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Effects of acidification on the survival of pathogens in reconstituted infant formula

by

Mufunwa Nemakonde

(1044345)

Dissertation

Submitted in fulfilment of the requirements for the degree

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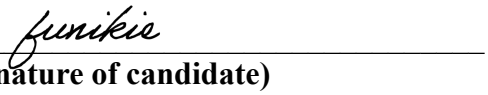
in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Prof Pieter De Maayer

October 2023

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

27th day of October 2023 at Johannesburg,

Preface

Infants are at a high risk of developing food-borne illnesses due to the consumption of powdered infant formulas (PIFs) contaminated by pathogenic microorganisms. These pathogens may be introduced during PIF production, transportation or during preparation and storage due to poor hygiene practices. Therefore, it is important to improve the microbiological safety of PIFs to reduce illness. Manufacturers have added further measures to ensure continued control of pathogenic microorganisms during the reconstitution and consumption, including the addition of prebiotics and probiotics, as well as acidification of the PIFs, which negatively affect the growth of pathogens. Different organic acids have been biologically or chemically added to commercial PIFs, however, little is known about the efficacy of the different acids in controlling pathogen growth post-reconstitution. The present study aimed to evaluate and compare the effects of two acids, namely citrate and lactate, integrated in commercial PIFs from two manufacturers on pathogen growth within the products.

In Chapter 1, we present a review of the current literature pertaining to PIF production and consumption, its microbiological safety and key problem pathogens, as well as measures to control these pathogens (with specific emphasis on PIF acidification). In Chapter 2, the methodology of the present study is discussed. The effects of lactate and citrate incorporated in commercial PIFs against eight key PIF pathogens was evaluated using plate-based assays. Furthermore, the efficacy of PIF acidification at different storage temperatures and over prolonged storage periods was determined. Finally, to validate the effect of acidity on controlling pathogen growth, a spectrophotometric approach was used.

Chapter 3 presents and discusses the key findings of the study. PIF acidification was found to negatively affect the growth of pathogens in comparison to the non-acidified infant formula. Acidification and storage at suboptimal temperatures resulted in little to no microbial growth. Lactate acidification of PIFs demonstrated greater inhibitory effects against most pathogens compared to citrate acidification. Further, lactate was found to have a bactericidal effect on the growth of some pathogens. Similarly, Luria Broth acidification resulted in the reduction of microbial growth. This validated that the acids and not other constituents of the infant formula were responsible for the inhibitory effect. Finally, Chapter 4 provides a summary of the key findings as well as recommendations and guidance on future studies which could be undertaken to enhance the effective control of pathogens in powdered infant formulas, thereby ensuring a safe and nutritious food source for infants.

Dedication

I dedicate this to my sister, Ndivhuwo, who has always been there for me, especially through the challenging days.

Acknowledgements

I would like to acknowledge my supervisor, Prof. Pieter De Maayer for giving me the opportunity to do this research and providing constant support throughout this journey. I would like to thank my brother Thabelo and my friends Wanga, Unanne, Phindile and Elsie for their encouragement and support throughout this research.

I would also like to acknowledge Dr. Angela Botes who acted as my academic advisor and kindly provided some of the equipment I used to carry out my research as well as my lab mate Michael Mol, who helped me find my feet when I started my research.

This endeavour would not have been possible without the funding from TATA International Postgraduate Scholarship and FoodBev SETA for my tuition fees.

Finally, I would like to express my sincere gratitude to the Almighty God who granted me health, a sound mind and strength to carry out my research.

Table of Contents

Declaration i

Preface ii

Dedication iii

Acknowledgements iii

List of figures vii

List of tables viii

List of appendices ix

List of abbreviations x

Chapter 1: Introduction 1

1.1. Powdered infant formulas 3

1.3. Bacterial pathogens commonly associated with powdered infant formulas 6

1.3.1. *Cronobacter sakazakii* 8

1.3.2. *Salmonella enterica* 11

1.3.3. Other pathogens known to contaminate powdered infant formulas 12

1.3.4. How do pathogenic bacteria survive in “sterile” PIF? 14

1.4. Measures to reduce powdered infant formula contamination 15

1.4.1. Inclusion of antimicrobial agents in PIFs 16

1.4.2. PIF acidification 17

1.5. Problem statement 18

Chapter 2: Materials and Methods 20

2.1. Materials 21

2.1.1. Bacterial strains 21

2.1.2. Culture media 22
2.1.3. Powdered infant formulas and reconstitution 24
2.2. Methods 24
2.2.1. Evaluation of inherent microbial contaminants in commercial PIFs 24
2.2.2. Standardisation of pathogen concentrations 24
2.2.3. Reconstituted PIF pathogen-spiking trials 25
2.2.4. Pathogen enumeration and statistical analyses 25
2.2.5. Validation of the effects of acids in LB broth on pathogen growth 25
Chapter 3: Results and Discussion 27
3.1. Analysis of inherent PIF contamination 28
3.2. Properties of the reconstituted PIFs 28
3.3. PIF acidification negatively affects the growth of pathogens 30
3.4. Lactate acidification is more effective than citrate acidification for most pathogens 33
3.5. Lactate acidification has bactericidal effects on *B. cereus* and *P. aeruginosa* 35
3.6. Interplay between acidification and storage temperature in controlling pathogen growth in reconstituted PIFs 36
3.7. Pathogen growth is directly affected by acidification, not other PIF constituents 36
3.8. Acidification pH is a key driver affecting pathogen growth 39
Chapter 4: Summary 42
References 45
Appendices 68

Appendix A: Media Preparation.....69

Appendix B: Mean pH measurements for the reconstituted PIFs without pathogens at the four different time points post-inoculation.....71

Appendix C: Mean pH measurements for the pathogen-spiked reconstituted PIFs at the four different time points post-inoculation 72

Appendix D: log (CFU/ml) values of the pathogen-spiked PIF over a 6-hour incubation period.....75

Appendix E: Effect of acidification and temperature on the growth of pathogens.....79

Appendix F: Absorbance (OD 600_{nm}) of pathogens in acidified and non-acidified LB Broth 83

Appendix G: Absorbance (OD 600_{nm}) of pathogens in citrate- and lactate- acidified LB Broth 87

List of figures

Figure 1.1: Schematic showing the three different approaches of PIF production

Figure 3.1: Effect of acidification on pathogen growth in reconstituted PIFs after 6 hours at 37 °C. The error bars show the standard error of the mean of the triplicates

Figure 3.2: Effect of citrate- and lactate-acidification on pathogen growth in reconstituted PIFs after 6 hours at 37 °C. The error bars show the standard error of the mean of the triplicates

Figure 3.3: Growth absorbance (OD at 600 nm) of PIF pathogens in citrate-, non- and lactate acidified LB broth at 37 °C 96 hours post-inoculation. The error bars show the standard error of the mean of the triplicates

Figure 3.4: Absorbance (OD 600nm) of pathogens in citrate- and lactate- acidified LB on the growth of the PIF pathogens. The error bars show the standard error of the mean of the triplicates

List of tables

Table 1.1: Categorization of pathogens associated with powdered infant formula

Table 1.2: Outbreaks of infections linked to *C. sakazakii*-contaminated PIFs

Table 2.1: Bacterial strains and their selective media

Table 3.1: Mean pH measurements for the reconstituted PIFs containing pathogens at the four different time points post-inoculation

Table 3.2: Growth conditions of the PIF pathogens

List of appendices

Appendix A: Media preparation

Appendix B: Mean pH measurements for the reconstituted PIFs without pathogens at the four different time points post-inoculation

Appendix C: Mean pH measurements for the pathogen-spiked reconstituted PIFs at the four different time points post-inoculation

Appendix D: log (CFU/ml) values of the pathogen-spiked PIF over a 6-hour incubation period

Appendix E: Effect of acidification and temperature on the growth of pathogens

Appendix F: Absorbance (OD 600_{nm}) of pathogens in acidified and non-acidified LB Broth

Appendix G: Absorbance (OD 600_{nm}) of pathogens in citrate- and lactate- acidified LB Broth

List of abbreviations

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
a_w	Water activity
CAC	Codex Alimentarius Commission
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FOS	Fructo-oligosaccharides
GIT	Gastrointestinal tract
GOS	Galacto-oligosaccharides
HACCP	Hazard analysis critical control points
HMO	Human milk oligosaccharides
LB	Luria Bertani
OD	Optical density
PBS	Phosphate-buffered saline
pH	Potential of hydrogen
PIF	Powdered infant formula
SC-CO_2	Supercritical carbon dioxide

spp.	Species
subsp.	Subspecies
TOS	Transgalacto-oligosaccharides
UV	Ultraviolet
VRBG	Violet Red Bile Glucose
WHO	World Health Organization

Chapter 1: Introduction

New-born infants and young children are at a higher risk of contracting food-borne diseases and illnesses as their immune systems, internal organs and gastrointestinal tracts (GITs) are not completely developed and therefore are not as effective as those of adults (Lönnerdal, 2012; Patrick *et al*., 2014; Sani *et al*., 2013; Song *et al*., 2018; Turck, 2012). Preterm infants delivered before twenty six weeks of gestation have a 7.5-fold higher incidence of these infections than those carried to full-term (Clapp *et al*., 1989; Clapp, 2006). This renders them vulnerable to diarrhoea and serious bacterial infections which may result in bacteraemia, meningitis, pneumonia and urinary tract infections (DePorre *et al*., 2017; Esposito *et al*., 2018; Sani *et al*., 2013).

Breast milk incorporates a range of protective measures against food-borne pathogenic microorganisms, including human milk oligosaccharides (HMOs), immune-regulatory antibodies and white blood cells (Gura, 2014; Joosten and Lardeau, 2004; Labuschagne *et al*., 2012). HMOs provide antimicrobial properties against bacteria including *Salmonella* and *Listeria* spp., by producing substances that bind these microorganisms thus decreasing the incidence of diarrhoea and infections associated with these pathogens (Agostoni *et al*., 2009; Gura, 2014; Martin *et al*., 2016). Immune-regulatory antibodies and white blood cells form part of the immune system and help fight infections and diseases (Hjelm *et al*., 2006). Breast milk further promotes the growth of bifidobacteria in the feeding infant's GIT, which create an acidic environment that inhibits the growth of infectious food-borne pathogens (Huërou-Luron *et al*., 2010; Labuschagne *et al*., 2012).

The World Health Organization (WHO) recommends breastfeeding of infants in their first six to twenty-four months of life as breast milk provides infants with the nutrition required for growth and development and provides a first line of defence against infection (Hanif, 2011; Kent *et al*., 2015; Motee and Jeewon, 2014; WHO, 2001; WHO, 2017). Additional advantages of breastfeeding include that it helps mothers form a bond with their young and to lose the weight gained during pregnancy (Bai *et al*., 2009; Kramer and Kakuma, 2012; WHO, 2011). Further, breastfeeding has been linked to a decreased risk of mothers developing breast, epithelial ovarian and endometrial cancers and type 2 diabetes (Bernier *et al*., 2000; Danforth *et al*., 2007; Motee and Jeewon, 2014; Rosenblatt and Thomas 1995).

Despite the countless benefits associated with breastfeeding, some mothers choose not to breastfeed, and in some cases, it is not an option (Chouraqui *et al*., 2004; Hudara *et al*., 2010; Labuschagne *et al*., 2012). Studies have shown that some mothers find it difficult to breastfeed

due to sore nipples, inadequate milk production, short maternity leave terms and lack of familial support, as well as a lack of guidance from healthcare professionals (Cherop *et al*., 2009; Gartner *et al*., 2005; Motee and Jeewon, 2014; Whalen and Cramton, 2010). Some mothers are unable to breastfeed due to transmissible diseases such as human immunodeficiency virus, human T-cell lymphotropic virus type I and II as well as cytomegalovirus (Lawrence, 2005). Since breastfeeding is not always feasible, a wide variety of powdered infant formulas, follow-up formulas and infant foods have been developed and serve as an alternative nutritional source (Hudara *et al*., 2010; Labuschagne *et al*., 2012).

1.1. Powdered infant formulas

Powdered infant formulas (PIFs) are nutrient-rich substitutes which represent a key nutritional source for both breastfed (as supplementary nutrition) and non-breastfed infants (often as primary nutrient source). They are used because they are affordable, easy to transport and store, and have a long shelf life. There are a large variety of PIFs available for infants and babies of all ages less than twelve months old and these include those for pre-term and full-term infants, as well as those with health problems (e.g., reflux). The composition of PIFs differs greatly depending on the nutritional needs of the infant (Martin *et al*., 2016; Masum *et al*., 2020; Ryan and Hay, 2016; Turck, 2012).

PIFs are produced using either a wet-mix approach, a dry-mix approach, or a combination of both (Figure 1.1) (Kent *et al*., 2015; Masum *et al*., 2020). The wet-mix approach processes all the ingredients of the PIF in a liquid state and involves homogenization, heat treatment and pasteurization while the dry-mix approach processes all the ingredients in their powdered state followed by blending to achieve the recommended composition of the final product. The combination approach uses both the wet and dry-mix methods and is the primary methodology employed by the majority of commercial PIF manufacturers (Masum *et al*., 2020; Montagne *et al*., 2009; Proudy *et al*., 2008).

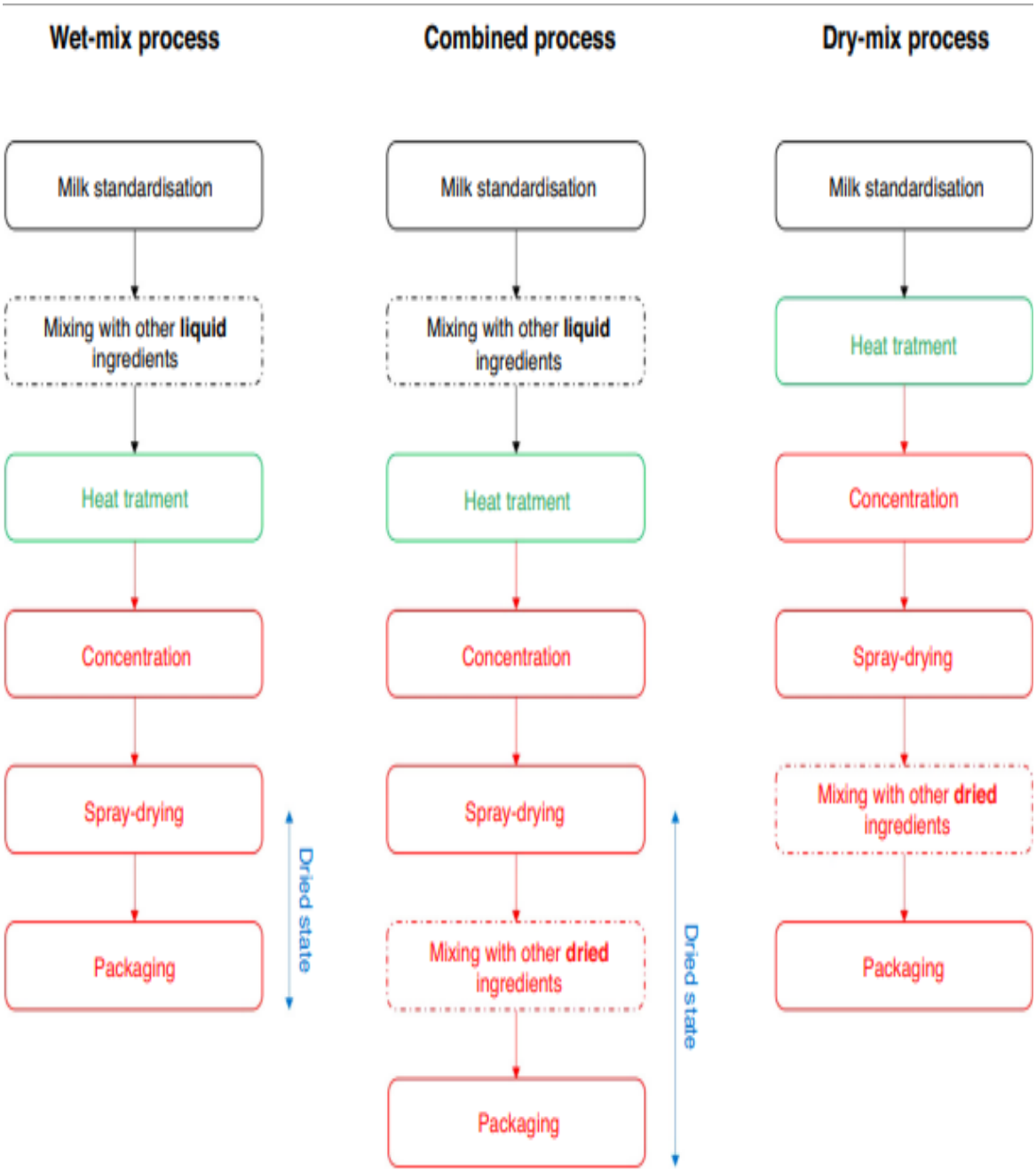


Figure 1.1: Schematic showing the three different approaches of PIF production (Lang and Sant'Ana, 2021).

Infant formulas are required to strictly contain ingredients that contribute towards the growth and development of the infant as the addition of unnecessary ingredients may hinder its metabolic and physiological functions (FAO/WHO, 2007; Koletzko *et al*., 2005). The ingredients include fats, proteins, carbohydrates, vitamins and minerals. These nutrients are required to be in concentrations recommended by the Codex Alimentarius Commission (CAC) and the recommended concentration range of each nutrient should be maintained throughout the product's shelf-life (CAC, 1979; FAO/WHO, 2007; Martin *et al*., 2016). The main aim of infant formula manufacturing companies is to produce PIFs that mimic, as much as possible, the nutritional composition of human breastmilk. However, some components of human breast milk cannot be reproduced. These include white blood cells, antibodies, growth factors and enzymes (Imboden *et al*., 2023; Martin *et al*., 2016).

As a consequence of their nutrient-rich nature, PIFs are hotspots for microbial growth and thus production requires high levels of microbiological quality control and standards (Turck, 2012). The production of PIFs is required to comply with the WHO and Food and Agricultural Organization (FAO) guidelines aimed to reduce intrinsic contamination (FAO/WHO, 2006; Forsythe, 2005; WHO, 2007a; WHO, 2007b). During production, PIF manufacturers are required to follow good manufacturing and hygienic practices and should have hazard analysis critical control point (HACCP) systems set in place. Manufacturers are required to ensure that their wet and dry production areas are separated and are regularly cleaned to reduce the risk of cross-contamination. Further, manufacturers are compelled to provide precise and comprehensible information that enables caregivers to reduce the risk of contamination of the PIFs during preparation, reconstitution and consumption (FAO/WHO, 2006; FAO/WHO, 2007; Kent *et al*., 2015; Lönnerdal, 2012; Martin *et al*., 2016; WHO 2007b).

The microbiological quality and nutritional composition of the final product is routinely assessed to ensure that it is safe for consumption. Suppliers, distributors, transporters and retailers are required to ensure that the PIFs are handled correctly and according to the manufacturers' instructions as improper handling may interfere with their physicochemical properties and stability (FAO/WHO, 2006; FAO, 2007; Forsythe, 2005; Masum *et al*., 2020; Nasirpour *et al*., 2006; Thompkinson and Kharb, 2007; Turck, 2012). Apart from intrinsic contamination, improper practices of caregivers whilst handling infant formulas may also favour the growth of harmful bacteria (Sani *et al*., 2012; WHO, 2007a; WHO, 2007b).

1.2. Powdered infant formula handling, storage and preparation

The WHO, in collaboration with the FAO, published guidelines containing recommendations that should be followed during the preparation, handling and storage of PIFs to inactivate intrinsic contaminants, reduce extrinsic contamination and thus the occurrence of PIF-related diseases in infants. Caregivers are required to frequently sanitize their hands and sterilize surfaces before preparing and handling infant formula. Prior to the reconstitution of PIFs in water, the water needs to be boiled to 100 °C to render potential water-borne microorganisms inactive. The water should be poured into a sterilized feeding bottle, cooled down to room temperature (~25 °C) prior to the reconstitution of the PIF. The reconstituted formula should be consumed directly after each preparation or be stored in a refrigerator at temperatures below 4 °C (Agostoni *et al*., 2009; FAO/WHO, 2006; Silano *et al*., 2016; Turck, 2012; WHO, 2007a; WHO, 2007b).

Poor hygienic practices and improper handling of PIFs such as unsatisfactory cleaning of infant formula bottles, multiple reheating and inappropriate rehydration procedures have been identified as factors that influence the establishment and proliferation of pathogens (Sani *et al*., 2012). For example, the use of contaminated utensils and improper cleaning of the preparation environment can influence the microbiological safety of both powdered and reconstituted infant formulas (Kent *et al*., 2015; WHO, 2007a; WHO, 2007b). Improper storage and the use of contaminated water outlets have also been identified as contributing factors facilitating the establishment and growth of pathogenic contaminants. To prevent the contamination of PIFs, caregivers are advised to store them in clean and dry containers in cool environments as humidity may facilitate the growth of microorganisms (Hedegaard and Skibsted, 2013; Cheng *et al*., 2017). In communities without proper sanitation and water supply systems, which could impact on PIF reconstitution, storage and consumption, breastfeeding is strongly encouraged because it reduces the exposure to pathogenic microorganisms (Joosten and Lardeau, 2004; Labuschagne *et al*., 2012; WHO, 2017).

1.3. Bacterial pathogens commonly associated with powdered infant formulas

A broad range of bacterial pathogens have been associated with the contamination of PIFs during and post-production.

On the basis of their prevalence in PIFs and the severity of infection, the WHO and FAO have compiled a list of bacterial pathogens of greatest concern in PIFs (Joosten and Lardeau, 2004;

Kent *et al*., 2015; Labuschagne *et al*., 2012; Wang *et al*., 2009; WHO, 2004; WHO, 2007b). These bacterial pathogens are further subdivided into three risk categories (Table 1.1). Category A comprises pathogens that have been identified as the cause of illness in infants and are directly linked to PIF consumption. Category B includes pathogens that cause illness in infants and have been found in PIFs, but their presence in PIFs has not been found as the source of illness. Category C is comprised of pathogens which despite causing illness in infants, have not been identified in PIFs or, although having been identified in powdered infant formula, have not been connected to causing illness in infants (Cahill *et al*., 2008; FAO/WHO, 2006).

Table 1.1: Categorization of pathogens associated with powdered infant formula

Category	**Pathogens**	**References**
Category A	*Cronobacter sakazakii* and *Salmonella* spp.	FAO/WHO, 2006;
Category B	*Acinetobacter* spp., *Citrobacter freundii*, *Citrobacter koseri, Enterobacter* spp., *Escherichia coli*, *Hafnia alvei*, *Klebsiella oxytoca, Klebsiella pneumoniae*, *Pantoea agglomerans*, *Pseudescherichia vulneris* and *Serratia* spp.	FAO/WHO, 2006
Category C	*Bacillus cereus*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Listeria monocytogenes*, *Staphylococcus aureus* and coagulase negative staphylococci	FAO/WHO, 2006

C. sakazakii and *Salmonella enterica* have been identified as the pathogens of greatest concern in infant formulas due to their capacity to cause severe diseases which often result in death in infants (Kent *et al*., 2015; WHO, 2007b). These species have been found to contaminate infant formula during manufacturing, transportation and preparation and have been linked to PIF-borne disease outbreaks (WHO, 2004; WHO, 2007b). A food-borne disease or illness outbreak is when two or more people contract the same disease or illness from the same food contaminated by the same pathogen(s) (Bintsis, 2017).

1.3.1. *Cronobacter sakazakii*

C. sakazakii is a Gram-negative, rod shaped, motile, facultative anaerobe and non-spore forming bacterium that belongs to the genus *Cronobacter* and the family *Enterobacteriaceae* (Farmer *et al*., 1980; Kalyantanda *et al*., 2015). It is an opportunistic pathogen that is capable of causing illness in all age groups. *C. sakazakii* has been reported to grow at temperatures ranging between 6 and 45 °C with the optimum temperatures ranging from 37 to 43 °C (FAO/WHO, 2006; Iversen *et al*., 2004b; WHO, 2007b). The disease reservoir of *C. sakazakii* has not been confirmed, however, it has been isolated from human stool, blood and sputum samples of ill and healthy individuals (Kent *et al*., 2015). *C. sakazakii* has also been identified in non-human sources such as soil samples, dairy products, meats, fruits, vegetables and spices (Baumgartner *et al*., 2009; Hunter and Bean, 2013; Lee *et al*., 2012; Song *et al*., 2018).

Despite the incidence of *C. sakazakii* infections in infants due to PIF consumption being relatively low, the consequences may be life-threatening, particularly in immunocompromised and pre-term neonates (FAO/WHO, 2006). *C. sakazakii* is assumed to contaminate PIFs after pasteurization as this step occurs at temperatures above the maximum that the pathogen can tolerate (Gurtler *et al*., 2005; Strydom *et al*., 2012). Although PIF production downstream of the pasteurisation step involves drying and heat treatment, the ability of *C. sakazakii* to survive in low water activity (a_w) and at high temperatures enable it to survive in the PIF environment (Arku *et al*., 2011; Hunter and Bean, 2013). Water activity is defined as the measurement of unbound water in a food substance which facilitates the growth of microorganisms and is available for chemical and biochemical reactions (Karel and Lund, 2003).

Most studies have shown that *Cronobacter* spp. contamination of PIF normally occurs during packaging and transportation. The capability of *C. sakazakii* to contaminate PIFs is attributed to its propensity to form biofilms (Brandl *et al*., 2014; Lee *et al*., 2012; Yang *et al*., 2013). Biofilms aid *C. sakazakii* to rigidly attach to the surfaces of packaging materials and manufacturing instruments thus increasing the possibility of contamination (Aly *et al*., 2019). The presence of biofilms provides a protective barrier for *C. sakazakii* against antibacterial products thus enabling the survival and proliferation of the pathogen in PIFs (Fei *et al*., 2022; Lehner *et al*., 2005).

C. sakazakii has been implicated as a causative agent of meningitis, bacteraemia, septicaemia and necrotizing enterocolitis as well as intestinal and extra-intestinal infections in infants due to contaminated PIFs (Hunter and Bean, 2013; Lai, 2001; Pakbin *et al*., 2022). Bacteraemia

caused by *C. sakazakii* tends to develop in pre-term infants in their first two months of life whereas meningitis develops in neonates (WHO, 2004). Although bacteraemia and meningitis are curable, there is a high likelihood of severe neurological sequelae with the most frequently reported sequelae being focal neurological deficits, hearing loss, cognitive impairment and epilepsy (Li *et al*., 2023; Lucas *et al*., 2016;)

The first association between PIF and *C. sakazakii* was identified in a case of neonatal meningitis in 1958 and it has since been connected to a multitude of reported cases and outbreaks (Table 1.2) (Bowen and Braden, 2006; Drudy *et al*., 2006; Henry and Fouladkhah, 2019; Jason, 2011; Song *et al*., 2018; Urmenyi and Franklin, 1961). In 1998, *C. sakazakii* was identified as the vehicle of infection in an outbreak of necrotizing enterocolitis among infants in Belgium due to the consumption of contaminated PIFs. In this outbreak, twelve neonates were admitted to a neonatal intensive care unit, of which two neonates died (Van Acker *et al*., 2001).

In 2004, a disease outbreak in France due to contaminated PIFs was linked to *C. sakazakii*. The outbreak involved nine cases of pre-term infants in a hospital setting, of which two resulted in death (Drudy *et al*., 2006; FAO/WHO, 2006; WHO, 2007b). A review of practices revealed that the recommended guidelines regarding the preparation and handling of PIFs were not adequately followed. Two cases linked to *C. sakazakii* infection were reported in China in 2017 where one infant developed fever and muscle spasms whilst the other suffered from anaemia, diarrhea and moderate dehydration following the ingestion of contaminated PIFs (Cui *et al*., 2017; Song *et al*., 2018). In 2022, eminent infant formula brand, Abbott Nutrition recalled its PIF due to two cases where one infant died from meningitis and bacteremia caused by this pathogen (Haston *et al*., 2023).

Table 1.2: Outbreaks of infections linked to *C. sakazakii*-contaminated PIFs

Country	Year	Number of individuals	References
United Kingdom	1958	2	Urmenyi and Franklin, 1961
Netherlands	1983	8	Muytjens *et al*., 1983; Muytjens and Kollee, 1990
Iceland	1986 - 1987	3	Biering *et al*., 1989; Clark *et al*., 1990
USA	1987	2	Willis and Robinson, 1988
USA	1988	4	Simmons *et al*., 1989
Israel	1993	4	Block *et al*., 2002
USA	1995	5	Lai, 2001
Belgium	1998	12	Van Acker *et al*., 2001
Israel	1997-2000	5	Block *et al*., 2002; Bar-Oz *et al*., 2001
USA	2003	6	Bowen and Braden, 2006
France	2004	2	Coignard *et al*., 2004; Bowen and Braden, 2006
USA	2004 - 2005	4	Bowen and Braden, 2006
Canada	2007	2	Pagotto and Farber, 2009
USA	2008	2	CDC, 2009
Mexico	2010	2	Flores *et al*., 2011
China	2017	2	Cui *et al*., 2017
USA	2021 – 2022	2	Haston *et al*., 2023

1.3.2. *Salmonella enterica*

Salmonella enterica belongs to the family *Enterobacteriaceae* and is a Gram-negative, rod-shaped and facultative anaerobic bacterium (Fàbrega and Vila, 2013; Su and Chiu, 2007). It comprises of six subspecies and over 2600 serovars (Chandel *et al*., 2022; Murray *et al*., 2009). *S. enterica* is an opportunistic pathogen known to cause mild to serious infections in a wide range of hosts including humans, poultry as well as domestic animals. It is capable of surviving temperatures between 2 and 54 °C depending on the serovar (Hanes, 2003). The pathogen reservoirs of *S. enterica* are raw eggs, meat and dairy products (Chandel *et al*., 2022). Similar to *C. sakazakii*, *S. enterica* is capable of forming biofilms which enable it to attach to various surfaces and give it the ability to survive under unfavourable conditions (Hall-Stoodley *et al*, 2004). Due to their xerotolerant nature, most *Salmonella enterica* spp. have the ability to survive in the dry environments of PIFs as they have an increased adaptability to low a_w (Sekhon *et al*., 2021). Xerotolerant organisms are extremophiles that can survive in environments with low a_w (Esbelin *et al*., 2018).

Salmonella is a pathogen of concern due to its ability to cause disease even when present in low concentrations in PIFs (Cahill *et al*., 2008; FAO/WHO, 2006). Consequently, some countries have a very low tolerance limit of *Salmonella* spp. in PIFs and other milk derived products. In South Africa, China and New Zealand, the tolerance limit is 0 CFU/ml (Wu *et al*., 2006). Infections caused by the consumption of *Salmonella* spp. - contaminated PIFs can occur in both the immune-compromised and healthy infants (FAO/WHO, 2006). In particular, *S. enterica* subsp. *enterica* serovars Poona and Agona have been linked to salmonellosis, bacteraemia and gastroenteritis in infants (Cahill *et al*., 2008; Jones *et al*., 2006; WHO, 2004).

Although *Salmonella* is rarely found in PIFs, *S. enterica* spp. have been connected to a number of disease outbreaks associated with this food source. Between 1985 and 2005, at least six outbreaks of salmonellosis were linked to the consumption of contaminated PIFs (Cahill *et al*., 2008; FAO/WHO, 2006; Toyofuku *et al*., 2006; WHO, 2004; WHO, 2007a; WHO, 2007b). However, salmonellosis cases in most developing and poor nations often go unreported, which implies that the number of PIF-linked *Salmonella* outbreaks may be grossly underestimated (FAO/WHO, 2006).

In 2005, two outbreaks of salmonellosis caused by *S. enterica* subsp. *enterica* serovar Agona were reported and linked to two PIF brands in France. The outbreaks involved a total of 141

confirmed cases, but no deaths were reported (Brouard *et al*., 2007; Cahill *et al*., 2008; Wang *et al*., 2012). The PIFs were found to have been contaminated during production but the source of contamination was never identified (Cahill *et al*., 2008). In 2011, there was a large national outbreak of salmonellosis due to *S. enterica* subsp. *enterica* serovar Poona in Spain which was associated with the consumption of contaminated PIFs. Strains of this pathogen were isolated from both sealed and opened packages of the PIF brand, showing that contamination occurred during the production process (ECDC and EFSA, 2019).

In December 2017, *S. enterica* subsp. *enterica* serovar Agona was implicated in an outbreak in France linked to the consumption of a commercial PIF, with a total of 37 confirmed cases in infants of median age four months (Jourdan-Da Silva *et al*., 2018). Seven PIF brands were identified as the vehicles of infection and were subsequently recalled (EFSA and ECDC, 2018; Jourdan-Da Silva *et al*., 2018; Losio *et al*., 2018). As a consequence of this outbreak, over twelve million PIF products were recalled causing a considerable loss of consumer confidence and raising concerns about the processing methods (Lang and Sant'Ana, 2021). In 2019, *S. enterica* serotype Poona was found to be the causative agent of salmonellosis in 32 cases reported in Belgium, France and Luxembourg linked to PIF contamination (ECDC and EFSA, 2019).

1.3.3. Other pathogens known to contaminate powdered infant formulas

Apart from *S. enterica* and *C. sakazakii*, there have been reports of other bacterial pathogens found to contaminate infant formula (FAO/WHO, 2006). These include *Bacillus cereus*, *Citrobacter* spp., *Clostridium* spp., *Escherichia vulneris*, *Klebsiella* spp., *Listeria monocytogenes* and *Staphylococcus aureus* (Forsythe, 2005; Gribble and Hausman, 2012; Wang *et al*., 2012; Xin *et al*., 2018). The presence of these pathogens in PIFs may be harmful to infants as some have been identified as the causative agents of neonatal meningitis, severe diarrhoea, necrotizing enterocolitis, systemic infections and food intoxication (e.g., with toxins produced by *Clostridium* spp.) (Jiang *et al*., 2019; Liu *et al*., 2006; Wang *et al*., 2012).

The Gram-negative, facultative anaerobe, rod-shaped, motile bacterium *Citrobacter freundii* is a ground-dwelling bacterium which can also be found in the intestinal tracts of both humans and animals (Aminharati *et al*., 2019; Bai *et al*., 2012; Murray *et al*., 2010). Whilst this bacterium rarely causes illness, some species have been associated with cases of neonatal meningitis, sepsis and urinary tract infections (Hidayatullah *et al*., 2020; Murray *et al*., 2010;

Ranjan and Ranjan, 2013). *C. freundii* grows optimally at a temperature of 37 °C and can survive at temperatures as low as 7 °C (Rogers *et al*., 2016).

In 1990, a PIF brand was identified as the vehicle of infection in an outbreak of *C. freundii* infection in a neonatal intensive care unit of a large hospital in Germany. It still remains unclear whether the infant formula was contaminated intrinsically or extrinsically (Thurm and Gericke, 1994). While no recent cases and outbreaks related to *C. freundii* have been reported, due to misidentification by the analytical profile index (API) 20E system, it is likely that *C. freundii* linked to PIF contamination may be underreported (Giammanco *et al*., 2011). Giammanco *et al*. (2011), reported that *C. freundii* isolated from PIFs is often misidentified as *Cronobacter* spp. and *Enterobacter hormaechei*.

Staphylococcus aureus is a Gram-positive, cocci-shaped and facultative anaerobic bacterium that is the world's leading cause of disease outbreaks as a result of consumption of contaminated food (Dai *et al*., 2019; Jamali *et al*., 2015). Even in the absence of live bacteria, it produces toxins, such as enterotoxins, toxic shock syndrome toxin and Panton–Valentine leukocidin that can cause disease (Dinges *et al*., 2000; Wang *et al*., 2012). The presence of *S. aureus* in high concentrations in PIFs threatens the health of infants as it may result in septicaemia and eventually death (Wang *et al*., 2012).

A study on PIFs and rice cereals in China demonstrated that *S. aureus* could be recovered from 11.2 % of 143 tested PIF products. Of further concern, 83.3 % of the *S. aureus* isolates were resistant to at least one antimicrobial agent (Wang *et al.*, 2012). The risk of developing *S. aureus* infections is increased when the PIFs are reconstituted at improper temperatures, and stored for extended periods (Bogdanovicova *et al*, 2017). Although infrequent, there have been reports of outbreaks associated with *S. aureus*-contaminated reconstituted PIFs in Japan and China (Asao *et al*., 2003; Xie and Liu, 2021). In 2004, *S. aureus* was implicated in an outbreak associated with contaminated PIFs which involved 150 infants in China (Wang *et al*., 2012).

The bacillus shaped, Gram-positive, toxin- and spore-forming bacterium *Bacillus cereus,* is an opportunistic food-borne pathogen that has been isolated from various food products including infant formula. A survey of sixty-eight infant formulas from Beijing supermarkets revealed a prevalence rate of 70.6 % and 52.9 % for domestic Chinese and imported brands, respectively (Wang *et al.*, 2022). *B. cereus* has been found to be the most frequent bacterial species present in PIFs and human milk even after pasteurization (Cormontagne *et al*., 2021; Lewin *et al*.,

2019). It has been reported to grow between temperatures 4 and 50 °C with the optimum temperatures ranging from 28 to 37 °C (FDA, 2012). Currently, most countries are in agreement that the limit of *B. cereus* in infant formula should be less than 10^2 CFU/g. Most studies indicate that the majority of outbreaks attributable to this pathogen have been linked to concentrations of 10^5 CFU/g in implicated products (Wang *et al*., 2022).

Other potential pathogens of concern include the Gram-positive bacterium *Listeria monocytogenes* and Gram-negative bacterium *Escherichia coli*. *L. monocytogenes* has been linked to multiple food-borne disease outbreaks, including in South Africa in 2017 - 2018, where an outbreak in processed meat resulted in 1 060 individuals becoming infected with a high mortality rate of 20.4 % (NICD, 2018). Similarly, *E. coli*, although generally commensal, is frequently associated with foods and serovars such as *E. coli* O104:H4 and O157:H7 have been linked to numerous disease outbreaks (Piérard *et al*., 2012). While neither pathogen has been linked to PIF consumption-related disease, both have been shown to survive for prolonged periods in PIFs. Despite the very low relative water activity (a_w = 0.28) of PIFs, *L. monocytogenes* was demonstrated to be able to survive for more than twelve months at 22 °C in infant formula (Koseki *et al*., 2015; Taylor *et al*., 2019).

1.3.4. How do pathogenic bacteria survive in "sterile" PIF?

Despite the strict guidelines underpinning safe PIF production practices, handling, storage and preparation, they represent a key source of infection with pathogens. These include pathogens that may have survived the production processes as well as those that are introduced during subsequent preparation, storage and consumption (Agostoni *et al*., 2009; Iversen *et al*., 2004a; Iversen *et al*., 2004b; Kent *et al*., 2015; Sani *et al*., 2012).

While PIF production involves heat treatment, PIF contamination can reoccur due to the presence of pathogens in the production environment and equipment (Lang and Sant'Ana, 2021). Most food-borne pathogens have been reported to be more heat resistant in low-moisture foods such as PIFs compared to high-moisture foods (Jin *et al*., 2018; Taylor *et al*., 2019). Low-moisture foods are food products that are low in moisture or have a low water activity (a_w < 0.85) while high moisture foods are those with a high water activity (a_w > 0.95) due to their high moisture content (Liu *et al*., 2022; Young *et al.* 2015). Although PIFs have a relatively low water activity (a_w = 0.28), some microorganisms can survive and adapt under these conditions (Lang and Sant'Ana, 2021).

Some food-borne pathogens may exist as vegetative cells or produce spores which once favourable growth conditions are established, grow and replicate, thus causing food-borne diseases (Beuchat *et al*., 2011). Another strategy food-borne pathogens may employ to survive unfavourable PIF environments is biofilm formation (Vestby *et al*., 2020). Biofilms are clusters of bacteria attached to a surface or to each other, which enable pathogens such as *S. aureus*, *Cronobacter* and *Salmonella* spp. to persist on the surfaces of equipment and instrumentation used in PIF production (Ling *et al*., 2020; Tasneem *et al*., 2018). The ability of pathogenic bacteria to survive in PIFs products is crucial to manufacturers and consumers and as such, manufactures of PIFs have developed and make use of various strategies to reduce contamination (Taylor *et al*., 2019).

1.4. Measures to reduce powdered infant formula contamination

Manufacturers of PIFs and the raw ingredients included in these products are required to maintain extremely high standards of hygiene during production to prevent contamination (WHO, 2007b). As such, PIF manufacturers have implemented several control measures to reduce intrinsic contamination (Kent *et al*., 2015). One such measure is the adjustment of good manufacturing practices and is based on four principles: 1) the avoidance of entry and establishment of pathogens, 2) the avoidance of proliferation of pathogens through the elimination of water, 3) regular hygienic practices in high-hygiene zones and 4) the use of *Salmonella* and *C. sakazakii* free dry-mix ingredients (Kent *et al*., 2015).

Currently, researchers are exploring additional approaches that may reduce intrinsic microbial contamination of PIFs. These include supercritical carbon dioxide (SC-CO_2) treatment, heat treatment and ultraviolet (UV) irradiation of the ingredients and finished products (Kim *et al*., 2010; Liu *et al*., 2012). SC-CO_2 treatment is a non-thermal and environmentally friendly sterilization method that relies on the diffusion pressure and solubility of the cell membrane of microorganisms (Efaq *et al*., 2015; Zhang *et al*., 2006). The use of SC-CO_2 treatment in PIFs notably reduces the levels of *Cronobacter* spp. in the final product (Kim *et al*., 2010). When the technique is used in combination with heat treatment using temperatures above 60 °C, it markedly inactivates *Cronobacter* spp. (Kim *et al*., 2010). This is because *Cronobacter* spp. cannot withstand temperatures above 60 °C (Arku *et al*., 2011; Hunter and Bean, 2013).

Similarly, UV treatment has been reported to considerably reduce intrinsic contamination of PIFs by *C. sakazakii*. UV radiation doses between 12.1 ± 0.30 kJ/m² and 72.8 ± 1.83 kJ/m² at ambient temperature exhibited a remarkable inactivation of *C. sakazakii* in dry infant formula

(Liu *et al*., 2012). Although irradiation is the only technology capable of generating sterile PIFs, it is prohibited by the CAC as its efficacy on powered foods has not been determined (FAO/WHO, 2006; FAO/WHO, 2007; Kent *et al*., 2015).

While these techniques ensure that PIFs are as sterile as possible during the production process, it is important to set control measures that ensure PIF safety after reconstitution.

1.4.1. Inclusion of antimicrobial agents in PIFs

Manufacturers have identified and employ a number of control measures to improve the sterility of PIFs post-reconstitution, including the incorporation of intrinsic antimicrobial agents such as probiotics, prebiotics and bioactive antimicrobial proteins (Joosten and Lardeau, 2004; Labuschagne *et al*., 2012).

Probiotics are living microorganisms that offer health benefits to the host when ingested in adequate amounts (FAO/WHO, 2002; Hill *et al*., 2014; Reid *et al*., 2019). Following ingestion, probiotics improve the levels of normal microflora in the digestive tract resulting in the reduction or neutralization of pathogenic microbes (Amara and Shibl, 2015). Probiotics commonly used in PIFs include the *Lactobacillus* spp. *L. rhamnosus*, *L. casei* and *L. reuteri*, as well as *Streptococcus thermophilus* and *Bifidobacterium animalis* subsp. *lactis* (Ackerberg *et al*., 2012; Labuschagne *et al*., 2012).

Prebiotics are selectively fermented fibres which beneficially influence the growth of gut microorganisms (Boehm *et al*., 2005; Flint *et al*., 2012; Gibson *et al*., 2017; Reid *et al*., 2019; Slavin, 2013). The most commonly used prebiotics are fructo-oligosaccharides (FOS), transgalacto-oligosaccharides (TOS) and galacto-oligosaccharide (GOS), which are inherently present in plants (Davani-Davari *et al*., 2019; Kent and Doherty, 2014; Sabater-Molina *et al.*, 2009). Prebiotics modulate the composition, growth and function of gut microflora in particular, *Lactobacilli* and *Bifidobacteria* spp.**,** through their provision of energy to these microorganisms (Davani-Davari *et al*., 2019; Flint *et al*., 2007). The inclusion of probiotics and prebiotics in PIFs has been found to significantly enhance the health of infants by promoting the growth of beneficial gut microflora (Cooper *et al*., 2010; Kent *et al*., 2015; Labuschagne *et al*., 2012; Shah, 2007).

Bioactive proteins are small peptides comprised of 12 - 50 amino acid residues released during enzymatic proteolysis of proteins (Benkerroum, 2010; Kent *et al*., 2015; Korhonen and Pihlanto, 2006; Pane *et al*., 2017). These peptides have various physiological properties such

as antimicrobial, antioxidant and antithrombic activity and can be of bacterial, plant or animal origin (Clare and Swaisgood, 2000; Memarpoor-Yazdi *et al*., 2012; Smacchi and Gobbetti, 2000). Currently, over a hundred bioactive peptides have been identified including lactoferrin, α-lactalbumin and osteopontin (Baldi *et al*., 2005; Expósito and Recio, 2006). These peptides cause bacterial cell death by making pores through cell membranes or by interacting with macromolecules inside the bacterial cells (Daliri *et al*., 2017; Farkas *et al*., 2017; Shah *et al*., 2016; Taniguchi *et al*., 2016; Zhang *et al*., 2017). Alternatively, manufactures may incorporate weak organic acids into the PIFs, which serve the purpose of controlling pathogen growth post-reconstitution.

1.4.2. PIF acidification

Acidification of food involves the use of organic acids to control the growth of food spoilage and pathogenic microorganisms (Barbut, 2005). It is one of the most important processes in the food industry because it influences the taste, consistency and shelf-life of both dairy and non-dairy products (Mudgil and Barak, 2019; Robles-Rodríguez *et al*., 2021). Acidification involves the use of organic acids such as acetic, sorbic, benzoic, formic, citric and propionic acid which are differentiated from other acids by their functional carboxylic group, which is usually attached to an organic group (Cherrington *et al*., 1991; Hazan *et al*., 2004).

Organic acids have been in use as additive and preservative strategies for inhibiting microbial contamination in drink, food and pharmaceutical products for decades (Back *et al*., 2009; Cherrington *et al*., 1991; Ricke, 2003; Zhu *et al*., 2013). Despite the fact that the antimicrobial mechanisms of organic acids are not fully understood, it is assumed that an undissociated form of the acid penetrates the cell membrane of pathogens to achieve its antimicrobial activity (Agostoni *et al*., 2007; Back *et al*., 2009; Oshima *et al*., 2012; Ricke, 2003).

In infant formulas, organic acids are introduced into the product using two approaches, either biologically or chemically (Joosten and Lardeau, 2004; Labuschagne *et al*., 2012). Biological acidification is the incorporation of lactic acid-producing bacteria such as *Lactobacillus amylolyticus* and *Lactobacillus fermentum* into infant formula during production (Hutzler *et al*., 2018). Here, lactic acid-producing bacteria ferment lactose in the PIF into lactic acid (Cooper *et al*., 2010; Szajewska *et al*., 2015). Chemical acidification involves the direct addition of organic acids to the PIFs during the production process (Joosten and Lardeau, 2004; Labuschagne *et al*., 2012; Velaphi *et al*., 2008).

Research has shown that infants fed acidified PIFs have a reduced incidence of diarrhoea and diarrhoeal-associated diseases compared to those that are fed non-acidified PIFs (Brunser *et al*., 1989; Cooper *et al*., 2010; Joosten and Lardeau, 2004). Organic acids such as acetic, butyric, citric, formic, lactic, and propionic acid have been shown to have antibacterial activities (Back *et al*., 2009; Oshima *et al*., 2012; Zhu *et al*., 2013). Further, studies show that acetic, butyric, propionic and lactic acid are effective inhibitors of the opportunistic pathogen, *C. sakazakii* whilst acetic, lactic and propionic acid are effective against the rapid growth of *B. cereus*, *C. sakazakii*, *E. coli*, *S. aureus* and *S. enterica* subsp. *enterica* serovar Typhimurium (Back *et al*., 2009; Joosten and Lardeau, 2004; Zhu *et al*., 2013).

1.5. Problem statement

Powdered infant formulas may become contaminated by pathogenic microorganisms during the production, packaging, transportation or preparation stages, resulting in food-borne illness and, infrequently, mortality in its infant consumers. As such, various methods to improve the microbiological safety of PIFs have been introduced. One such approach involves the acidification with organic acids, where these acids can control the growth of pathogens post-reconstitution of the PIFs. However, the efficacy of different organic acids at controlling the growth of key PIF pathogens remains poorly understood. In this study, we thus set out to evaluate the efficacy of chemical acidification with lactate and citrate against a range of common PIF-contaminating pathogens. The data from this research can inform on future acidification practices in commercial PIF production.

1.6. Aims and Objectives

This study aimed to compare the bacteriostatic and bactericidal effects of lactate and citrate acidification in commercial PIFs against eight common PIF pathogens. Furthermore, by using these acids in common microbial growth medium, we set out to validate that the effects of each acid on the pathogens were due to acidification and not additional components of the PIF or differences in acidity levels (relative pH).

These aims were achieved according to the following objectives:

- Reconstitution of individual PIFs and determination of inherent contamination
- PIF-pathogen inoculation with 10^4 CFU/ml of eight common PIF pathogens
- Incubation of pathogen-spiked powdered infant formulas for up to 6 hours and at different storage temperatures

- Enumeration of pathogens inoculated in individual PIFs by plate counts at different time points.
- Validation of the effects of acids on pathogen growth in citrate and lactate acidified Luria Bertani broth.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Bacterial strains

A total of eight relevant bacterial pathogens were selected for the study (Table 2.1). These included both representatives of risk category A stipulated by the FAO and WHO (FAO/WHO, 2006; Cahill *et al*., 2008), while two representatives each of risk categories B and C were also included (Table 1.1). Further, we included *Listeria innocua* ATCC 33090 as representative of *L. monocytogenes*. Given the inherent risk of working with *L. monocytogenes*, the non-pathogenic *L. innocua* serves as a model for the pathogen, having similar phenotypic characteristics and physiological behaviour to *L. monocytogenes* (Milillo *et al*., 2012; Kaszoni-Rückerl *et al*., 2020). The eighth pathogen, *Pseudomonas aeruginosa* is not included in the FAO/WHO risk category list. However, an outbreak with this pathogen was observed in a neonatal intensive care unit in Spain, and it was linked to prepared infant formula and bottles (Sánchez-Carrillo *et al.*, 2009). As such, it was decided to include this pathogen in the study.

All strains were obtained from Thermo Fisher Scientific (Walton, MA, USA), either as ready-to-use Culti-loops, or as freeze-dried cultures. All cultures were initially grown by streak-plating on Luria Bertani (LB) agar (Appendix A) and incubated at 37 ºC overnight. Single colonies were selected and sub-cultured in 50 ml of LB broth (Appendix A), with rotary shaking at 120 rpm and incubation at 37 ºC overnight. Subsequently 25 % glycerol stocks of each culture were prepared by mixing 500 µl 50 % glycerol (Appendix A) and 500 µl culture in 1.5 ml screw-cap tubes and these were stored at -80 ºC until use.

Table 2.1: Bacterial strains and their selective media

Pathogen	Strain	Risk category	Selective medium
Cronobacter sakazakii	ATCC 29544	A	*Cronobacter* ChromoSelect Agar
Salmonella enterica subsp. *enterica* serovar Enteritidis	ATCC 13076	A	Violet Red Bile Glucose Agar
Citrobacter freundii	ATCC 8090	B	Violet Red Bile Glucose Agar
Escherichia coli	ATCC 25922	B	Violet Red Bile Glucose Agar
Bacillus cereus	ATCC 11778	C	*Bacillus cereus* Selective Agar
Staphylococcus aureus	ATCC 25923	C	Baird Parker Agar
Listeria innocua	ATCC 33090	-	Luria Bertani Agar
Pseudomonas aeruginosa	ATCC 10145	-	Cetrimide Agar

2.1.2. Culture media

All routine culturing of the pathogens was done in LB broth or LB agar as required. However, for pathogen enumeration, selective agar media suitable for each pathogen were used. A selective medium is used for the selective isolation or growth of a specific species while inhibiting the growth of undesired microorganisms (Bonnet *et al*., 2020).

For *C. freundii* ATCC 8090*, E. coli* ATCC 25922 and *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, all three members of the family *Enterobacteriaceae*, Violet Red Bile Glucose (VRBG) agar (Millipore, India; 102499722), was used. VRBG agar enables the growth of *Enterobacteriaceae* while inhibiting the growth of other bacterial species (Mossel *et al*., 1978). VRBG works on the principle of its constituents, where the bile salts and crystal violet inhibit Gram-positive bacteria, while sodium chloride retains the osmotic balance (Mossel *et al*., 1978). The selectivity of the medium can further be enhanced by incubation at

certain temperatures (below 4 °C or above 37 °C) or under anaerobic conditions (Corry *et al*., 1995).

Bacillus cereus selective agar (Millipore, India; 22310) was used to select for the selective growth of *Bacillus cereus* ATCC 11778. It allows the growth of *B. cereus*, while inhibiting the growth of Gram-negative bacteria due to the addition of the antibiotic polymyxin B supplement (Holbrook and Anderson, 1980). Further, it differentiates *B. cereus* from other *Bacillus* spp. based on their ability to ferment mannitol as well as their sensitivity to polymyxin (Holbrook and Anderson, 1980) *S. aureus* ATCC 25923 was selectively grown on Baird Parker agar (Millipore, India; 11750) (Baird-Parker, 1962; Holbrook and Anderson, 1980). Baird Parker agar is used for the isolation and differentiation of coagulase-positive staphylococci based on their ability to produce the enzyme coagulase (Baird-Parker, 1962). Lithium chloride and tellurite inhibit the growth of other microorganisms while glycine and sodium pyruvate promote the growth of *S. aureus* (Baird-Parker, 1962).

Cronobacter ChromoSelect agar (Millipore, India, 92324) was used for the selective growth of *C. sakazakii* ATCC 29544. Due to the presence of desoxycholate, the medium is selective against most Gram-positive organisms (Muytjens *et al*., 1983). *Cronobacter* spp. possess the enzyme glucosidase which cleaves the chromogenic mixture thus resulting in blue-green colonies (Muytjens *et al*., 1983). *P. aeruginosa* ATCC 10145 was selectively grown on Cetrimide agar (Millipore, India; 22470). Cetrimide agar is both a selective and differential medium to isolate and identify Gram-negative bacterium *Pseudomonas aeruginosa* (King *et al*., 1954). Cetrimide serves as a selective agent that inhibits the growth of Gram-positive and Gram-negative microorganisms including other *Pseudomonas* spp. while allowing the growth of *P. aeruginosa* (Brown and Lowbury, 1965). Further, it enhances the production of pyocyanin, a blue pigment that gives *P. aeruginosa* colonies a blue-green colour (Brown and Lowbury, 1965).

No suitable selective medium could be obtained for *L. innocua* ATCC 33090. As such, given the lack of contaminating growth from the reconstituted infant formula (see section **2.2.1** below), it was decided to utilise LB agar for the latter pathogen. All media were prepared as indicated in Appendix A.

2.1.3. Powdered infant formulas and reconstitution

Three commercial PIFs were evaluated in this study and were purchased in the supermarket. Two of the commercial formulas were chemically acidified during manufacturing with citric acid (citrate) and lactic acid (lactate), respectively. A non-acidified PIF, with similar nutritional composition to the acidified formulas, was included in the study as control. All three PIFs were reconstituted by adding four scoops of PIF in 100 ml of autoclaved deionized water which was initially boiled (~ 100 °C) then cooled to 70 °C before reconstitution, followed by cooling to room temperature prior to spiking trials. The initial pH of the different reconstituted PIFs were determined using a pH meter (Basic 20; Crison, Spain).

2.2. Methods

2.2.1. Evaluation of inherent microbial contaminants in commercial PIFs

Aliquots (100 µl) of each of the reconstituted PIFs were spread-plated onto LB agar plates in triplicate. The agar plates were incubated at 4, 25 and 37 °C for 24 hours and subsequently evaluated for the growth of contaminating microorganisms originating from the reconstituted PIFs.

2.2.2. Standardisation of pathogen concentrations

In order to standardise the analysis of pathogen survival in the different reconstituted PIFs, dilution series were performed for each pathogen to determine the dilution and volume that would be needed to achieve a standard of 10^4 (10,000) CFU/ml in the reconstituted PIFs at the onset of the experimental trials.

Each of the pathogens were grown in 50 ml LB broth for 16 hours at 37 °C on a rotary shaker at 120 rpm. Subsequently, serial dilutions were carried out using phosphate buffered-saline (PBS) (pH 7.4) as diluent (Appendix A). Dilutions of each pathogen (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) were prepared in volumes of 10 ml. A volume of 100 µl of each pathogen dilution was spread plated onto LB agar plates in triplicate followed by incubation at 37 °C for 16 hours. Following incubation, visible colonies present on the plates were counted and the CFU/ml values were calculated for plates that had detectable colonies. The formula used to estimate the number of colony forming units (CFUs) per ml is $\frac{number\ of\ colonies\ x\ dilution\ factor}{volume\ cultured\ on\ plate}$ (Breed and Dotterrer, 1916). All initial standardization experiments were performed in triplicate.

2.2.3. Reconstituted PIF pathogen-spiking trials

On the basis of the results from section 2.2.2., the reconstituted PIFs were spiked with approximately 10^4 CFU/ml of each pathogen (10^{-6} serial dilution). The pathogen-spiked infant formulas were incubated at three different temperatures, 4, 25 and 37 °C, for a totality of six hours to evaluate pathogen concentrations in the reconstituted infant formulas under refrigeration, ambient and optimal storage temperatures. Samples were taken 0-, 1-, 3-, and 6-hours post-inoculation to evaluate pathogen growth in the reconstituted PIFs. The pH of the reconstituted formulas was monitored at each of the four different time points using the Crison Basic 20 pH meter.

Aliquots (100 µl) were taken from the pathogen-spiked PIFs, and serial dilutions (10^{-2}) were carried out for all pathogens as 10 000 (10^4) CFU would be too numerous to count on selective agar. For these serial dilutions, 900 µl PBS was used as a diluent. Thereafter, 100 µl was spread-plated onto the selective media specific to each bacterial pathogen (Table 2.1.). The spread plates were incubated 37 °C for 24 hours. For each pathogen, this protocol was repeated in triplicate to validate the reproducibility of the results.

2.2.4. Pathogen enumeration and statistical analyses

Pathogen colonies derived from the spiked reconstituted PIFs were counted on the selective agar plates at the appropriate dilution. The colony counts were then converted to their CFU/ml values as discussed in section 2.2.2. These values were further converted to their respective logarithmic values (log10) and graphed using Microsoft 365 Excel.

For the statistical analysis, we took the complete set of logarithmic CFU/ml results for each acidification technique across all temperatures and compared all values to one another. An Analysis of variance (ANOVA) test was then performed to inform the subsequent independent t-test. The standard deviation was also calculated for each triplicate dataset to ensure there was no large enough deviation between the replicate results which would skew any results of statistical tests.

2.2.5. Validation of the effects of acids in LB broth on pathogen growth

To validate that the pathogen growth trends in the reconstituted PIFs was due to chemical acidification and not due to other PIF constituents, pathogens were grown in non-selective Luria Bertani broth which was acidified with either citric monohydrate (USA, Sigma-Aldrich;

C1909) or L (+) lactic acid (South Africa, ACE Chemicals; L1249CC), respectively, to similar pH values as observed in PIFs upon reconstitution. The pH was measured using the Crison Basic 20 pH meter.

Based on the results from section 2.2.2., 9.9 ml of the citrate and lactate acidified as well as non-acidified LB broth was spiked with 100 µl of each pathogen (to a final concentration of ~ 10^4 CFU/ml) and incubated at 4, 25 and 37 °C in triplicate. The absorbance (optical density) was measured with a spectrophotometer (Germany, BOECO S-22 UV/Vis) at a wavelength of 600 nm every 24 hours over a period of 96 hours post-inoculation.

As discrepancies in the pH of the reconstituted citrate- and lactate acidified formulas were observed, the pH of the lactate acidified LB broth was also adjusted to that observed for the citrate acidified PIF, and vice versa. The above process for pathogen spiking and spectrophotometric analyses was repeated for these inverse pH values. The data of each triplicate were statistically analysed as discussed in 2.2.4.

Chapter 3: Results and Discussion

3.1. Analysis of inherent PIF contamination

Samples taken from each reconstituted infant formula were grown on non-selective LB medium and evaluated for inherent contaminant bacteria. No growth was observed for any of the three evaluated formulas, and as such, all three products were free from culturable contaminants.

3.2. Properties of the reconstituted PIFs

The pH of each reconstituted formula prior to pathogen addition, and at the different storage time points (and temperatures) post pathogen-spiking was evaluated. The mean initial pH of the reconstituted citrate- and lactate acidified infant formulas were 4.70 and 4.50, respectively, while that of the non-acidified infant formula was 6.77. No change in the pH was observed in the pathogen-free PIFs stored at 4, 25 and 37 °C across the six-hour storage period (Appendix B). By contrast, a slight increase in the pH of the reconstituted formulas was observed during the 6-hour incubation period post pathogen inoculation, increasing with time, regardless of whether the infant formulas were acidified or not (Table 3.1; Appendix C). This was observed for all eight pathogens. For example, for *B. cereus* ATCC 11778 the pH of the citrate acidified infant formula increased from 4.62 to 4.68 at 4 °C while at 25 and 37 °C it increased from 4.62 to 4.70 and 4.62 to 4.71, respectively. This indicated that the increase in pH of the formulas over the incubation period across the three storage temperatures was likely due to the metabolic activities of the pathogens and not other reactions taking place in the reconstituted PIFs.

The concentration of protons (H^+ ions), generally referred to as pH, is one of the most crucial environmental variables that influence the growth and survival of microbes (Lund *et al*., 2020). Most bacteria grow optimally in environments with neutral pH (7.0). However, they generally have the propensity to grow and proliferate at pH values ranging from 4.6 – 9.0 (Table 3.2) (Bintsis, 2017). Bacteria counter acid stress (the effects of low pH) by preventing the decline of the threshold cytoplasmic pH level (Lund *et al*., 2020). Most microorganisms have the ability to modify the pH of the environment they inhabit in order to adapt when conditions are unfavourable (Ratzke and Gore, 2018). Bacterial cells often secrete urease, an enzyme which hydrolyses urea into ammonia and as a consequence, increase the pH of the environment they inhabit (Ratzke and Gore, 2018; Solé *et al*., 2010). Alternatively, microorganisms may use amine-containing amino acids to neutralize the acidity or remove the protons from the cells by consuming adenosine triphosphate (ATP) (Lund *et al*., 2020; Pennacchietti *et al*., 2018).

Table 3.1: Mean pH measurements for the pathogen-spiked reconstituted PIFs at the four different time points post-inoculation

Storage temperature	Citrate Acidified Formula			
	0	**1**	**3**	**6**
4 °C	4,70	4,76	4,70	4,76
25 °C	4,70	4,75	4,75	4,76
37 °C	4,70	4,74	4,76	4,76
	Non-Acidified Formula			
	0	**1**	**3**	**6**
4 °C	6,77	6,78	6,81	6,80
25 °C	6,77	6,82	6,80	6,80
37 °C	6,77	6,77	6,75	6,73
	Lactate Acidified Formula			
	0	**1**	**3**	**6**
4 °C	4,50	4,53	4,52	4,53
25 °C	4,50	4,54	4,49	4,54
37 °C	4,50	4,54	4,53	4,53

Table 3.2: Growth conditions of the PIF pathogens

Bacterial species	pH range	Temperature range (°C)	Optimal temperature (°C)	References
Bacillus cereus	4.9 – 9.3	4 – 48	28 – 35	FDA, 2012
Citrobacter freundii	5.0 – 8.0	5 – 45	37	Rogers *et al*., 2016
Cronobacter sakazakii	5.0 – 10	6 – 45	34 – 37	Iversen *et al*., 2004 ; Iversen *et al*., 2008
Escherichia coli	4.5 – 9.0	23 – 40	37	Wilks and Slonczewski, 2007
Listeria innocua	4.0 – 9.5	<1 – 50	30 – 37	Batt, 2014
Pseudomonas aeruginosa	4.5 – 9.5	4 – 42	37	Klein *et al*., 2009; LaBauve and Wargo, 2012
Salmonella enterica	4.0 – 9.0	5 – 47	32 – 35	Jajere, 2019
Staphylococcus aureus	5.7 – 8.6	6.5 – 46	37 – 40	Medveďová and Valík, 2012

3.3. PIF acidification negatively affects the growth of pathogens

The inclusion of weak organic acids such as citric, acetic and lactic acid in foods offers various antimicrobial benefits against a broad range of bacterial and fungal pathogens (Eswaranandam *et al*., 2004; Musyoka *et al*., 2018; Raftari *et al*., 2009 Samelis *et al*., 2001). These acids act against microbial cells by penetrating the lipid bilayer of the cell membrane and releasing H^+ ions in the cytoplasm (Lund *et al*., 2020). This triggers cytoplasmic acidification and the transmembrane proton motive force collapses, thus resulting in a decline in the bacterial population (Lund *et al*., 2020; Mira and Teixeira, 2013).

Powdered infant formula acidification, regardless of whether lactate or citrate was added, resulted in a marked reduction in the growth of the eight evaluated pathogens in comparison to the non-acidified formula (Figure 3.1). This was supported by statistical analyses where the non-acidified infant formula (M = 5.80 SD = 0.17) supported significantly greater growth of

the pathogens than the acidified infant formulas (M = 4.59 SD = 1.78), $p < 0.05$. For instance, in the non-acidified infant formula, *C. freundii* ATCC 8090 attained a maximum of ~ 10^6 CFU/ml) at 37 °C after six hours, whereas this pathogen reached a maximum of ~10^5 CFU/ml) in the acidified PIFs under the same conditions (Appendix D). A similar trend was observed across all three storage temperatures for all eight evaluated pathogens, demonstrating the antimicrobial effects of lactic and citric acid on the reduction of bacterial growth in reconstituted PIFs. This corroborates previous *in vitro* (in acidified media) studies where lactate and citrate both negatively affected the growth of *C. sakazakii*, *E. coli L. monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Gaminara, *Shigella* spp. and *S. aureus*. (Arvizu-Medrano and Escartín, 2005; Back *et al.*, 2009; Eswaranandam *et al*., 2004; In *et al.*, 2013).

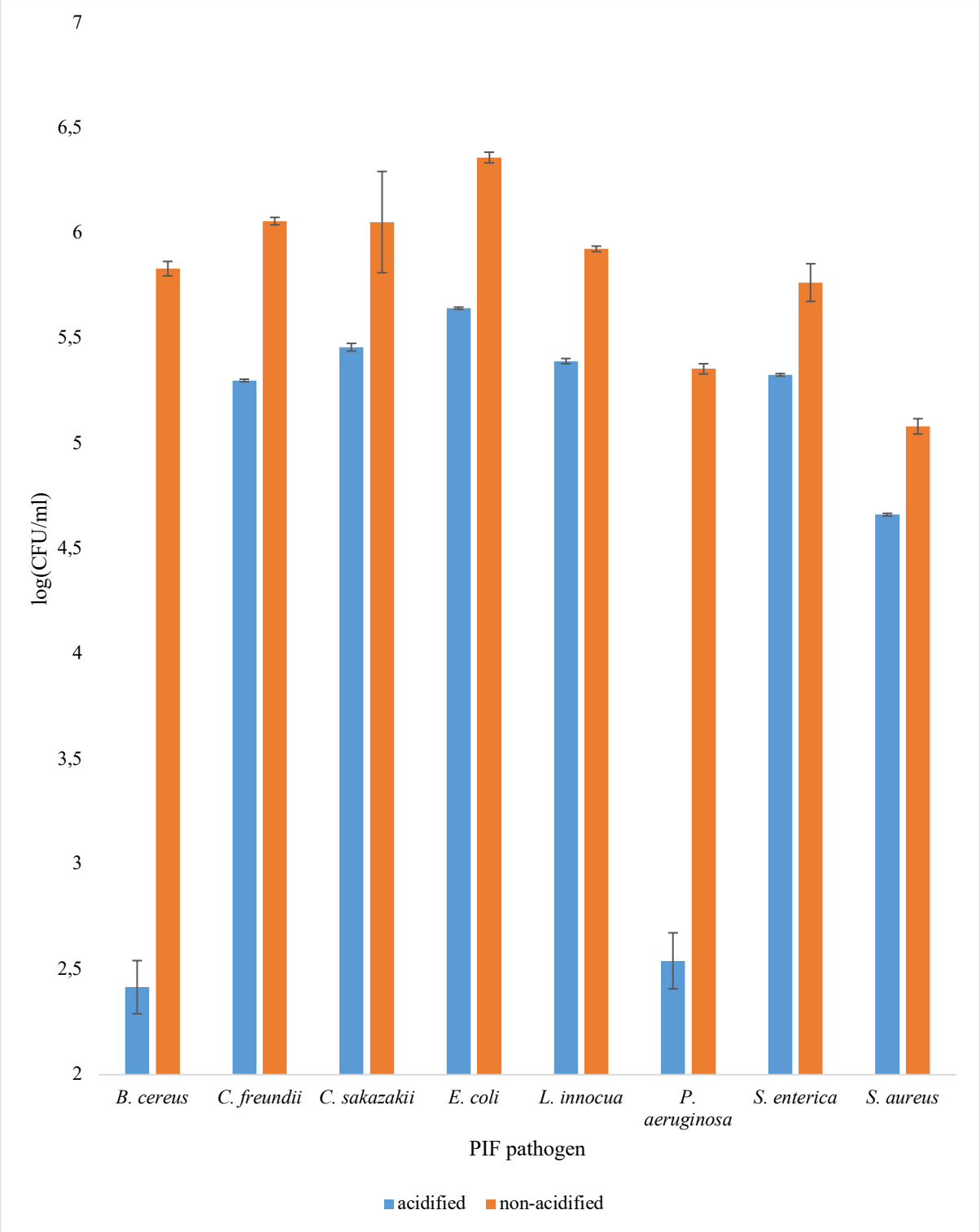


Figure 3.1: Effect of acidification on pathogen growth in reconstituted PIFs after 6 hours at 37 °C. The error bars show the standard error of the mean of the triplicates. The mean of the two acidified PIFs was used to construct the graph.

3.4. Lactate acidification is more effective than citrate acidification for most pathogens

While PIF acidification resulted in a marked reduction in the growth of all eight evaluated pathogens, differences in pathogen reduction between the two acids incorporated in the PIFs could be observed. On average, significantly lower growth was observed for pathogens grown in the lactate acidified PIF compared to the citrate acidified formula acidification (lactate M = 3.90 SD = 0.74 and citrate M = 5.29 SD = 0.02, $p < 0.05$). Lactate acidification exhibited the greatest antimicrobial activity against six out of the eight evaluated pathogens, namely *B. cereus* ATCC 11778, *C. sakazakii* ATCC 29544, *E. coli* ATCC 25922, *L. innocua* ATCC 33090, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 10145 (Figure 3.2).

Lactate is a weak organic acid comprising a carboxylic acid and a hydroxyl group. The asymmetrical α-carbon in its carboxyl group induces inhibitory effects in a wide range of microorganisms (Martyn, 2011; Wang *et al*., 2014). Further, due it being a water-soluble molecule it is able to gain access to the periplasm of bacterial cells by using porin proteins of the outer membrane (Alakomi *et al*., 2000). Small organic acids penetrate bacterial cell membranes more efficiently in comparison to large organic acid and thus may effect greater antimicrobial activities (Doores, 1993; In *et al*., 2013). The higher inhibitory effect of lactate acidification compared to citrate acidification is often attributed to the difference in the sizes between lactate (90.06 Da) and citrate molecules (210.14 Da) (Litchfield, 2009; Papagianni, 2007). Bjornsdottir *et al*., (2006) demonstrated that lactic acid provided a greater inhibitive effect against the pathogen *Escherichia coli* O157:H7 compared to citric acid, which did not exhibit any inhibitory effect at similar concentrations. Additionally, Abu-Ghazaleh (2013) reported that lactic acid showed superior antimicrobial activity against *S. aureus* and *E. coli* compared to citric acid which is consistent with the present study.

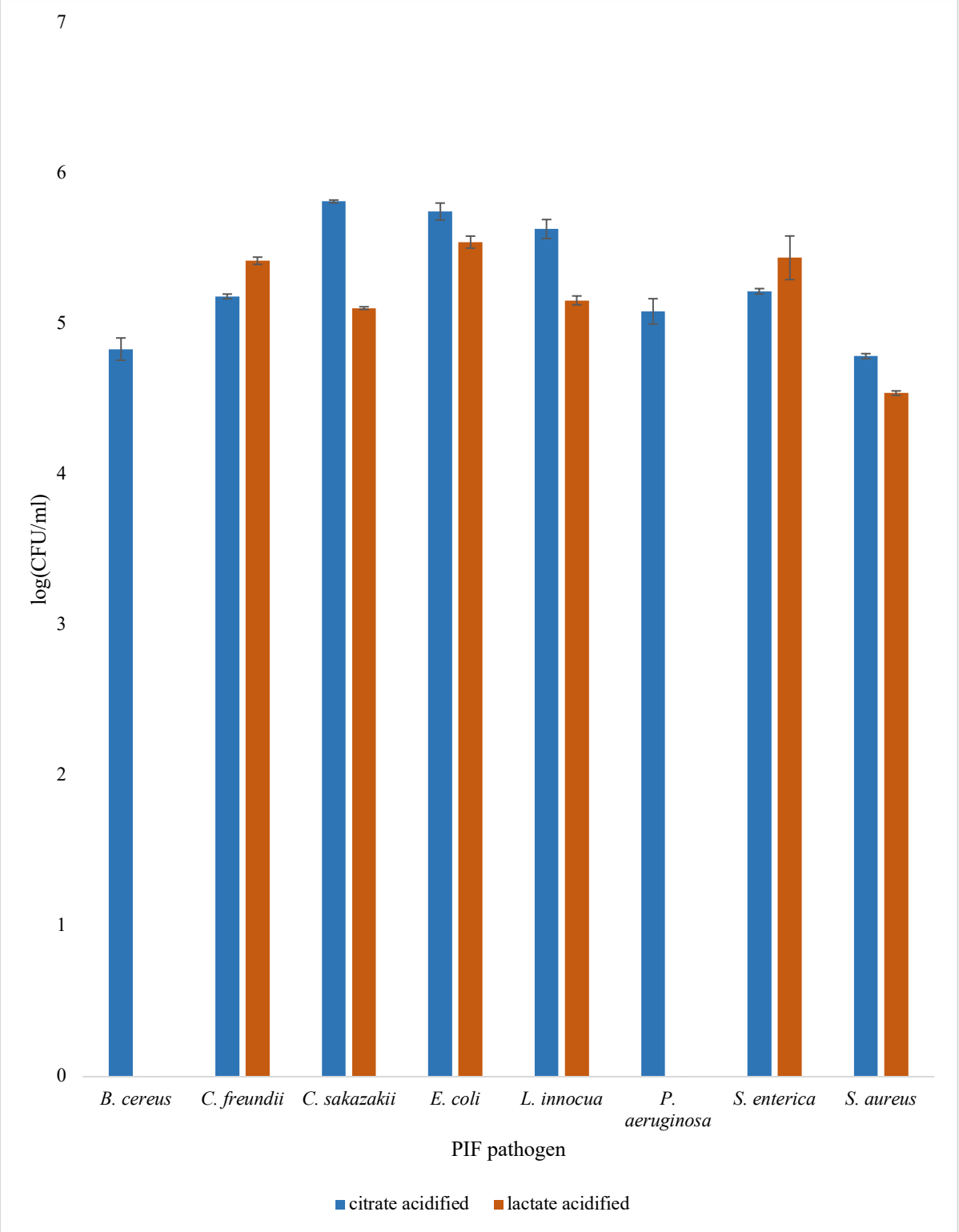


Figure 3.2: Effect of citrate- and lactate-acidification on pathogen growth in reconstituted PIFs after 6 hours at 37 °C. The error bars show the standard error of the mean of the triplicates.

Citrate displayed a greater inhibitory effect than lactate on two of the pathogens evaluated in this study, the enterobacteria *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 and *C. freundii* ATCC 8090 (Figure 3.2). Some bacterial species, at certain pH ranges, may employ the enzyme lactate dehydrogenase, to convert the undissociated form of lactate back to pyruvic acid (Brul and Coote, 1999; Červenka *et al*., 2004). This reduces the concentration of intracellular lactate and as such, prevents the lactate from triggering intracellular acidification in bacterial cells. Similar to our study, Hussain *et al*., (2015) reported that citric acid was more effective than lactic acid in reducing the growth of *Salmonella* spp. on beef at similar concentrations.

3.5. Lactate acidification has bactericidal effects on *B. cereus* and *P. aeruginosa*

Despite the reduction in pathogen number in the acidified reconstituted PIFs respective to the non-acidified formula, the pathogens continued to grow from the initial 10^4 CFU/ml to up to 1.5 x 10^5 CFU/ml and 2.6 x10^5 CFU/ml in the citrate- and lactate acidified reconstituted formula respectively, as observed for *C. freundii* ATCC 8090 grown at 37 °C six hours post-inoculation (Appendix D). This suggests that both acids have a bacteriostatic effect, retarding the growth of most pathogens, and that pathogen recovery occurs with prolonged storage times. That said, none of the pathogens recover to the same numbers as observed in the non-acidified reconstituted PIF.

For two pathogens, recovery over time was not observed, namely *B. cereus* ATCC 11778 and *P. aeruginosa* ATCC 10145, when grown in lactate acidified PIF. No growth was observed for *B. cereus* after 3 and 6 hours at 25 and 37 °C, respectively (Appendix E). This suggested that, rather than being bacteriostatic, lactate is bactericidal for this pathogen. Wong and Chen (1988) reported that lactic acid produced by the lactic acid bacterium *Streptococcus lactis* exhibited bactericidal inhibition and prevented germination of *B. cereus* spores in non-fat milk medium. Similar inhibition of *B. cereus* was described by Byaruhanga *et al*. (1999), where the growth was reduced from 10^6 CFU/ml to 10^2 CFU/ml. In another study, lactate acidification resulted in the reduction of *B. cereus* from 900 CFU/ml to 100 CFU/ml (Yang *et al*., 2008).

A similar inhibitory effect of lactate on *P. aeruginosa* ATCC 10145 growth was observed after 6 hours of PIF storage at 37 °C (Appendix E). The number of colony forming units decreased from 10^4 CFU/ml to < 1 CFU/ml in 6 hours (Appendix D). A study by Jamalifar *et al*. (2011) showed that lactic acid has the capability to suppress the growth of *P. aeruginosa*. Cherrington

et al. (1992) reported that the bactericidal effect of organic acids is enhanced by increasing temperature and incubation time. This proved true for these two pathogens as the bactericidal activity of lactic acid was found to be much higher after six hours of incubation at 37 °C than after the same time period at 4 and 25 °C.

3.6. Interplay between acidification and storage temperature in controlling pathogen growth in reconstituted PIFs

The temperature at which the reconstituted infant formulas were stored was found to have an influence on the growth of the pathogens (Appendix E). Temperature is one of the most crucial factors that influence the growth and survival of bacteria. Whilst every bacterial species has its own optimal growth temperature, most human bacterial pathogens usually flourish at 37 °C (Gutierrez *et al*., 2018). The increase or decrease in temperature above or below an organism's optimal range often leads to the loss of function of enzymes thus slows down their metabolic processes (Sauer and Wang, 2019). Low temperatures usually have a bacteriostatic effect on the growth (Nedwell, 1999).

In the present study, all the pathogens grew substantially better at 25 and 37 °C compared to growth at 4 °C in all three formulas. Negligible increases (or slight decrease) in cell numbers at the lower temperature indicates that refrigeration at 4 °C had a bacteriostatic effect, regardless of acidification. Slight decreases in number of colonies (CFU/ml) in the lactate acidified formula were observed at 4 °C for *B. cereus* ATCC 11778, *L. innocua* ATCC 33090 and *P. aeruginosa* ATCC 10145 (Appendix E). However, more effective control of pathogen growth with respect to acidification was observed when the pathogen-spiked PIFs were stored at 25 and 37 °C. This suggests that acidification is more effective in metabolically active pathogen cells. Overall, pathogen growth was lower at 25 °C compared to 37 °C and lower CFU/ml values were observed (Appendix E). This suggests that sub-optimal temperatures and pH in combination most effectively controlled the growth of the eight pathogens in the reconstituted PIFs.

3.7. Pathogen growth is directly affected by acidification, not other PIF constituents

Pathogen growth in acidified reconstituted infant formulas was demonstrably lower than the non-acidified control. However, it could not be excluded that this effect was due to differences in PIF constituents other than the added acids. To confirm the direct effects of the acids on pathogen growth, each pathogen was grown in non-selective LB broth which was chemically acidified through the addition of citrate and lactate, respectively, to pH values reflecting those

of the PIFs upon reconstitution. The effects of the acids on pathogen growth (consistently at optimal physiological temperature for the pathogens – 37 °C) were then evaluated by spectrophotometric analysis 24-hourly over a period of 96 hours.

Demonstrably lower growth, as indicated by lower absorbance (OD_{600}) values, was observed for both lactate and citrate acidified LB medium compared to the non-acidified control (pH = 6.64) (Figure 3.3; Appendix F). This was further confirmed by statistical analysis where the growth of the pathogens in the lactate (M = 0.88 SD = 0.43) and citrate acidified LB medium (M = 1.41, SD = 0.19) was significantly reduced compared to the non-acidified control (M = 1.88 SD = 0.01), $p < 0.005$. This corroborates that the acid and not other PIF constituents or properties affect pathogen growth. Increases in absorbance (OD_{600}) are observed throughout the experiment regardless of acidification, suggesting a bacteriostatic, rather than bactericidal, effect for the acids. However, absorbance values of pathogens grown in acidified LB medium remain lower than those observed in the non-acidified control, indicating that the bacteriostatic effect is retained even 96 hours post-inoculation (Appendix F).

The disparate efficacies of lactate and citrate in controlling pathogen growth in the acidified LB medium are also largely consistent with those observed for the differently acidified PIFs. As such, lactate is more effective in controlling the growth of *B. cereus* ATCC 11778, *C. sakazakii* ATCC 29544, *E. coli* ATCC 25922, *L. innocua* ATCC 33090, *P. aeruginosa* ATCC 1045 and *S. aureus* ATCC 25923. In particular, lactate acidification was found to have bactericidal effects on *B. cereus* ATCC 11778 and *S. aureus* ATCC 25923. For *E. coli* ATCC 25922, little difference in the absorbance values between the citrate and the non-acidified control medium was observed at all four sample time points, while substantially lower growth was observed in the lactate acidified LB medium (Appendix F). Also consistent with the results from the reconstituted PIF spiking trials, growth of *C. freundii* was substantially lower for the citrate acidified LB medium compared to the lactate acidified and control media.

One exception was observed for *S. enterica subsp. enterica serovar* Enteritidis ATCC 13076, where lactate acidification was more effective at controlling growth of this pathogen compared to citrate acidification when used in LB medium (Figure 3.3; Appendix F). This is in contrast to what was observed in the acidified PIFs. The reason for the discrepancy between the acidified PIF and LB medium for this pathogen remains unclear. *Salmonella* spp. have the ability to grow on citrate (Andino and Hanning, 2015). They utilize the enzyme citrate lyase to catabolize citrate and use it as a carbon source. This leads to the decrease of citrate in the

intracellular matrix and thus a reduction in the inhibitory activity of citrate (Andino and Hanning, 2015). In the LB medium, this activity may be repressed by other available nutrients. Nevertheless, from this analysis it can be concluded that the growth of the pathogens evaluated was directly affected by acidification and this effect was not due to other components of the infant formulas.

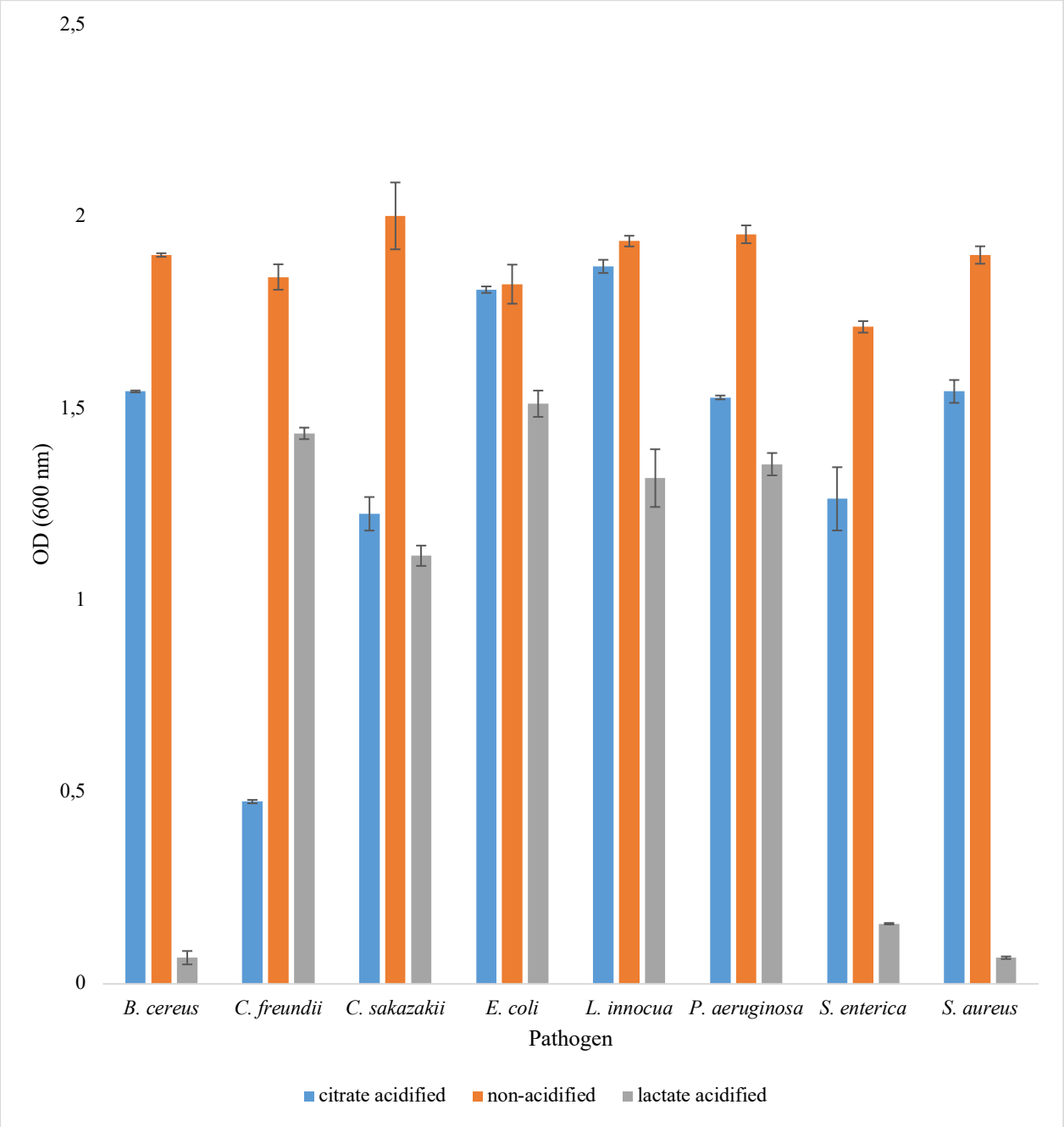


Figure 3.3: Growth absorbance (OD at 600 nm) of PIF pathogens in citrate-, non- and lactate acidified LB broth at 37 °C 96 hours post-inoculation. The error bars show the standard error of the mean of the triplicates.

3.8. Acidification pH is a key driver affecting pathogen growth

Measurement of the pH of the reconstituted PIFs prior to addition of the pathogens indicated that the pH of the lactate acidified formula (pH = 4.45) was consistently lower than that of the citrate acidified (pH = 4.60) formula. To ascertain whether the type of acid or the discrepancy in pH value (~0.15) was responsible for the observed differences in efficacy in controlling pathogen growth, LB medium was prepared with the inverse pH values, with citrate acidified LB medium at pH 4.45 and lactate acidified medium at pH 4.60. Spectrophotometric analysis of the absorbance values of pathogens grown in the inversely acidified formulas was again undertaken.

This analysis showed that, 96 hours post-inoculation, all pathogens treated with citrate at pH 4.45 (M = 0.67 SD = 0.07) showed lower absorbance (OD_{600}) and concomitantly lower growth than those treated with citrate at pH 4.60 (M = 1.41 SD = 0.19) $p < 0.05$ (Figure 3.4; Appendix G). Similarly, significantly lower absorbance values were observed for pathogens grown in LB medium containing lactate at pH 4.45 (M = 0.69 SD = 0.41) compared to lactate at pH 4.60 (M = 1.20 SD = 0.10) $p < 0.05$. This suggests that the level of acidity is key in determining the bacteriostatic effects of acidification. This is further supported by comparing the effects of the citrate acidified medium at pH 4.45 to the lactate acidified medium at pH 4.6, where absorbance values were consistently lower for the former compared to the latter treatment (Figure 3.4).

A comparison of the LB medium acidified with citrate and lactate at the same pH of 4.45 was undertaken. Both the acidified media exhibited inhibitory effects against the growth of the pathogens however, the differences in their antimicrobial activity was statistically insignificant (citrate = M = 0.67 SD = 0.07 and lactate M = 0.69 SD = 0.40, $p > 0.005$). The lactate acidified LB medium demonstrated greater inhibitory effects against *B. cereus* ATCC 11778*, C. sakazakii* ATCC 29544, *S. enterica* subsp. *enterica serovar* Enteritidis ATCC 13076*,* and *S. aureus* ATCC 25923, while citrate was found to have superior antibacterial effects against *C. freundii* ATCC 8090, *E. coli* ATCC 25922, *L. innocua* ATCC 33090 and *P. aeruginosa* ATCC 10145 (Figure 3.4)

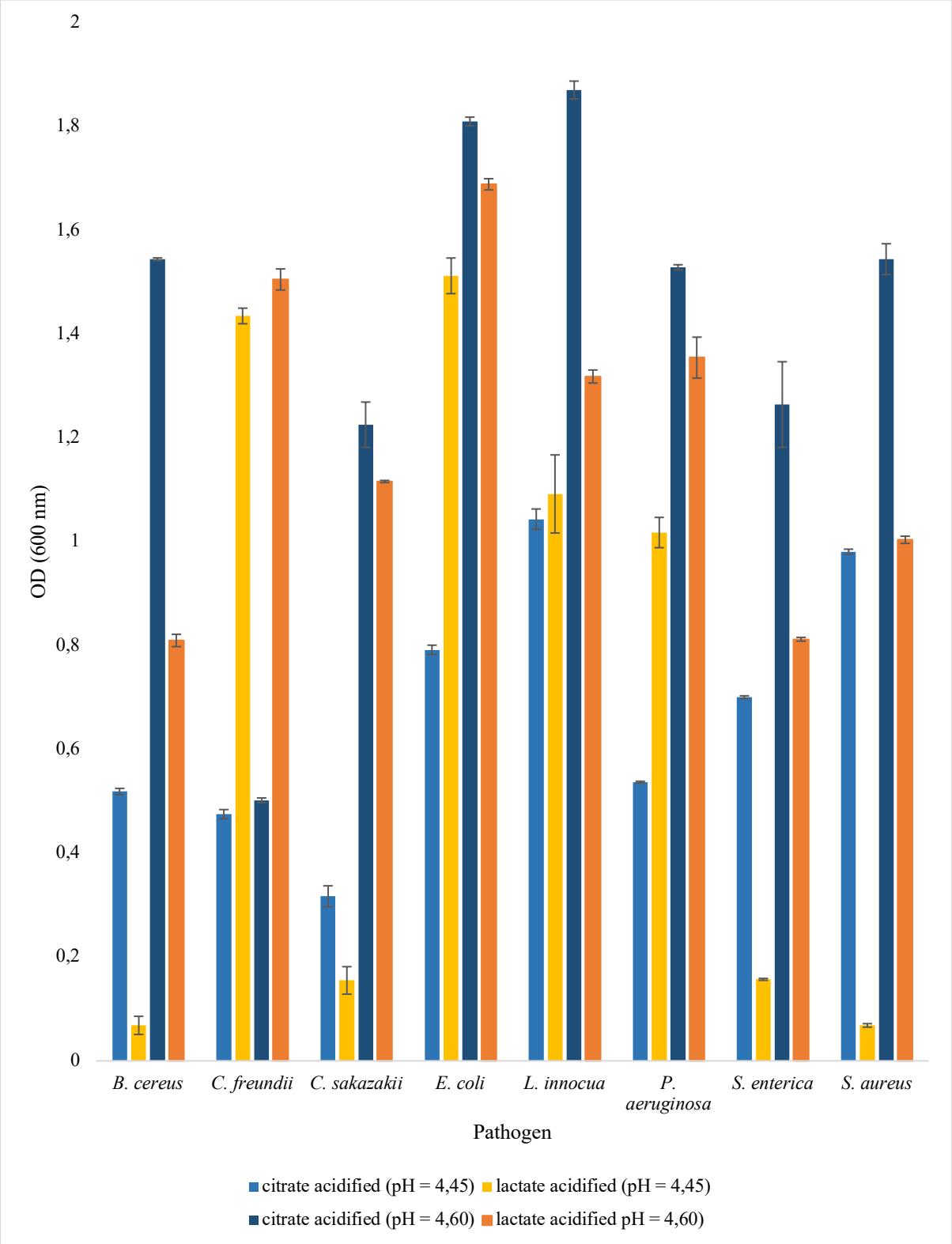


Figure 3.4: Growth absorbance (OD at 600 nm) of PIF pathogens in citrate-, and lactate acidified LB broth at 37 °C 96 hours post-inoculation. The error bars show the standard error of the mean of the triplicates

The growth of pathogens were treated with citrate and lactate at pH 4.60 revealed that lactate acidification exhibited the greatest antimicrobial activity against seven out of the eight evaluated pathogens, namely *B. cereus* ATCC 11778, *C. sakazakii* ATCC 29544, *E. coli* ATCC 25922, *L. innocua* ATCC 33090, *P. aeruginosa* ATCC 10145, *S. enterica* subsp. *enterica serovar* Enteritidis ATCC 13076 and *S. aureus* ATCC 25923 while citrate was more effective against the growth of *C. freundii* ATCC 8090 (Figure 3.4; Appendix G). This was further supported by statistical analysis where the lactate acidified LB medium (M = 1.20 SD = 0.10) resulted in a significantly reduced the growth of the pathogens compared to the citrate (M = 1.41 SD = 0.19), $p < 0.005$.

The pH of an organic acid determines its antimicrobial activity as well as the proportions of the undissociated ion of that acid. (Hsiao and Siebert, 1999; Ullah *et al*., 2012). At low pH units, more undissociated ions of the acid are present and thus can easily permeate cell membranes resulting in the inhibition of pathogens (Ullah *et al*., 2012). This suggests that the level of acidity plays a role in controlling the growth of microorganisms. This was observed in both the lactate and citrate acidified LB broth where a lower pH (4.45) resulted in greater antimicrobial activity compared to acidification at a higher pH (4.60).

Chapter 4: Summary

Infant formula represents a pivotal nutrient source for infants when breast feeding is not an option. Given their propensity to develop food-borne illnesses, ensuring the microbiological safety of PIFs is imperative. Due to their high nutrient content, PIFs serve as hotpots for microorganisms. This was demonstrated in our evaluation of a commercial non-acidified infant formula, where extensive growth of eight key PIF pathogens could be observed. Here, *E. coli* ATCC 25922 increased from 10^4 CFU/ml up to 2.3 x 10^6 CFU/ml

Various techniques have been introduced into PIF manufacturing processes to reduce inherent contamination of the PIFs during production process. However, due to improper hygiene practices, contaminated utensils and water, pathogens may be introduced post-production. One effective means to control pathogen growth post-production which has been introduced by several companies in commercial PIFs is the addition of weak organic acids, which effectively controls the growth of pathogens post-reconstitution.

In the present study, we evaluated three commercial PIFs; two acidified with L-lactate and citrate and one non-acidified. We sought out to determine the efficacy of these acids at controlling the growth of eight key PIF pathogens post-reconstitution. It demonstrated that non-acidified infant formulas may promote the growth of pathogenic bacteria while acidification is effective in inhibiting the growth of pathogenic microorganisms. Additionally, the duration and temperature at which the reconstituted PIFs were stored was found to play an important role in the growth of these organisms. Microbial growth was promoted when the reconstituted formulas were stored at optimal temperatures for prolonged periods following reconstitution. For this reason, reconstituted infant formulas should only temporarily be stored at optimal temperatures or in refrigerators to prevent microbial growth.

Regardless of whether lactate or citrate was used, acidification proved to reduce microbial growth. This suggests that PIF acidification has the potential to inhibit the growth of pathogenic bacteria known to contaminate PIFs. Additionally, acidification and storage at suboptimal temperatures resulted in little to no microbial growth, thus suggesting that the combination of both these strategies may prevent the risk of microbial growth.

To validate whether the reduction in growth was due to acids and not other components, we chemically acidified LB broth to pH units mimicking those observed in the reconstituted infant formula. The growth of pathogens, similarly to PIF acidification, was greatly reduced thus suggesting that the antimicrobial activity is attributed to the acids and not other constituents present in the infant formulas.

A comparison of the effect of the two acids further highlighted that the inhibitory effect of these acids is dependent on the pH of the acids rather than the acids themselves. At pH 4.45 both acids were more effective in controlling the growth of PIF than at 4.60. This suggests that to improve microbiological safety of PIFs, the pH should be carefully evaluated. Further, there should be an interplay between microbiological safety and safety for the infant.

Regardless of the pH, one acid was more effective against a particular pathogen. For instance, citrate acidification was more effective against *C. freundii* ATCC 8090 while lactate was more effective against *B. cereus* ATCC 11778*, C. sakazakii* ATCC 29544 and *S. aureus* ATCC 25923. Hence, acidification with a combination of different acids should be taken into consideration to provide most effective control.

To further understand the inhibitory effects of organic acids on PIF pathogens, further research focusing on evaluating the efficacy of other organic acids such as malic, acetic, formic and oxalic acids can be carried out. These studies can be done regardless of the availability of PIFs that incorporate these acids. They could chemically acidify non-acidified infant formula or similarly to what was done in the study acidify LB broth as the inhibitory effect is solely due to the organic acid and not influenced by constituents of infant formulas.

Another aspect to consider is to investigate the efficacy of biological acidification when compared to chemical acidification. Given the efficacy of the different organic acids against the different pathogens, one could consider biological acidification using a mixed acid fermenter such as *Lactobacillus* or *Pediococcus* spp., which could effectively provide the broadest control.

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Appendices

Appendix A: Media Preparation

500 ml Luria Bertani broth and agar

- 5 g Casein hydrolysate
- 2.5 g Yeast Extract
- 2.5 g NaCl
- 497.5 ml dH_2O
- 625 µl 10 % (w/v) NaOH
- For LB agar, add 7.5 g agar

Selective media preparation

All the selective media were prepared by dissolving the agar powder in distilled water and autoclaved at 121 ^oC for fifteen minutes with the exception of VRBG agar which was boiled over a Bunsen burner to dissolve the powder.

22310 *Bacillus cereus* Selective Agar

- 43 g Cereus Selective agar powder
- 900 ml of dH_2O
- 100 ml egg-yolk suspension

92324 *Cronobacter* spp. ChromoSelect Agar

- 51.67 g Cronobacter spp. ChromoSelect Agar powder
- 1000 ml dH_2O

70189 Violet Red Bile Glucose Agar

- 39.5 g Violet Red Bile Glucose Agar powder
- 1000 ml dH_2O

11705 Baird Parker Agar

- 58.9 g Baird Parker Agar powder
- 1000 ml dH_2O
- 5 ml egg-yolk emulsion

700887 Cetrimide Agar

- 46.7 g Cetrimide Agar
- 1000 ml dH_2O
- 10 ml glycerol

500 ml Phosphate Buffered-Saline

- 0.72 g Na_2HPO_4
- 0.1125 g KH_2PO_4
- 4 g NaCl
- 0.1 g KCl
- 497.5 ml dH_2O

50 % glycerol

- 50 ml dH_2O
- 50 ml 100 % glycerol

25 % glycerol stock solution

- 500 µl 50 % glycerol
- 500 µl cell culture

Appendix B: Mean pH measurements for the reconstituted PIFs without pathogens at the four different time points post-inoculation

Storage temperature	Citrate Acidified Formula			
	0	**1**	**3**	**6**
4 °C	4.68	4.68	4.68	4.69
25 °C	4.68	4.68	4.69	4.69
37 °C	4.68	4.69	4.69	4.69
	Non-Acidified Formula			
	0	**1**	**3**	**6**
4 °C	6.70	6.70	6.70	6.70
25 °C	6.70	6.71	6.71	6.71
37 °C	6.70	6.70	6.71	6.71
	Lactate Acidified Formula			
	0	**1**	**3**	**6**
4 °C	4.50	4.49	4.50	4.50
25 °C	4.50	4.50	4.50	4.50
37 °C	4.50	4.50	4.50	4.50

Appendix C: Mean pH measurements for the pathogen-spiked reconstituted PIFs at the four different time points post-inoculation

***B. cereus* ATCC 11778**												
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.62	4.80	4.66	4.68	6.66	6.75	6.75	6.75	4.48	4.57	4.52	4.54
25 °C	4.62	4.71	4.68	4.70	6.66	6.78	6.78	6.75	4.48	4.54	4.52	4.53
37 °C	4.62	4.72	4.71	4.71	6.6	6.78	6.77	6.72	4.48	4.54	4.52	4.53

***C. freundii* ATCC 8090**												
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.61	4.68	4.68	4.76	6.70	6.74	6.78	6.79	4.42	4.52	4.49	4.49
25 °C	4.61	4.69	4.72	4.73	6.70	6.78	6.70	6.77	4.42	4.50	4.50	4.51
37 °C	4.61	4.70	4.70	4.72	6.70	6.76	6.74	6.68	4.42	4.50	4.50	4.50

***C. sakazakii* ATCC 29544**												
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.66	4.87	4.77	4.82	6.75	6.75	6.85	6.75	4.43	4.54	4.54	4.51
25 °C	4.66	4.77	4.73	4.75	6.75	6.85	6.82	6.78	4.43	4.54	4.54	4.52
37 °C	4.66	4.72	4.75	4.72	6.75	6.84	6.67	6.73	4.43	4.54	4.50	4.50

***E. coli* ATCC 25922**												
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.76	4.79	4.81	4.81	6.77	6.78	6.78	6.82	4.52	4.49	4.45	4.52
25 °C	4.76	4.77	4.83	4.80	6.77	6.84	6.81	681	4.52	4.53	4.53	4.54
37 °C	4.76	4.77	4.80	4.80	6.77	6.84	6.76	6.74	4.52	4.51	4.52	4.53

***L. innocua* ATCC 33090**												
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.75	4.81	4.82	4.83	6.79	6.79	6.87	6.82	4.52	4.51	4.54	4.53
25 °C	4.75	4.76	4.75	4.81	6.79	6.83	6.83	6.83	4.52	4.53	4.54	4.57
37 °C	4.75	4.73	4.81	4.81	6.79	6.79	6.70	6.75	4.52	4.54	4.55	4.55

***P. aeruginosa* ATCC 10145**												
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	1	3	6	0	1	3	6	0	1	3	6
4 °C	4.66	4.65	4.67	4.66	6.82	6.88	6.88	6.87	4.52	4.54	4.53	4.53
25 °C	4.66	4.67	4.68	4.67	6.82	6.81	6.85	6.85	4.52	4.52	4.52	4.54
37 °C	4.66	4.66	4.69	4.69	6.82	6.81	6.79	6.74	4.52	4.51	4.53	4.53

***S. enterica* subsp. *enterica serovar* Enteritidis ATCC 13076**												
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.75	4.77	4.76	4.77	6.80	6.77	6.79	6.78	4.55	4.56	4.56	4.58
25 °C	4.75	4.79	4.78	4.78	6.80	6.79	6.79	6.77	4.55	4.55	4.55	4.55
37 °C	4.75	4.77	4.76	4.77	6.80	6.46	6.74	6.71	4.55	4.55	4.55	4.55

***S. aureus* ATCC 25923**												
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.78	4.73	4.71	4.73	6.76	6.78	6.8	6.81	4.54	4.54	4.53	4.53
25 °C	4.78	4.85	4.83	4.85	6.76	6.84	6.82	6.83	4.54	4.55	4.54	4.54
37 °C	4.78	4.87	4.83	4.85	6.76	6.85	6.80	6.78	4.54	4.55	4.53	4.53

Appendix D: Mean log (CFU/ml) values of the pathogen-spiked PIF over a 6-hour incubation period

***B. cereus* ATCC 11778**												
	log(CFU/ml)											
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	1	3	6	0	1	3	6	0	1	3	6
4 °C	4.009	3.962	4.050	4.036	4.007	4.035	4.032	4.043	4.010	4.014	4.029	3.851
25 °C	4.012	4.049	4.225	4.439	4.004	4.153	4.405	5.123	4.007	4.068	4.233	0.000
37 °C	4.017	4.016	4.246	4.833	4.009	4.109	5.139	5.833	4.021	3.977	0.000	0.000

***C. freundii* ATCC 8090**												
	log(CFU/ml)											
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.002	4.023	4.012	4.004	4.012	4.008	3.979	3.986	3.988	3.977	4.035	4.039
25 °C	3.999	3.964	4.056	4.818	4.015	4.028	4.179	5.027	4.000	4.032	4.104	4.828
37 °C	4.016	3.987	4.242	5.182	4.014	4.066	4.788	6.058	4.006	4.027	4.328	5.419

	***C. sakazakii* ATCC 29544**											
	log(CFU/ml)											
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.009	4.004	3.987	3.995	3.997	4.114	4.131	4.091	4.009	4.087	4.011	4.001
25 °C	4.013	4.181	4.153	4.592	4.002	4.163	4.487	5.245	4.031	4.149	4.063	4.279
37 °C	3.981	4.265	4.617	5.813	4.014	4.287	5.215	6.054	4.033	4.194	4.245	5.105

***E. coli* ATCC 25922**												
	log(CFU/ml)											
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.015	4.064	4.090	4.074	4.023	4.097	4.175	4.232	4.019	4.017	4.076	4.032
25 °C	4.032	3.986	4.113	4.705	4.039	4.166	4.290	5.650	4.019	4.032	4.089	4.431
37 °C	4.022	4.038	4.164	5.746	4.020	4.244	4.682	6.361	4.032	4.107	4.118	5.543

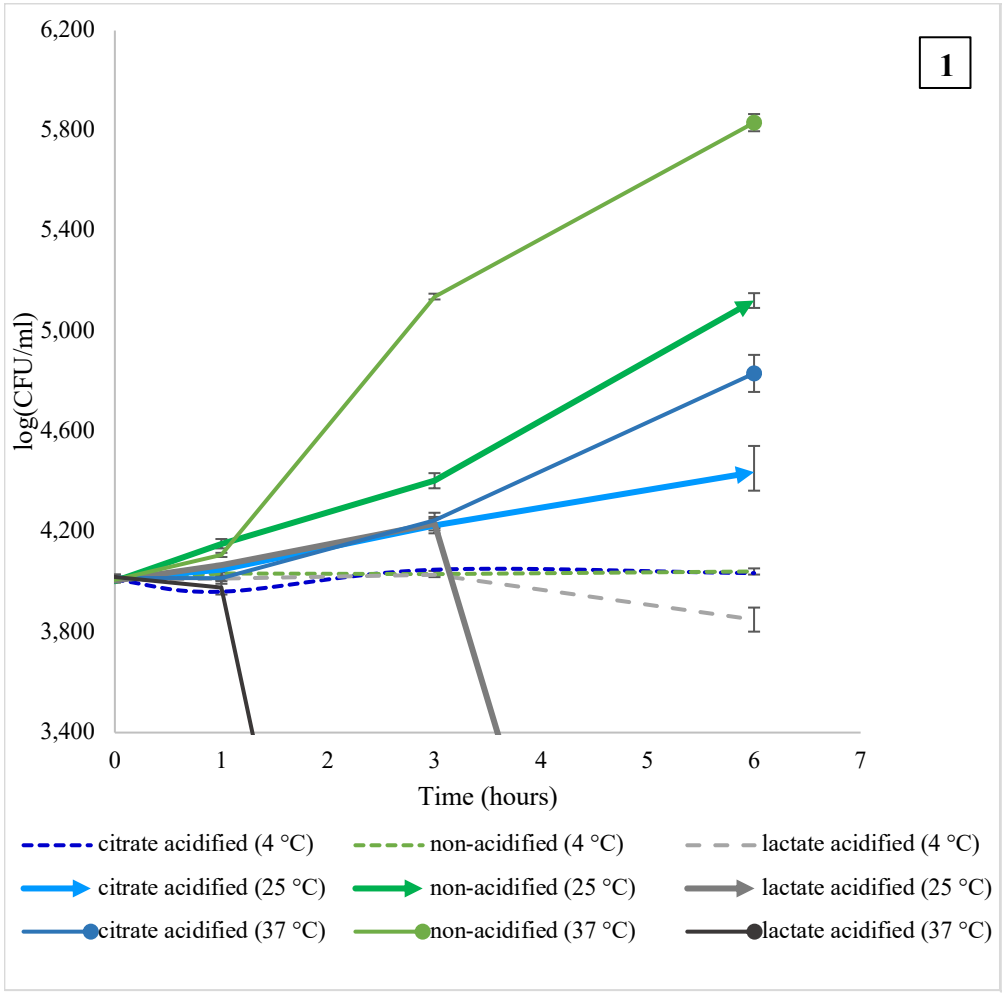
	***L. innocua* ATCC 33090**											
	log(CFU/ml)											
Storage temperature	**Citrate acidified**				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.011	4.123	4.009	3.983	4.022	4.065	4.086	4.025	4.030	3.956	3.956	3.898
25 °C	4.004	4.036	4.293	4.747	4.020	4.037	4.403	5.015	4.024	3.948	4.167	4.564
37 °C	4.008	4.070	4.632	5.630	4.005	4.065	4.854	5.927	4.003	3.970	4.276	5.156

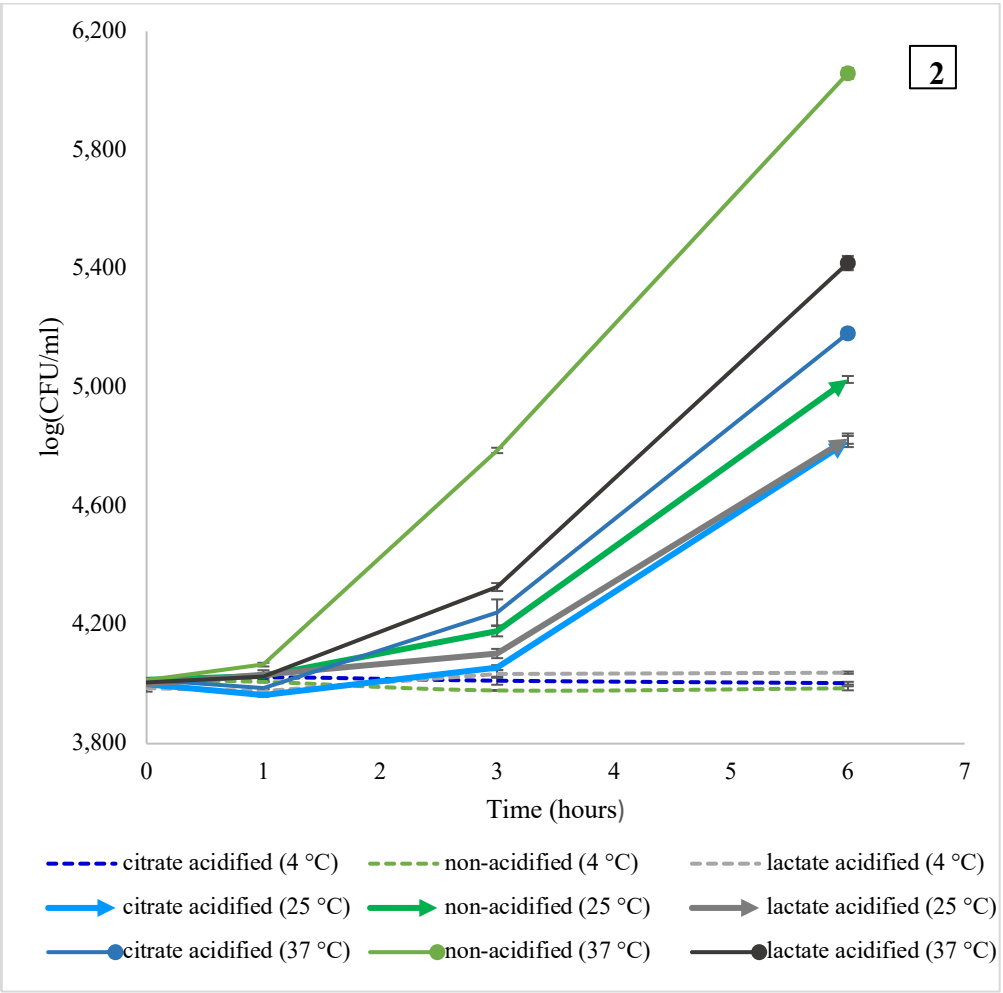
***P. aeruginosa* ATCC 10145**												
Storage temperature	**log(CFU/ml)**											
	Citrate acidified				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.030	3.969	4.022	3.967	4.000	3.981	4.094	3.982	4.005	3.845	3.895	3.834
25 °C	4.024	4.005	4.153	4.349	4.019	4.017	4.286	4.798	4.022	3.831	3.865	3.643
37 °C	3.998	4.016	4.494	5.083	4.021	4.046	4.612	5.356	4.005	3.929	3.710	0.000

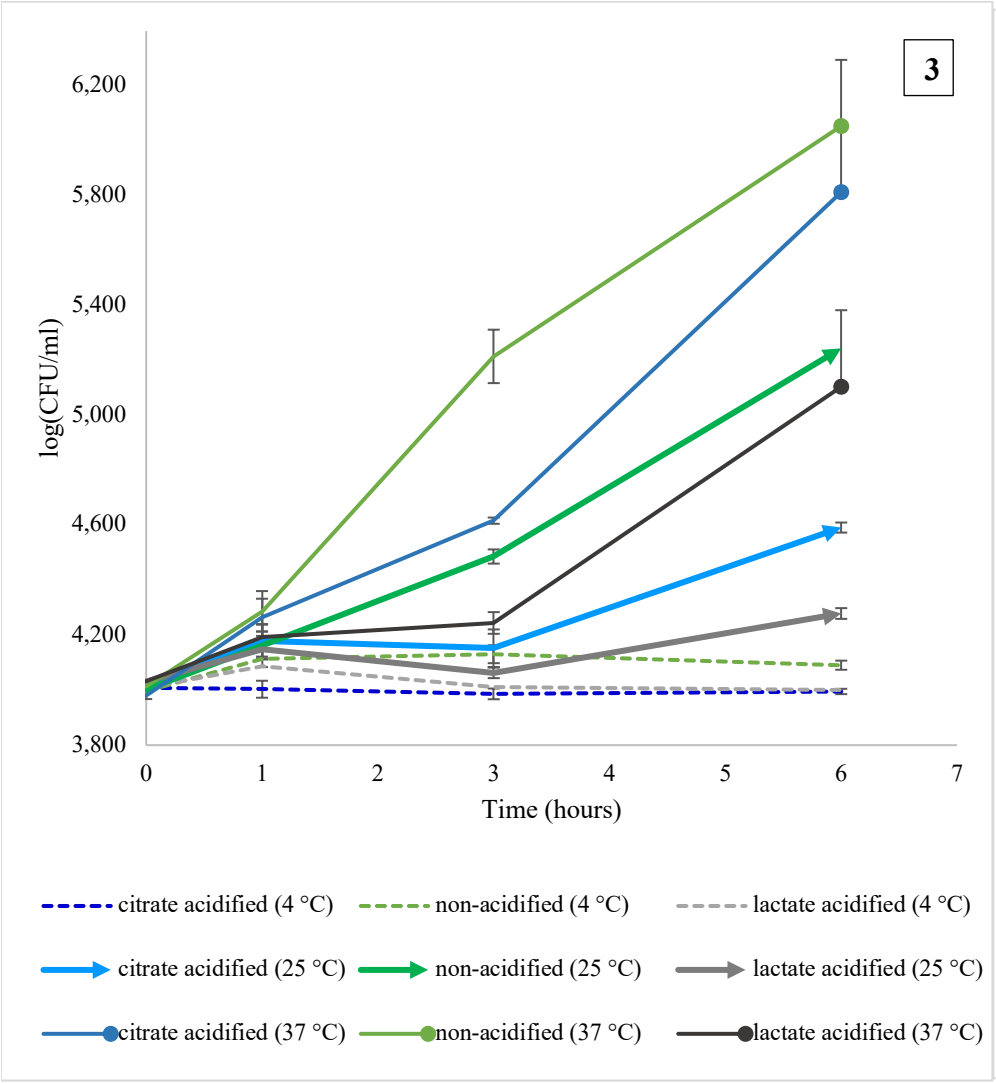
***S. enterica* subsp. *enterica serovar* Enteritidis ATCC 13076**												
Storage temperature	**log(CFU/ml)**											
	Citrate acidified				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.016	4.018	3.974	4.018	4.023	4.000	4.036	3.965	4.035	4.017	3.929	3.942
25 °C	4.011	4.008	4.509	4.681	4.022	3.974	4.781	5.141	4.022	3.987	4.317	4.690
37 °C	4.015	3.967	4.561	5.216	4.020	4.015	5.103	5.766	4.024	3.994	4.643	5.439

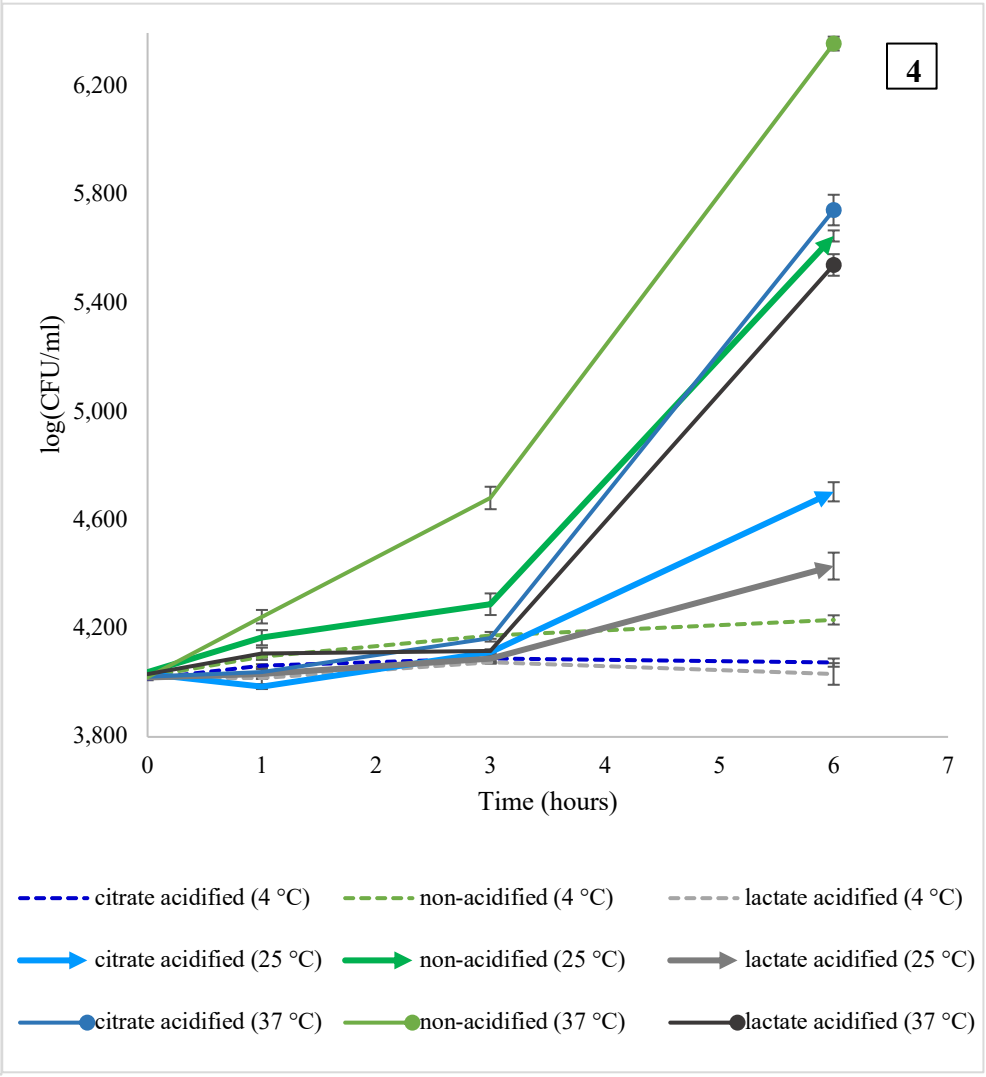
	***S. aureus* ATCC 25923**											
Storage temperature	**log(CFU/ml)**											
	Citrate acidified				**Non-acidified**				**Lactate acidified**			
	0	**1**	**3**	**6**	**0**	**1**	**3**	**6**	**0**	**1**	**3**	**6**
4 °C	4.025	4.136	4.206	4.273	4.029	4.186	4.278	4.336	4.021	4.109	4.119	4.228
25 °C	4.046	4.181	4.309	4.537	4.028	4.241	4.354	4.682	4.025	4.160	4.262	4.551
37 °C	4.030	4.183	4.351	4.786	4.045	4.291	4.658	5.083	4.040	4.161	4.310	4.540

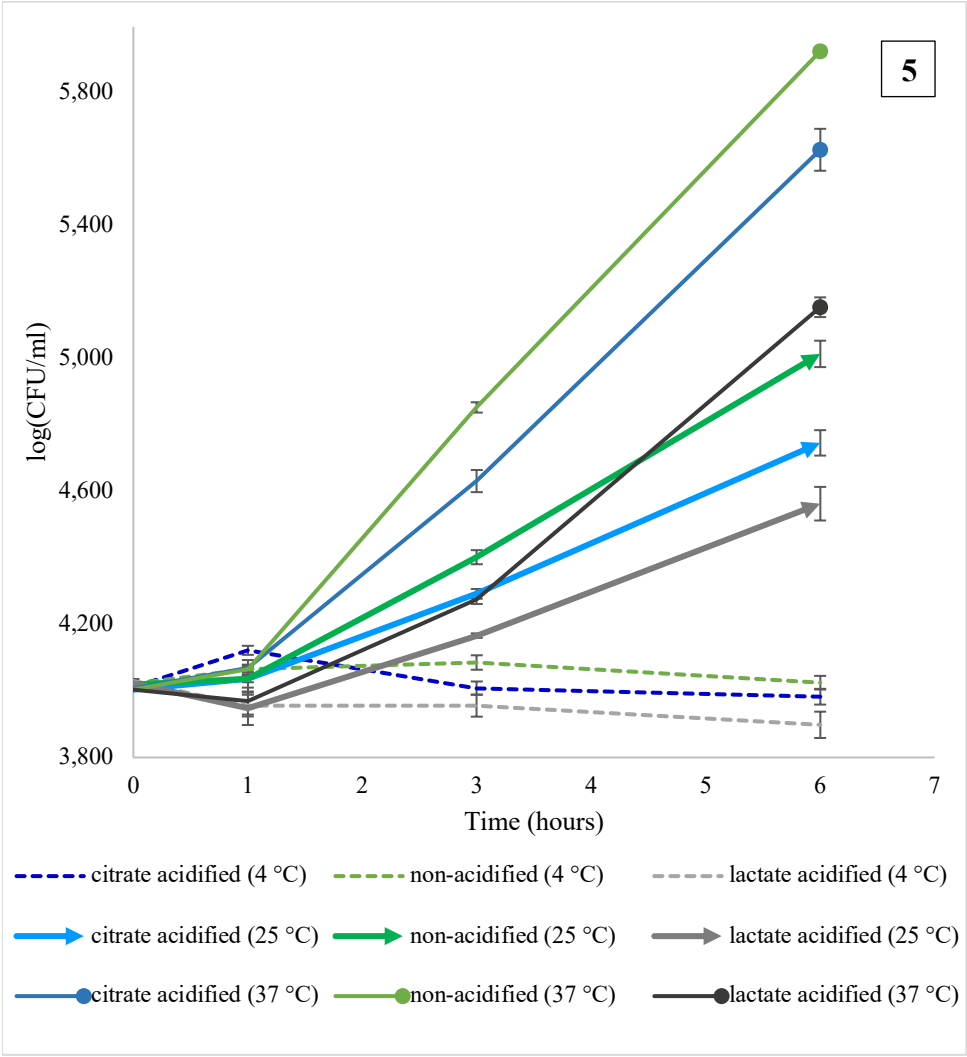
Appendix E: Effect of acidification and temperature on the growth of pathogens

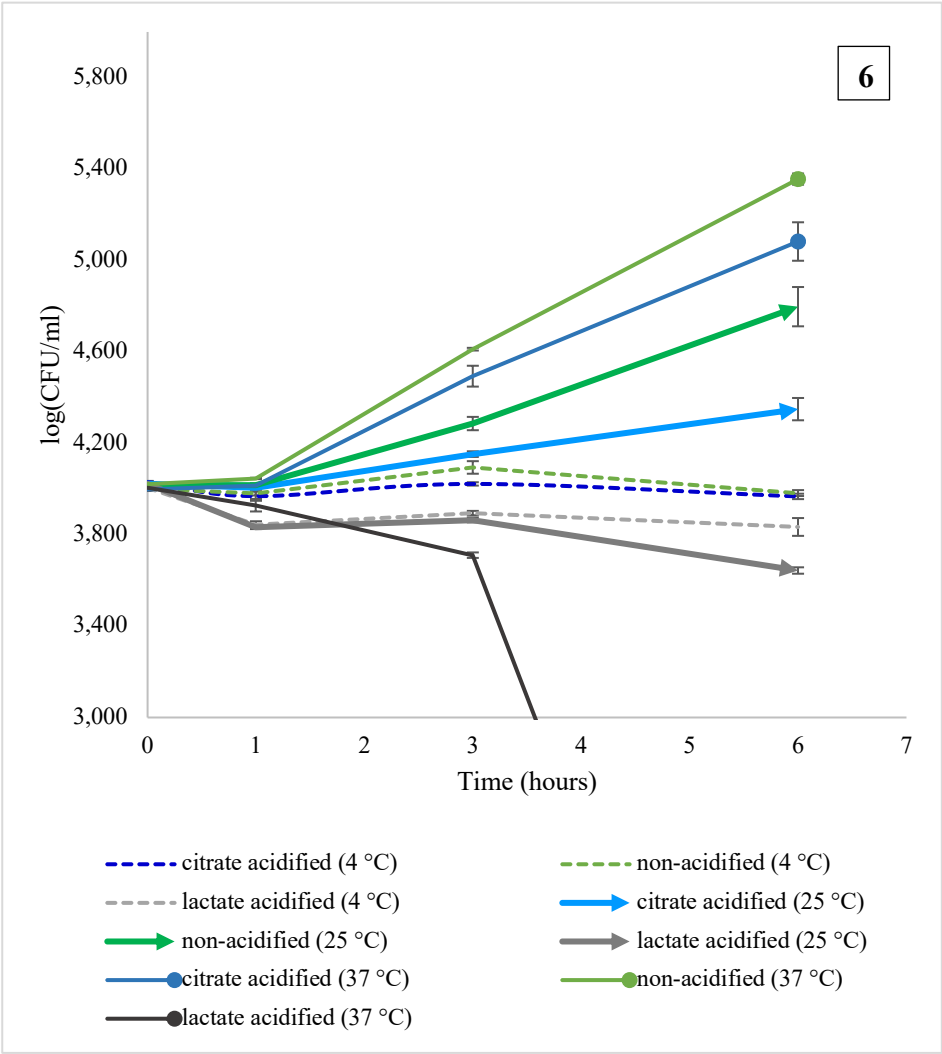


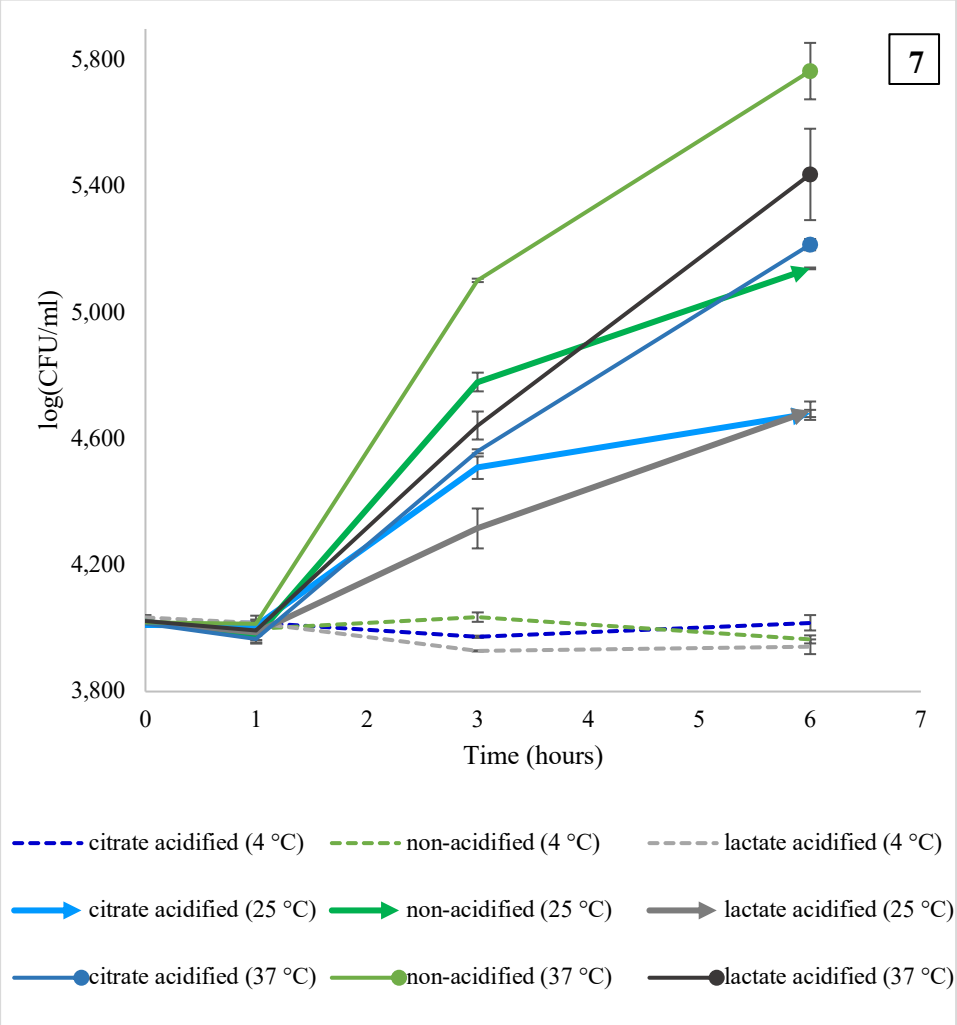


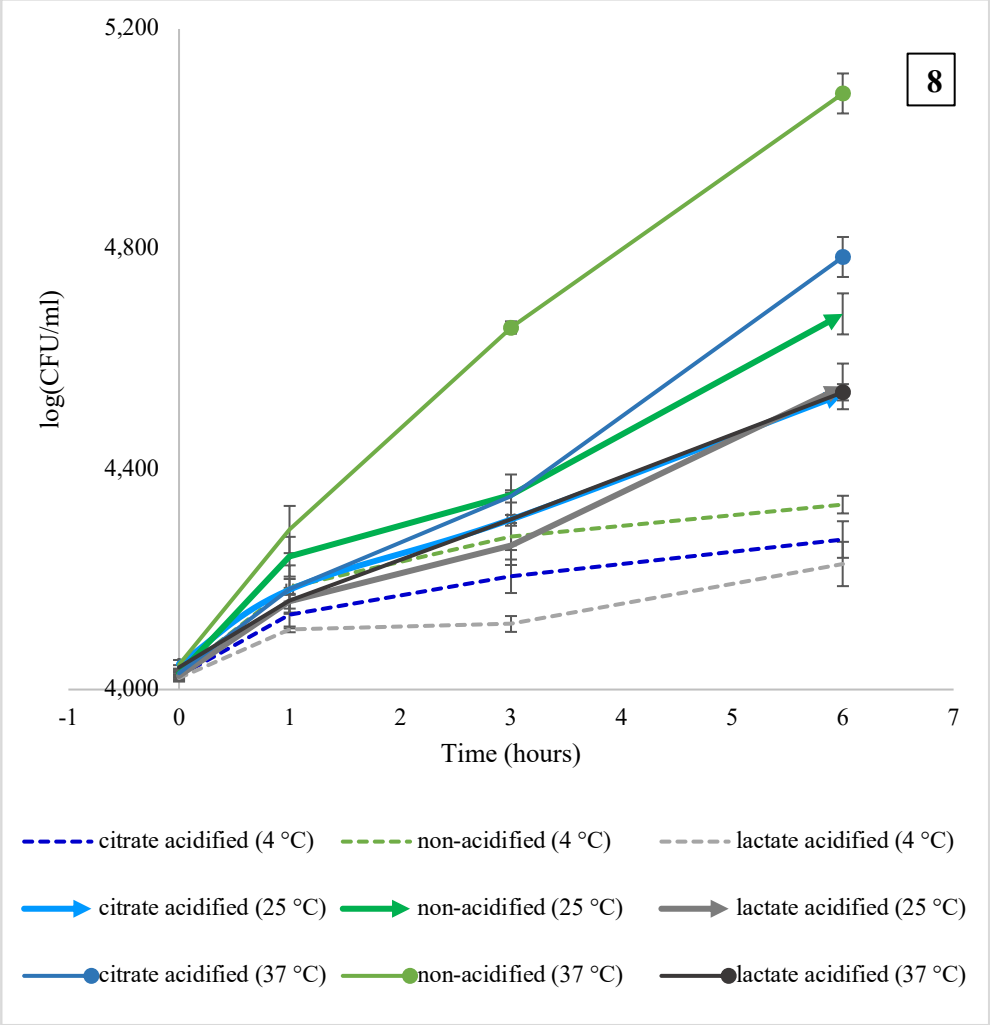

3
6,200
5,800
5,400
5,000
4,600
4,200
3,800
log(CFU/ml)
0
1
2
3
4
5
6
7
Time (hours)
citrate acidified (4 °C)
non-acidified (4 °C)
lactate acidified (4 °C)
citrate acidified (25 °C)
non-acidified (25 °C)
lactate acidified (25 °C)
citrate acidified (37 °C)
non-acidified (37 °C)
lactate acidified (37 °C)


4
6,200
5,800
5,400
5,000
4,600
4,200
3,800
log(CFU/ml)
0
1
2
3
4
5
6
7
Time (hours)
citrate acidified (4 °C)
non-acidified (4 °C)
lactate acidified (4 °C)
citrate acidified (25 °C)
non-acidified (25 °C)
lactate acidified (25 °C)
citrate acidified (37 °C)
non-acidified (37 °C)
lactate acidified (37 °C)


5
5,800
5,400
5,000
4,600
4,200
3,800
log(CFU/ml)
0
1
2
3
4
5
6
7
Time (hours)
citrate acidified (4 °C)
non-acidified (4 °C)
lactate acidified (4 °C)
citrate acidified (25 °C)
non-acidified (25 °C)
lactate acidified (25 °C)
citrate acidified (37 °C)
non-acidified (37 °C)
lactate acidified (37 °C)

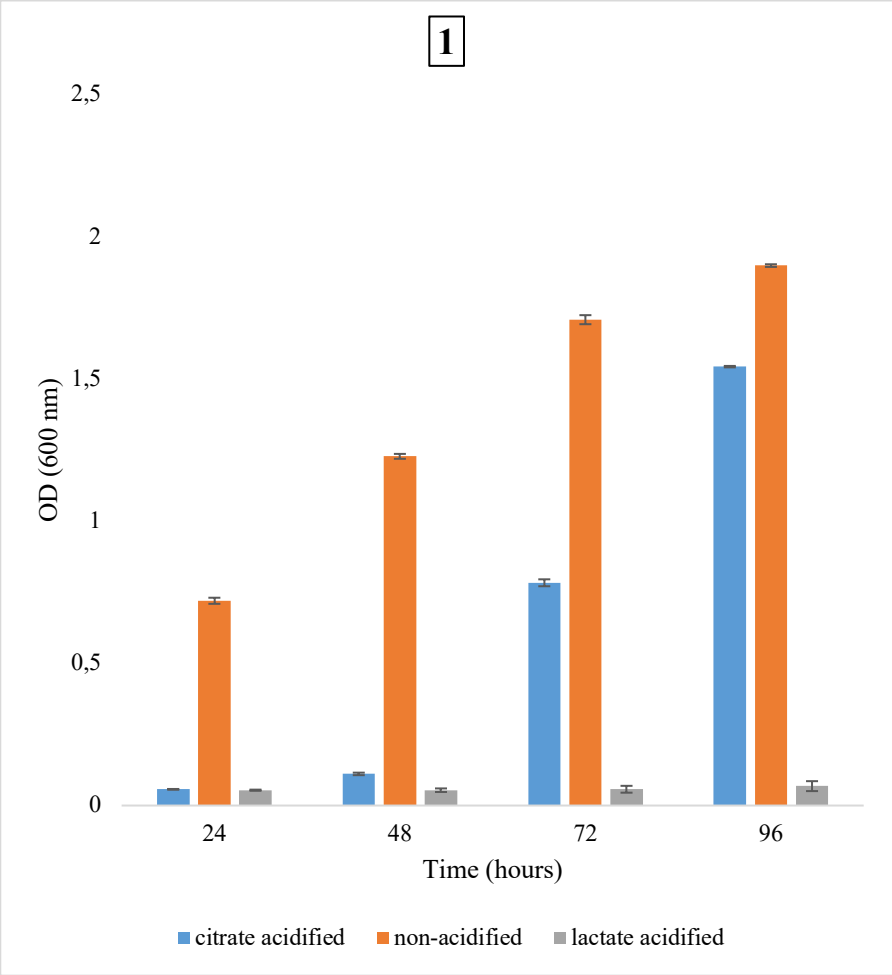

6
5,800
5,400
5,000
4,600
4,200
3,800
3,400
3,000
log(CFU/ml)
0
1
2
3
4
5
6
7
Time (hours)
citrate acidified (4 °C)
non-acidified (4 °C)
lactate acidified (4 °C)
citrate acidified (25 °C)
non-acidified (25 °C)
lactate acidified (25 °C)
citrate acidified (37 °C)
non-acidified (37 °C)
lactate acidified (37 °C)

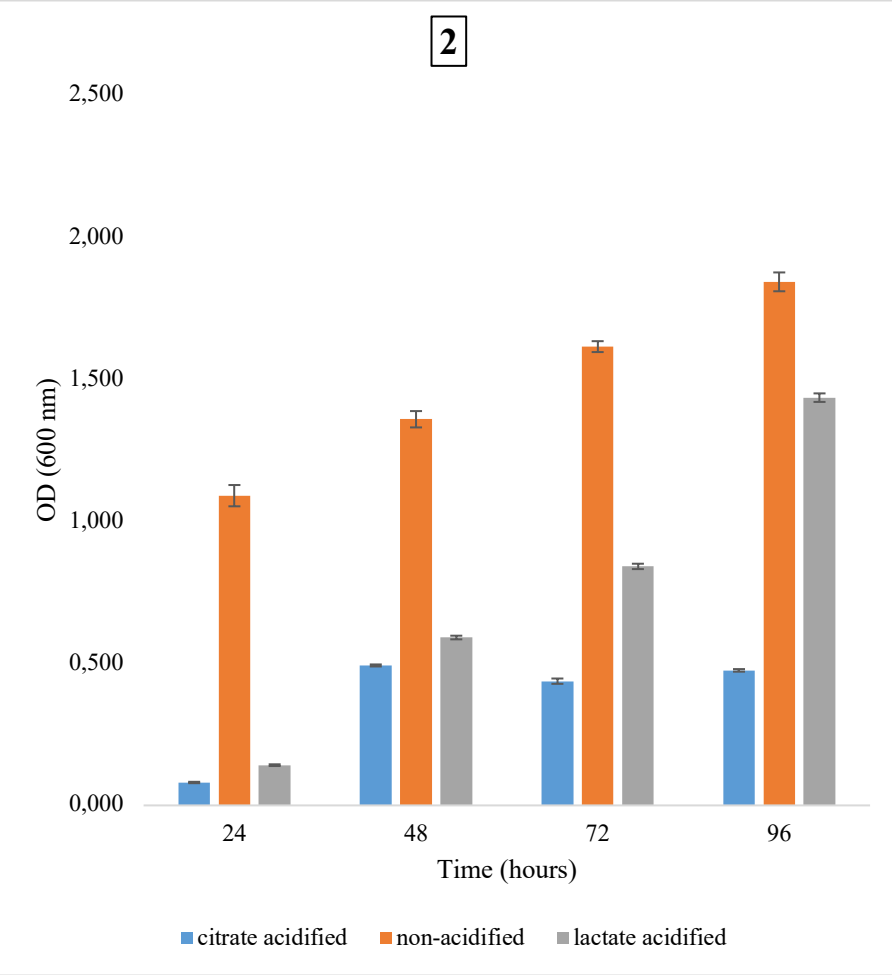


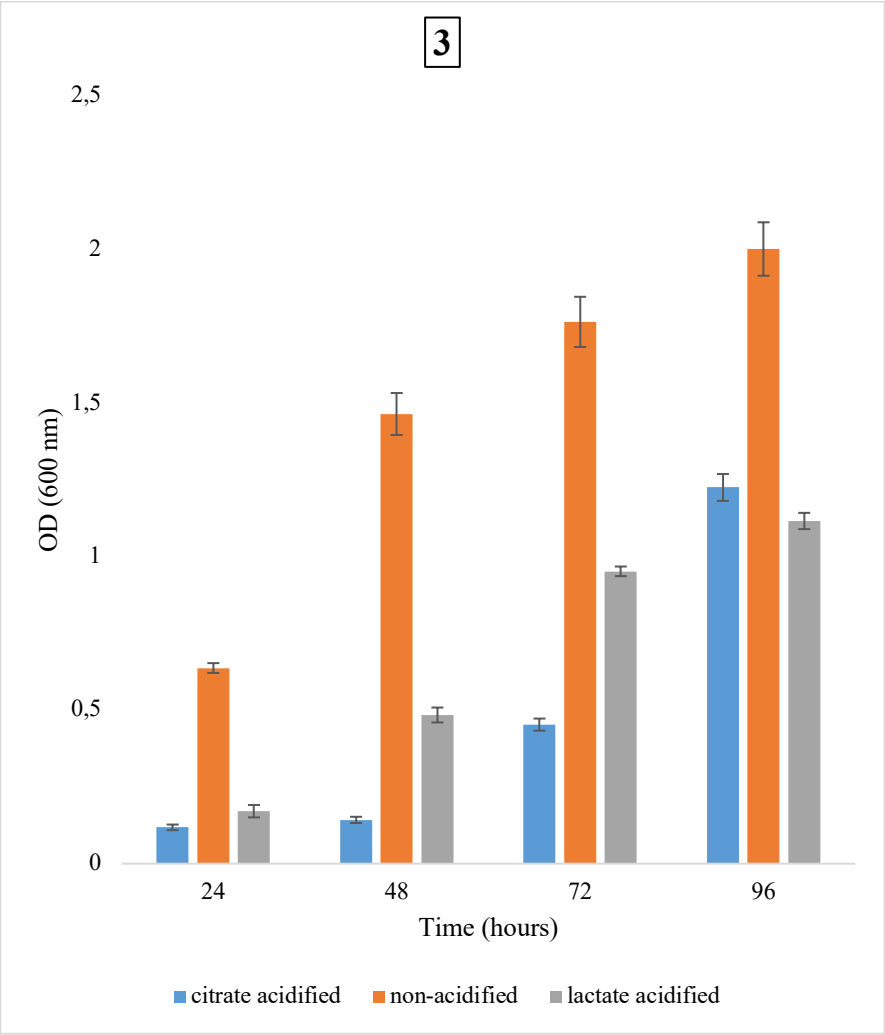


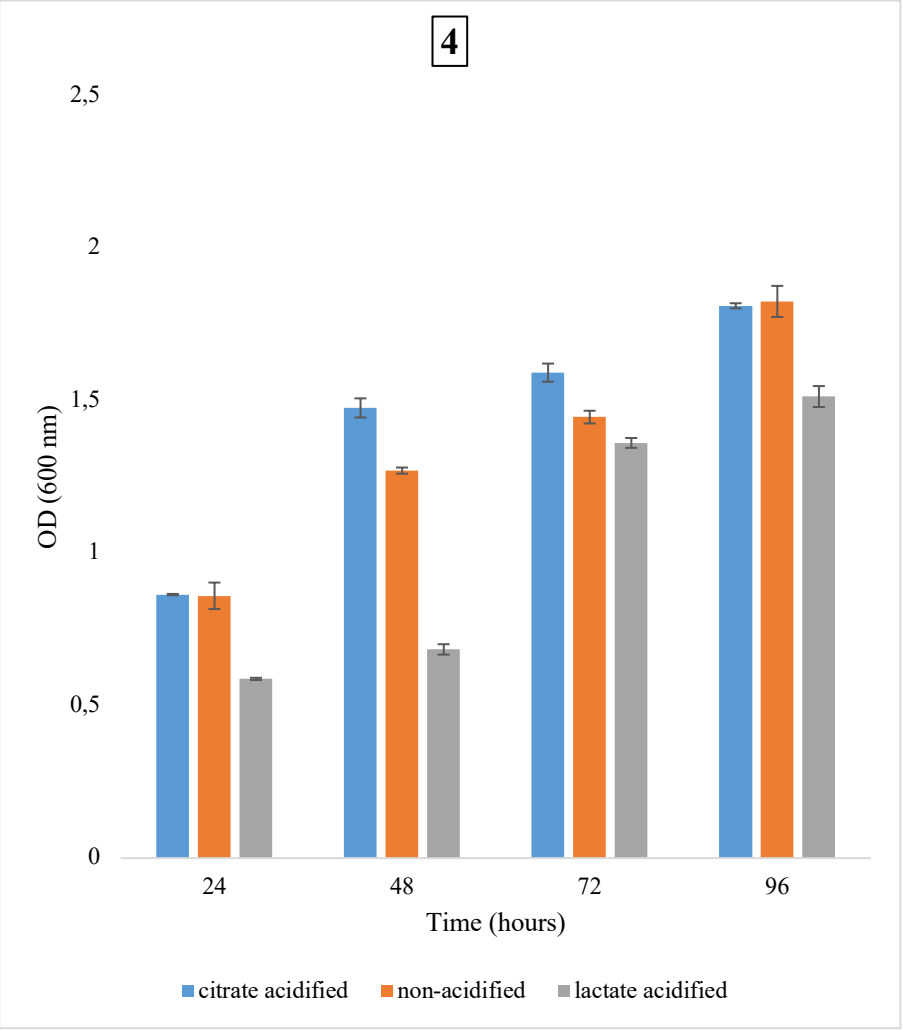
Appendix E: Effect of acidification and temperature (4, 25 and 37 °C) of reconstituted infant formula on the growth of 1) *B. cereus* ATCC 11778, 2) *C. freundii* ATCC 8090, 3) *C. sakazakii* ATCC 29544, 4) *E. coli* ATCC 25922, 5) *L. innocua* ATCC 33090, 6) *P. aeruginosa* ATCC 10145, 7) *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 and 8) *S. aureus* ATCC 25923 respectively. The error bars show the standard error of the mean of the triplicates at each time point

Appendix F: Absorbance (OD 600_{nm}) of pathogens in acidified and non-acidified LB Broth

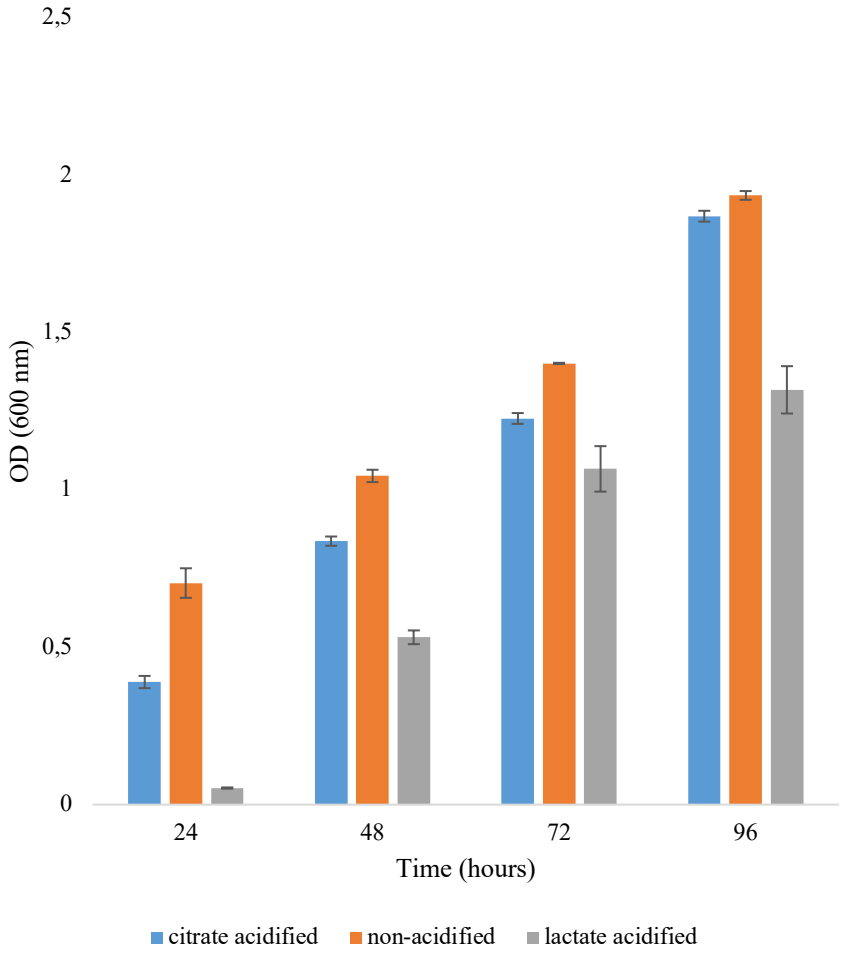




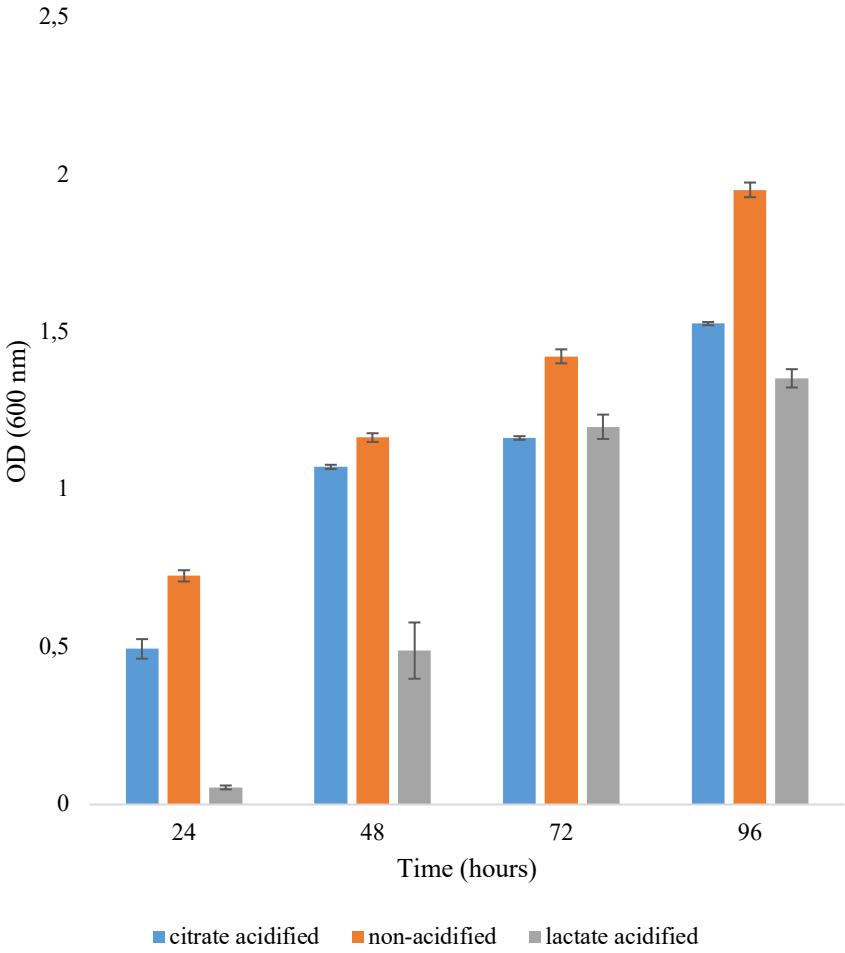

3
2,5
2
1,5
1
0,5
0
OD (600 nm)
24
48
72
96
Time (hours)
citrate acidified
non-acidified
lactate acidified

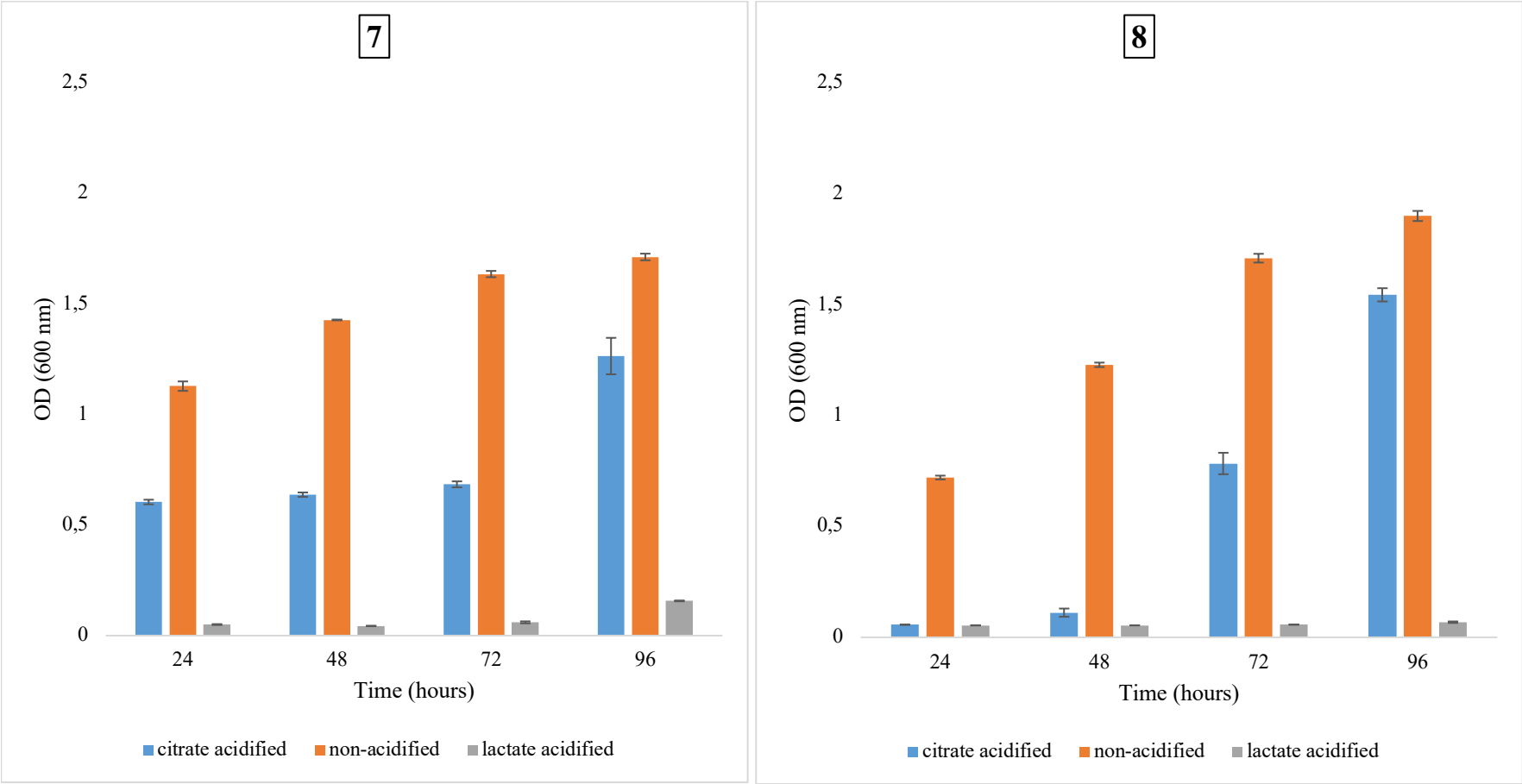

4
2,5
2
1,5
1
0,5
0
OD (600 nm)
24
48
72
96
Time (hours)
citrate acidified
non-acidified
lactate acidified

5


2,5
2
1,5
1
0,5
0
OD (600 nm)
24
48
72
96
Time (hours)
citrate acidified
non-acidified
lactate acidified

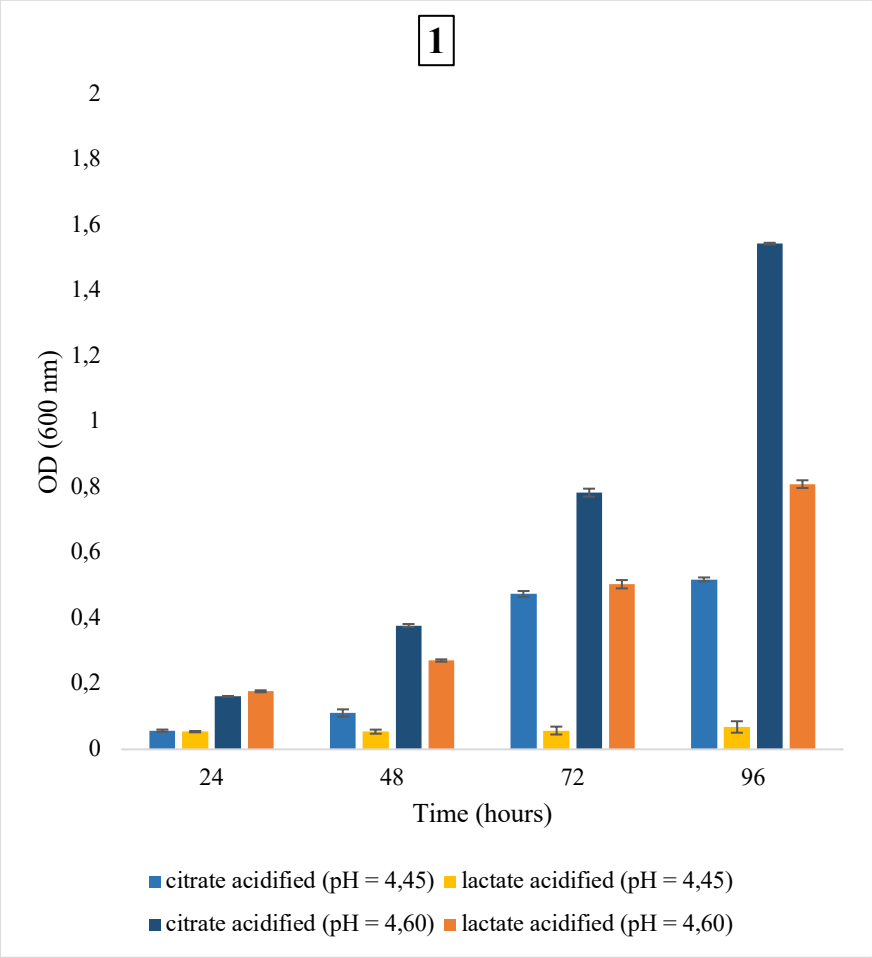
6

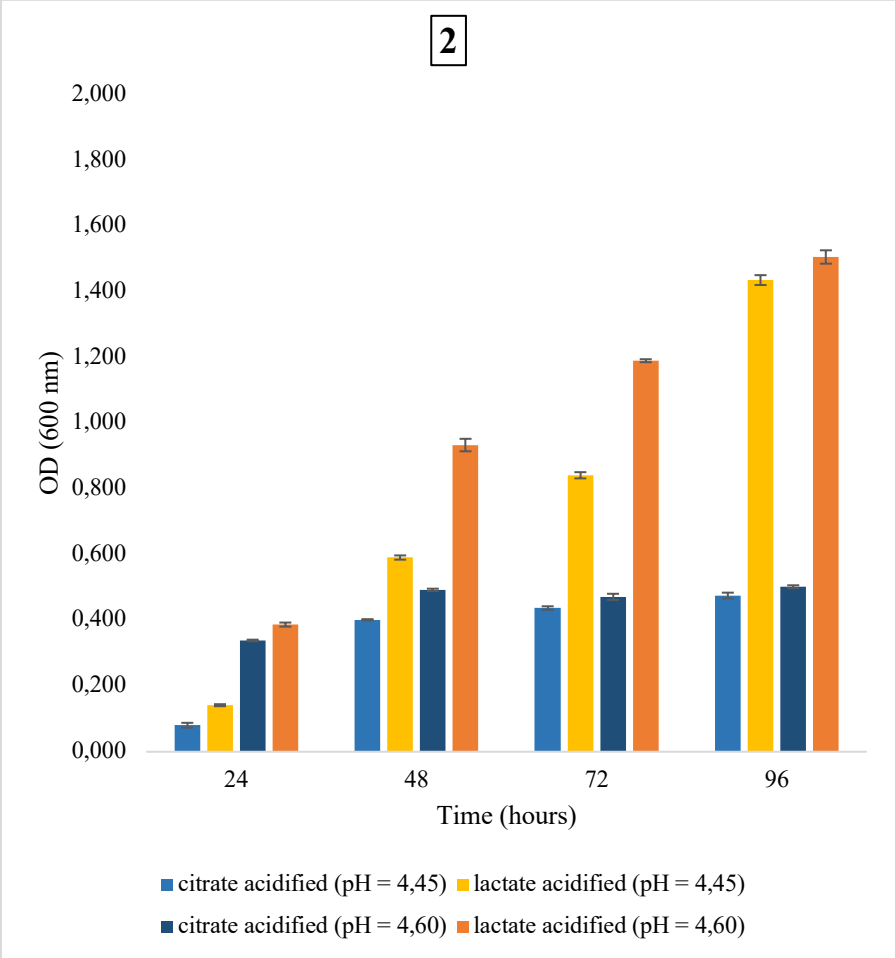

2,5
2
1,5
1
0,5
0
OD (600 nm)
24
48
72
96
Time (hours)
citrate acidified
non-acidified
lactate acidified

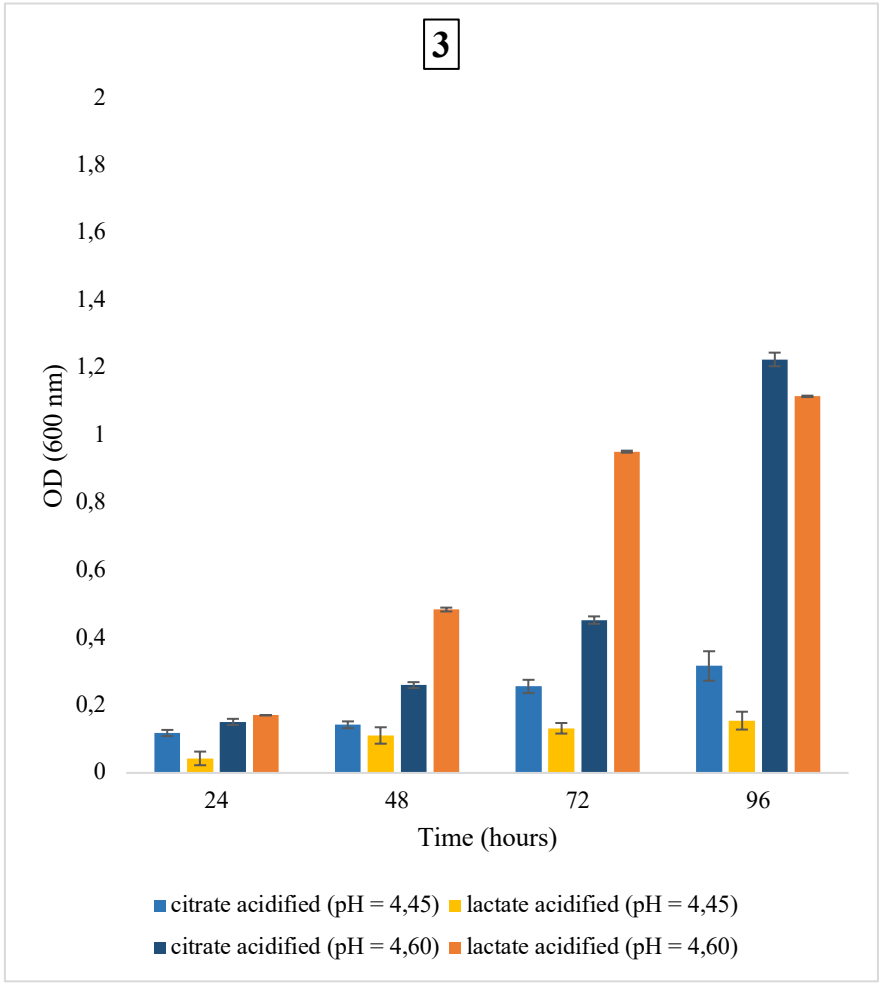


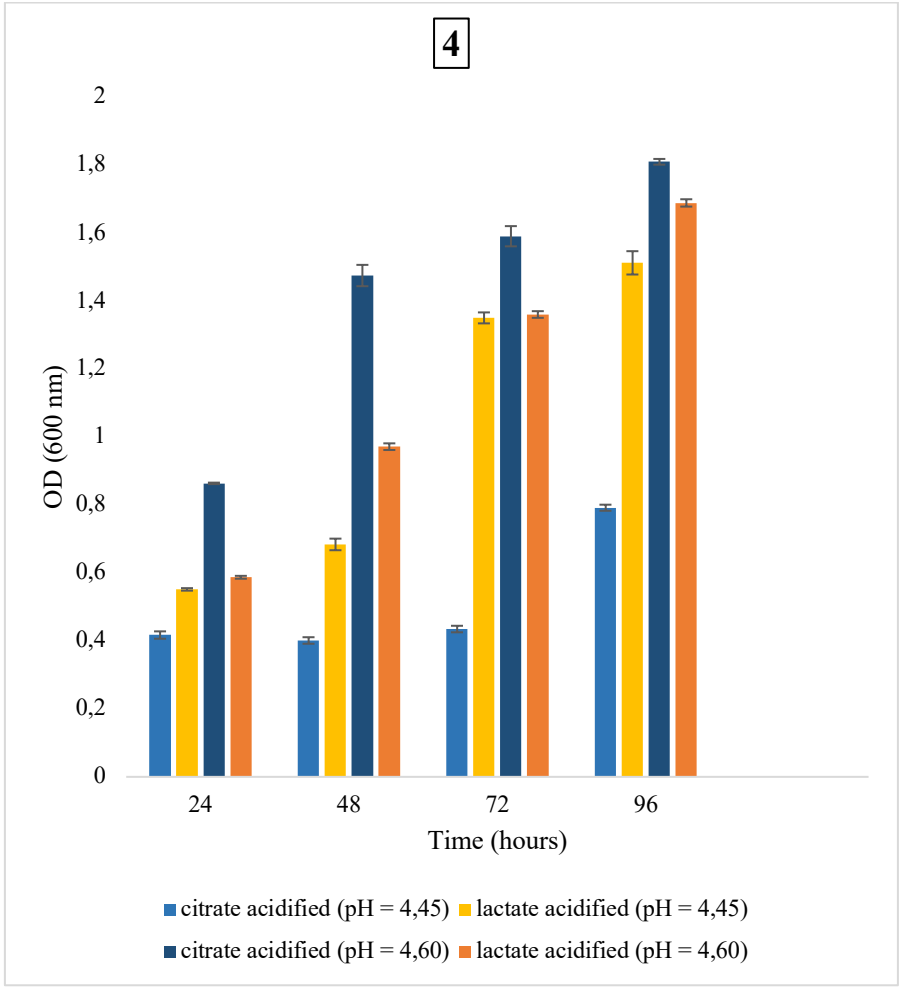
Appendix F: Absorbance (OD 600_{nm}) of pathogens in acidified and non-acidified LB on the growth of 1) *B. cereus* ATCC 11778, 2) *C. freundii* ATCC 8090, 3) *C. sakazakii* ATCC 29544, 4) *E. coli* ATCC 25922, 5) *L. innocua* ATCC 33090, 6) *P. aeruginosa* ATCC 10145, 7) *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 and 8) *S. aureus* ATCC 25923 respectively. The error bars show the standard error of the mean of the triplicates.

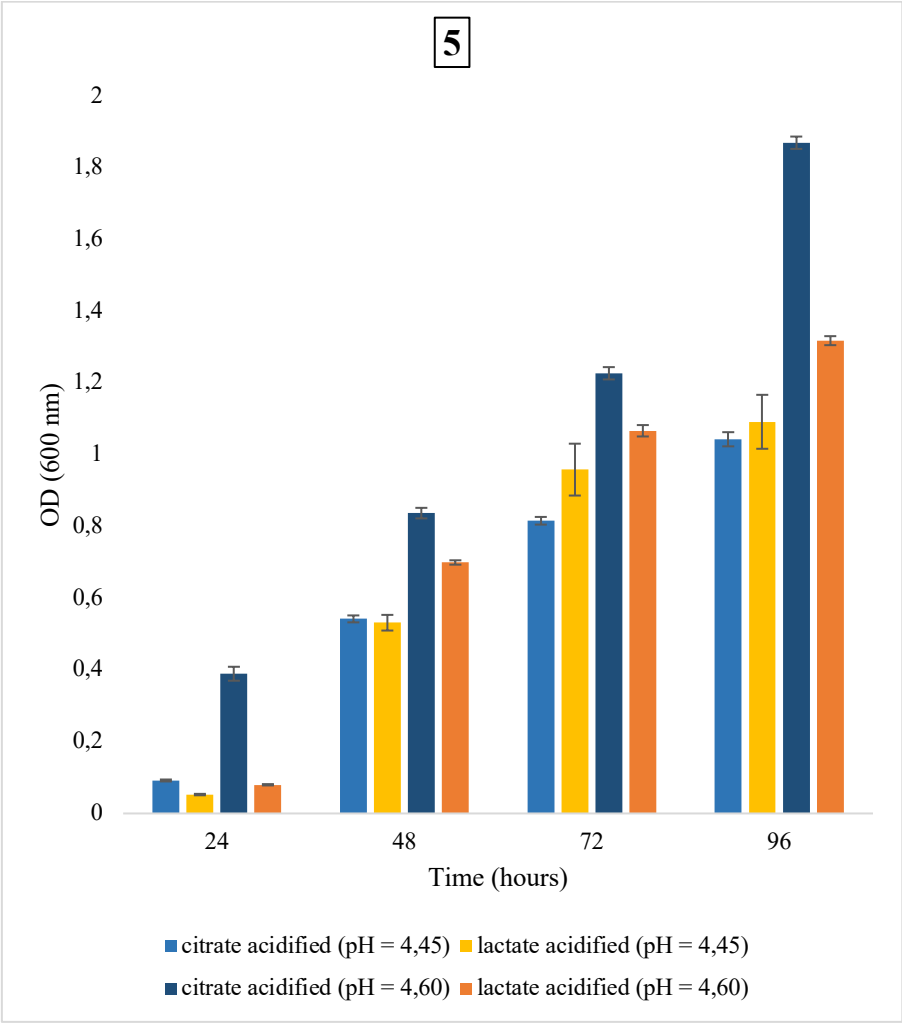
Appendix G: Absorbance (OD 600_{nm}) of pathogens in citrate- and lactate- acidified LB Broth

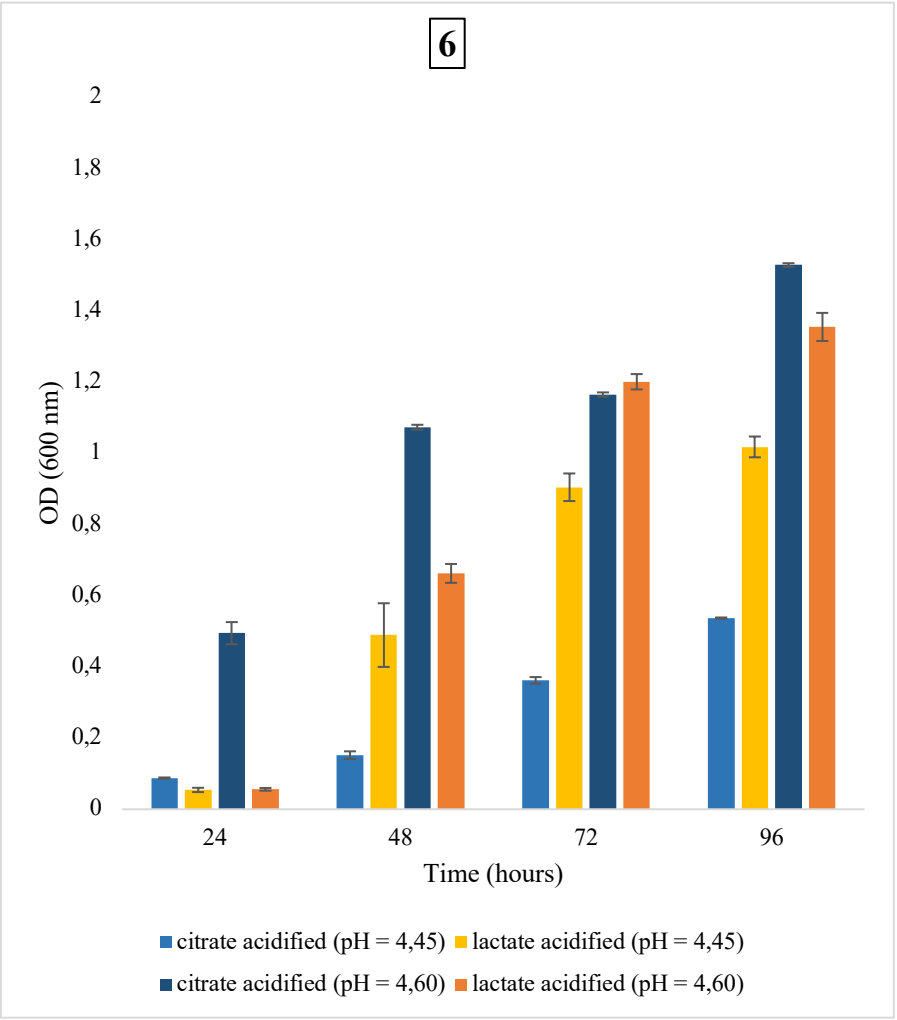


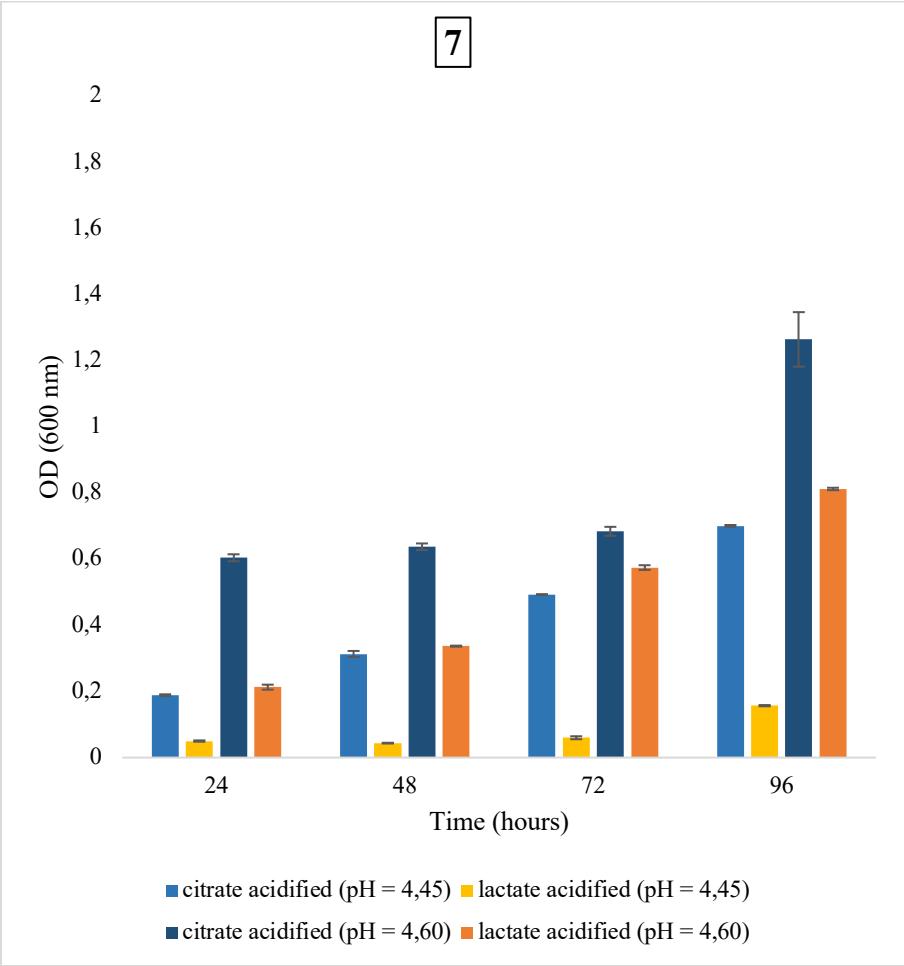


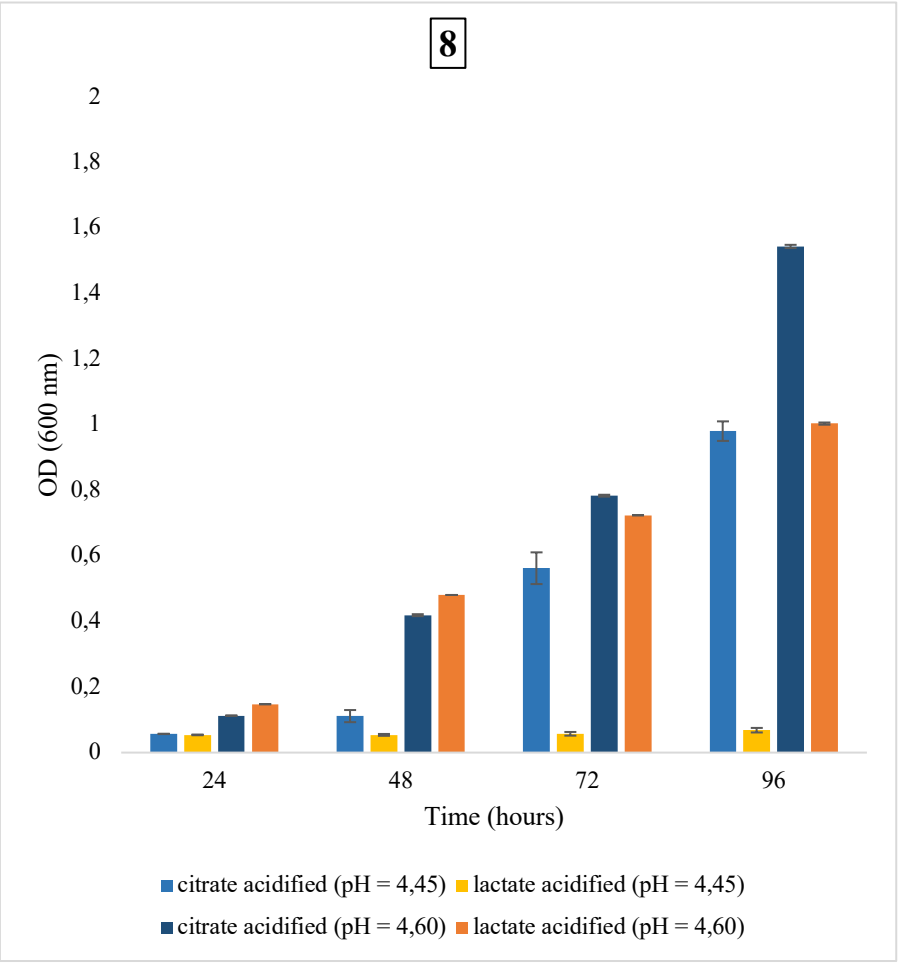

3
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
OD (600 nm)
24
48
72
96
Time (hours)
citrate acidified (pH = 4,45)
lactate acidified (pH = 4,45)
citrate acidified (pH = 4,60)
lactate acidified (pH = 4,60)


4
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
OD (600 nm)
24
48
72
96
Time (hours)
citrate acidified (pH = 4,45)
lactate acidified (pH = 4,45)
citrate acidified (pH = 4,60)
lactate acidified (pH = 4,60)


5
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
OD (600 nm)
24
48
72
96
Time (hours)
citrate acidified (pH = 4,45)
lactate acidified (pH = 4,45)
citrate acidified (pH = 4,60)
lactate acidified (pH = 4,60)


6
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
OD (600 nm)
24
48
72
96
Time (hours)
citrate acidified (pH = 4,45)
lactate acidified (pH = 4,45)
citrate acidified (pH = 4,60)
lactate acidified (pH = 4,60)





Appendix G: Absorbance (OD 600_{nm}) of pathogens in citrate- and lactate- acidified LB on the growth of 1) *B. cereus* ATCC 11778, 2) *C. freundii* ATCC 8090, 3) *C. sakazakii* ATCC 29544, 4) *E. coli* ATCC 25922, 5) *L. innocua* ATCC 33090, 6) *P. aeruginosa* ATCC 10145, 7) *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 and 8) *S. aureus* ATCC 25923 respectively. The error bars show the standard error of the mean of the triplicates.