w/v) in 0.5x TBE buffer which contained 5 µL ethidium bromide.

3.6.3 Electrophoresis

The gel was electrophoresed at 90 V for 80 minutes until the sample had migrated at least 10 cm down the agarose gel. The gel was carefully removed from the gel box and placed into the Uvidoc (UVItech, Whitehead Scientific, South Africa) and visualised under UV light.

3.7 Results and Discussion

3.7.1 *Propionibacterium freudenreichii* growth on conventional media

Conventional phenotypic identification methods are critical for taxonomic characterisation of microorganisms, but they are time-consuming and often yield unclear findings even though they remain widely used (Adzitey et al., 2013:98; Tortone et al., 2018:58). These findings include observation of growth and colony morphology on various media. In this study, propionibacteria showed growth on all media, namely PCA, YEL and Pal propiobac, using the streak plate method. Figures 3.3a and 3.3b show the growth of PAB on Pal propiobac, which was incubated for 6 days and 8 days respectively. Both plates showed discolouration of the Pal propiobac from violet to yellow around the cream / beige colonies. This is in line with the literature by Gautier (2014:234), who found that propionibacteria colonies on Pal propiobac

appear as brownish colonies larger than 0.5 mm in diameter, surrounded by a yellow area, which is caused by a decrease in pH due to glycerol fermentation. The surface colonies were shiny, convex, and beige with darker grey centres. Colonies on Pal propiobac after 8 days was larger and raised, ranging from 2 to 4 mm. The agar discoloured to a brown /orange colour upon longer incubation, while the colonies became darker in colour. However, 6 days of incubation under anaerobic conditions at 30°C were needed to form visible, mature colonies for all media tested.

According to previous studies (Theirry et al., 1995:317; Britz & Riedel, 1994:258; Turgay et al., 2020:3), PAB can be maintained and isolated in YEL, incubated at 30°C under anaerobic conditions. The colour of the colonies on YEL agar can be expected to range from light-cream to orange or red brown, depending on the species. PAB colonies in this study formed colonies ranging in size from 1 to 4 mm in diameter. Colonies obtained on YEL after 6 days of incubation anaerobically at 30°C, were beige colonies ranging from 2-4 mm in diameter. This is in line with YEL N agar employed by Britz & Riedel (1994:260), which consisted of filter-sterilised nalidixic acid added to the YEL agar to enhance the selectivity for PAB. The pigmentation of the colonies was described as white/cream, yellow/orange and red/brown. The colonies obtained in the current study were 0.5 to 1 mm in diameter compared to the larger (2 to 4 mm), raised colonies found on YEL N-media. Britz & Riedel (1994:264) concluded that YEL was an unsuitable selective medium for propionibacteria as it allowed the growth of certain mesophilic lactobacilli and Gram-negative organisms. De Freitas et al. (2013:272), employed YEL as a non-selective growth medium for PAB and recorded small red colonies isolated from Emmental cheese.

PAB streaked on PCA (Figure 3.3c) appeared pale, creamy, and slightly smaller colonies than on YEL (Figure 3.3d) after the incubation period of 6 days at 30°C. Colonies were smooth, clear, and circular with an entire margin. The growth rate also appeared slower on PCA than on YEL and Pal propiobac, as the colonies appeared smaller in size after the same incubation period. This could be due to media composition, which affects the growth rate. PAB requires various substrates, such as specific carbon and energy sources, to grow, which is not present in PCA. Sucrose, lactose, glucose, galactose, and amino acids are among PAB's required sources of carbohydrates. The faster growth on YEL can thus be attributed to lactate, which has been described as its preferred carbohydrate source (Huang et al., 2016:4645).

The growth of propionibacteria on Pal propiobac, when compared to YEL and PCA agar, facilitated easier identification of colonies due to the bromocresol indicator responsible for the colour change in the media. Since Pal propiobac is a patented chromogenic media, this study also confirms that no further differentiation methods were required this media to isolate PAB selectively. Pal propiobac is more expensive and there is no significant time saving than YEL and PCA when growth is expected on the agar plates. Total viable counts obtained on

PCA and YEL, however, require further confirmation tests like the Analytical profile index 50 CH for carbohydrate metabolism of PAB. Anaerobic bacteria including *Corynebacterium* can be detected with the Vitec 2 ANC card, however the media used to subculture the bacteria for the VITEK 2 system may lead to misidentification of the results. PAB furthermore required a lengthy incubation period to observe visible growth on all three media. Traditional phenotypic approaches and other commercial systems utilising fatty acid and carbon utilisation profiles are less accurate than 16S rRNA gene sequencing methods using PCR. It was reported that no one method was reliable, and that incomplete and obsolete databases hampered all methods. Additional research using genotypic, phenotypic, and proteomics investigations is needed to develop reliable and repeatable tests (Cloud et al., 2010:1444). Conventional methods are regarded as the gold standard for enumeration in the probiotic industry due to its simplicity and being easily implemented. Traditional microbiological plate count methods is a measurement of the bacterial cell's ability to form detectable colonies on agar media. However, colony forming units (CFU) is not regarded as a true count of the number of viable microorganisms present, but merely as an estimation and relies heavily on the growth media and incubation conditions (Boyte et al., 2023:2). In agreement with previous studies, it can be concluded that the conventional culture-based methods used to enumerate PAB were time-consuming and labour-intensive (Joo et al., 2019:2; Bücher et al., 2021:4312).

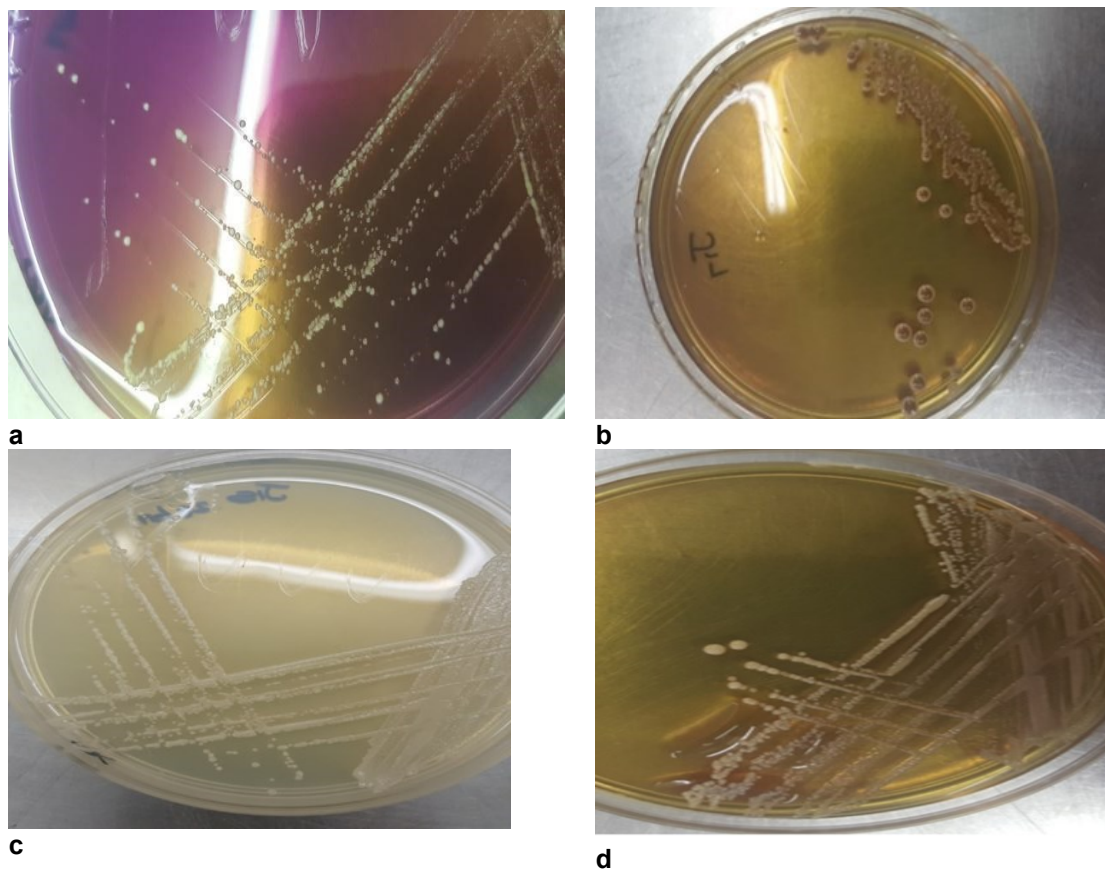


Figure 3.3 Propioni bacteria streaked on Pal propiobac at various incubation periods; (a) 6 days on Pal propiobac, (b) 8 days on Pal propiobac; (c) Propioni bacteria streaked on PCA, (d) Propioni bacteria streaked on YEL agar

3.7.2 Optimised polymerase chaise reaction protocol to detect *P. freudenreichii*

PCR is reported to be a sensitive detection tool and often requires optimisation. The effectiveness of the amplification of genes by PCR is dependent on several factors, such as Mg²⁺ ions concentration, primers concentration and annealing temperatures, which are also dependent on the melting temperatures of the respective primers (Obradovic, 2013: 489). The Taq polymerases also play an important role in the formation of primer-dimer artefacts. The PCR amplification was carried out using species-specific primers Prop1 (5'GATACGGGTGACTTGAGG-3') and Prop2 (5'GCGTTGCTGATCTGCGATTAC-3'), procured from IDT (White head Scientific, South Africa), targeting 750 bp. The 50 µL PCR reaction mixture 25 µL Ampliqon Master mix (Lasec, Cape Town, South Africa), 1 µL Prop1, 1 µL Prop2, 20 µL PCR grade water and 3 µL DNA sample. The optimised PCR amplification conditions consisted of initial denaturation at 95°C for 5 minutes, followed by 98°C for 20 s, annealing at 52°C for 15 s and chain elongation at 72°C for 1 minutes to complete the cycle, followed by a chain elongation at 72°C for 5 minutes. The cycle was repeated 35 times and completed by a final chain elongation step at 72°C for 5 minutes and cooling of the reaction products to 4°C (Kulkarni et al., 2017:278). The PCR product was electrophoresed in 1% agarose gel containing ethidium bromide in TE buffer at 100 V for 1 h. The DNA marker was a mixture of 2 µL bromothymol blue loading dye and 8 µL DNA ladder (Promega 1kb, Anatech). A 3 µL loading dye was mixed with 10 µL DNA ladder. Figure 3.4b showed that the DNA marker, which consisted of 2 µL loading dye and 10 µL DNA ladder, was still too much as the bands did not separate clearly. Gelstar staining gel was used to stain the agarose gel before electrophoresis and in the electrophoresis buffer.

Figures 3.4A and 3.4B show very high-intensity bands that appear as a solid fluorescent dye, making it impossible to see where they separate. Figure 3.4C shows amplification products for 5 µL and 10 µL PCR products of J15 and J17 DNA. The reactions showed high fluorescence, indicating that the amount of DNA used was too high. The electrophoresis time was extended to 70 minutes, which showed a slight increase in the separation of the marker. It was also suggested to increase the annealing temperature from 50°C to 52°C. Lane 1 contained a DNA marker, which consisted of an 8 µL DNA ladder mixed with 2 µL loading dye, and Lane 10 contained a DNA marker, which consisted of a 4 µL ladder and 1 µL loading dye. The latter combination provided the desired results. Gelstar stain (Figure 3.4C) was exchanged with ethidium bromide since better and clearer bands were evident. Lanes 6 and 8 contained 5 µL PCR products, which resulted in obtaining clear and more defined bands in combination with the ethidium bromide stain. In Figure 3.5, the annealing temperature of 52°C was used and the Gelstar stain was substituted for ethidium bromide. Five µL ethidium bromide was mixed with 100 mL agarose gel and the same amount was added to the TBE electrophoresis buffer. Running the gel inside the mould resulted in straight

bands. DNA marker consisting of 4 μL DNA ladder and 1 μL loading dye was used and the electrophoresis time was extended to 70 minutes. Clear separation of all bands could be visualised. Bands were obtained at the 750 base pair mark.

To ensure that the method employed was optimised and exclusively detected, PAB, DNA of a non-target organism was introduced.

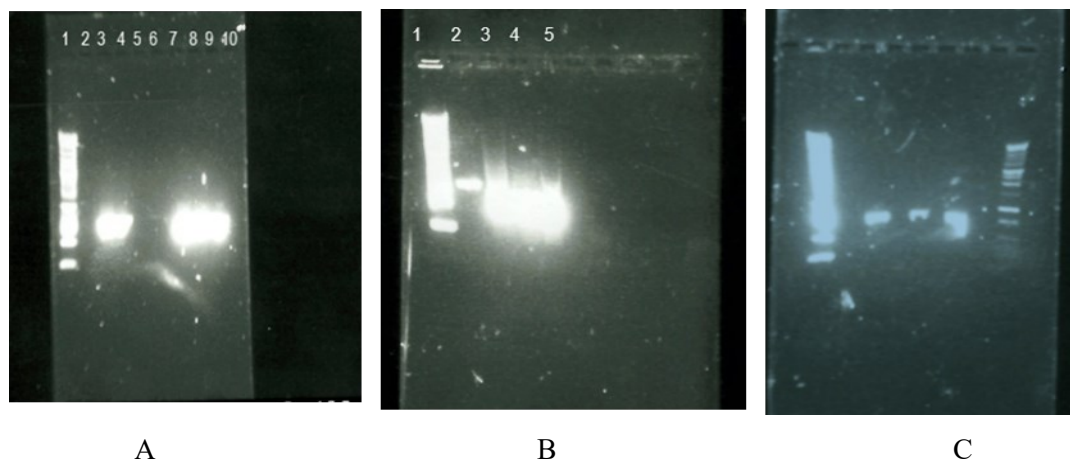


Figure 3.4 (a) 1% Agarose gel with overloaded marker. Lane 1: DNA marker (3 μL loading dye + 10 μL DNA ladder); Lane 2: empty; Lane 3: J15 control, 5 μL ; Lane 4: J17 control, 5 μL ; Lane 5: empty; Lane 6: negative control; Lane 7: J15 10 μL ; Lane 8 and Lane 9 J17; (b) High intensity bands with no clear separation. Lane 1: DNA marker (3 μL loading dye + 10 μL DNA ladder); Lane 2: J15 5 μL ; Lane 3: J15 10 μL , 5 μL ; Lane 4: J17 5 μL ; Lane 5: J17 10 μL ; (c) Lane 1: empty; Lane 2: DNA marker (2 μL loading dye + 8 μL DNA ladder); Lane 3: negative control; Lane 4: J15 control, 3 μL ; Lane 5: empty; Lane 6: J17 control 3 μL ; Lane 7: empty; Lane 8 J17 5 μL ; Lane 9: empty; Lane 10: DNA marker 2 (1 μL loading dye + 4 μL DNA ladder)

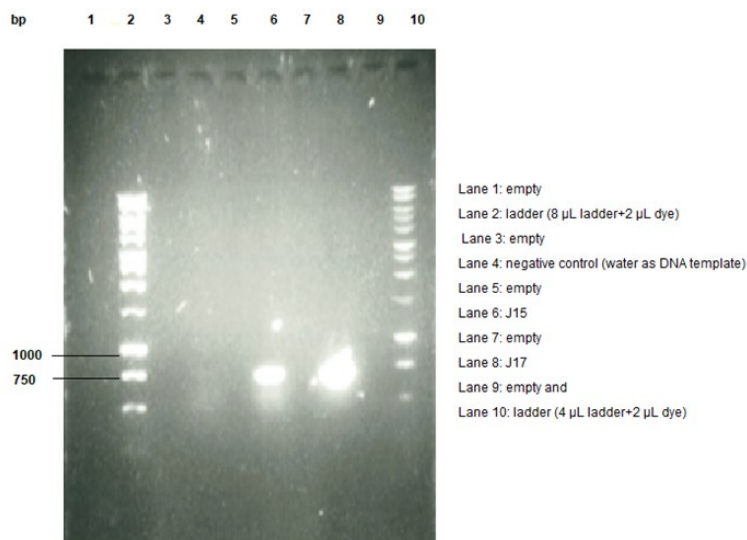


Figure 3.5 1% Agarose gel using ethidium bromide as a stain following the PCR protocol with an annealing temperature of 52°C

Figure 3.6 depicts a pure culture of *Escherichia coli*, introduced as a non-target organism. Since the primer set used was specifically designed to detect PAB, the DNA of the

non-target organism was not amplified. This can be observed in Lane 10, which was inoculated with extracted DNA from *E. coli*. Lane 6 and Lane 8

contained DNA of PAB J15 and J17, respectively, as evident in the fluorescent bands at 750 bp. This proved that the method was successfully optimised to detect and amplify PAB DNA.

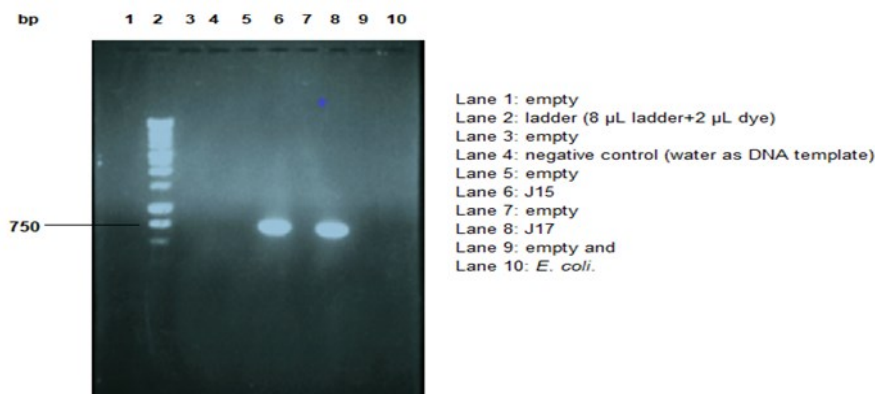


Figure 3.6 Validation of the PCR-based method based on a response from target (PAB) and non-target (*E. coli*) DNA using pure bacterial strains.

3.7.3 Treatment of viable and non-viable cell DNA using PMA

Combining PMA-PCR outcomes with conventional plate count techniques allows for the determination of whether a molecular signal is obtained from growth-capable and metabolically active cells or from dead, compromised or injured, viable-but-nonculturable cells (VBNC). These VBNC cells often are unable to grow on conventional media. In microbiology, intercalating dyes such as PMA are used to distinguish viable from non-viable cells before DNA extraction (Joo et al., 2019:2). The protocol for testing the efficacy of the PMA treatment was slightly modified. The 10-minute heat treatment at 70°C, as recommended by the manufacturer, was insufficient to inhibit the bacteria, as a very faint line remained when the sample was treated with PMA. This study obtained the “Dead plus PMA” after 15 minutes at 85°C. A pure freeze-dried culture of PAB was reconstituted and divided into samples 1 and 2. Sample 1 was heat-treated to represent the non-viable cell control, while sample 2 was not heat-treated. Sample 1 was further divided into two smaller samples, 1a and 1b. Sample 1a was treated with PMA, while sample 1b was left untreated. After DNA amplification using conventional PCR, Sample 1a was loaded into Lane 5 of the agarose gel, while Sample 1b was loaded into Lane 6 and Sample 2 into Lane 7.

Figure 3.7 showed no amplification of DNA for sample 1a (“non-viable plus PMA”), however, the amplification of DNA occurred for sample 1b (“non-viable no PMA”). This proved that the PMA with dead-cell masking was successful. These results are in line with a study from Yu et al. (2017:7874), where a PMA-multiplex-PCR assay was successfully employed to detect *viable C. sakazakii*, *Salmonella enteritidis*, and *B. cereus* in milk and milk products. The DNA used in the amplification process in Lane 10 and Lane 11 was of pure cultures of PAB

J15 and J17, respectively, and treated with PMA. The amplification occurred in the correct region, proving that PMA masking of cells may be analysed using conventional PCR. Figure 3.8 depicts the successful amplification of DNA of a pure culture of PAB exposed to PMA treatment to establish if the PMA is compatible with conventional PCR. The DNA of a pure culture of PAB was exposed to the lightbox containing two 250 W fluorescent lamps, rather than the prescribed Blu-V box, as per manufacturer instructions. Similar light sources were reportedly employed in previous studies (Yu et al., 2017:7875; Joo et al., 2019:3). Lane 8 showed amplification product for DNA of PAB that was not exposed to PMA but was placed inside the lightbox, vortexed and placed on the ice every 2 minutes according to the photo treatment as outlined in the standard PMA treatment protocol (Nocker et al., 2007:5112). In this study, PMA treatment combined with conventional PCR was performed on live and non-viable PAB cells, as well as on a mixture of live and non-viable PAB cells, to validate PMA's ability to detect only live PAB in a microbial sample. Similarly, the DNA of dead cell of *Staphylococcus aureus* was completely masked by pre-treating the DNA with intercalating dyes like EMA and PMA before extraction, preventing the multiplication thereof and without inhibiting the PCR

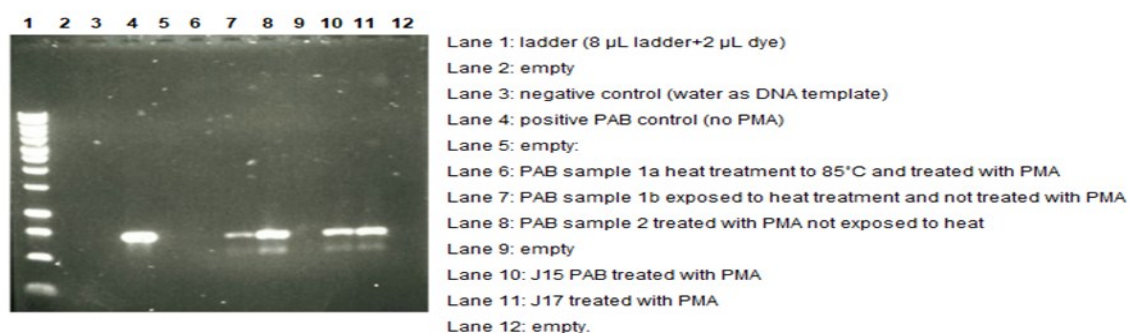


Figure 3.7 DNA treated with PMA

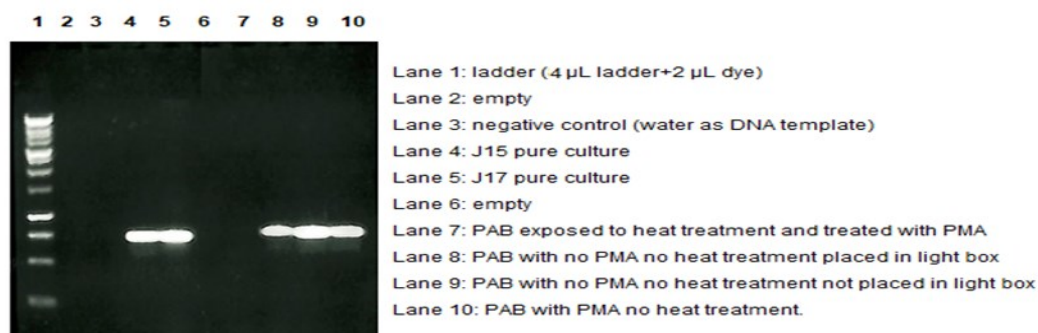


Figure 3.8 PMA treated DNA of PAB exposed to fluorescent light

amplification process. However, it was found that EMA to some degree penetrated viable cells as well as dead cells. Fan et al. (2021:15) compared existing culture-based plating methods and flow cytometry with qPCR and proved that qPCR methods provided reliable and reproducible results that could provide rapid detection of contaminating pathogenic bacteria in foods. This study provided qualitative and quantitative detection and the distribution of live and dead bacteria of the six kinds of LAB in yogurt. PMA and photo treatment were effective in masking the DNA of dead bacterial cells in the samples before PCR amplification. This is in line with a study by Joo et al. (2019:5) where PMA was successfully employed to discriminate between live and non-viable cells in phytoplankton studies in freshwater systems followed by downstream qPCR. The absence of fluorescent bands in the gel of the heat-treated sample after electrophoresis showed that no amplification of DNA occurred. The PAB sample was treated with PMA before DNA extraction. A PCR protocol was optimised for the detection of the PMA-treated PAB cells by adjusting parameters like annealing temperature from 50 to 52°C, primer concentrations and using ethidium bromide in the agarose and the electrophoresis buffer. Very sharp, intense bands of PCR amplification were obtained at the correct base-pair size of 750. After amplified PCR products were separated in agarose gel electrophoresis, using 1% agarose gel stained with 10% ethidium bromide solution. The gel was placed in a UV doc to visualise bands and images were captured. Fluorescent bands were only visible in the region of the gel where the DNA of viable bacterial cells were inoculated. DNA of non-viable cells showed no fluorescence in the gel which means that no DNA was amplified. In a study by Mito et al. (2020:5), it was concluded that PMA-qPCR was successfully used as a detection and quantification tool for viable and viable but not culturable (VBNC) E.coli cells in food. VBNC poses a concern due to misinterpretation of results is a major concern when qPCR and conventional methods are used respectively.

3.7.4 Conclusion

The media selected in this study, namely Pal propiobac, YEL agar and Plate Count agar, supported the growth of a pure PAB culture. The desired microbial growth characteristics of the PAB were exhibited by employing these culture media using freeze-dried strains with colonies on Pal propiobac being more easily distinguishable than YEL and PCA. Thus, it was concluded that Pal propiobac is a more reliable option for the growing and quantifying PAB as the colonies are more clearly defined and identified. However, an incubation period of 6 days under anaerobic conditions was required to obtain mature colonies based on the characteristic color and morphology of PAB on the conventional and chromogenic media. A molecular-based method like PCR was employed to overcome the limitations of culture dependent techniques like long incubation times and labor intensiveness. The DNA of non-viable cells was completely intercalated by PMA, resulting in the inhibition of amplified DNA during the PCR protocol. Hence, the masking of non-viable cells enabled the successful amplification of viable DNA.

The findings obtained were satisfactory and it is concluded that the photo treatment employed was effective. *P. freudenreichii* was successfully identified using the combination of PMA and conventional PCR. *P. freudenreichii* was more rapidly detected using PCR as compared to conventional culturing methods. This study confirmed that molecular biology-based methods, such as PCR, are ideal tools for PAB identification. Furthermore, molecular viability approaches, such as viability PCR, can differentiate viable from non-viable cells in samples without relying on microbiological culture methods, which have numerous limitations.

References

- Adzitey, F., Huda, N. & Ali, G.R.R. 2013. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated ducks. *Biotech* 3: 97-107
- Ahmadi, N., Khosravi-Darani, K. & Mortazavian, A.M. 2017. An overview of biotechnological production of propionic acid: From upstream to downstream processes. *Electronic Journal of Biotechnology*, 28: 67-75.
- Bairoliya, S., Koh, J., Xiang, Z. & Cao, B. 2022. Extracellular DNA in environmental samples: Occurrence, extraction, quantification, and impact on microbial biodiversity assessment. *Applied and Environmental Microbiology*, 88(3): 1-13.
- Boyte, M.E., Benkowski, A., Pane, M. & Shehata, H.R. 2023. Probiotic and postbiotic analytical methods: a perspective of available enumeration techniques. *Frontiers in Microbiology*, 14: 1-15. Frontiers Media SA.
- Britz, T.J. & Riedel, K.H. 1994. Propionibacterium species diversity in Leerdammer cheese. *International journal of food microbiology*, 22(4): 257-267.
- Bücher, C., Burtscher, J. & Domig, K.J. 2021. Propionic acid bacteria in the food industry: An update on essential traits and detection methods. *Comprehensive Reviews in Food Science and Food Safety*, 20: 4299-4323.
- Chamlagain, B., Deptula, P., Edelmann, M., Kariluoto, S., Grattepanche, F., Lacroix, C., Varmanen, P. & Pironen, V. 2016. Effect of the lower ligand precursors on vitamin B12 production by food-grade Propionibacteria. *LWT*, 72: 117-124.
- Chang, B., Taguri, T., Sugiyama, K., Amemura-Maekawa, J., Kura, F. & Watanabe, H. 2010. Comparison of ethidium monoazide and propidium monoazide for the selective detection of viable *Legionella* cells. *Japanese Journal of Infectious diseases*, 63: 119-123.

- Chang, C., Hungd, N. & Chena, N. 2017. Optimization and application of propidium monoazide-quantitative PCR method for viable bacterial bioaerosols. *Journal of Aerosol Science*, 104: 90-99.
- Chen, J., Heale, S., Regan, P., Laksanalamai, P. & Hu, Z. 2017. PCR-based methodologies for detection and characterization of *Listeria monocytogenes* and *Listeria ivanovii* in foods and environmental sources. *Food Science and Human Wellness*, 6: 39-59.
- Cloud, J.L., Harmsen, D., Iwen, P.C., Dunn, J.J., Hall, G., LaSala P.R., Hoggan, K., Wilson, D., Woods, G.L. & Mellmann, A. 2010. Comparison of traditional phenotypic identification methods with partial 16S rRNA gene sequencing for species-level identification of nonfermenting Gram-negative bacilli. *Journal of Clinical Microbiology*, 48: 1442-1444.
- Coronas, R., Zara, G., Gallo, A., Rocchetti, G., Lapris, M., Petretto, G.L., Zara, S., Fancello, F. & Mannazzu, I. 2023. *Propionibacteria* as promising tools for the production of probiotic scotta: a proof-of-concept study. *Frontiers in Microbiology*, 14: 1-17.
- Cousin, F.J., Louesdon, S., Maillard, M., Parayre, S., Falentin, H., Deutsch, S., Boudry, G. & Jan, G. 2012. The first dairy product exclusively fermented by *Propionibacterium freudenreichii*: A new vector to study probiotic potentialities *in vivo*. *Food Microbiology*, 32: 135-146.
- Danilova, I.V., Lee, H., Tourova, T.P., Ryzhkova, E.P. & Netrusov, A.I. 2012. *Propionibacterium freudenreichii* strains as antibacterial agents at neutral ph and their production on food-grade media fermented by some lactobacilli. *Journal of Food Safety*, 32: 48–58.
- Dasen, G., Smutny, J., Teuber, M. & Meile, L. 1998. Classification and identification of propionibacteria based on ribosomal RNA genes and PCR. *Systematic and Applied Microbiology*, 21: 251-259.
- de Freitas, R., Madec, M.N., Chuat, V., Maillard, M.B., Mukdsi, M.C.A., Falentin, H., de Carvalho, A.F., Valence, F. & Thierry, A. 2015. New insights about phenotypic heterogeneity within *Propionibacterium freudenreichii* argue against its division into subspecies. *Dairy Science and Technology*, 95(4): 465-477.
- de Freitas, R., Pinheiro, L.M., Luiz, L.M., Alves, M.P., Valence-Bertel, F., Nero L.A. & de Carvalho, A. F. 2013. Selective enumeration of propionibacteria in Emmental-type cheese using Petrifilm™ Aerobic count plates added to Lithium glycerol broth. *Journal of Dairy Research*, 80: 270-275.

- Duarte, G.F., Rosado, A.S., Seldin, L., Keijzer-Woltersb, A.C. & van Elsas, J.D. 1998. Extraction of ribosomal RNA and genomic DNA from soil for studying the diversity of the indigenous bacterial community. *Journal of Microbiological Methods*, 32:21-29.
- Fan, X., Li, X., Zhang, T., Xu, J., Shi, Z., Wu., Z., Wu, J., Pan, D. & Du, L. 2021. A Novel qPCR method for the detection of *Lactic Acid Bacteria* in Fermented Milk. *Foods*, 10(3066): 1-17.
- Faye, F., Holo, H., Langsrud, T., Nes, I.F. & Brede, A.D. 2011. The unconventional antimicrobial peptides of the classical propionibacteria. *Applied Microbiology and Biotechnology*, 89: 549-554.
- Gaucher, F., Rabah, H., Kponouglo, K., Bonnassie, S., Pottier, S., Dolivet, A., Marchand, P., Jeantet, R., Blanc, P. & Jan, G. 2020. Intracellular osmoprotectant concentrations determine *Propionibacterium freudenreichii* survival during drying. *Applied Microbiology and Biotechnology*, 104(7): 3145-3156.
- Gautier, M. 2014. *Propionibacterium*. *Encyclopedia of Food Microbiology*, 2:232-237.
- Guggisberg, D., Schuetz, P., Winkler, H., Amrein, R., Jakob, E., Fröhlich-Wyder, M.T., Irmeler, S., Bisig, W., Jerjen, I., Plamondon, M., Hofmann, J., Flisch, A. & Wechsler, D. 2015. Mechanism and control of the eye formation in cheese. *International Dairy Journal*, 47: 118-127. <https://doi.org/10.3389/fmicb.2023.1304621>
- Huang, S., Rabah, H., Jardin, J., Briard-Bion, V., Parayre, S., Maillard, M.B., Loir, Y. Le, Chen, X.D., Schuck, P., Jeantet, R. & Jan, G. 2016. Hyperconcentrated sweet whey, a new culture medium that enhances *Propionibacterium freudenreichii* stress tolerance. *Applied and Environmental Microbiology*, 82(15): 4641-4651.
- Joo, S., Park, P. & Park, S. 2019. Applicability of propidium monoazide (PMA) for discrimination between living and dead phytoplankton cells. *PLoS ONE* 14(6): 1-10. e0218924. <https://doi.org/10.1371/journal.pone.0218924>
- Josephson, K.L., Gerba, C.P. & Pepper, L. 1993. Polymerase Chain Reaction detection of nonviable bacterial pathogens. *American Society for Microbiology*, 59(10): 3513-3515.
- Karimi, R., Mortazavian, A.M. & Amiri-Rigi, A. 2012. Selective enumeration of probiotic microorganisms in cheese. *Food Microbiology*, 29:1-9.
- Kulkarni, S.S., Madalgi, R., Ganavalli S., Raghavendra, A. & Kulkarni, D. 2017. Identification of genus *Acinetobacter*: Standardization of in-house PCR and its comparison with conventional phenotypic methods. *Journal of Laboratory Physicians*, 9(4): 279-282

- Kumaran, D., Laflamme, C. & Ramirez-Arcos, S. 2023. A multiphasic approach to solve misidentification of *Cutibacterium acnes* as *Atopobium vaginae* during routine bacterial screening of platelet concentrates using the VITEK 2 system. *Access Microbiology*, 5(6): 1-17.
- Le Maréchal, C., Peton, V., Plé, C., Vroland, C., Jardin, J., Briard-Bion, V., Durant, G., Chuat, V., Loux, V., Foligne, B., Deutch,., Falentin, H. & Jan, G. 2015. Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *Journal of Proteomics*, 112: 447-461.
- Miotto, M., Barretta, C., Ossai, S.O., da Silva, H.S., Kist, A., Vieira, C.R.W. & Parveen, S. 2020. Optimization of a propidium monoazide-qPCR method for *Escherichia coli* quantification in raw seafood. *International Journal of Food Microbiology*, 318: 1-6.
- Nocker, A., Sossa- Fernandez, P. Burr, M.D. & Campher, A.K. 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. *Applied and Environmental Microbiology*, 73: 5111-5117.
- Obradovic, J., Jurisic, V., Tomic, N., Mrdjanovic, J., Perin, B., Pavlovic, S. & Djordjevic, N. 2013. Optimization of PCR conditions for amplification of GC-rich EGFR promoter sequence. *Journal of Clinical Laboratory Analysis*, 27(6):487-493.
- Riedel, K.H., Wingfield, I. & Britz, T.J. 1998. Identification of classical *Propionibacterium* Species using 16S rDNA - Restriction Fragment Length Polymorphisms. *System Applied Microbiology*, 21: 419-428.
- Rossi, F., Torriani, S., Dellaglio, F. 1999. Genus- and species-specific PCR-based detection of dairy propionibacteria in environmental samples by using primers targeted to the genus encoding 16S rRNA. *Applied and Environmental Microbiology*, 65: 4241-4244.
- Schoeman, T. 2001. Characterisation and identification of the active microbial consortium present in Kepi grains. Masters' Thesis, University of Stellenbosch, South Africa.
- Thierry, A., Madec, M.N. 1995. Enumeration of propionibacteria in raw milk using a new selective medium. *Lait*, 75: 315-323.
- Tortone, A.C., Zumárraga, M.J., Gioffr, A.K. & Oriani, D.S. 2018. Utilization of molecular and conventional methods for the identification of nontuberculous mycobacteria isolated from different water sources. *International Journal of Mycobacteriology*, 7(1): 53-60.

- Turgay, M., Bachmann, H., Irmeler, S., Ah, U., Fröhlich-Wyder, M., Falentin, H., Deutsch, S., Jan, G. & Thierry, A. 2020. *Propionibacterium* spp. and *Acidipropionibacterium* spp. Reference Module in Food Science, 1-13.
- Van Wyk, J., Witthuhn, R.C. & Britz, T.J. 2011. Optimisation of vitamin B12 and folate production by *Propionibacterium freudenreichii* strains in kefir. *International Dairy Journal*, 21(2): 69-74.
- Wang, M., Asam, S., Chen, J., Ehrmann, M. & Rychlik, M. 2021. Production of four ¹⁵N-labelled cobalamins via biosynthesis using *Propionibacterium freudenreichii*. *Frontiers in Microbiology*, 12: 1-13.
- Witthuhn, R.C., Schoeman, T. & Britz, T.J. 2005. Characterisation of the microbial population at different stages of kefir production and kefir grain mass cultivation. *International Dairy Journal*, 15: 383-389.
- Yu, S., Yan, L., Wu, X., Li, F., Wang, D. & Xu, H. 2017. Multiplex PCR coupled with propidium monoazide for the detection of viable *Cronobacter sakazakii*, *Bacillus cereus*, and *Salmonella* spp. in milk and milk products. *Journal of Dairy Science*, 100: 7874-7882.
- Yuan, Y., Zheng, G., Lin, M. & Mustapha, A. 2018. Detection of viable *Escherichia coli* in environmental water using combined propidium monoazide staining and quantitative PCR. *Water Research*, 145: 398-407.
- Zi, C., Zengi, D., Ling, N., Dai, J., Xue, F., Jiang, Y. & Li, B. 2018. An improved assay for rapid detection of viable *Staphylococcus aureus* cells by incorporating surfactant and PMA treatments in qPCR. *BMC Microbiology*, 18: 1-18.

CHAPTER 4

THE DETECTION OF *PROPIONIBACTERIUM SPP.* IN KEFIR USING PCR AND MICROBIOLOGICAL METHODS UNDER VARIOUS STORAGE CONDITIONS

Abstract

Kefir is an ancient dairy beverage, obtained by the fermentation of milk with kefir grains to obtain a thin, yoghurt-like product. Kefir grains are a mixture of various mesophilic and symbiotic bacteria and yeast in a polysaccharide matrix. Kefir has many beneficial properties, such as promoting gut health, anti-cancer, and healing properties. In this study, kefir was further enriched with two different *Propionibacterium freudenreichii* strains to further enhance its probiotic and vitamin B producing properties. This work aimed to investigate the survival of propionibacteria in the enriched kefir during storage under refrigerated, frozen and freeze-drying conditions after 7, 14, 21 and 28 days. General and selective media a Polymerase chain reaction (PCR), were used to obtain the enumeration and survival of propionibacteria. Kefir was analysed using Plate count agar, Yeast extract lactate agar and Pal propiobac at 30°C for 6 days under anaerobic conditions. There was a significant difference for the microbial count obtained using PCA than YEL and PAC for KJ15 Log 6.3cfu/mL. There was a significant decrease for total microbial load obtained from day 0 to day 28 for all samples stored under the various storage conditions. A faster and more reliable method, Polymerase chain reaction, was used as an analytic tool to successfully identify the V13 region of the 16S RNA of propionibacteria in the enriched kefir. Propidium monoazide is an intercalating dye which encapsulates the DNA of dead bacterial cells, thus preventing them from multiplying during the PCR protocol. The kefir samples were exposed to Propidium monoazide treatment before extraction of the DNA for the PCR protocol. Conventional culture methods were more labour intensive and time consuming than PCR, rendering PCR a more suitable method to detect PAB in kefir. However, PAB was successfully detected in all kefir variants employed in this study during the 28-day storage period.

4.1. Introduction

The health-related features of Kefir and its capacity to suppress some cancer types, lower cholesterol, and impact the immune system have been the subject of several research, many of which have shown promising outcomes (Kasenkass et al., 2016:339). Kefir is a complex probiotic fermented milk beverage historically valued as a wholesome and nutritious dairy product. However, Morkel (2016:126) showed that kefir was successfully enriched with propionibacteria to enhance vitamin B12 and folate levels. Hence, the microbiota in kefir and propionibacteria can exist in symbiosis.

Over the years, dairy-origin *Propionibacterium* has played an important role in the dairy industry, mainly as a starter culture. However, they can also contribute to food spoilage as potential pathogens. Dairy PAB are typically found in ruminants such as dairy cattle, milk, and milk products and are widely utilised in producing vitamins, cheese ripening, and probiotics (Quigley et al., 2013:664). They are Gram-positive, non-motile, rod-shaped or coryneform, catalase variable organisms (Piwowarek, 2018:516). PAB can grow on a variety of substrates as carbon and energy sources, such as sucrose, lactose, glucose, galactose, inositol, erythritol, and adonitol, which are all examples of sugars and esculine, as well as amino acids, glycerol, and lactate, are its preferred carbon sources (Huang et al., 2016:4645). *Propionibacterium freudenreichii* (PAB) prefers microaerophilic to anaerobic growth conditions, growing optimally at 30-37°C. Optimal growth occurs in a pH range from 6.5 to 7.0. Certain strains of PAB may grow very slowly at a pH of 4.6 but decrease drastically in the range of 5.5 to 5.0 (Carcano et al., 1995:415; Ahmadi et al., 2017:71).

In previous studies, classical propionibacteria were successfully cultured in lactate-rich media like sodium lactate broth (SLB) and Yeast extract lactate agar (YEL-agar), which was incubated under anaerobic conditions using an Anaerocult A system at 30-32°C for 2 to 10 days (Schoeman et al., 2001:17; Tharmaraj et al., 2003:2288; Witthuhn et al., 2005:384; Karimi, 2012:7). However, conventional methods were cumbersome and more rapid, convenient methods of detection of PAB were required. A popular molecular technique in microbial ecology is the genotypic profiling of samples. Primers designed based on aligned 16S rRNA gene sequences of the specific PAB reference strains were used to successfully detect PAB in previous studies (Riedel et al., 1998:427; Morkel et al., 2016:91). However, one of the requirements to get valuable data is to restrict the study to the active component of the microbial communities in the sample. The challenge for DNA-based methods is that DNA is exceedingly consistent and persistent for days to weeks after cell death (Nocker et al., 2007:5111). Propidium monoazide (PMA), an intercalating dye, has been utilised in previous studies to selectively restrict the PCR to the live RNA and DNA of bacteria (Chang et al., 2010:122, Askar et al., 2019:6463).

P. freudenreichii has demonstrated a wide range of probiotic potentials regarding pH and bile salt tolerance, antibacterial, non-haemolytic activities and is considered GRAS (Generally Recognised as Safe). It is also susceptible to the antibiotics commonly used in medicine. Additionally, this could spark research into the origins of dairy propionibacteria and contribute to clarifying their ecology (Chamlagain et al., 2016:3; Kumar et al., 2020:35). Thus, propionibacteria are of great economic importance to both the food industry as well as the medical profession and rapid methods are required for their enumeration and detection. Some health benefits may be lost in commercial production due to reduced microbial diversity and a lack of beneficial exopolysaccharides. In this study, kefir was enriched with PAB to further increase its probiotic potential, vitamin B12 and folate content. In the previous chapter, PAB

was successfully grown on YEL, PCA and Pal propiobac. A PCR protocol was also successfully optimised to detect PAB from a freeze-dried sample. This study aimed to use these media and an optimised PCR protocol to determine PAB's viability in kefir under storage conditions such as refrigeration, freezing, and freeze-drying over time.

4.2. Materials and Methods

4.2.1 Source of materials

Kefir grains were obtained from West Coast Cultures in the Western Cape, South Africa. PAB J15 was obtained from a subculture of Damlitter cheese. UHT Clover full-cream milk was purchased locally and has a fat level of 2-5%. The second culture, PAB J17, was obtained from an ATCC13637 culture. Both cultures were received in freeze-dried form from the culture collection of Cape Peninsula University of Technology (CPUT), South Africa (Morkel, 2016:31).

4.3 Activation of kefir grains and PAB

Kefir grains were mixed into 100 mL milk and incubated in an orbital shaking incubator at 25°C for 8-12 hours. The kefir grains were separated from the liquid milk, by straining it into a clean, sterile Schott bottle with a sterile stainless-steel strainer for five minutes. Using a precision pipette, the PAB cultures were rehydrated by adding 1 mL of sterile Ringer's solution (Merck, Johannesburg) to the vial containing the freeze-dried culture (Van Wyk et al., 2011:70).

4.4 Production of Kefir Control (KC) and kefir inoculated with PAB

Figure 4.1 briefly outlines the kefir production process adapted from Van Wyk et al., 2011:70 and Morkel, 2016:110. Kefir grains were inoculated into milk to a final concentration of 10% w.v⁻¹ in an empty sterile 1000 mL Schott bottle. Reconstituted freeze-dried PAB culture (8 vials) was gently mixed by swirling into 80 g activated kefir grains in a clean, sterile 1000 mL Schott bottle. Aseptically, 100 mL of milk was added, and the suspension was incubated at 30°C for 4 days. Thereafter, 800 mL milk was added and incubated at 30°C for 4 days in an orbital shaking (MRC, United Scientific). The kefir grains were separated from the liquid milk by straining them into a beaker for 5 minutes using a sterile strainer. The kefir was discarded, and the process was repeated by adding 8 vials of reconstituted PAB culture into the grains and gently mixed. Eight hundred mL milk was added to the suspension and incubated at 30°C for 4 days in an orbital shaking incubator. Using a sterile strainer, the kefir grains were separated from the kefir by straining for 5 minutes. The kefir obtained was discarded. This process was repeated for a final time by mixing 8 vials of reconstituted PAB culture to the

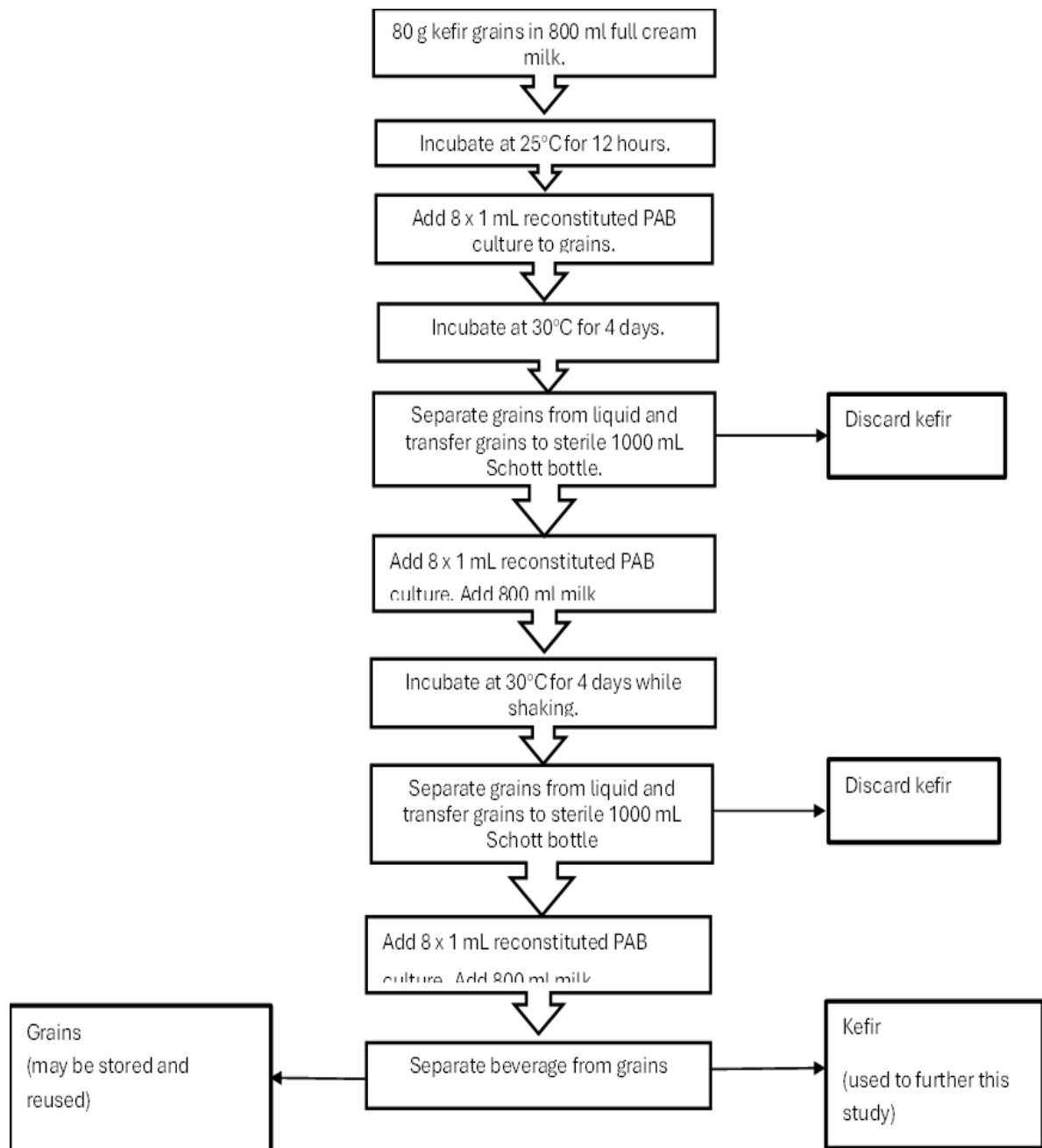


Figure 4.1 Schematic diagram of the production of kefir

grains. Eight hundred mL milk was added to the suspension and incubated at 30°C for 4 days in an orbital shaking incubator. The grains were separated from the beverage in 250 mL Schott bottles and stored for reuse. The liquid was retained and used to complete the analysis for this study. Kefir inoculated with J15 and J17 was labelled KJ15 and KJ17, respectively. Kefir, not inoculated with PAB, was labelled Kefir Control (KC).

4.5 Storage of kefir samples

The prepared 800 ml kefir was divided into three smaller batches containing 250 mL kefir each. These 250 mL kefir aliquots were further divided into five samples of 50 mL each. The

remaining 50 mL was analysed as a sample reference for day 0. Five samples were stored at 5°C, five at -20°C, while the third set was freeze-dried using a Virtice benchtop freeze-dryer before storage at -20°C. The samples were stored in airtight plastic centrifuge jars at the respective storage conditions and analysed for total viable counts and PCR at intervals of 7 days: 0, 14, 21 and 28 days. The refrigerated sample was analysed directly from the refrigerator, while the frozen sample was thawed at room temperature for 5 hours before analysis. The freeze-dried sample was rehydrated by adding sterile milli Q water to the freeze-dried kefir sample to obtain a volume of 50 mL. This suspension was mixed thoroughly by shaking and used for analyses described in this study.

4.6 Preparation of growth media and reagents

4.6.1 Preparation of YEL agar

YEL agar was prepared using distilled water and consisted of gL⁻¹ yeast extract 5 (Biolab, Merck Gauteng, South Africa), peptone powder 2 (Biolab), KH₂PO₄ 10 (Merck, Gauteng, SA), sodium lactate syrup (50% v/v) 20 mL⁻¹ (Sigma Aldrich, Germany), and Tween 80 20 mL⁻¹ (Saarchem, Merck SA) and bacteriological agar 15 (Biolab). The pH was adjusted to pH 7 using 1M NaOH with a pH meter. The media was dissolved in distilled water by boiling, autoclaved for 15 minutes at 121°C and cooled to 48°C in a water bath (Mettler, Lasec, Cape Town) (Van Wyk et al., 2011:70).

4.6.2 Preparation of Plate Count Agar (PCA)

PCA (Biolab, Merck), was prepared by weighing 23 g of dehydrated media into 1 L distilled water in a Shott bottle and boiled until all solids were dissolved, autoclaved at 121°C for 15 minutes and cooled to 48°C.

4.6.3 Preparation of Pal propiobac

Pal propiobac base media (Standa Laboratoires Laboratories, France) was melted in a boiling water bath until the agar had melted and cooled to 48°C. Per the manufacturer's instructions, the selective supplement was reconstituted with 7 mL sterile distilled water, mixed gently, and dispensed evenly into six bottles of molten agar before use.

4.6.4 Preparation of Quarter strength ringer's solution

Quarter strength ringer's solution (Merck, Cape Town, South Africa) was prepared by dissolving 1 tablet into 500 mL distilled water. The Ringer's solution was dispensed into 90 mL and 9.0 mL aliquots and autoclaved at 121°C for 15 minutes.

4.7 Preparation of PCR reagents

4.7.1 Tris/Borate/EDTA (TBE) buffer

TBE (Cat No 50843, Lonza, South Africa) buffer 0.5 X concentration was prepared in a Schott bottle by adding 50 mL concentrated 10 X TBE buffer with 950 mL MilliQ water and mixed by inverting the bottle several times.

4.7.2 Preparation of 1% Agarose gel tray with wells

Agarose gel was prepared by dissolving 1 g of agarose powder into 100 mL 0.5 x TBE buffer by heating in a microwave on high power until all the granules were dissolved. The 1% agarose gel was cooled in a water bath (Memmert, Lasec, South Africa) set at 55°C. The agarose gel was gently mixed with five µL of ethidium bromide (Cat No BP1302-10, Jansen Pharmaceuticals, Thermofisher, South Africa) before casting into a gel mould containing a comb. The gel was allowed to solidify at room temperature. The combs were carefully removed after the solidification of the gel.

4.7.3 Preparation of primers

The primers (Integrated DNA Technologies, Whitehead Scientific, South Africa) were obtained in the form of a transparent, thin film and named Prop 1 and Prop 2 primers and were dissolved in 349 µL and 390 µL TE (10 mM Tris pH8, 1 mM EDTA) (Cat No 51235, Accugene, Lonza, Whitehead Scientific, South Africa) respectively to obtain 100 µM suspension.

4.8 Treatment of kefir samples with PMA reagent

Kefir samples were treated with Blu-V PMA (Cat No296015, Qiagen, Whitehead Scientific, SA). One mL of kefir was each transferred to a sterile 2 mL safe seal microtube and centrifuged for 5 minutes at 13,000 x g. After the supernatant was discarded, the pellet was re-suspended in 500 µL buffer EB and vortexed vigorously. A volume of 10 µL PMA reagent was added to the samples and vortexed for 5 to 6 seconds. The tubes were wrapped in foil and incubated for 10 minutes at room temperature in the dark. The samples were placed into an incubation box which contained two bright 230 V and 250 W halogen lights for 10 minutes. The samples were removed, placed on ice and vortexed to allow homogenous sample illumination at two-minute intervals.

The samples were centrifuged at 13,000 x g for 5 minutes, and the supernatant was discarded. The sample was ready for DNA isolation, as described by the protocol of the Mobio Powerfood Microbial DNA isolation kit (Cat No# 21000-50, Whitehead Scientific, Cape Town, South Africa).

4.9 Isolation of DNA of PMA-treated kefir samples

The PMA-treated kefir sample (1.8 mL) was added to a 2 mL collection tube and centrifuged at 13,000 × g for 1 minutes at room temperature. The supernatant was discarded, and the sample was centrifuged again under the same conditions. Any remaining supernatant was removed with a pipette tip. The pellet was re-suspended in 450 µL of solution MBL. The re-suspended cells were transferred to a micro-bead tube. The micro-bead tube was vortexed horizontally at maximum speed for 10 minutes and again for 1 minutes .

The supernatant was transferred to a clean 2 mL collection tube and briefly mixed with 100 µL of Solution IRS by vortexing. The mixture was incubated at 4°C for 5 minutes, then centrifuged at 13,000 × g for 1 minute. The entire volume of the resulting supernatant was transferred to another clean 2 mL collection tube, followed by the addition of 900 µL of Solution MR, and vortexed thoroughly. The mixture was then loaded onto a spin filter and centrifuged at 13,000 × g for 1 minute. This step was repeated until all supernatant had been processed through the spin filter. The final supernatant was discarded, and the spin filter basket was transferred to a clean 2 mL collection tube.

Solution PW was mixed thoroughly before adding 650 µL of this solution into the tube and centrifuged at 13,000 × g for 1 minutes at room temperature, followed by adding d 650 µL of ethanol to the tube. The suspension was centrifuged at 13,000 × g for 1 minutes , the flow-through was discarded, and the pellet was centrifuged at 13,000 × g for 2 minutes to remove the residual wash. The spin filter basket was placed into a clean 2 mL collection tube provided. Solution EB with a volume of 100 µL was placed into the centre of the white filter membrane and centrifuged at 13,000 × g for 1 minutes and the spin filter basket was discarded. The extracted DNA from each sample was either used immediately for further analysis in this study or stored at -20 °C for future use.

4.10 PCR analysis of kefir

4.10.1 PCR

The PCR protocol optimised in Chapter 3 was used to detect *P. freudenreichii* in kefir. The amplification of the V3 variable region within the 16S rRNA gene was obtained using the primers known as Prop1 (5'GATACGGGTGACTTGAGG-3') and Prop2 (5'GCGTTGCTGATCTGCGATTAC-3'). All PCR amplifications were prepared in duplicate on ice. The PCR reaction was prepared to a final volume of 50 µL, which contained: 25 µL Ampliqon Master mix (Lasec), 1 µL Prop1, 1 µL Prop2, 17 µL PCR grade water and 6 µL DNA sample. The positive control PAB DNA template contained no kefir. A negative blank reaction was prepared with each PCR run, where the DNA template was substituted with PCR grade water. The Taq DNA Polymerase master-mix contained 1.5 mM Ampliqon Taq DNA Polymerase, the NH₄⁺ buffer system, dNTPs and magnesium chloride. The master mix is a-

ready-to-use system. The PCR reaction mix was amplified in 0.2 mL thin-walled tubes using the Eppendorf Master cycler (Merck, Germany).

The PCR amplification conditions consisted of initial denaturation at 95°C for 5 minutes followed by 98°C for 20 s, annealing at 52°C for 15 s and chain elongation at 72°C for 1 minutes to complete the cycle, followed by a chain elongation at 72°C for 5 minutes. The cycle was completed by a final chain elongation at 72°C for 5 minutes . The amplified PCR products were cooled to 4°C.

4.10.2 Inoculation of wells

The gel was placed into the electrophoresis tray without removing the gel from the mould. The tray was filled with 0.5 × TBE buffer until the gel was covered entirely. The first well of the gel was inoculated with a DNA marker, which consisted of 4 µL 1 kB DNA ladder (Promega, Anatech) and 1 µL bromophenol loading dye (Ref# G190, Promega. Anatech). PAB bands must fluoresce in the region of 750 Base pairs (BP) when comparing the fluorescent bands to the DNA ladder as this is the size of the DNA fragment of interest. Three µL of amplified DNA product was applied to each well. Before electrophoresis was started, a volume of 5 µL of ethidium bromide was added to the bottom of the tray.

4.10.3 Electrophoresis

The gel was electrophoresed at 90 V for 80 minutes until the sample had migrated at least 10 cm. The gel was carefully removed from the box, placed into the Uvidoc box (Uvitech, Whitehead Scientific, South Africa), and visualised under ultraviolet light (UV) light. The visualised gel was imaged.

4.10.4 Enumeration of kefir samples

To quantify the total viable counts in kefir, serial dilutions were using quarter strength Ringer's solution. Aliquoted 1 ml dilutions were plated into sterile petri dishes and PCA, YEL agar and Pal propiobac was added aseptically. All the plates were incubated anaerobically at 30°C for 6 days. After incubation, colonies were counted.

4.11 Data Analysis

Microbial load was expressed as mean ± standard deviation of triplicate measurements of microbial load. Multivariate analysis of variance (MANOVA) was performed with SPSS® version 29.0 (SPSS® Inc., Chicago, Illinois, USA) to estimate the main effects of sample, treatment, storage of 28 days and their interaction. Duncan's multiple-range test was used to separate the means when differences exist at the 5% confidence level.

4.12 Results and Discussion

4.12.1 Enumeration of kefir on conventional media

Kefir is a fermented milk drink produced with grains composed of a diverse and complex matrix of bacteria and yeasts embedded symbiotically in a polysaccharide-protein matrix (Kalamaki et al., 2020:138). In this study, kefir was inoculated with *Propionibacterium freudenreichii* (PAB). Another kefir sample was not inoculated with PAB and labelled the control (KC) sample. Due to the sample composition of dairy products like kefir, traditional plating methods for identifying dairy PAB have been reported as difficult to culture (Bucher et al., 2021:4308). PAB were successfully enumerated in kefir using Pal propiobac, however it was challenging to identify colonies as PAB using PCA and YEL. Variable levels of selectivity across the multiple media were observed, with PAL propiobac suppressing the growth of various bacteria. Chromogenic media like PAL propiobac was easy to read, straightforward and more specific than classical agar media like YEL agar and PCA, which need further identification methods. This aligns with findings by Turgay et al. (2016:38), where it was stated that conventional methods to detect PAB were very time consuming, labour intensive and inaccurate. Chromogenic media was relevant for use in microbiological controls performed by the dairy industry to detect PAB in this study. This aligns with findings from Sohier et al. (2014:5), who concluded that chromogenic media was straightforward and more specific than classical agar media. Pal propiobac changed colour from its original violet to yellow due to acid production. PAB colonies that grew on the surface of the medium were shiny, convex and beige with darker grey centres. PAB colonies in Pal propiobac ranged from 1 to 2 mm in size with yellow discoloration in the media around the colonies. These findings aligned with previous studies by Theiry et al. (1995: 317) and Schoeman (2001:52). It was easy to distinguish PAB from other bacteria on plates with lower total bacterial counts on Pal propiobac. Non-PAB colonies were not accompanied by the yellow discolouration in the media, as shown in Figure 4.2a. However, in Figure 4.2b, it was difficult to distinguish PAB from other bacteria when the plates contained a higher number of PAB populations as the media in the entire plate was discoloured to yellow due to the higher amount of PAB present in the sample. Gautier (2014:233) also reported PAB visible on Pal Propiobac medium as brown colonies with a diameter of more than 0.5 mm, encircled by a yellow region, reportedly due to the pH reduction brought on by glycerol fermentation.

Figure 4.3a shows the total viable count of the control kefir sample not inoculated with PAB before fermentation. Various types of bacteria are evident from the different colony morphologies observed on the plates, except for the dark cream to grey colonies, which represent PAB colonies, as shown in Figure 4.3b. Figures 4.3c and 4.3d were plated to a dilution of 1×10^{-6} and 1×10^{-7} respectively, showing a difference in PAB colonies' size and colour intensity. PCA is a general growth media and supports PAB growth but is not selective for PAB in kefir, as a variety of other bacteria was evident. Kefir consists of various yeasts and

lactobacilli, which are also supported by PCA and thus will not be suppressed. Amongst other bacterial flora, PAB also appeared as round dark cream colonies in PCA. Beloti et al. (1999:139) and Zhang et al. (2021:2875) reported that it is challenging to enumerate milk samples in PCA due to the lack of contrast between the media and the colony, thus leading to possible inaccurate total vial counts. Zhang et al. (2021:2935) recommended that imaging analysis tools be used for microbiological counting, while Beloti et al. (1999:139) proposed the use of dyes like 2,3,5-triphenyltetrazolium chloride (TTC) added to PCA to create contrast between the colonies and food particles. TTC is recommended for milk samples as the opacity in the media due to the added sample makes counting inaccurate. TTC could be added to PCA or in the rehydratable film.

PAB on YEL agar appears as larger dark cream to grey, spherical-shaped colonies from 1.5 to 2 mm in size. However, this media does not sufficiently suppress non-PAB colonies' growth, as there is evidence of small and large white colonies. This aligns with findings from Dasen, 1998:11. There are also white colonies surrounded by discolouration in the media. These colonies can be yeasts and or lactobacilli. Figures 4.4a, b and c show various morphological characteristics of the colonies obtained in YEL agar. The findings obtained in this study were in line with the description of PAB colonies using Sodium Lactate agar under anaerobic conditions at 30°C (Kumar et al. (2020:31) and (Tharmaraj et al., 2003:2293).

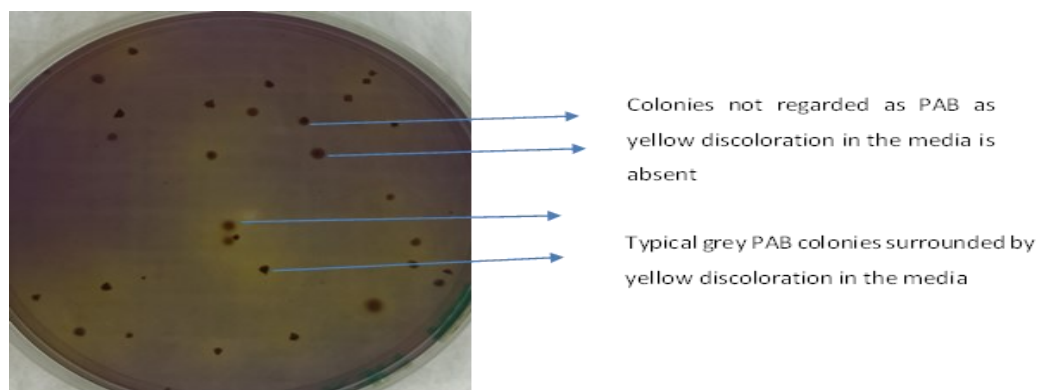


Figure 4.2a PAB in kefir on Pal propiobac

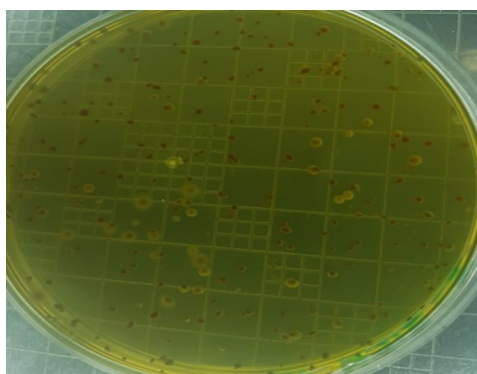


Figure 4.2b High number of PAB in Pal propiobac

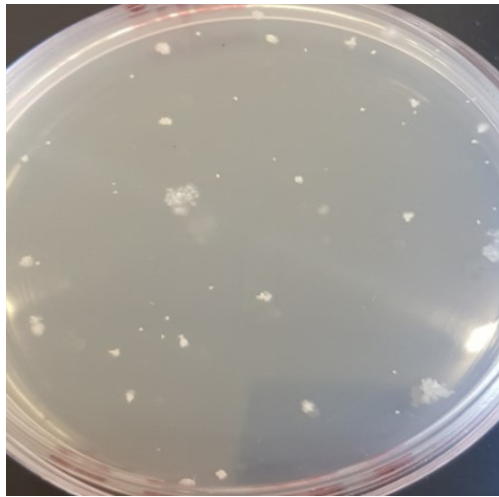


Figure 4.3a Kefir control in PCA

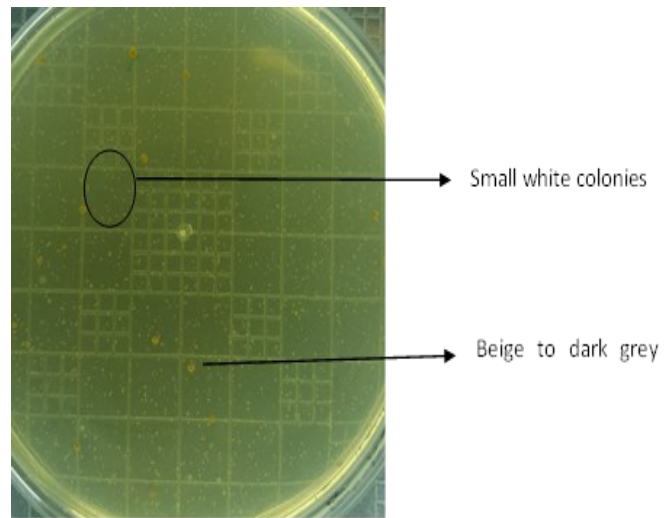


Figure 4.3b Kefir with PAB in PCA

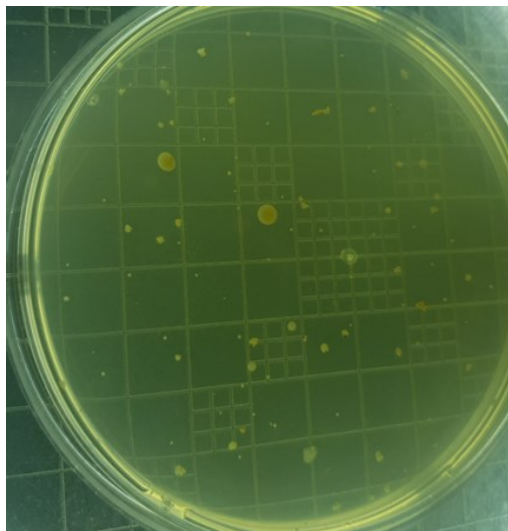


Figure 4.3c Kefir in PCA (Dilution 1×10^{-7})

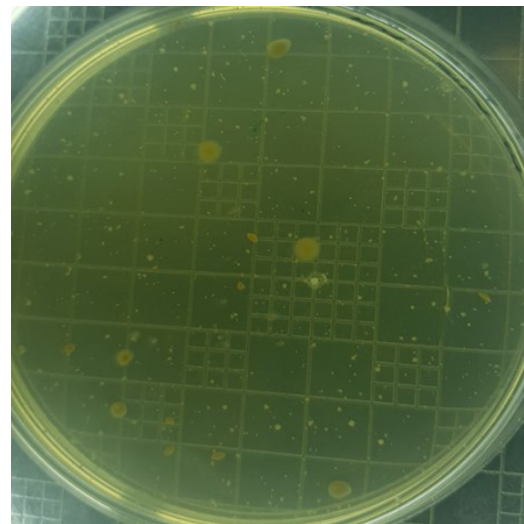


Figure 4.3d Kefir in PCA (Dilution 1×10^{-6})

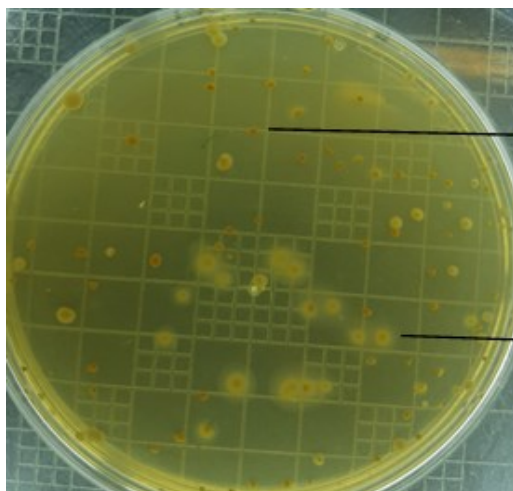


Figure 4.4 a Kefir in YEL agar

Dark grey colonies

White colonies with
discoloration in media

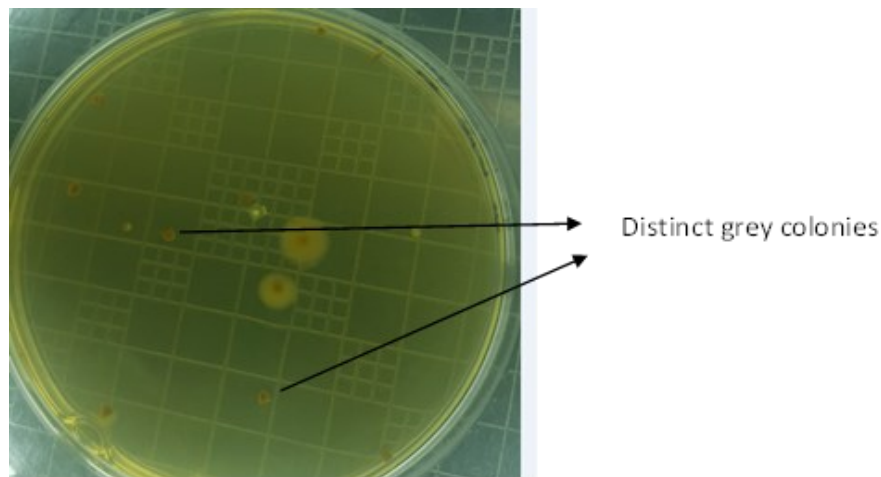


Figure 4.4 b Kefir in YEL agar

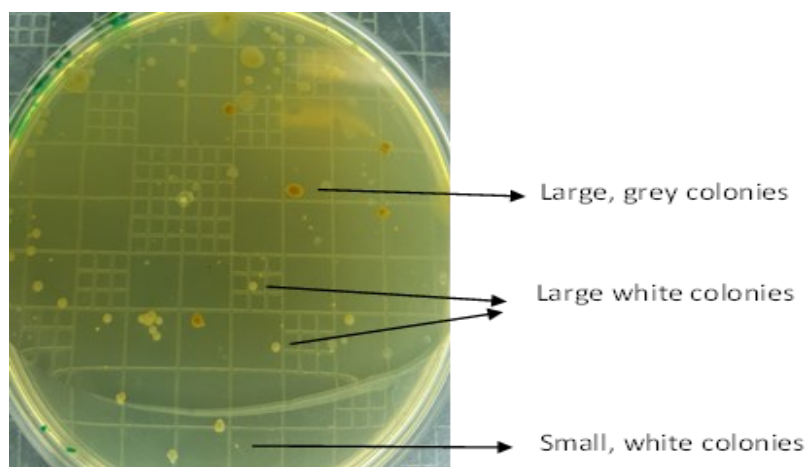


Figure 4.4 c Kefir in YEL agar

4.12.2 Effect of PAB type, storage type and storage time on the kefir microbial load

The main effect of PAB type was significant ($p < 0.001$) on the microbial count of PAB from Pal propiobac but not on the total microbial count from PCA and YEL agar. The main effects of both storage type (refrigerated, frozen, freeze-dried) and storage time on the microbial load obtained were significant ($p < 0.05$) on PCA, YEL and Pal propiobac ($p < 0.001$). The interaction between the kefir and storage type was significant ($p = 0.0001$) for Pal propiobac only. The interaction between PAB type and storage types was significant ($p = 0.0001$) for Pal propiobac. The interaction between PAB type and storage time, storage type and time were significant ($p < 0.05$) for YEL and Pal propiobac. The interaction between sample, treatment and day was significant ($p < 0.05$). In this study, PAB was observed in only kefir samples inoculated with the target organism during production. Table 4.1 depicts the main effect of the total viable count in kefir (KC, KJ15 and KJ17) in comparison in PCA, YEL and characteristic colonies of PAB in Pal propiobac. Microbial load obtained on

Table 4.1 Main effect of PAB inoculation on the kefir microbial load¹

Kefir	Total microbial load in kefir (Log cfu/mL)		
	TVC in PCA	TVC in YEL-agar	PAB in PAL
KC	5.09 ± 3.80 ^a	3.96 ± 3.59 ^a	0.00 ± 0.00 ^a
KJ15	4.41 ± 3.68 ^a	3.37 ± 3.44 ^a	2.27 ± 3.19 ^b
KJ17	4.45 ± 3.72 ^a	3.77 ± 3.58 ^a	2.23 ± 3.15 ^b

The values are mean ± standard deviation of three independent trials. KC = Kefir control, KJ15 = Kefir inoculated with J15 and KJ17 = Kefir inoculated with J17. TVC = Total viable count, PCA = Plate count agar, YEL = Yeast extract lactose agar, Pal = Pal propiobac, PAB = *Propionibacterium freudenreichii*. No significant ($p > 0.05$) difference exists for the values with the same superscript in the same column.

TVC obtained on PCA and YEL counted total colony forming units in kefir, whereas only characteristic colonies were counted on Pal propiobac. It was difficult to distinguish and identify PAB on YEL agar and PCA as further confirmation of suspect colonies gave variable microbial load with PCR as a detection method for PAB. Presumptive colonies on Pal propiobac were confirmed with PCR and microscopic examination (findings not shown). There was no significant difference for the TVC obtained on PCA and YEL-agar for all three samples. However, there was a significant difference ($p \leq 0.05$) in the counts obtained on the Pal propiobac for KC compared to the microbial load for KJ15 and KJ17. There was no growth of PAB for KC on Pal propiobac, but there was growth for KJ15 and KJ17 on Pal propiobac. This is because no PAB was naturally present in the KC sample. PAB J15 and J17 was inoculated into kefir grains during the production of kefir, KJ15 and KJ17 respectively. PAB was detected in KJ15 and KJ17. This is in line with a previous study which found that PAB was only present in the treated samples and not in the kefir control sample where no PAB was included during fermentation. (Morkel, 2016: 92).

4.12.3 Effect of storage time on the kefir microbial load based on PAB type

The individual rates of microbiological changes were compared in this study. The total viable count (TVC) was expressed as cfu/mL. As shown in Figure 4.5a, the KC microbial count on PCA exhibited a significant decrease ($p < 0.05$) in microbial counts from day 0 (9.17 Log cfu/mL) to day 14 (1.39 Log cfu/mL), followed by a significant increase ($p < 0.05$) by day 21 (5.0 Log cfu/mL). After that the microbial load decreased significantly ($p < 0.05$) to day 28. However, there was no significant difference ($p > 0.05$) for day 7 (6.41 Log cfu/mL) and 21 (5.0 Log cfu/mL). YEL showed a similar significant decrease ($p < 0.05$) in the total viable count as obtained in PCA from day 0 (8.07 Log cfu/mL) to day 14 (1.33 Log cfu/mL). This was followed by a significant increase ($p < 0.05$) to day 21 (4.82 Log cfu/mL) with no significant decrease (p

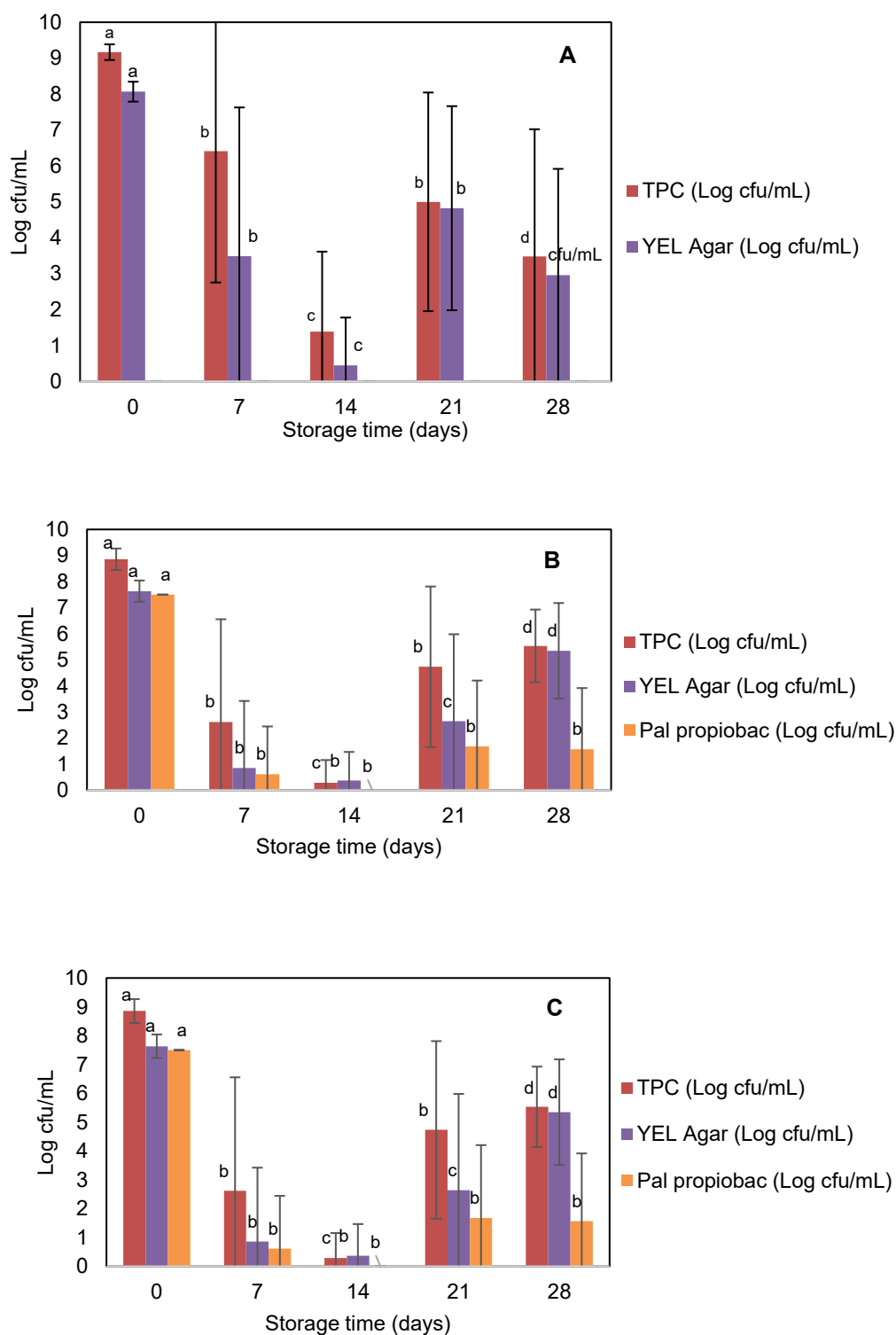


Figure 4.5: The values are mean $\pm$ standard deviation of three independent trials showing the succession microbial counts (Log cfu/mL) over the storage time of 28 days for: (A) KC = Kefir control, kefir not inoculated with PAB. (B) KJ15 = Kefir inoculated with J15h PAB. (C) KJ17 = Kefir inoculated with J17 PAB, TPC = Total plate count, YEL=Yeast extract lactate, PAL = Pal propiobac. There is no significant ($p > 0.05$) difference for the values with the same letter in the same set.

> 0.05) to day 28 (2.96 Log cfu/mL). Pal propiobac showed no growth from day 0 until day 28, as no PAB was added to the KC sample. There was also no PAB naturally present in the kefir.

The TVC obtained for KJ15 on PCA, as indicated in Fig 4.5b, showed a significant ($p < 0.05$) decrease from day 0 (8.86 Log cfu/mL) to day 14 (0.29 Log cfu/mL), followed by a significant ($p < 0.05$) increase in day 28 (5.53 Log cfu/mL). KJ15 on YEL agar also showed a similar pattern as for PCA, where a significant ($p < 0.05$) decrease for TVC obtained for day 0 (7.63 Log cfu/mL) till day 14 (0.37 Log cfu/mL), followed by a significant ($p \leq 0.05$) increase in day 21 (2.64 Log cfu/mL). After that, the microbial load increased significantly ($p \leq 0.05$) to day 28 (5.34 Log cfu/mL). Pal propiobac for KJ15 showed a significant ($p < 0.05$) decrease between day 0 (7.50 Log cfu/mL) to day 14, where no growth was detected. The increase in microbial counts until day 21 (1.68 Log cfu/mL), followed by a decrease at day 28 (1.57 Log cfu/mL) was not significant ($p > 0.05$).

PCA for KJ17 in Figure 4.5c showed a significant ($p < 0.05$) decrease between day 0 (8.23 Log cfu/mL) and day 14 (1.41 Log cfu/mL). There was no significant difference ($p > 0.05$) on day 21 (4.61 Log cfu/mL), followed by a decrease on day 28 (4.17 Log cfu/mL), which was also not significant. Total viable count on YEL agar showed a significant ($p < 0.05$) decrease between day 0 (8.17 Log cfu/mL) and day 14 (0.44 Log cfu/mL) followed by a significant ($p < 0.05$) increase on day 28 (4.26 Log cfu/mL); however, there was no significant difference on day 21 (2.69 Log cfu/mL) and day 28. Colonies counted on Pal propiobac for KJ17 showed a significant ($p < 0.05$) decrease between day 0 (6.57 Log cfu/mL) and day 14, where no growth was observed. This was followed by a non-significant increase to day 21 (0.22 Log cfu/mL), followed by a significant ($p < 0.05$) increase on day 28 (1.76 Log cfu/mL). Mesophilic microbiological counts, most of which were reported as lactic acid bacteria and yeasts in kefir and milk products, obtained on day 0 were in line with initial counts of up to 10^8 cfu/mL and 10^7 cfu/mL in a previous study (O'Brien, 2016:7045). Overall, TVC obtained for all three sample interactions with storage time over 28 days showed significant decreases ($p < 0.05$) for all the kefir samples in PCA, YEL- and Pal propiobac. For (KC) in Figure 4.6a, there was no naturally occurring PAB in the kefir control and no PAB was added during the kefir fermentation process. Therefore, there were no colony-forming units in the Pal propiobac for the three storage types. Figure 4.6a shows that for PCA, there was no observable significant difference in the cell counts for the three storage types. On the Yel-agar there was no significant difference ($p > 0.05$) in the cell counts for the refrigerated (5.07 Log cfu/mL), frozen (3.21 Log cfu/mL) and freeze-dried (3.59 Log cfu/mL) treatments.

In Figure 4.6b the refrigerated (6.32 Log cfu/mL) KJ15 was significantly ($p < 0.05$) different to the frozen (3.21 Log cfu/mL) and freeze-dried (3.69 Log cfu/mL) kefir. No significant difference was found between the treatments on YEL-, (2.58; 2.71, 4.82 Log cfu/mL), respectively for the frozen, freeze-dried and refrigerated samples. KJ15 on Pal propiobac showed (1.5, 1.50, 3.81 Log cfu/mL), respectively for the same storage conditions.

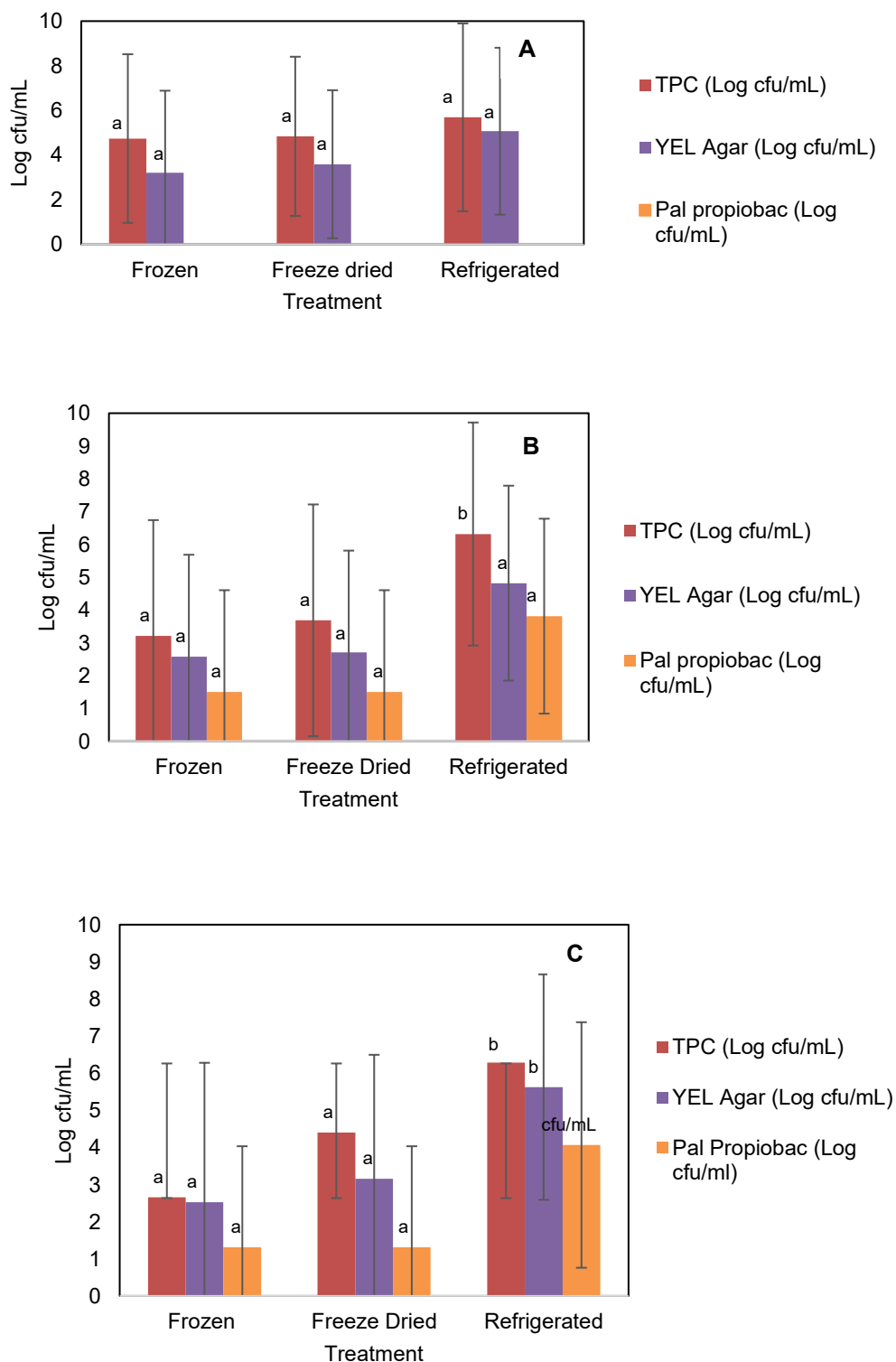


Figure 4.6 Mean microbial succession counts (cfu/ mL) showing the effect of the interaction of: (A) Kefir control, (B) KJ15, (C) KJ17 with treatment refrigerated, frozen and freeze-dried. (A) KC = Kefir control, kefir not inoculated with PAB. (B) KJ15 = Kefir inoculated with J15h PAB. (C) KJ17 = Kefir inoculated with J17 PAB TPC = Total plate count, YEL=Yeast extract lactate , PAL = Pal propiobac. There is no significant ($p > 0.05$) difference for the values with the same letter in the same set.

It was observed in Figure 4.6c that for KJ17 on PCA, no significant difference was found for the frozen (2.67 Log cfu/mL) and refrigerated (6.29 Log cfu/mL) treatments; however, there was a significant ($p < 0.05$) difference after they were freeze-dried (4.40 Log cfu/mL). The YEL agar showed no significant difference for the refrigerated (5.63 Log cfu/mL) and freeze-dried (3.15 Log cfu/mL) kefir, but a significant difference ($p < 0.05$) existed in the frozen (2.53 Log cfu/mL) kefir. On Pal propiobac, the frozen (1.33 Log cfu/mL) and freeze-dried (1.31 Log cfu/mL) kefir differed significantly ($p < 0.05$) compared to the refrigerated (4.07 Log cfu/mL). The refrigerated treatment was the optimum treatment for the storage of kefir. This is in line with the report of Teijeiro et al. (2018:169,173), which states that refrigeration treatment was a better alternative for the storage of dried products.

It was challenging to enumerate successfully from a frozen sample. A study by Clark et al. (2014:538) observed a potential decrease in viable organism concentration during freezing, storage and thawing, however, the micro-organisms found were robust before and after thawing. Slow-growing bacteria like propionibacteria require longer incubation detection times due to their slow generation time. Among classical nutrients, Pal propiobac consists of lithium, inhibiting the growth of various lactic acid bacteria and a cocktail of antibiotics to inhibit Gram-negative organisms. Pal propiobac also contains glycerol, which further decreases the pH of the medium.

However, it has been reported that Pal PAB does not grow well on solid media under reducing pH conditions (Bücher et al., 2021:4308). In this study, the pH of kefir after four days was between 3.2 and 3.7 after the three treatments, respectively (finding not shown). A previous study by Abdel-Mogheith et al. (2017:760) showed that kefir has a pH of 4.2 to 4.6 after 48 hours fermentation. It was proven that there was a significant ($p < 0.05$) decrease of pH as fermentation time increased, where a total of 12% after 12 hours and 43.4% reduction in pH was recorded after 48 hours fermentation (Anyiam et al., 2023:4). This is in line with findings from Teijeiro et al. (2018:169), who recommended the use of spray-drying as an alternate drying process for the preservation of probiotics as it is rapid, cost-effective and yields better end-product in terms of attributes like moisture control, flowability and uniform shape. Furthermore, it will be a suitable method for mass production.

A study conducted by Coronas et al. (2023:14) stated that due to factors like the denaturation of proteins, cell membrane and DNA damage, cell viability was affected during freeze-drying. It was observed that the PAB isolated employed in their study proved resistant to freeze-drying as cell counts of 8.5 log/ml were obtained. Reportedly, this result was also 88% higher than the counts obtained in an earlier study. It was proven that PAB is able to adapt different salt concentrations of different culture media. The stress tolerance of PAB can be greatly enhanced by adapting growth conditions, particularly by fine tuning trehalose and glycine betain. While such adaptations can improve survival under specific industrial

conditions, it may not successfully protect tolerance against all technological stresses (Gaucher et al., 2020:3154).

4.13 Molecular detection of PAB in kefir using polymerase chain reaction

In this study, PAB was detected using conventional PCR combined with propidium monoazide (PMA) treatment of all kefir samples inoculated with PAB during production thereof. This was evident by the PCR products, which were amplified in the 750bp region as shown in Figures 4.7a, b and c, as observed on days 0, 14 and 28, respectively. Thus, the successful amplification of the targeted sequence of the 16S ribosomal DNA gene of the *P. freudenreichii* was possible. The method successfully identified the presence of PAB in kefir samples treated with PMA, supporting the use of the PMA with PCR to detect PAB in kefir. The PCR products from all the kefir control (KC) samples showed no bands in the 750 bp region. The TVC of the conventional culture methods showed no PAB growth in the KC sample. This indicated that no PAB was present in these samples. There was a significant decrease in the TVC obtained for KJ15 and KJ17 for PCA and YEL agar from day 0 to day 28. There was also a significant decrease for the PAB results obtained on Pal Propiobac media throughout this storage period. The rapid evolution and integration of molecular, genomic and viability-specific methods has enhanced detection sensitivity while also enabling the differentiation between viable and non-viable cells in complexed food matrices like dairy products (Farid et al., 2025:8). It has been proven that PCR, specifically qPCR assays in conjunction with PMA, is a sensitive and precise method for identifying and amplifying live bacterial cells (Guo et al., 2024:10). The non-viable cells were entirely masked by the PMA treatment before the extraction of the DNA. The details of sample treatment using surfactants like intercalating dyes to enhance the effectiveness of dead cell DNA removal in PCR essays were proven by Zi et al. (2018:6). Zheng et al. (2014:1625) concluded that microscopic and phenotypic culture methods for detecting and detecting bacteria are often labour-intensive, time-consuming and tedious. However, as established in this and previous studies, conventional methods and PCR methods can be used in conjunction with each other (Kralik & Ricchi, 2017:3).

While the PCR detected the presence or absence the DNA of live PAB cells, the conventional methods enabled their quantification in kefir. The conventional culturing methods also showed no PAB growth in the KC sample. There was a significant decrease in TVC for KJ15 and KJ17 on PCA and YEL agar as well as for the PAB on Pal propiobac from day 0 to day 28. Thus, this study demonstrated that the application of PMA treatment effectively masks non-viable cells, while ensuring the selective removal of these dead cell DNA and further enhancing the accuracy of PCR assays.

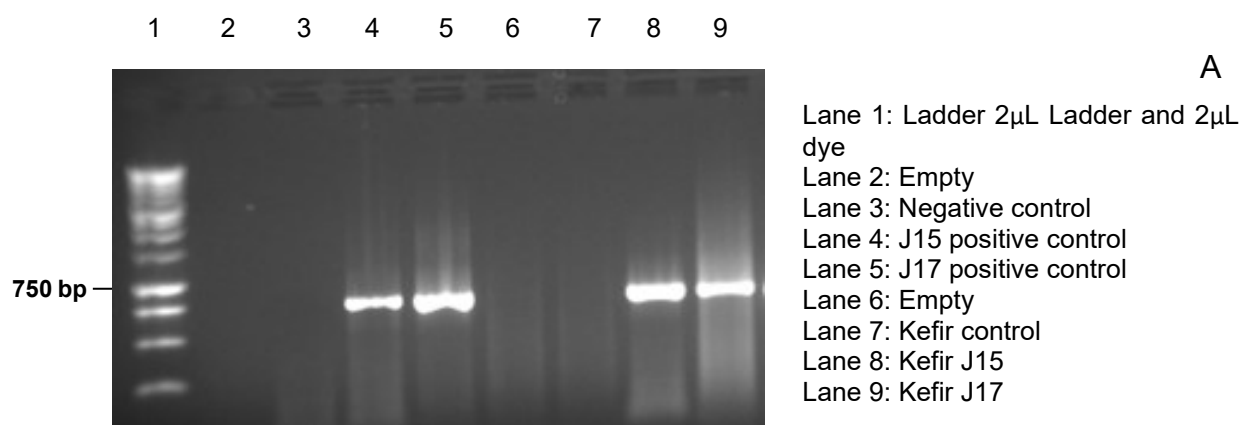


Figure 4.7 PCR images for kefir under various storage conditions: (A) Day 0, (B) day 14, (C) day 28

4.14 Conclusion

Identification of PAB in the samples was possible within 2 days after fermentation of the final product, compared with TVC of PAB obtained after 6 days on Pal propiobac and presumptive TVC from PCA and YEL agar. PMA was a powerful tool in this study, ensuring that the DNA of compromised and dead cells was removed from the PCR amplification process, allowing only the DNA of live cells to be amplified. The conventional plating methods were still required for complete chromogenic culture media identification. Kefir contained a matrix of various organisms, evident in all three growth media; however, Pal propiobac was more selective for PAB as most other bacterial growth was suppressed. Refrigeration storage at 5°C was the optimum storage condition as it yielded the highest microbial load between the three storage treatments. Freeze drying was not a suitable storage method, as it was the most time-consuming and expensive method for preserving kefir.

References

- Abdel-Mogheith, S., El-Gendy, A.O., Sultan, Serageldeen, S. & El-Nesr K. 2017. Exploring the antimicrobial and hepatoprotective effects of kefir: a probiotic fermented milk. *Journal of Pure and Applied Microbiology*, 11: 759-772.
- Ahmadi, N., Khosravi-Darani, K. & Mortazavian, A.M. 2017. An overview of biotechnological production of propionic acid: From upstream to downstream processes. *Electronic Journal of Biotechnology*, 28: 67-75.
- Anyiam, P.N., Nwuke, C.P., Uhwo, E.N., Ije, U.E., Salvador, E.M., Mahumbi, B.M. & Boyiako, B.H. 2023. Effect of fermentation time on nutritional, antinutritional factors and in-vitro protein digestibility of *Macrotermes nigeriensis*-cassava mahewu. *Measurement: Food*, 11: 1-9.
- Askar, M., Sajid, M., Nassif, Y., Ashraf, W., Scammell, B. & Bayston, R. 2019. Propidium monoazide-polymerase chain reaction of residential periprosthetic joint infection in two stage revision. *Molecular Biology Reports* 46: 6463-6470.
- Beloti, V., Barros, M.A.F., de Freitas, J.C., Nero, L.N., de Souza, J.A., Santana, E.H.W. & Franco, B.G.D.M. 1999. Frequency of 2,3,5-triphenyltetrazolium chloride (TTC) non reducing bacteria in pasteurised milk. *Revista de Microbiologia* 30:137-140.
- Bücher, C., Burtscher, J. & Domig, K.J. 2021. *Propionic acid bacteria* in the food industry: An update on essential traits and detection methods. *Comprehensive Reviews in Food Science and Food Safety*, 20: 4299-4323.

- Carcano, M., Todesco, R., Lodi, R. & Brasca, M. 1995. *Propionibacteria* in Italian hard cheese. *Lait*, 415-426.
- Chamlagain, B. 2016. Fermentation fortification of active vitamin B12 in food matrices using *Propionibacterium freudenreichii*: Analysis, production and stability. PhD thesis. Helsinki: University of Helsinki.
<https://researchportal.helsinki.fi/en/publications/fermentation-fortification-of-active-vitamin-b12-in-food-matrices> [Accessed 15 December 2025]
- Chang, B., Taguri, T., Sugiyama, K., Amemura-Maekawa, J., Kura, F. & Watanabe, H. 2010. Comparison of ethidium monoazide and propidium monoazide for the selective detection of viable *Legionella* cells. *Japanese Journal of Infectious diseases*, 63: 119-123.
- Clark, P., Trickett, A., Saffo, S. & Stark, D. 2014. Effects of cryopreservation on microbial contaminated cord-food. *Transplantation and cellular engineering*, 54: 532-540.
- Coronas, R., Zara, G., Gallo, A., Rocchetti, G., Lapris, M., Petrto, G.L., Zara, S., Fancello, F. & Mannazzu, I. 2023. *Propionibacteria* as promising tools for the production of probioactive scotta: a proof-of-concept study. *Frontiers in Microbiology*, 1-17.
- Farid, A., Khan, M.U., Wang, Z., ZhiJie, W., BaoQiu, C., KaiYue, W., Liu, H. & Chen, Z. 2025. Mapping research trends in milk microbiology: Rapid detection methods and bibliometric analysis. *LWT*, 233: 1-12.
- Gaucher, F., Rabah, H., Kponouglo, K., Bonnassie, S., Pottier, S., Dolivet, A., Marchand, P., Jeantet, R., Blanc, P. & Jan, G. 2020. Intracellular osmoprotectant concentrations determine *Propionibacterium freudenreichii* survival during drying. *Applied Microbiology and Biotechnology*, 104(7): 3145–3156.
- Gautier, M., 2014. *Propionibacterium*. *Encyclopedia of food Microbiology*, 2: 232-237.
- Guo, L., Ze, X., Jiao, Y., Song, C., Zhao, X., Song, Z., Mu, S., Liu, Y., Ge, Y., Jing, Y. & Yao, S. 2024. Development and validation of a PMA-qPCR method for accurate quantification of viable *Lactocaseibacillus paracasei* in probiotics. *Frontiers in Microbiology*, 15: 1-12.
- Huang, S., Rabah, H., Rabah, H., Jardin, J., Briard-Bion, V., Parayre, S., Maillard, M., Le Loir, Y., Chen, X. d., Shuck, P., Jeantet, R. & Jan, G. 2016. Hyperconcentrated sweet whey, a new culture medium that enhances *Propionibacterium freudenreichii* stress tolerance. *Applied and Environmental Microbiology*, 82:4641-4651.

- Kalamaki, S.M. & Angelidis, A.S. 2020. High throughput, sequence-based analysis of microbiota of Greek kefir grains from two geographic regions. *Food Technology and Biotechnology*, 58: 138-146.
- Karimi, R., Mortazavian, A.M. & Amiri-Rigi, A. 2012. Selective enumeration of probiotic microorganisms in cheese. *Food Microbiology*, 29: 1-9.
- Kasenkas, H., Gursoy, O. & Ozbas, H. 2016. Kefir. *Fermented foods in health and disease prevention*. (edited by J. Frias, C. Martinez-Vilaluenga and E. Peñas). 339-359.
- Kumar, K.H., Suvarna, V.C., Sarvani, B.H., Abhishek, R.U. & Anil, V.S. 2020. In-Vitro Assessment of Probiotic Attributes of *Propionibacterium freudenreichii* Isolated from dairy cheese. *Current Journal of Applied Science and Technology*, 28-37.
- Morkel, R.A. 2016. Vitamin B12 and folate enrichment of kefir by *Propionibacterium freudenreichii* and *Streptococcus thermophilus* strains. Unpublished Masters' dissertation, Cape Peninsula University of Technology, Bellville.
- Nocker, A., Sossa- Fernandez, P. Burr, M.D. & Campher, A.K. 2007. Use of propidium monoazide for live/ dead distinction in microbial ecology. *Applied and Environmental Microbiology*, 73: 5111-5117.
- O'Brien, K.V., Aryana, K.J., Prinyawiwatkul, W., Ordonez, K.C. & Boeneke, C.A. 2016. The effects of frozen storage on the survival of probiotic microorganisms found in traditionally and commercially manufactured kefir. *Journal of Dairy Science*, 99(9): 7043-7048.
- Piwowarek, K., Lipińska, E., Hać-Szymańczuk, E., Kieliszek, M. & Ścibisz, I. 2018. *Propionibacterium spp.*—source of propionic acid, vitamin B12, and other metabolites important for the industry. *Applied Microbiology and Biotechnology*, 102(2): 515-538.
- Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F. & Cotter, P.D. 2013. The complex microbiota of raw milk. *FEMS Microbiology Reviews*, 37(5): 664-698.
- Riedel, K. H., Wingfield, I. & Britz, T.J. 1998. Identification of Classical *Propionibacterium species* using 16S rDNA - Restriction fragment length polymorphisms. *System Applied Microbiology*, 21: 419-428.
- Schoeman, T. 2001. Characterisation and identification of the active microbial consortium present in kepi grains. Unpublished master's dissertation, Stellenbosch University, Cape Town.

- Sohier, D., Pavan, S., Riou, A., Combrisson, J. & Postollec, F. 2014. Evolution of microbiological analytical methods for dairy industry needs. *Frontiers in Microbiology*, 5: 1-11.
- Teijeiro, M., Péres, P.F., de Antoni, G.L. & Golowczyc, M.A. 2018. Suitability of kefir powder production using spray drying. *Food Research International*, 112: 169-174.
- Tharamaraj, N. & Shah, N.P. 2003. Selective enumeration of *Lactobacillus delbrueckii* spp. *Bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Bifidobacteria*, *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Propionibacteria*. *Journal of Dairy Science*, 86: 2288-2296.
- Thierry, A. & Madec, M.N. 1995. Enumeration of propionibacteria in raw milk using a new selective medium. *Lait*, 75: 315-323.
- Turgay, M., Schaeren, W., Wechsler, D., Bütikofer, U. & Graber, H.U. 2016. Fast detection and quantification of four dairy propionic acid bacteria in milk samples using real-time quantitative polymerase chain reaction. *International Dairy Journal*, 61: 37-43. <http://dx.doi.org/10.1016/j.idairyj.2016.03.014>.
- Van Wyk, J., Witthunn, C.R. & Britz, T.J. 2011. Optimisation of vitamin B12 and folate production by *Propionibacterium freudenreichii* strains in kefir. *International Dairy Journal*, 21: 69-79.
- Witthuhn, R.C., Schoeman, T. & Britz, T.J. 2005. Characterisation of the microbial population at different stages of kefir production and kefir grain mass cultivation. *International Dairy Journal*, 15: 383-389.
- Yu, S., Yan, L., Wu, X., Li, F., Wang, D. & Xu, H. 2017. Multiplex PCR coupled with propidium monoazide for the detection of viable *Cronobacter sakazakii*, *Bacillus cereus*, and *Salmonella* spp. in milk and milk products. *Journal of Dairy Science*, 100: 7874-7882.
- Zhang, J., Li, C., Rahaman, M.M., Yao, Y., Ma, P., Zhang, J., Zhao, X., Jiang, T. & Grzegorzec, M. 2021. A comprehensive review of image analysis methods for microorganism counting from classical image processing to deep learning approaches. *Artificial Intelligence Review*, 55 :2875-2944.
- Zi, C., Zengi, D., Ling, N., Dai, J., Xue, F., Jiang, Y. & Li, B. 2018. An improved assay for rapid detection of viable *Staphylococcus aureus* cells by incorporating surfactant and PMA treatments in qPCR. *BMC Microbiology*, 18(132): 1-8.

CHAPTER 5

SUMMARY, CONCLUSIONS and RECOMMENDATIONS

5.1. Summary

Food producers are searching for ways to improve the survivability of probiotics in various food products, which has led to an increase in research in the field of PCR compared to traditional phenotypical approaches. Probiotics in the food industry are subject to various limitations regarding processing, growth, purification, and quantification. Nevertheless, adding naturally occurring protective properties to fermented products, such as milk, can significantly improve product quality without incurring additional costs for the producer. Previous studies have proven that enriching kefir with PAB will also enhance the microbial flora of the end-product with no additional financial implications, thus also increasing the economic marketability of enriched kefir and possibly other dairy products.

The first objective of this study was to establish an optimum growth medium for the cultivation of *P. freudenreichii* subsp. *Shermanii*. In Chapter 3, reconstituted PAB strains, J15 and J17, were inoculated into 9 mL YEL broth and incubated for 48 hours at 32°C. These cultures were used as control cultures throughout the study. The cultures were streaked onto PCA, YEL agar, and Pal Propiobac and incubated anaerobically for 6 days at 30°C. The control culture was treated with PMA and the DNA was subsequently isolated. A PCR protocol was optimised to successfully detect PAB. PAB was cultivated on PCA, YEL agar and Pal propiobac, however Pal propiobac showed clear characteristic colonies of PAB. The colonies were cream surrounded by yellow discoloration of the purple media. On PCA, PAB appeared pale and creamy in colour, but the colonies were smaller than on YEL. Colonies on YEL were smooth, beige and circular with an entire margin. The hypothesis was proven that Pal propiobac was a better medium for the cultivation of PAB. PMA treated PAB was successfully detected with the optimised PCR method.

The second objective of this study was to find an optimum growth media to enumerate PAB and the total bacterial count of kefir stored under different storage conditions via conventional microbiological techniques over 28 days using PCA, YEL and Pal propiobac. Kefir was fortified with PAB and stored under refrigerated, frozen conditions. Samples of kefir was freeze-dried and stored in a freezer at -20°C. The kefir was analysed on day 0, 7, 14 and 28. Refrigeration storage was found to be the optimal storage condition while also maintaining high microbial loads on PCA and YEL. The main effect of refrigeration on the microbial load was 5.69 Logcfu/mL and 5.07 Logcfu/mL respectively on PCA and YEL for KC, 6.32 Logcfu/mL and 4.82Log cfu/mL for J15 and 6.3Log cfu/mL and 5.6Log cfu/mL for J17. Microbial growth using Pal propiobac showed significantly lower bacterial counts of 3.81 Log cfu/mL and 4.07 Log cfu/mL. Thus, while Pal propiobac showed less microbial growth, it was found to be the most selective media of the 3 different media employed in this study for PAB as other microbes were

suppressed during incubation. It was difficult to count PAB exclusively as PCA and Yel are not specifically selective growth media for PAB only, whereas Pal Propiobac is selective for PAB. This could be the reason for the difference in bacterial counts between the different media employed as conventional methods were cumbersome and time-consuming to detect PAB. The hypothesis was proven that Pal propiobac was the optimum media to enumerate PAB in kefir and . PCR was employed as an analytical tool to identify PAB in kefir. A conventional PCR method was specifically optimised to detect viable PAB in the samples stored over 28 days. DNA was extracted using Mobio Powerfood DNA-extraction kit. It was important in this study to amplify only the DNA of live PAB cells, as it is possible to amplify the DNA of dead cells of the same region. To combat this, kefir samples were treated with PMA before the extraction of DNA. Treating the kefir samples with PMA before PCR detection proved effective for detecting viable PAB by selectively masking the DNA of dead PAB cells, thereby preventing them from multiplying. PAB was detected throughout the storage period in all samples that was inoculated with PAB using Prop1 and Prop2 primers. The fragment size of the amplified 16S ribosomal DNA amplified in the region of 750bp. The PCR method employed was faster and less labour intensive, however it was unable to quantify the microbial load of PAB in kefir. Conventional methods employed allowed for quantification of the microbial flora in kefir. The hypothesis was proven that viable DNA was detected in kefir over 28 days. Overall, the successful detection of PAB in kefir during this study has contributed to valuable insights into the detection and storage methods for PAB in kefir.

5.2 Conclusion

PAB in kefir were successfully enumerated using Pal propiobac. Pal propiobac is a suitable selective medium for the enumeration of kefir fortified with PAB over 28 days. Viable PAB was effectively detected in PMA-treated kefir samples prior to amplification of DNA using conventional PCR.

Storage of kefir at refrigeration temperatures was proven to be the optimum storage temperature to store kefir over 28 days as the microbial load was culturable.

In this study, freeze drying was proven to be the least effective storage condition for kefir, as the bacterial count was low, or the viable bacteria present was not culturable.

Further studies could explore the microbial diversity and probiotic functionality of kefir over longer storage periods. This could be achieved, by combining conventional methods with realtime PCR. Future studies could include other methods like spray drying or modified atmospheric storage conditions.

5.3.1 Recommendations

Further studies exploring the viability and enumeration of qPCR in kefir will be of great importance and helpful in determining viability without the labour-intensive conventional methods.

Storage of fermented beverages, which contains a matrix of other microbes along with other proteins and sugars is also an avenue for further studies. Research in the quantity and type of inoculum for example, broth or freeze-dried origin of PAB added to the fermentation process, as PAB survives, but is not easily enumerated after frozen and freeze-dried storage will be advantageous.

Further recommendations for this study could include investigating other types of selective or differential media, using antibiotics.

Since this study was performed using conventional PCR. Further studies could explore PMA-PCR combinations using realtime PCR approaches which could include multiplex PCR in detecting live *Propionibacterium* along with other species in complex matrices like kefir. PCR primers and sequences could be further modified for better specificity and sensitivity.

Since storage at refrigeration temperatures was proven to be the optimum storage temperature, further studies could explore the microbial diversity and probiotic functionality of kefir over longer storage periods. These studies could further enhance the understanding of propionibacteria in kefir, improve quality control in production and during storage, and potentially lead to better methods for enhancing the health benefits of kefir.

Detection of PAB using PCR offers significant advances over conventional plating to the industry due to the reduction of labour, media usage and is less time-consuming, thus reducing cumulative cost, while providing rapid, sensitive and reliable results.

Further investigation regarding the initial inoculation dosage of PAB into kefir and other dairy products will contribute to the development of PAB as a consistent and reliable source of probiotics.