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Evaluation of Postbiotic Supernatants from Selected Lactic Acid Bacteria Against the Survival of *Cronobacter* spp. in Infant Foods

Moyne Institute of Preventive Medicine
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ABSTRACT

Cronobacter is an opportunistic foodborne pathogen associated with severe neonatal infections such as meningitis, sepsis, and necrotizing enterocolitis. It poses a safety concern in infant nutrition due to its ability to persist in dry environments such as powdered infant formulas (PIF), which are not manufactured to be sterile. *Cronobacter* can remain viable for extended periods of time in low-moisture conditions and resist heat stress during rehydration, making it difficult to eliminate through standard preparation methods. *Cronobacter* is also able to tolerate and survive in acidic environments, however, the low pH of the gastric fluid in adults is usually enough to kill the bacteria. Unfortunately, infants have a higher gastric fluid pH, allowing *Cronobacter* to survive and reach the intestinal tract, increasing the likelihood of systemic infection. The use of postbiotics from lactic acid bacteria (LAB) as prophylactic additions to PIF, offer a promising alternative to traditional antimicrobial strategies. The aim of this study was to evaluate the antimicrobial effects of *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* cell-free supernatants (CFS) against *Cronobacter* as well as assess their stability under different conditions.

To assess the antimicrobial effect of *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFS, reconstituted dried baby food was mixed with the autoclaved CFS from a 48 h culture diluted 1:5 (CFS : baby food) from the LAB strains and then inoculated with approx. 1 CFU/10 ml of varying *Cronobacter* strains. After incubation for 24 h at 37℃, CFS from all four LAB strains displayed an inhibition rate of ≥99% against all the *Cronobacter* strains tested, showing their potential as postbiotics. *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFS (10 mg/ml) were also used as treatments to ready to feed formula inoculated with approx. 5 CFU/10 ml of *C. sakazakii* ES5. The solution was then put through simulated gastrointestinal conditions with a time frame of 6 h and temperature 37℃, although incubation of the solutions went up to 24 h. It was then determined that, at least in the simulated condition, 10 mg/ml of *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFS added to ready to feed infant milk was enough to inhibit *C. sakazakii* ES5 growth for at least 6 h that it could not be detected when samples of the milk were drip plated onto the Chromogenic Cronobacter Isolation (CCI) agar, no *C. sakazakii* ES5 colonies could be isolated. *C. sakazakii* ES5 colonies, however,

were isolated from samples taken at t=24, thus the antimicrobial activity of the *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFS diminished between t=6 and t=24, and that 10 mg/ml of CFS may have a bacteriostatic effect rather than a bactericidal effect.

The initial of pH of the solution *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFS are added into also affect the LAB CFS's antimicrobial activity. The MIC for all four LAB CFSs was determined to be 10 mg/ml, however when the CFS pH were altered to 7 and 9, *C. sakazakii* ES5 growth was not inhibited in L broth despite the increased concentration (25 mg/ml). In cultures where the CFS pH was not altered and CFS that were pH adjusted to 3 and 5, *C. sakazakii* ES5 showed no growth in the L broth. Likewise, when *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFS from a 48 h culture were adjusted to pH 5, 7 and 9, and then mixed into melted L agar at a 1:5 dilution, *C. sakazakii* ES5 growth was also detected after incubation at 37℃ for 18 h, whereas the unaltered CFS as well as pH 3 adjusted CFS showed no *C. sakazakii* ES5 growth. This comes to relevance when six dried baby foods from different manufacturers were used to test *L. plantarum* CFS against *C. sakazakii* ES5. In four of the dried baby foods, *C. sakazakii* ES5 could not be detected in treated samples. While it was detected in the other two albeit at lower CFU than its control. The acid buffering capacity of all six dried baby foods were checked, and while the two that allowed *C. sakazakii* ES5 growth did not have the most acid buffering capacity, it had, however, the highest initial pH at approx. pH 6.25.

While *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFS have shown potential to be effective postbiotics against *Cronobacter*, it is important to note that its effects vary between strain and the food matrix.

INTRODUCTION

Cronobacter spp. as a Cause of Disease

The *Cronobacter* genus is consists of facultatively anaerobic, rod-shaped bacteria belonging to the *Enterobacteriaceae* family. It was formerly known as *Enterobacter sakazakii*, and was reclassified in 2008 based on molecular and phenotypic analyses (Iversen et al., 2008). The genus currently has seven recognised species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis*, and *C. condimenti*. *Cronobacter* was first isolated from hospitalised infants in the United Kingdom in 1961. Then in 1979, cases of *Cronobacter* bacteremia were documented in newborns in Macon, USA. Additional cases were reported in the Netherlands in 1983 and 1987, Greece in 1987, Iceland in 1989, and the United States in 1989 and 2001. In 2001, a cluster of infections prompted the U.S. Centers for Disease Control and Prevention (CDC) to conduct a traceback investigation, which linked contaminated powdered infant formula (PIF) as a primary source of infant infections (Strysko et al. 2020). *C. sakazakii* and *C. malonaticus* have been reported to cause severe illnesses, while *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis*, and *C. condiment* are of less clinical importance (Ling et al. 2022). *Cronobacter* has been known to cause rare but severe infections in infants such as neonatal meningitis, necrotizing enterocilitis (NEC) and septicimia. Premature, immunocompromised or low birth weight infants are particularly at risk (Bow and Braden, 2006). While PIF is considered the primary vehicle for transmission, infections have also been linked to contaminated expressed breast milk, and *Cronobacter* have been isolated from neonatal feeding tubes for infants not fed reconstituted PIF as well as from milk preparation areas (Parra-Flores et al., 2018). Reported mortality rates in infected neonates range from 40% up to 80%, and surviving infants often suffer long-term neurological complications such as hydrocephalus, brain abscesses, and developmental delays (Drudy et al., 2006).

Unlike ready-to-feed formula, PIF is not sterile and may harbour *Cronobacter* spp. even after processing. Contamination can occur at various stages, including during manufacturing, packaging, and especially post-opening if poor hygiene is practiced during preparation and storage (CDC, 2023).

Persistence of *Cronobacter* in Food Production

The ability of *Cronobacter* spp. to persist in dry environments contributes significantly to its survival in powdered products and processing environments. Studies have shown that *Cronobacter* can remain viable even under desiccated conditions for extended periods, from several months to over one year, while retaining infectious potential (Barron & Forsythe, 2007). This desiccation tolerance is due to a combination of physiological adaptations *Cronobacter* has such as stress response regulators and, in some strains, the production of carotenoid pigments that enhance membrane stability and oxidative stress resistance (Joseph & Forsythe, 2012; Johler et al., 2010). Some *Cronobacter* species also possess a heteropolysaccharide capsule that provides resistance to osmotic stress and desiccation. As a result, some *Cronobacter* strains can persist in a dried state, such as in powdered infant formula (PIF) and other powdered foods, for extended periods of time reaching up to two years. On the other hand, strains that lack this capsule tend to survive for shorter durations and exhibit lower pathogenicity (Kalyatanda et al., 2015).

The optimum growth temperature for *Cronobacter* ranges from 5.5 to 47.0 °C, and it can survive thermal conditions between 54 and 64 °C (Ling et al., 2022). In addition to desiccation resistance, *Cronobacter* spp. also exhibit tolerance to thermal and acid stress. Certain species within the genus can produce a heat-stable enterotoxin that withstands pasteurization, as well as a lipopolysaccharide endotoxin that enables *Cronobacter* to penetrate the intestinal wall and cross the blood-brain barrier. This endotoxin is also heat-resistant and can persist in reconstituted powdered infant formula (PIF) for extended periods (Kalyatanda et al., 2015). Thermal inactivation studies have reported a wide range of D-values (time for a one-log reduction), varying from 2 to 30 minutes depending on temperature, strain, and food matrix (Osaili et al., 2009). Sublethal heat exposure can also induce cross-protection, increasing resistance not only to subsequent heat stress but also to acidic environments, an effect that complicates food safety control strategies (Niu et al., 2022). This is particularly relevant when PIF is reconstituted using water at suboptimal temperatures (<70°C), which may be insufficient to achieve significant microbial inactivation (Kalyatanda et al., 2015).

Moreover, *Cronobacter* demonstrates the ability to survive in highly acidic conditions, such as those encountered in the gastric environment. This acid tolerance may enhance the

pathogen's ability to reach and colonize the intestinal tract, increasing the likelihood of systemic infection. In general, the pH of gastric fluid in adults is below 2, and while this is enough to kill *Cronobacter*, newborns within the first two months of life, especially premature and underweight infants, have a higher gastric fluid pH. While fed PIF, an infant's gastric fluid pH is approx. 3.6. This higher pH is significant, as *Cronobacter* can survive and continue to grow even after 90 minutes of exposure to a pH of 3.0 (Ling et al., 2022).

Sialic acid is an additive to PIF during production due to its reported role in supporting healthy brain development in newborns. In addition to its desiccation, heat and acid resistances, *C. sakazakii* is distinct within its genus for its ability to utilise sialic acid as a nutrient source. This trait allows *C. sakazakii* to remain viable in PIF products that are enriched with sialic acid (Kalyatanda et al., 2015).

Cronobacter can adhere to a variety of materials commonly used in food preparation and healthcare settings, including silicone, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride. Additionally, they can produce capsules that facilitate biofilm formation, which can enhance their resistance to disinfectants (Forsythe & Holý, 2014). Biofilm formation is especially relevant in *Cronobacter* spp. for its persistence in processing environments, on feeding equipment, and potentially even in PIF residues or preparation surfaces. In a study by Hurrell et al. in 2009, it was determined that when compared with *Salmonella* serovars, *Acinetobacter* spp. and other *Enterobacteriaceae*, *Cronobacter* spp. tend to have the highest biofilm cell density. This is especially relevant in the context of enteral feeding tubes, as clumps of *Cronobacter* cells could detach and potentially survive passage through a baby's stomach. As a result, biofilm formation on these tubes represents a risk factor for vulnerable babies.

Lactic Acid Bacteria and *Cronobacter*

Lactic acid bacteria (LAB) are a diverse group of Gram-positive, acid-tolerant microorganisms that ferment sugars primarily into lactic acid. They are commonly found in fermented foods such as yogurt, kefir and sauerkraut. LAB are generally known for their antimicrobial effects against many food-borne pathogens in various food products and are

Generally Regarded as Safe (GRAS), and thus many strains are used as probiotics in the food industry (Charchoghlyan et al., 2016).

Lactiplantibacillus plantarum (*L. plantarum*) has shown some promising, but still limited, data activity against *C. sakazakii*. In a 2023 study by Fatemizadeh et al. *L. plantarum* strains M17 and ATCC 8014 significantly reduced *C. sakazakii* adhesion to intestinal epithelial cells, though the mechanism is not clear. In a study by Wei et al. in 2023, *L. plantarum* NCU001261 inhibited *C. sakazakii* translocation across Caco-2 cell monolayers by 66.85% as well as reduced claudin-1 degradation, preserving epithelial barrier function. *L. plantarum* NCU001261, however, did not tolerate gastrointestinal fluids well. While, these effects do not directly kill *C. sakazakii*, they help limit *C. sakazakii* colonisation and spread in the gut.

Lacticaseibacillus rhamnosus (*L. rhamnosus*) has also been studied for its antimicrobial effects against *C. sakazakii*. A strain referred to as *L. casei rhamnosus* in a study by Campana et al. in 2019 completely inhibited *C. sakazakii* growth after 4 hours of incubation with its crude Cell Free Supernatant (CFS), and only 2 hours when 2.5× concentration of the CFS was used. Flow cytometric analysis revealed that *L. casei rhamnosus* CFS had altered the permeability of the cell membrane of *C. sakazakii*.

Among some of the LAB studied, *Lactobacillus casei* (*L. casei*) has some of the strongest evidence supporting its antagonistic activity against *C. sakazakii*. *L. casei*, which has been isolated from feces of healthy infants, has been found to have inhibitory effect against multiple strains of *C. sakazakii*. This effect was observed in both agar plates and in rehydrated infant milk formula, highlighting its potential relevance for food safety. Its inhibitory effect was believed to be due to proteinaceous substances such as bacteriocins that had pH-dependent activity (Awaisheh et al., 2013).

In comparison to *L. plantarum*, *L. rhamnosus*, and *L. casei*, *Lactobacillus helveticus* (*L. helveticus*) has less evidence against *Cronobacter* spp. growth. However, some *L. helveticus* strains have been shown to produce antimicrobial compounds and inhibit pathogens such as *Listeria monocytogenes* or *Salmonella* (Georgiva et al., 2014). No strong studies have yet directly showed its effect against *Cronobacter* spp. in vitro or in food systems. A few broader screening studies that included *L. helveticus* showed

antimicrobial potential, but *Cronobacter* spp. was not among the pathogens tested (Du et al., 2025). *L. helveticus* may have probiotic and antimicrobial properties, but its antimicrobial effect against *Cronobacter* remains unclear and underexplored.

Postbiotics

The concept of using probiotics to inhibit pathogens has a long history. Recently, attention has shifted toward postbiotics, which differ from probiotics (live microorganisms) in that they are non-living (killed/inactivated), or are components/metabolites produced by bacteria that have activity (Golen, 2021). According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), a postbiotic is "a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host." This includes non-viable bacterial products or metabolic by-products (such as cell free supernatants, inactivated cells and metabolites), which may carry many of the benefits of probiotics but with fewer risks (e.g. for immunocompromised infants) and have better stability, making them easier to preserve and transport.

Multiple studies have highlighted the postbiotic potential of LAB strains, including their antimicrobial, antioxidant, immunomodulatory, and metabolic effects. In a study by Jalali et al. in 2024, *L. rhamnosus* had shown significant antimicrobial activity as a postbiotic, inhibiting pathogens like *Escherichia coli* and *Staphylococcus aureus* on food surfaces. *L. rhamnosus* supernatants have also been found to have immune-modulating effects. As a postbiotic, it has been found capable of modulating obesity-associated inflammation via TLR4/MAPK/NF- κB pathways (López-Almada et al., 2024). Postbiotic fractions (heat killed and extracted proteins) derived from both *L. plantarum* and *L. helveticus* have also been found to modulate inflammation in intestinal epithelial cells and restore metabolic markers in animal models of metabolic syndrome (Urbonaviciene et al., 2019). Another study also found that heat-killed *L. casei* (strain K-1) improved skin condition in healthy women over an eight-week period, suggesting efficacy even in the absence of live microbial activity (Nakamura et al., 2017).

The compounds released by these LAB strains can suppress pathogen colonisation, modulate host immune responses, and enhance intestinal barrier function. These effects are not limited to live cells as postbiotic forms have been shown to retain or sometimes even

enhance these properties, while offering advantages in terms of stability, safety, and regulatory compliance, especially in more vulnerable populations (Aguilar-Toalá et al., 2021).

Aims and Experimental Approach

In order to evaluate and better understand the antimicrobial potential of Cell Free Supernatants (CFS) derived from selected LAB strains, *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Lactobacillus helveticus*, against *Cronobacter* spp., its antimicrobial activity was investigated by adding them into reconstituted dried baby foods inoculated with *Cronobacter* spp. at approx. 1 CFU/10 ml and then incubating them for 24 h aat 37℃ as it is generally considered that *Cronobacter* multiplying in baby foods before consumption is needed to cause illness and while the infectious dose is currently unknown, approx. 1000 CFU has been suggested (Parra-Flores et al., 2015). The CFU reduction rate of CFS treated vs untreated baby food was then determined by drip plating samples onto Chromogenic Cronobacter Isolation (CCI) agar plates to ensure only *Cronobacter* were accounted for and not any other bacteria when determining the CFU as PIF is not a sterile product. pH, proteinase and an in vitro simulated gastrointestinal conditions test were also used to determine its stability against compounds and conditions commonly found in the gastric environment as well as characterise the CFS. A gentamicin protection assay, using both the CFS and the live LAB strains, was also conducted to examine the possible mechanisms of inhibition against *C. sakazakii*. *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* also underwent a Kirby-Bauer test to check for possible antibiotic resistances and avoid transferring them to *Cronobacter*, especially when live bacteria were to be used.

The biofilm morphology of multiple *Cronobacter* strains were also examined using a variety of agars such as overexpression agar, milk agar, calcofluor agar, and congo red + Coomassie blue agars in order to better understand how certain strains persist in certain environments such as feeding tubes. By combining these microbiological, biochemical, and genomic analyses, this study investigates both the functional efficacy and the safety profile of these postbiotic preparations as prophylactic agents in infant nutrition.

MATERIALS AND METHODS

Preparation of Bacterial Cultures and Cell Free Supernatant (CFS)

Overnight cultures of *Cronobacter* strains were made by inoculating 5 ml of L medium of pH 7.5 with the respective strain in a shaking incubator for 16-17 h at 37℃. Exponential phase cultures of *Cronobacter* strains were prepared by taking 50 µl from the overnight cultures and then added to 5 ml L medium and incubated for 2 h at 37℃ in a shaking incubator. The *Cronobacter* from these exponential phase cultures should then be approx. at log phase of growth.

Lactiplantibacillus plantarum (*L. plantarum*), *Lacticaseibacillus rhamnosus* (*L. rhamnosus*), *Lacticaseibacillus casei* (*L. casei*), and *Lactobacillus helveticus* (*L. helveticus*) overnight cultures were prepared by inoculating 14 ml of MRS medium with a loop of the strain and then grown anaerobically in a shaking incubator at 37℃ for 20 h. 48 h cultures were also made by allowing the cultures to incubate for approx. 48 h in a shaking incubator 37℃.

The cell free supernatant (CFS) of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* were prepared by centrifugation at 4000 rpm for 15 min for the overnight cultures and 1 h for the 48h cultures and then sterilised using a syringe with a 0.22 µm filter. Some of the CFS were then further sterilised via autoclaving.

Preparing Chromogenic *Cronobacter* Isolation (CCI) Agar Plates

In a 1 litre glass bottle with a lid with a magnetic stirrer, 14.75 g of ChromoCult® CCI medium granules were added along with 0.5 g of Millipore agar powder, and mixed in 500 ml of deionised water before being autoclaved. The liquid media were then poured onto square petri dishes for use in cfu determination. Typical *Cronobacter* colonies appear green/dark green on CCI agar, while non-*Cronobacter* species often turn up white or yellow.

Estimating the Amount of *Lactobacillus* Cell Free Supernatant Needed to Inhibit *Cronobacter* Growth

To determine the needed concentration of CFS to inhibit or affect *Cronobacter* growth for use as basis for further experiments, sterile filtered only/autoclaved CFS from a 48 h culture of *L. plantarum* were mixed with melted L-agar and then poured into square petri dishes. There were two conditions: one set of plates contained 10 ml of sterile filtered only/autoclaved *L. plantarum* CFS mixed with 40 ml of melted L-agar for a 1:5 dilution, while the other set of plates contained 5 ml of sterile filtered only/autoclaved *L. plantarum* CFS mixed with 45 ml of melted L-agar for a 1:10 dilution. 3 µl of *Cronobacter* strains (table 1) from overnight cultures were spotted onto the plates and then incubated for 24 h at 37℃. Results were determined by eye. Three technical and two biological replicates were made.

Table 1: List of *Cronobacter* strains used in this experiment.

Strain	**Species**	**Origin**	**Serotype**
MHI 975	*Cronobacter sakazakii* ATCC 29544	Human	O1
MHI 996	*C. sakazakii*	Baby food	O1
MHI 21011	*C. sakazakii*	Baby food	O1
MHI 21038	*C. sakazakii* ATCC BAA 893-1	Milk powder	O1
MHI 21039	*C. sakazakii* ATCC BAA 894	Human	O1
MHI 21086	*C. sakazakii*	Milk powder	O1
MHI 977	*C. sakazakii* NCTC 8155	Tin of dried milk	O2
MHI 995	*C. sakazakii*	Baby food	O2
MHI 21003	*C. sakazakii*	Baby food	O2
MHI 21004	*C. sakazakii*	Baby food	O2
MHI 21029	*C. sakazakii* E785	Human O2	
MHI 21042	*C. sakazakii* ES5	Human	O2
MHI 21122	*C. sakazakii* ATCC 12868	Unknown	O2

MHI 21006	*C. sakazakii*	Baby food	O3
MHI 21014	*C. sakazakii*	Baby food	O3
MHI 21051	*C. sakazakii*	Milk powder	O4
MHI 21170	*C. sakazakii*	Baby food	O4
MHI 21066	*C. sakazakii*	Milk powder	O7
MHI 21171	*C. sakazakii*	Baby food	O7
MHI 21097	*C. condimenti* LMG 26250	Food	
MHI 21093	*C. dublinensis* subsp. d*ublinensis* LMG 23823	Milk powder	
MHI 21094	*C. dublinensis* subsp. *lausannensis* LMG 23824	Water	O2
MHI 21095	*C. dublinensis* subsp. *lactariid* LMG 23825	Milk powder	O1
MHI 21091	*C. malonaticus* DSM 18702	Human	O2
MHI 21096	*C. muytjensii* DSM 21870	Uknown	O2
MHI 21213	*C. muytjensii* E888	Milk powder	O1
MHI 21026	*C. turicensis* 3032 LMG 23827	Neonate	O1
MHI 21049	*C. turicensis* E625	Baby food	O3
MHI 981	*C. universalis* NCTC 9529	Fresh water	O1

Note: Table was adapted from "Simultaneous Rapid Detection and Serotyping of Cronobacter sakazakii Serotypes O1, O2, and O3 by Using Specific Monoclonal Antibodies" by Eva J. Scharinger, Richard Dietrich, Ina Kleinsteuber, Erwin Märtlbauer, Kristina Schauer, 2016, American Society for Microbiology (1).

Measuring Inhibition Rates

Effects of postbiotics are often strain specific and thus may exhibit varying levels of inhibition against *Cronobacter* and a comparative analysis may allow identification for the most effective strain. In this experiment *Cronobacter* strains *C. sakazakii* MHI 996, *C. sakazakii* MHI 21038, *C. sakazakii* MHI 977, *C. sakazakii* MHI 995, *C. sakazakii* MHI

21006, *C. sakazakii* MHI 21014, *C. sakazakii* MHI 21051, *C. sakazakii* MHI 21170, *C. sakazakii* MHI 21066, *C. sakazakii* MHI 21171, *C. condiment* MHI 21097, *C. dublinensis* MHI 21093, *C. dublinensis* MHI 21095, *C. malonaticus* MHI 21091, *C. muytjensii* MHI 21096, *C. muytjensii* MHI 21213, *C. turicensis* MHI 21049 and *C. universalis* MHI 981 from food isolates were chosen to account for inter-strain variability in susceptibility.

Glycerol stocks of these *Cronobacter* strains were prepared from overnight cultures, and then stored at -20℃ for at least an overnight. The stocks were prewarmed at 37℃ for 10 min before use. Cfu of the stocks were determined via adding 500 µl of PBS to 100 µl of the stock in an Eppendorf tube, spun in a centrifuge for 3 min at 13,000 rpm, this washing step was then repeated three times before the *Cronobacter* pellets were resuspended with 900 µl of PBS, diluted up to six tenfold dilutions, and then drip plated on square L-agar plates and incubated overnight at 37℃. Four technical replicates were made. This was to help estimate the amount of *Cronobacter* to be added to the glass bottle.

A volume of 50 ml sterile glass bottles with a lid were used for this experiment. In each bottle, 7 ml of buffered peptone water (BPW) prewarmed at 60℃ was added onto 1 g of powdered infant formula (PIF) for infants aged 0-6 months and then thoroughly mixed with a sterile magnetic stirrer. 2 ml of *L. plantarum* autoclaved CFS prepared from a 48 h culture was then added and mixed before inoculating the milk solution with a *Cronobacter* strain for approx. 1 cfu/10 ml. The same solution was made for *L. rhamnosus*, *L. casei*, and *L. helveticus* CFS. A negative control bottle was prepared by replacing the 2 ml of CFS with 2 ml of MRS broth. The solutions were mixed well before being incubated at 37℃ for 24 h. The stocks used were also drip plated onto square L-agar plates to redetermine its cfu and confirm the number of *Cronobacter* added into each glass bottle.

After 24 h, samples were taken from each bottle, diluted with PBS and then drip plated onto square Chromogenic *Cronobacter* Isolation (CCI) agar plates, which was incubated overnight at 37℃, for cfu determination. This experiment was repeated three times.

The growth inhibition rates of each CFS against the 18 *Cronobacter* strains were determined by using the cfu from the treated bottles with CFS as a percentage of the cfu of the control bottles.

Inhibition rate: $100 - \left(\frac{\text{Cfu of treated bottles}}{\text{Cfu of control bottles}} \times 100\right)$

Identifying the Genus of the Bacterial colonies in CCI agar by using PCR

Cronobacter spp. isolates from the experiment above regarding inhibition of *Cronobacter* growth using *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS had their genomic DNA extracted for confirmation of its genus, and in turn to further confirm the use and reliability of CCI agar as a *Cronobacter* selection medium.

A single colony was inoculated into 5 ml L broth and incubated in a shaking incubator for 18 h at 37℃. 1.5 ml of this overnight culture was then pipetted into a 2 ml microtube and centrifuged for 2 minutes at 13,000 × g and the supernatant removed. 400 µl of the lysis buffer (table 2.1) was then added into the tube and the bacterial pellet resuspended by vortexing. 100 µl of lysozyme solution (100 mg/ml) was then added. The tube was then inverted several times to mix and then incubated on ice for 15 minutes.10 µl of 10% SDS solution and 5 µl of Proteinase K (10 mg/ml) was added and the tube inverted 10 times to mix. The tube was then incubated overnight at 55℃ in a slowly rotating hybridisation oven. Afterwards, the tube was allowed to cool to room temperature before 500 µl of room temperature isopropanol was added. The tube was inverted several times to gently mix the solutions until thread-like strands of DNA became a visible mass. The DNA precipitates were then transferred to a new 1.5 ml microtube containing 500 µl of room temperature 96% EtOH. The tube was then inverted several times to wash the DNA pellet. This wash step was repeated again with 70% (v/v) EtOH. The tube was then centrifuged at 13,000 × g for 2 minutes. Most of the EtOH was carefully removed by pipetting and the DNA pellet was allowed to air dry for 10-15 minutes. Afterwards, 500 µl Tris-EDTA buffer (pH 8.0) and 1 µl of RNase A (10 mg/ml) were added and mixed by carefully pipetting up and down. The tube was then incubated overnight at room temperature to facilitate homogenization of the DNA.

For genus identification using PCR (Tables 2.2 and 2.3), the following primers were used to specifically amplify the gene putatively encoding the α-glucosidase activity (3):

EsAgf: 5'- TGA AAG CAA TCG ACA AGA AG -3'

EsAgr: 5'- ACT CAT TAC CCC TCC TGA TG -3'

PCR product = 1680 bps (GenBank accession number AM075208).

Table 2.1: Solutions used in this experiment and their components.

Solutions	Components
Tris-EDTA buffer pH 8.0	10 mM Tris-Cl 0.1 mM EDTA
Lysis buffer pH 8.0	100 mM Tris-Cl 5 mM EDTA 100 mM NaCl

Table 2.2: Conditions for PCR

Components	Standard PCR	Positive Control	Negative Control
PCR Buffer (10x)	2.5 µl	2.5 µl	2.5 µl
MgCl 2 (25 mM)	1.5 µl	1.5 µl	1.5 µl
dNTPs (100 µM)	0.5 µl	0.5 µl	0.5 µl
Primer EsAgf (0.1 µM)	1 µl	1 µl	1 µl
Primer EsAgr (0.1 µM)	1 µl	1 µl	1 µl
Taq Polymerase (2 U)	0,1 µl	0,1 µl	0,1 µl
Sample DNA (25-50 ng)	25-50 ng	0 ng	0 ng
Positive Control DNA	0 ng	25-50 ng	0 ng
dH_2O	up to 25 µl	up to 25 µl	43.4 µl
Total	50 µl	50 µl	50 µl

Table 2.3: PCR cycle settings

Step	Temperature	Time	No. of cycles
Denaturation	95°C	5 minutes	1x
Denaturation	95°C	30 seconds	29x
Annealing	58°C	60 seconds	
Extension	72°C	90 seconds	
Extension	72°C	5 minutes	1x

Examining the Effectiveness of *L. plantarum* Cell Free Supernatants Against *C. sakazakii* ES5 in Different Dried Baby Foods

The composition of different dried baby foods can affect both the survival and growth of *C. sakazakii* ES5 and the activity and/or stability of antimicrobial compounds present in *L. plantarum* CFS. Different brands of dried baby foods intended for different ages (initial formula, follow up formula, and toddler milk formula) were used to account for the differences in formulations that could either enhance or inhibit *Cronobacter* growth and/or affect the antimicrobial efficacy of the *L. plantarum* CFS.

Glycerol stocks of *C. sakazakii* ES5 was prepared from an overnight culture, which were then stored at -20℃ for one overnight. The cfu of the stock was determined the same way as the other *Cronobacter* stocks made in the experiment above.

Six different dried baby foods were used in this experiment. Two were for infants aged 0-6 months, two were ages 6-12 months, and the last two were plant-based baby food suitable for ages 1-3 years. 1 g of dried baby foods was dissolved in 9 ml of BPW prewarmed at 60℃ by mixing with a sterile magnetic stirrer in a 50 ml sterile glass bottles with a lid. Afterwards, 2 ml of autoclaved *L. plantarum* CFS prepared from an overnight culture was added and thoroughly mixed into the milk solution. For the negative control bottle, 2 ml of MRS broth was added instead of CFS. Each bottle was then inoculated with *C. sakazakii* ES5 for an approx. 5 cfu/12 ml. The bottles were mixed well before being incubated at 37℃ for 20 h. The stocks used were also drip plated onto square L-agar plates to redetermine its cfu and confirm the amount of *Cronobacter* added into each glass bottle. The stocks used were also drip plated onto square L-agar plates to redetermine its cfu and confirm the number of *Cronobacter* added into each glass bottle.

After 20 h, samples were taken from each glass bottle. The samples were then diluted and drip plated onto square CCI agar plates and then incubated overnight at 37 ℃. Cfu of both the test and control were then determined from the plates. The experiment was repeated three times.

Examining the Acid Buffering Capacity of Different Powdered Infant Formulas

To determine a possible cause of the differing results from the experiment above, the acid buffering capacity of each of the dried baby foods used in the previous experiment was examined.

In a 100 ml glass bottle, the dried baby food was reconstituted according to manufacturers' instructions:

- Freshly run water was boiled and allowed to cool for 30 mins. Artificially softened or repeatedly boiled water was not used.
- The correct amount of powder was added to the warm water.
- The bottle was closed properly and held horizontally before shaking it from left to right for 10 s. Afterwards, it was held vertically and then shaken up and down for a further 10 s.

The reconstituted dried baby food was then allowed to cool to room temperature for accurate measuring of its pH. Once it was cool and its pH measured, 0.3 ml of 0.1 M HCl was added to each sample at a 2-3 min interval and mixed well using a magnetic stirrer. The pH was measured three times after each HCl addition until a pH of approx. 4.5 was reached.

Gentamicin Protection Assay

Cronobacter is known to be able to invade intestinal epithelial cells and translocate across the intestinal barrier, contributing to neonatal sepsis and meningitis (Wei et al., 2023). A gentamicin protection assay can evaluate the protective or anti-invasive effect of *L. plantarum* and its CFS against *C. sakazakii* ES5.

Three conditions were set up to examine how *L. plantarum* might prevent *Cronobacter* infection, using *C. sakazakii* ES5 to represent *Cronobacter* spp.. For all three conditions, a 24 well culture plate was seeded with Caco-2 cells with a density of approx. 10^5 cells/well, and the infection solution was made by adding *C. sakazakii* ES5 from an exponential phase culture into RPMI media with an MOI = 10 (10:1) per well.

The three conditions were 1) Using *L. plantarum* CFS from an overnight culture, which was allowed to preincubate for 2 h in the wells containing Caco-2 cells, as treatment to prevent *C. sakazakii* ES5 (MOI = 10 or 10:1) infection, 2) Using live *L. plantarum* cells (with an MOI = 100/well or 100:1) from an overnight culture, which was allowed to preincubate for 2 h, as treatment, 3) Live *L. plantarum* cells (with an MOI = 100/well or 100:1) were mixed with the infection solution and then the mixed solution inoculated into the wells. Before the assay, one well was treated with trypsin and used for Caco-2 cell counting.

For the first and second conditions, 12 out of 24 wells of the culture plate, the RPMI medium was removed and replaced with 1 ml of *L. plantarum* CFS from an overnight culture diluted 1:5 with RPMI media or 1 ml of live *L. plantarum* bacteria from an overnight culture diluted with RPMI media so that the solution had an MOI = 100, and then allowed to preincubate for 2 h at 37℃ and 5% CO_2. The remaining 12 wells received no treatment and acted was negative controls (fig. 2). After 2 h, all media were removed from all 24 wells and washed with PBS once. 500 µl of the infection solution was then added into each well. As for the third condition, live *L. plantarum* cells, also from an overnight culture diluted with RPMI media so that the solution had an MOI = 100, was mixed together with the infection solution and then 500 µl of this solution was added into the 12 wells. There was no 2 h preincubation. A serial dilution of the infection solution was also made and drip plated onto square L-agar plates and then incubated overnight at 37℃ for cfu counting.

For the adhesion prevention assay, the 12 out of 24 wells were allowed to incubate for 30 min at 37℃ and 5% CO_2 before being washed with 500 µl PBS three times. Of the 12 wells washed, 6 were for the adhesion prevention test and the other 6 were its controls. 1 ml of cold 0.1% Triton-X solution was added into the wells, pipetting up and down to resuspend lysed Caco-2 cells.

For the invasion prevention assay, the remaining 11 wells were allowed to incubate for 1 h 30 min at 37℃ and 5% CO_2 before being washed with 500 µl PBS three times and then treated with an RPMI-gentamicin solution, with a gentamicin final concentration of 100 µl/ml. It was then allowed to incubate for a further 1 h at 37℃ and 5% CO_2. After

incubation, the wells were washed twice with 500 µl PBS before adding 1 ml of cold 0.1% Triton-X solution to lyse and resuspend the Caco-2 cells.

For both the adhesion and invasion prevention assays, the lysed cells were then transferred into 2 ml safe lock tubes which were then vortexed horizontally using a horizontal microtube holder on a Vortex Genie 2 at maximum speed for 5 min. The solutions were then serially diluted in 10-fold dilutions and then drip plated onto L-agar plates and then allowed to incubate overnight at 37℃ for cfu counting. The adhesion and invasion rates of *C. sakazakii* ES5 on Caco-2 cells were then calculated. For the adhesion rate, the cfu of *C. sakazakii* ES5 from the adhesion assay was used as a percentage of the cfu of the infection solution. For the invasion rate, the cfu of *C. sakazakii* ES5 from the invasion assay was used as a percentage of the cfu from the adhesion assay.

$$\text{Adhesion rate: } \frac{\text{Cfu from adhesion assay}}{\text{Cfu of infection solution}} \times 100 \qquad \text{Invasion rate: } \frac{\text{Cfu from invasion assay}}{\text{Cfu from adhesion assay}} \times 100$$

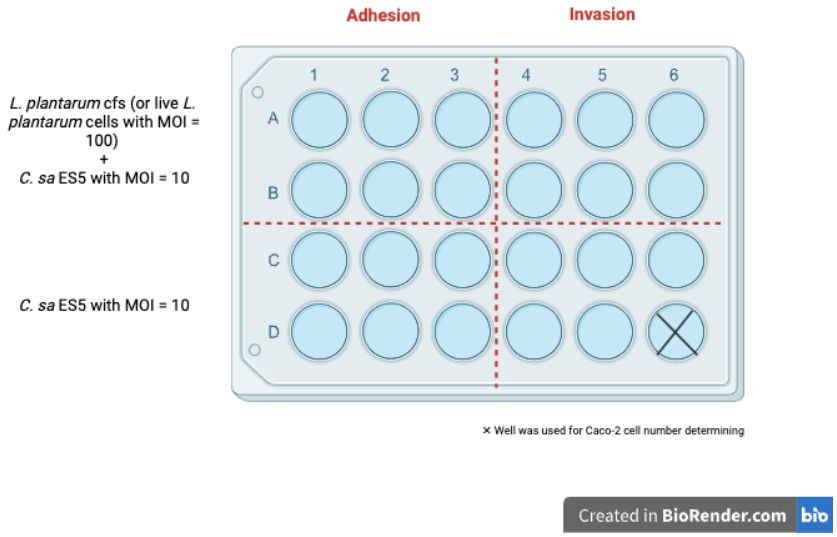

Fig. 2. The gentamicin protection assay was set up using 24 well culture plate divided in such a way that the top part of the plate consisted of treated wells, while the bottom part acted as controls. Similarly, the left side of the plate consisted of wells designed for the adhesion assay, while the right side was for the invasion assay.

Figure created in https://BioRender.com

L. plantarum and *C. sakazakii* ES5 Growth Test

To examine how the presence of live *L. plantarum* cells might affect growth of *C. sakazakii* ES5, whether it had a bactericidal or bacteriostatic effect, a 6 well culture plate similar to the one used in the gentamicin protection assay above was set up with three conditions: 1) two wells with RPMI media were inoculated with *L. plantarum* cells from an overnight culture to an MOI = 100; 2) two wells with RPMI media were inoculated with *C. sakazakii* ES5 exponential phase cultures to an MOI = 10; 3) and the last two wells with RPMI media were inoculated with both *L. plantarum* cells from an overnight culture to an MOI = 100 and *C. sakazakii* ES5 exponential phase cultures to an MOI = 10 mixed together (fig. 3). The MOIs were determined from Caco-2 cells with a density of 2.75×10^5. Each well totalled to a volume of 6 ml. The culture plate was then incubated for 2.5 h at 37℃ and 5% CO_2. Every 30 min, each well was, mixed thoroughly by pipetting up and down and 200 µl were taken and then drip plated onto square MRS-agar plates and incubated anaerobically at 37℃ overnight for the first condition, and L-agar plates for the second and third conditions, which were incubated aerobically at 37℃ overnight. Three biological replicates were made.

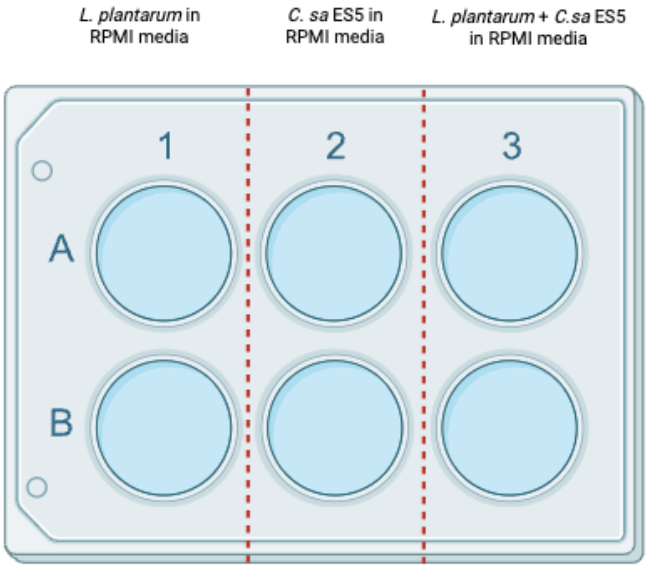

Fig. 3. The growth test was set up in a 6 well culture plate with three differing conditions. The first column were *L. plantarum* cells alone grown in RPMI media, the second column were *C. sa* ES5 cells grown alone in RPMI media, and the third column were both *L. plantarum* and *C. sa* ES5 cells grown together in RPMI media. The culture plate contained no Caco-2 cells and an imaginary density was used for consistency among replicates.

Figure created in https://BioRender.com

Determining the Minimum Inhibitory Concentration (MIC) – Broth Microdilution Method

An MIC and MBC test would allow for a quantitative assessment of the antimicrobial potency of the *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFSs. Such data are important for evaluating the therapeutic or preservative potential of CFS in dried baby foods, where precise control of microbial contamination is essential.

Both sterile filtered only and sterile filtered then autoclaved CFS from *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* prior to the experiment were lyophilised and then diluted in 6 ml of Mueller-Hinton broth (MH broth) resulting in a highly concentrated solution.

To determine the MIC of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS needed to inhibit *C. sakazakii* ES5 growth, a 96-well plate was prepared as in fig. 4a. 100 µl of MH broth was added to all wells except to wells in row 12. 200 µl of CFS, with a concentration of 25 mg/ml were added to wells in column 12. Afterwards, 100 µl of the 25 mg/ml concentration CFS were added to the wells on its left (column 11) and mixed well. This sequential dilution was repeated until column 3, where the final 100 µl was discarded. Wells in columns 1 and 2 were filled with 100 µl of MH broth only. An overnight culture of *C. sakazakii* ES5 was prepared and adjusted to McFarland 0.5 standard, where 5 µl of the solution was then inoculated to all wells except those from column 1. Wells from column 1 were used as sterility controls (SC) while wells from column 2 were used as growth controls (GC). The optical density (OD) at a wavelength of 600 nm was measured for t = 0 before the 96-well plate was incubated for 18 h at 37℃, where the OD was measured again for t = 18. Two biological replicates were made.

Afterwards, experiment was repeated again with modified concentrations for increased accuracy of results (fig. 4b)

The results were analysed both by eye and by measuring the OD at 600 nm using a plate reader.

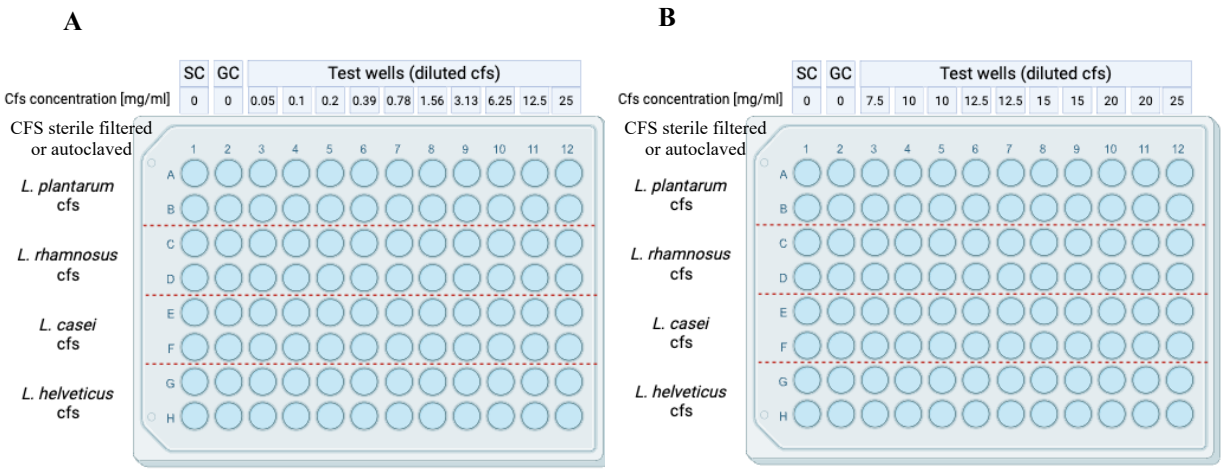

Fig. 4. The layout designed on a 96-well plate for the MIC assay. 4a was the first dilution and allowed for two technical replicates per CFS for all dilutions of CFS and controls. 4b was the modified dilution for more accurate results.

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Determining the Minimum Bactericidal Concentration (MBC)

To determine the MBC of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS, 10 µl was spotted from the last two wells with visible growth and all other wells without growth of the modified 96-well plate (fig. 4b) onto MH agar plates. The plates were then incubated for 18 h at 37℃. Two biological replicates were made.

Characterisation of the Cell Free Supernatants

Dried baby foods, especially milk formulas, often have an acid buffering effect and have a pH range of approx. 6.55 to 7.01 once reconstituted (Murphy et al., 2020). Lactic acid, which is produced by LAB, have their antimicrobial effect reduced at pH close to neutral, making them less membrane permeable (Russell & Diez-Gonzalez, 1998; Alakomi et al., 2000).

Examining How a Change in pH Affects MIC and MBC of the CFS

pH Test in a 96-well Plate

Sterile filtered only CFS (25 mg/ml), diluted in L broth, were adjusted to pH 3, 5, 7 and 9 using 1 M HCl and 1 M NaOH. CFS with unaltered pH were used as control. Sterility and growth controls were also used. 100 µl of the solutions were then added onto all wells of the 96-well plate except for the sterility and growth control wells which were filled with L broth instead (fig. 5). The wells, except for sterility control wells, were then inoculated with 5 µl of *C. sakazakii* ES5 from an overnight culture adjusted to McFarland 0.5 standard. The OD at 600 nm of the solutions were measured before incubation for t = 0. The plate was then incubated for 18 h at 37℃, where the OD at 600 nm was measured again for t = 18.

The results were analysed both by eye and by measuring the OD at 600 nm using a plate reader.

Afterwards, 10 µl from the last two wells with visible growth and all other wells without growth were spotted onto MH agar plates and then incubated for 18 at 37℃ to determine the MBC. Two biological replicates were made.

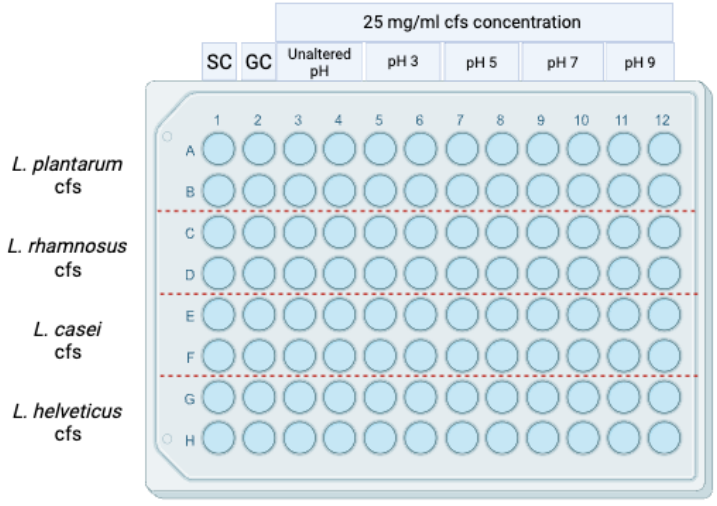


Fig. 5. The layout designed on a 96-well plate for the pH test. This allowed for 4 technical replicates per test and control pH, and two technical replicates for both sterility controls (SC) and growth controls (GC).

Figure created in https://BioRender.com

pH Test on Agar Plates

The *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS used were not from the lyophilised stock prepared beforehand and were instead from fresh 48 h cultures sterile filtered and adjusted to pH to pH 3, 5, 7 and 9 using 1 M HCl and 1 M NaOH. The unadjusted CFS were kept as control. The sterile CFS were then diluted 1:5 with melted L-agar, which were then poured into 60 mm diameter petri dishes and allowed to cool and solidify. 5 µl of *C. sakazakii* ES5 from both overnight and exponential phase cultures were spotted onto the plates. Using pH strips, the pH of the agar plates were measured for t = 0 before being incubated or 18 h at 37℃. The pH was measured again using pH strips at t = 18. The pH of the undiluted CFS were measured using a pH meter.

Examining Proteinase Treatment Effects on *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS

If *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS were to be used as postbiotics, it is important to know whether their antimicrobial activity can be retained despite proteinase treatment.

Trypsin, a proteinase found in the small intestine, and proteinase K, known for its broad-spectrum proteolytic activity, were chosen as proteinase treatments on *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFSs, and then tested against *C. sakazakii* ES5. The CFSs (25 mg/ml) were treated with 1 mg/ml or 0.025 mg/ml trypsin, or 0.5 mg/ml (15 U) or 0.01 mg/ml (0.3 U) proteinase K and then incubated for 2 h at 37℃. The enzymes were then deactivated by heating the CFS up to 65℃ for 15 min and then allowed to cool down to room temperature. A 96-well plate was then set up as in fig. 6. All outer wells of the 96-well plate were filled with 200 µl of L broth. The other wells, except for wells in columns 2 and 3, were filled with 200 µl of L broth mixed with or without treated CFS. 200 µl of untreated CFS were added into wells in column 2 and were used as controls. Wells in column 3 were added with 200 µl of L broth and were used for growth control. *C. sakazakii* ES5 from an overnight culture was diluted into a McFarland 0.5 standard, and 5 µl of this was then added into all wells, except the outer wells. The plate was then incubated at 37℃ for 24 h and 200 rpm in a microplate reader and the OD measured at 600 nm.

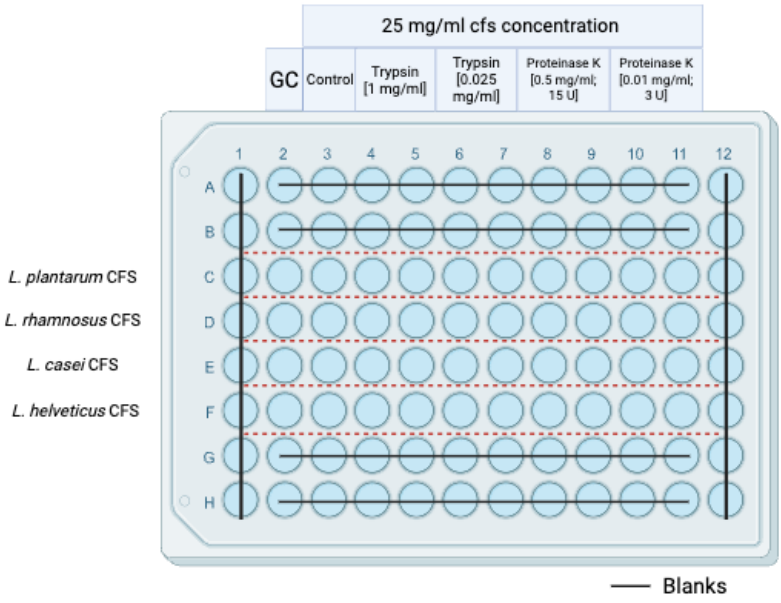


Fig. 6. The layout for the proteinase test on a 96-well plate, allowing for two technical replicates per test and one replicate for the controls. The outer wells were filled with L broth were used as blanks.

Figure created in https://BioRender.com

Preliminary Tests for In Vitro Simulated Gastrointestinal Conditions Experiment

Two preliminary tests were done prior to doing a simulated gastrointestinal experiment as the pH of the dried baby foods could affect food quality and/or irritate the oesophagus or stomach mucosa of infants or even contribute to metabolic acidosis (2).

C. sakazakii ES5 Growth Test Using Different CFS Concentrations

Despite dried baby foods having a buffering capacity, it would still be preferable not to add too much acids and alter its pH. This experiment aimed to determine the smallest concentration of CFS from *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* needed to inhibit *C. sakazakii* ES5 growth in L broth.

L. plantarum, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS were diluted with L broth into differing concentrations: 10 mg/ml, 15 mg/ml and 20 mg/ml. 200 µl of these dilutions were then added into columns 4-12 in the 96-well plate, while columns 1-3 were filled with 200 µl of L broth to use as growth controls (fig. 7). 5 µl of *C. sakazakii* ES5 from an overnight culture adjusted to McFarland 0.5 standard was then inoculated to all wells. The plate was then incubated at 37℃ for 24 h and 200 rpm in a microplate reader and the OD measured at 600 nm. Two biological replicates were made.

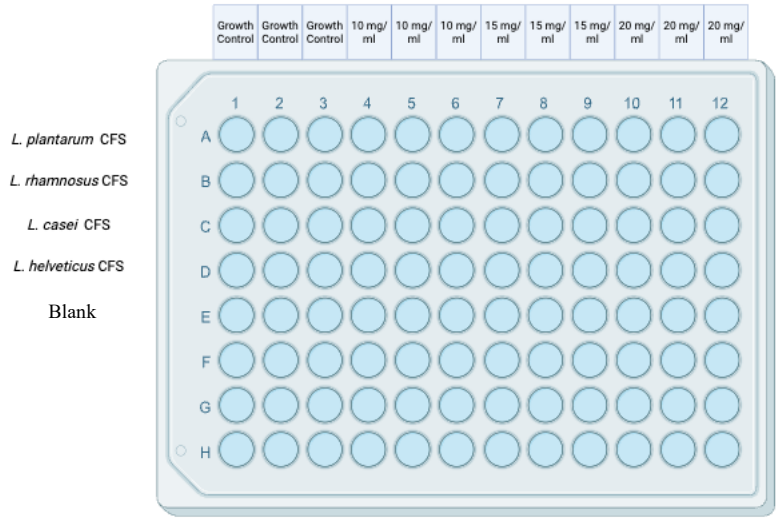


Fig. 7. The 96-well plate layout designed for the *C. sa* ES5 growth test with differing CFS concentrations. The layout allowed for three technical replicates for both tests and controls as well as space to fill in blanks.

Figure created in https://BioRender.com

Determining pH Value Changes in Dried Baby Food when Cell Free Supernatant is Added

A 0.5% (w/v) NaCl solution was prewarmed at 60℃ before being used to dissolve PIF, the amount according to the manufacturer. This 0.5% (w/v) NaCl + PIF solution was meant to represent the postprandial gastric juices in infants aged 0-4 weeks. Another 0.5% (w/v) NaCl solution adjusted to pH 3.5 using 1 M HCl was also used to dissolve PIF according to manufacturer directions. This solution was meant to instead represent the postprandial gastric juices of infants older than 4 weeks. 10 ml of the PIF solutions were then aliquoted into 100 ml glass bottles and CFS were then added in concentrations: 0 mg/ml, 10 mg/ml,

15 mg/ml, 20 mg/ml and 25 mg/ml. The solutions were then thoroughly mixed before its pH were measured three times using a pH meter.

In Vitro Simulated Gastrointestinal Conditions Experiment

The experiments prior all examined *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS and its effects on *Cronobacter* under controlled, non-representative conditions. After determining many of the characteristics of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS and the conditions they work in, it was time to examine how effective its antimicrobial activity was in more realistic environments.

In 50 ml polypropylene tubes, CFS was added to sterile ready to feed baby formula to a final concentration of 10 mg/ml and a total volume of 10 ml. The pH of baby formula and CFS solution was set to pH 5 using a 0.1 M HCl solution. The tubes were then inoculated with *C. sakazakii* ES5, from glycerol stocks made from an overnight culture, to an approx. 5 cfu/10 ml.

To simulate the gastric phase, pepsin (70 U) was added into the tubes and mixed well by inverting the tubes several times. Then lipase (0.0009 mg/ml) was added and the tube was inverted several times to mix. The tubes were then incubated at 37℃ for 2 h with shaking (200 rpm) before 120 µl were taken as samples to be serially diluted and drip plated onto square CCI agar plates for cfu counting for t = 2.

Simulating enteric phase 1, the solution in the tubes were then adjusted to pH 4.7 with an alkaline solution (table 3). Bovine bile (0.01 g/ml) was then added into the tubes, which were then inverted several times before adding pancreatin (1 mg/ml). The tubes were mixed well by inverting and then incubated for 2 h at 37℃ with shaking (200 rpm). Afterwards, 120 µl was taken as a sample for serial dilution and then drip plated onto square CCI agar plates for cfu counting for t = 4.

To simulate enteric phase 2, the solution was adjusted to pH 7.1 with alkaline solution and the concentrations of both the bovine bile (0.01 g/ml) and pancreatin (1 mg/ml) were adjusted to the same final concentration as in enteric phase 1. The tubes were then

incubated for 2 h at 37℃ with shaking (200 rpm) and 120 µl was taken as a sample for serial dilution and then drip plated onto square CCI agar plates for cfu counting for t = 6. The tube was then allowed to incubate at 37℃ with shaking (200 rpm) for a further 18 h. Samples were then serially diluted and then drip plated onto square CCI agar plates for cfu counting for t = 24.

Table 3: Solutions used in the in vitro gastrointestinal conditions experiment

Solutions	Components	Final concentration
Pepsin	Stock: 10 mg/ml (dissolved in 0.1 M HCl)	70 U/ml
Lipase	Stock: 1 mg/ml (dissolved in sterile deionised water)	0.0009 mg/ml
Bovine Bile	Stock: 0.5 g/ml (dissolved in sterile deionised water)	0.01 g/ml
Porcine Pancreatin	1 capsule of Pankreatin Mikro-ratiopharm® 20.000 with 195.2 mg dissolved in 1 ml of alkaline solution	1 mg/ml
Alkaline Solution	1.4 g NaH_2PO_2 dissolved in 85 ml sterile deionised water. Add 15 ml of 1 M NaOH then syringe filtered	
0.1 M HCl Solution	5 ml of 1 M HCl added and mixed into 45 ml of sterile deionised water then syringe filtered	

Infant Formula in Different Storage Conditions

Often times, infant milk are prepared in advance and given to the baby at a later time. The time between storage and feeding provides sufficient time for *Cronobacter* to multiply and grow, increasing chances for it to infect and cause disease to the infant. To examine the effectiveness of the CFS of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* in inhibiting *C. sakazakii* ES5 growth, 10 mg/ml of CFS was added to 5 ml of sterile ready to

feed baby formula in 15 ml polypropylene tubes. Approx. 5 CFU/10 ml of *C. sakazakii* ES5, prepared from a glycerol stock, was the inoculated into the milk solution. The milk were then stored for 24 hours in three different conditions: 1) at 5℃ in the fridge, 2) at room temperature (22℃), and 3) at 37℃. Samples were taken from the tubes at t = 2, 4, 6 and 24 and then drip plated onto CCI agar.

Comparing Biofilm Morphology of Different *Cronobacter* Strains

Biofilm morphology can contribute to increase in resistance against disinfectants, survival in dry environments and risk to infant health via biofilm formation in feeding tubes or during processing of infant milk. By comparing the morphology of different strains of *Cronobacter* we can better understand how certain strains may or may not persist in certain environments.

For this experiment, *Cronobacter* strains from food isolates (highlighted in table 1) were used: *C. sakazakii* MHI 996, *C. sakazakii* MHI 21038, *C. sakazakii* MHI 977, *C. sakazakii* MHI 995, *C. sakazakii* MHI 21006, *C. sakazakii* MHI 21014, *C. sakazakii* MHI 21051, *C. sakazakii* MHI 21170, *C. sakazakii* MHI 21066, *C. sakazakii* MHI 21171, *C. condimenti* MHI 21097, *C. dublinensis* MHI 21093, *C. dublinensis* MHI 21095, *C. malonaticus* MHI 21091, *C. muytjensii* MHI 21096, *C. muytjensii* MHI 21213, *C. turicensis* MHI 21049 and *C. universalis* MHI 981. All strains were streaked onto Overexpression agar and onto Milk agar (table 4) for biofilm comparison. All plates were then incubated overnight at 37℃ before examining results. Afterwards, the milk agar plates were left to continue growing at room temperature (22℃) for one week before results were noted down. The autoinduction agar plates were incubated at 37℃ for one more overnight before leaving it to grow at room temperature for 2 more weeks.

Table 4: Media Compositions

Media	Components
Overexpression agar	Novagen® Overnight Express™ Instant LB Medium granules 45g/L Agar powder 15 g/L
Milk agar	$(NH_4)_2SO_4$ 0.4 g Distilled water 40 ml Agar powder 3.6 g Milupa Aptamil Pronutra Pre ready-to-drink infant formula (sterile) 200 ml

The media used in this experiment were prepared externally and provided for experimental use by Kristina Schauer.
The milk agar was prepared by dissolving 0.4 g $(NH_4)_2SO_4$ in 40 ml distilled water before adding 3.6 g of agar powder and then autoclaved. The medium was then cooled to approx. 60℃. 200 ml of Milupa Aptamil Pronutra Pre ready-to-drink infant formula, which was preheated to 40-50℃, was then added and mixed into the medium.

The overexpression agar was made by dissolving the Overnight Express Instant LB Medium granules (45 g/L) in deionised water, the pH adjusted to 7, and agar powder (15 g/L) added then autoclaved. The medium was a ready-to-use granulate and the exact compositions were not disclosed.

Analysis of the Biofilm Matrix of Different *Cronobacter* Strains

Cronobacter forming biofilms on surfaces such as enteral feeding tubes and milk processing areas contribute to its spread and persistence in such environments. Thus, it is relevant to analyse their biofilms and detect PNAG (Poly-β-(1→6)-N-acetylglucosamine), cellulose and Curli as such components have major roles in biofilms. PNAG mediates intercellular adhesion and thus helps bacteria to accumulate and stick together and onto surfaces as well as contribute to evasion of antimicrobials and host defences (Balducci et al., 2023). Cellulose has structural role in biofilms by providing a polysaccharide scaffolding, helping matrix cohesion, retaining moisture, and contributing to mechanical

strength (Tursi et al., 2018). While the protein Curli, forms amyloid fibrils and are important for the early stages of biofilm formation and also contributes to overall matrix structure. It also mediates adhesion to surfaces as well as cell aggregation (Tursi et al., 2018).

To further analyse the biofilm by detecting biofilm components such as PNAG, cellulose and proteins, *C. sakazakii* MHI 996, *C. sakazakii* MHI 21038, *C. sakazakii* MHI 977, *C. sakazakii* MHI 995, *C. sakazakii* MHI 21006, *C. sakazakii* MHI 21014, *C. sakazakii* MHI 21051, *C. sakazakii* MHI 21170, *C. sakazakii* MHI 21066, *C. sakazakii* MHI 21171, *C. condimenti* MHI 21097, *C. dublinensis* MHI 21093, *C. dublinensis* MHI 21095, *C. malonaticus* MHI 21091, *C. muytjensii* MHI 21096, *C. muytjensii* MHI 21213, *C. turicensis* MHI 21049, *C. universalis* MHI 981, *C. sakazakii* MHI 21039, *C. sakazakii* ES5, *C. sakazakii* MHI 21029, *C. sakazakii* MHI 21122 and *C. turicensis* MHI 21026 were grown overnight in 5 ml L broth and then adjusted to OD_{600} = 1.0 in PBS before 5 µl of the cell suspensions were then spotted on Congo Red Agar plates (CRA) 1 and 2, and 10 µl were spotted on Calcofluor plates (table 5). The CRA plates were incubated at room temperature (22℃) in a bag, to prevent the plates from drying out, for six days. Colonies were photographed on days 3 and 6. The Calcofluor plates were placed in a bag, to prevent the plates from drying out, and then incubated at 28℃ for 48 h before the colonies were analysed under UV light and then photographed. The plates were then incubated at 5℃ for 24 h and then analysed under UV light again to note any changes.

Table 5: Media Compositions

Media	**Components**
Congo Red Agar 1	Casamino acids 2 g Yeast extract 0.3 g Millipore® Agar powder 3 g Congo red solution (25 mg/ml) 0.32 ml Coomassie brilliant blue solution (25 mg/ml) 80 µl 1 M $MgSO_4$ 80 µl Deionised water 200 ml
Congo Red Agar 2	Tryptone 2 g Yeast extract 1 g

	NaCl 1 g Glucose 2 g Millipore® Agar powder 3 g Congo red solution (25 mg/ml) 1.6 ml Coomassie brilliant blue solution (25 mg/ml) 0.8 ml Deionised water 200 ml
Calcofluor Agar	Tryptone 2 g Yeast extract 1 g Millipore® Agar powder 3 g Calcofluor 20 mg

Measuring the Protein Concentration in the Cell Free Supernatants of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus*

LAB CFS contain many postbiotic compounds such as organic acids, short-chain fatty acids, enzymes, antimicrobial peptides and bacteriocins. Many postbiotic effects are mediated by proteinaceous components in the CFS, and thus measuring its total protein provides a baseline for understanding the potency or activity of the CFS as well as determine whether proteins are the major active components.

A Pierce™ BCA Protein Assay Kit was used to estimate the protein concentration in the CFS of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* cultures grown for 20 h, 24 h and 48 h. In a 96-well microplate, 25 µl of either the BSA standard (working range = 5 – 250 µg/ml) or the CFS sample, which were diluted with PBS, were pipetted into the wells. 200 µl of the working reagent was then added to each well and mixed thoroughly via a plate shaker for 30 seconds. The plate was then incubated at 37℃ for 30 minutes. Afterwards, the plate was allowed to cool to room temperature before the OD were measured at a plate reader at 562 nm.

Kirby-Bauer Test on *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus*

While the aim of this study was to use supernatants as prophylactic additions to powdered infant formulas, it is still important to know the characteristics of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus*. If living bacterial cells were to be used, knowing whether or not the LAB strains carried resistance genes would be important. If antibiotic resistance genes were present, it is possible for it to be transferred to other bacteria both in the reconstituted baby food or in the intestine. Even when using CFS, there is still a chance for the genomic DNA from dead lysed LAB to be taken up by other bacteria.

To measure the sensitivity of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* to certain antibiotics, two types of media were prepared: Mueller-Hinton (MH) agar and Mueller-Hinton agar mixed with lysed horse blood. The lysed erythrocytes were added to see if it had any effect on the sensitivity of the four strains to the antibiotics used. *L. casei* and *L. helveticus* were plated on both Mueller-Hinton (MH) agar and Mueller-Hinton agar with lysed erythrocytes. *L. plantarum* and *L. rhamnosus*, however, were only plated on Mueller-Hinton agar with lysed erythrocytes due to time constraints.

The lysis of the horse blood was done by freeze-thaw method. 10 ml of the horse blood was poured into a 50 ml polypropylene tube and then stored at -20℃ overnight. It was then allowed to thaw at room temperature the following day. This was repeated until the blood has been thawed three times. 10 ml of sterile deionised water was then added to the tube and inverted several times to mix before incubating it at room temperature for 30 minutes. The tube was then centrifuged at 4000 rpm for 1 hour at 4℃. The supernatant was then decanted into a new tube and the pellet thrown away.

Preparation of the MH with lysed erythrocytes was done by adding 10 ml of lysed horse blood to 200 ml of melted MH agar cooled to 60℃, swirling the flask to mix. The media was then poured into petri dishes and allowed to solidify.

Overnight cultures of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* were adjusted to McFarland 0.5 standard, and then cotton swabbed onto the MH agar and MH + lysed blood agar plates. Using a dispenser, twelve antibiotic discs were stamped onto the agar plates with only six discs dispensed per plate. The antibiotics used were: vancomycin,

linezolid, gentamicin, chloramphenicol, doripenem, imipenem, meropenem, piperacillin/tazobactam, STX (sulfamethoxazole-trimethoprim), ticarcillin, erythromycin and tetracycline. The plates were then anaerobically incubated at 37℃ for 48 h before the any zones of inhibition were measured.

Isolating the Genomic DNA of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus*

The genomic DNA of *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* were isolated and sequenced to identify functional genes associated with postbiotic production, assess strain safety by ensuring the absence of transferable resistance genes, and confirm species identity. All of which are impotant for the development of safe and effective postbiotic candidates.

For *L. plantarum* and *L. helveticus*, isolation of genomic DNA was done by centrifuging 3 ml of an overnight culture for 2 minutes at 13,000 rpm. The supernatant was then discarded and the bacterial pellets were resuspended in 500 µl of AKI solution (table 6) containing 10 mg/ml of lysozyme. The tube was then incubated horizontally in a hybridization oven at 37℃ for 90 minutes. After incubation, 25 µl of Proteinase K solution (10 mg/ml) was added and the tube was inverted several times to mix. 200 µl of 5% SDS was added next and then mixed by inverting the tube several times again. The tube was then incubated for 10 minutes at 70℃ and then allowed to cool to room temperature afterwards. 200 µl of neutral phenol-chloroform was added and then homogenized by inverting the tube several times. The tube was then centrifuged for 60 minutes at 4,000 rpm and at 4℃. The aqueous phase was then transferred to a new tube without taking the interphase. In the new tube, 200 µl of neutral phenol-chloroform was added and homogenized by inverting the tube a few times. The tube was then centrifuged for 60 minutes at 4,000 rpm and at 4℃. In a 2 ml Eppendorf tube, 600 µl of isopropanol was added and the upper phase from the centrifuged tube taken and added to the 2 ml Eppendorf tube. 60 µl of 3 M Na acetate with pH 8.0 was then added and mixed by inverting. The precipitated DNA was then transferred to a new 1.5 ml Eppendorf tube containing 500 µl 70% (v/v) ethanol, and then inverted several times. The tube was then centrifuged for 15 minutes at 13,000 rpm. Most of the ethanol was then removed, leaving only approx. 30 µl. 500 µl 70% (v/v) ethanol was added again, and the tube inverted

several times and left to stand at room temperature for 10 minutes. Once more, The tube was centrifuged for 15 minutes at 13,000 rpm and the ethanol removed to approx. 30 µl. The tube was then transferred to a heat block at 55-60℃ with the lid kept open to allow the EtOH to evaporate. 100-300 µl of Tris-EDTA buffer (pH 8.0) was then added into the tube depending on the size of the DNA pellet, and then incubated for 60 minutes at 50℃ and 500 rpm. Afterwards, the tube was stored at -20℃.

The method above proved ineffective in isolating genomic DNA for both *L. rhamnosus*, and *L. casei*, and so a modified approach was used. An overnight culture was diluted 1:5 with MRS broth and then incubated anaerobically at 37℃ for 3 hours. Then by centrifuging for 2 minutes at 13,000 rpm, approx. 20 mg of bacterial pellets were transferred into a 1.5 ml Eppendorf tube. The pellets were then resuspended in 120 µl lysostaphin buffer (table 6), and then 60 µl of lysostaphin (10 mg/ml) and 20 µl of lysozyme (100 mg/ml) was added and mixed well. The tube was then incubated at 37℃ for 2 hours. Afterwards, 25 µl of Proteinase K (10 mg/ml) was added, and the tube incubated at 56℃ for 60 minutes and 1000 rpm. 25 µl of 20% SDS was added and the tube incubated at 56℃ for 60 minutes and 1000 rpm. 400 µl of lysis buffer, from a Thermo Fisher Scientific Genomic DNA Extraction kit, was added and the tube incubated at 65℃ for 15 minutes and 1000 rpm. It was then further incubated at 80℃ for 10 minutes and 1000 rpm and then allowed to cool to room temperature. 600 µl of chloroform was added and the tube inverted several times to mix before it was centrifuged at 10,000 × g for 5 minutes. The upper aqueous phase was then transferred to a new 2 ml Eppendorf tube. 800 µl of precipitation solution, also from a Thermo Fisher Scientific Genomic DNA Extraction kit, was added to the tube and then inverted at room temperature for 5 minutes on ice. The tube was then centrifuges at 10,000 × g for 5 minutes. The supernatant was then removed, and the DNA pellet was dissolved in 100 µl of NaCl solution. 2 µl of RNase A (10 mg/ml) was then added and then the tube was incubated at 37℃ for 10 minutes. 300 µl of cold 100% EtOH was added and the tube was inverted several times and then stored at -20℃ overnight to allow the DNA to precipitate. The tube was then centrifuged at 10,000 × g for 5 minutes to pellet the DNA. The EtOH was removed, and the tube was allowed to stand at room temperature with the lid open for 15 minutes or until the DNA pellet is dry. The DNA pellet was then dissolved in 80 µl of sterile deionised water and then incubated

overnight at room temperature. It was then mixed gently with a pipette tip and then incubated at 4℃ for 3 days before being stored in at -20℃.

All samples from *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* were loaded into an agarose gel to confirm the presence of genomic DNA in the tubes, and its concentration and purity furthered assessed using a NanoDrop Microvolume Spectrophotometer.

Table 6: Solutions and their components

Solution	**Components**
AKI solution	6.7% (w/v) Saccharose 50 mM Tris-HCl, pH 8.0 1 mM EDTA 20 µg/ml RNase
Lysostaphin buffer	145 mM NaCL 50 mM Tris-HCl, pH 7.5

The procedure described in this section was carried out by Anna Ershova and is included to provide context for subsequent analyses. The genomic DNA samples were then sequenced by nanopore sequencing. The Nanopore sequencing libraries were prepared following the protocol provided with the Ligation Sequencing Kit V14 (SQK-NBD114-24; Oxford Nanopore Technologies, Oxford Science Park, OX4 4DQ, UK). The libraries were loaded onto R10.4 MinION flow cells and sequenced using the MinION Mk1B device with Kit 14 chemistry, operated via MinKNOW software version 23.07.8.

Raw signal data from Oxford Nanopore sequencing were basecalled using Dorado v0.8.3 with the super accuracy (sup) model dna_r10.4.1_e8.2_400bps_sup@v5.0.0 (5 kHz). Demultiplexing was carried out using the dorado demux command with the kit specified as --kit-name SQK-NBD114-24. Genome assembly was performed with Flye v2.9.4-b1799 using the --nano-hq option.

RESULTS

All of the powdered infant formula and ready to feed baby formula used in this study were purchased from standard drugstores. The three kinds of dried baby foods used, two first infant formula (for 0-3 months old), two follow-on formulas (for 6-12 months old), and two plant-based baby food (for toddlers) were first tested for *Cronobacter* contamination in the diagnostic laboratory in accordance with DIN regulations for baby food before use. All showed negative (1).

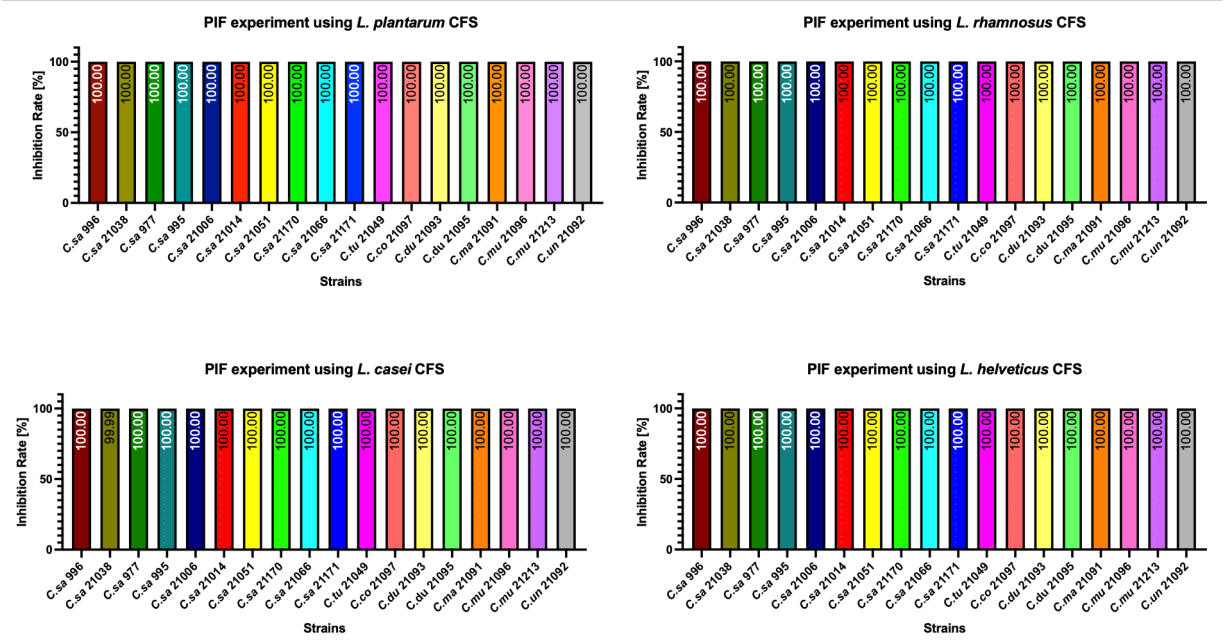
A 1:10 Dilution of *L. plantarum* Cell Free Supernatant is Enough to Inhibit *Cronobacter* Growth

L. plantarum CFS, sterile filtered only and autoclaved, were mixed with melted L-agar at a 1:5 and 1:10 dilution. The plates were then spotted with 3 µl of *Cronobacter* and then incubated at 37℃ for 24h. On the CFS mixed L-agar plates, *C. sakazakii* MHI 996, *C. sakazakii* MHI 21038, *C. sakazakii* MHI 977, *C. sakazakii* MHI 995, *C. sakazakii* MHI 21006, *C. sakazakii* MHI 21014, *C. sakazakii* MHI 21051, *C. sakazakii* MHI 21170, *C. sakazakii* MHI 21066, *C. sakazakii* MHI 21171, *C. condiment* MHI 21097, *C. dublinensis* MHI 21093, *C. dublinensis* MHI 21095, *C. malonaticus* MHI 21091, *C. muytjensii* MHI 21096, *C. muytjensii* MHI 21213, *C. turicensis* MHI 21049, *C. universalis* MHI 981, *C. sakazakii* MHI 975, *C. sakazakii* MHI 21011, *C. sakazakii* MHI 21039, *C. sakazakii* MHI 21086, *C. sakazakii* MHI 21003 *C. sakazakii* MHI 21004, *C. sakazakii* MHI 21029, *C. sakazakii* ES5 MHI 21042, *C. sakazakii* MHI 21122, *C. dublinensis* MHI 21094 and *C. turicensis* MHI 3032 (table 1) from overnight cultures showed no visible colonies for both plates with 1:5 and 1:10 dilution of sterile filtered only and autoclaved *L. plantarum* CFS in melted L agar after 24 h of incubation at 37℃. This shows that a dilution of 1:10 or a 10% concentration of *L. plantarum* CFS, either sterile filtered only or also autoclaved, mixed in L agar would be enough to inhibit *Cronobacter* growth for at least 24 h. This was the basis that the experiments after were constructed from.

L. plantarum, *L. rhamnosus*, *L. casei* and *L. helveticus* Cell Free Supernatant All Had ≥99% Growth Inhibition Rates Against Multiple *Cronobacter* Strains

L. plantarum, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS were diluted 1:5 in PIF solution which had been inoculated with approx. 1 cfu/10 ml of *Cronobacter*. After incubation for 24 h at 37℃, and samples drip plated onto CCI agar for cfu counting, all CFSs from the LAB strains showed a high growth inhibition rate against the *Cronobacter* strains tested (fig. 8A). While a lot of *Cronobacter* strains showed no growth at all or the cfu were so low it wasn't possible to determine when plated on CCI agar, *C. sakazakii* MHI 21006, *C. sakazakii* MHI 21014, *C. sakazakii* MHI 21051, *C. sakazakii* MHI 21170, *C. dublinensis* MHI 21095, *C. malonaticus* MHI 21091, *C. muytjensii* MHI 21096 and *C. muytjensii* MHI 21213 had approx. 10^3 cfu/ml concentration in the PIF solutions for PIF solutions treated with *L. plantarum*, *L. rhamnosus* and *L. helveticus* CFS (fig. 8B). While PIF solutions treated with *L. casei* CFS showed *C. sakazakii* MHI 996, *C. sakazakii* MHI 21038, *C. sakazakii* MHI 995, *C. sakazakii* MHI 21006, *C. sakazakii* MHI 21014, *C. sakazakii* MHI 21051, *C. sakazakii* MHI 21170, *C. sakazakii* MHI 21171, *C. dublinensis* MHI 21095, *C. malonaticus* MHI 21091, *C. muytjensii* MHI 21096 and *C. muytjensii* MHI 21213 growth. This showed PIF's ability to promote *Cronobacter* growth possibly by buffering acidic conditions, providing a carbon source for growth and/or the powder physically shielding them from antimicrobial agents.

A



n=3

B

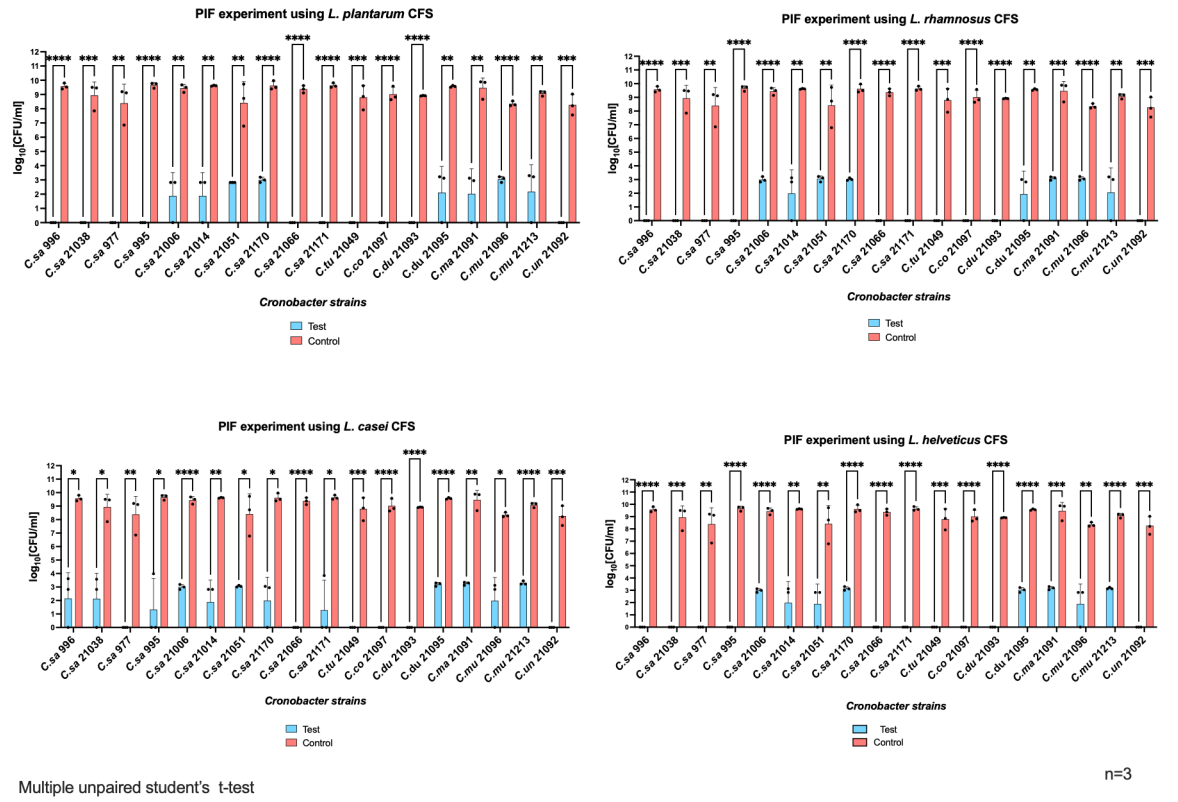


Fig. 8. PIF solution treated with *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFS inhibit *Cronobacter* growth for 24 h.
The inhibition rates shown in figure 8A were all ≥99% and thus have been rounded up in the graph. Figure 8A shows that the CFS from all lactic acid bacteria strains mostly inhibit *Cronobacter* growth in reconstituted PIF. Statistical analysis shown on figure 8B, however, show that only a small number of results were statistically significant.

CFU values in figure 8B were $\log_{10}$-transformed prior to statistical analysis to normalize data distribution and meet assumptions of homogeneity of variance.

Genus Confirmation by PCR

The isolated colonies from the CCI agar from the experiment above (fig. 8B) were all confirmed to be belong to the genus *Cronobacter* through PCR. All lanes, except for the negative control, on the agarose gel have a band between 1500-2000 bps strongly suggesting that the gene putatively encoding the α-glucosidase activity have been amplified (fig. 9). Thus, the CCI agar prepared and used in this study was further confirmed to be a reliable *Cronobacter* selection medium.

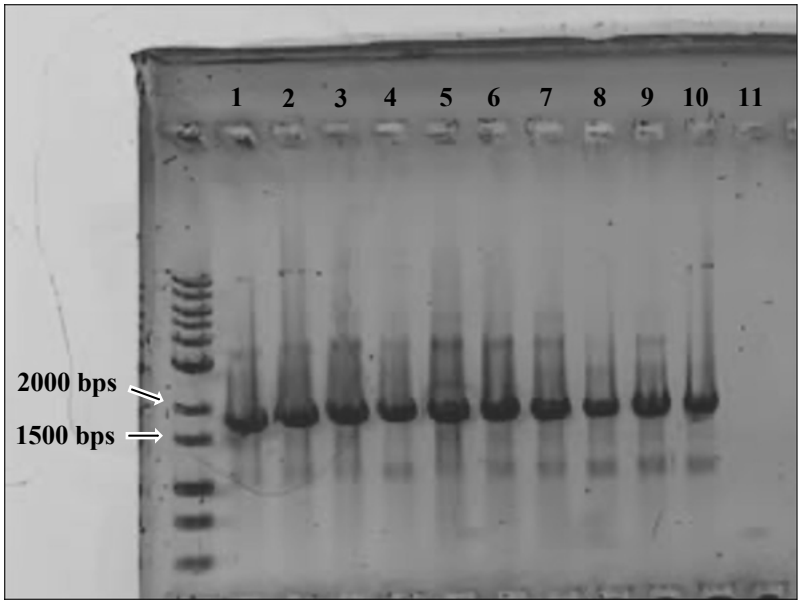
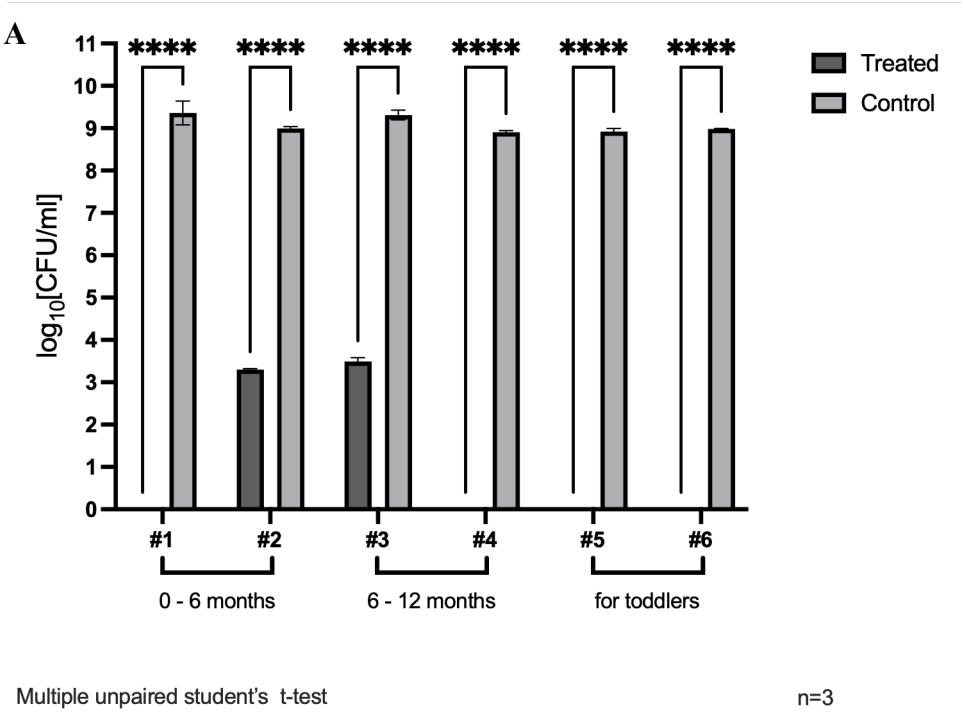


Fig. 9. ***Cronobacter*** **isolates from the prior PIF experiment had their genus confirmed through PCR and the use of CCI agar as a** ***Cronobacter*** **selection medium validated.**
The expected PCR product size is 1680 base pairs. The bands in the agarose gel confirms that all of the colonies taken from the CCI agar were from the genus *Cronobacter*.
The *Cronobacter* strains in each column are as follows:
Column 1: *C. sakazakii* 21006, column 2: *C. sakazakii* 21014, column 3: *C. sakazakii* 21051, column 4: *C. sakazakii* 21170, column 5: *C. sakazakii* 211711, column 6: *C. dublinensis* 21095, column 7: *C. malonaticus* 21091, column 8: *C. muytjensii* 21096, column 9: *C. muytjensii* 21213, column 10: positive control (*C. sakazakii* ES5 gDNA), and column 11: negative control (dH_2O).

Antimicrobial Effect of *L. plantarum* Supernatant Less Effective in Certain Dried Baby Food

C. sakazakii ES5 was inoculated in reconstituted dried baby foods for varying ages (initial formula, follow on formula and toddler milk) and from different manufacturers. The reconstituted dried baby foods were mixed with *L. plantarum* CFS with a *C. sakazakii* ES5 CFU of approx. 5 in 12 ml total volume. After 20 h of incubation at 37℃, results showed that for dried baby food #1, #4, #5 and #6, a 1:6 dilution or 16.7% concentration of *L. plantarum* CFS in solution was enough to inhibit *C. sakazakii* ES5. Dried baby food #2 and #3 showed *C. sakazakii* ES5 growth albeit at a much slower rate when compared to its control (fig. 10A). To look at one possible reason for such results, the acid buffering capacity of each reconstituted dried baby food was determined (fig. 10B). While it was determined that dried baby food #5 and #6 had the most acid buffering capacity, #2 and #3 were less acidic than the other reconstituted baby food and also had a good acid buffering

capacity. On the other hand dried baby food #4 was found to be the most acidic of the six tested baby foods as well as having the least buffering capacity. The type of dried baby food, initial formula, follow-on formula or plant based for toddler, appeared to have little or no effect against the antimicrobial effect of *L. plantarum* CFS against *C. sakazakii* ES5.



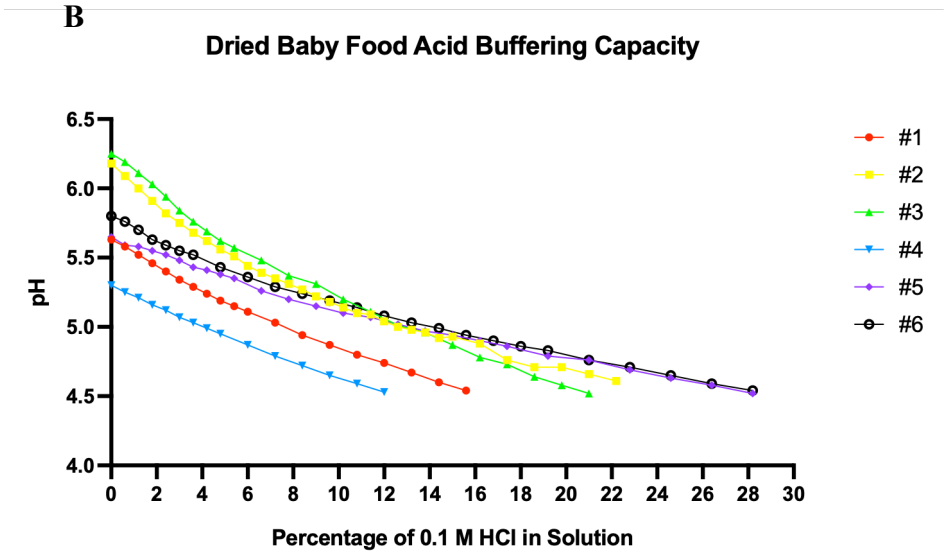


Fig. 10. Different types of dried baby were reconstituted and treated with LAB CFS, and then its acid buffering capacity tested.
Figure 10A shows that #2 and #3 allowed for *C. sa* ES5 growth while the others didn't. Figure 10B looks at one possible explanation for the results in figure 10A, showing baby food #2 and #3 to be less acidic and have good buffering capacity.

CFU values in figure 10A were $\log_{10}$-transformed prior to statistical analysis to normalize data distribution and meet assumptions of homogeneity of variance.

L. plantarum Cell Free Supernatant Decreases Cell Invasion Rate

In the gentamicin protection assay, it was found that *L. plantarum* CFS from an overnight culture, both with and without a 2 h preincubation in wells containing CaCo-2 cells, and *L. plantarum* bacterial cells did not decrease *C. sakazakii* ES5 adhesion rate on the epithelial cells. In fact, the *L. plantarum* CFS appeared to have increased *C. sakazakii* ES5 adhesion rate slightly (fig. 11A). For all three conditions, that *L. plantarum* CFS with and without a 2 h preincubation and using live cells, the invasion rate of *C. sakazakii* ES5 were decreased. *L. plantarum* CFS with a 2 h preincubation proved the most effective at decreasing *C. sakazakii* ES5 invasion rate, bringing it down to less than 1% (fig. 11A).

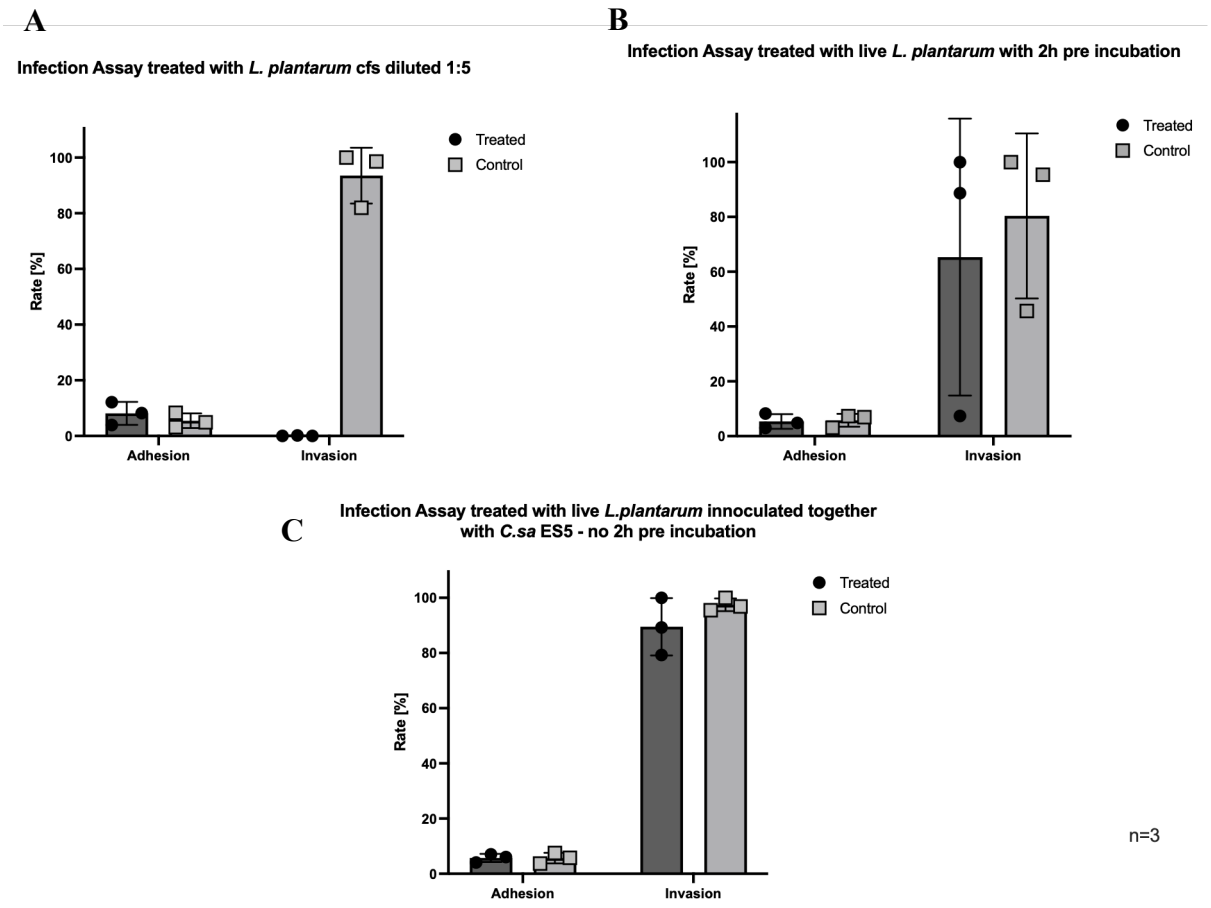


Fig. 11. Gentamicin protection assay was used to assess the protective capabilities of *L. plantarum* and its CFS against adhesion and invasion of *C. sa* ES5 into intestinal epithelial cells.

L. plantarum had little effect at preventing *C. sa* ES5 adhesion to the Caco-2 cells. However, it has a noticeable effect of decreasing the capability of *C. sa* ES5 at invading the epithelial cells. The conditions in figure 11B and 11C show a small decrease in invasion rate of *C. sa* ES5, figure 11A shows the capability of *L. plantarum* CFS to greatly prevent *C. sa* ES5 invasion if given enough time to preincubate first.

L. plantarum Cells Had No Visible Effect on *C. sakazakii* ES5 Growth

C. sakazakii ES5 and *L. plantarum* were co-incubated in RPMI media for 2.5 h, and samples taken and plated every 30 min. Results show that the *L. plantarum* cells showed no significant antimicrobial activity against *C. sakazakii* ES5. The rate of growth of *C. sakazakii* ES5 did not decrease despite the presence of *L. plantarum* in the RPMI media it was incubated in. Its growth rate remained similar to *C. sakazakii* ES5 grown without the presence of *L. plantarum* within the 2.5 hour incubation period (fig. 12). The *L. plantarum* cells grown aerobically in RPMI media (in red) grew poorly as expected likely due to the lack of components essential for optimal lactic acid bacterial metabolism. Its inclusion was to provide verification of its capability to survive in RPMI media within the 2.5 h incubation and that it did grow together with *C. sakazakii* ES5 in the mixed culture.

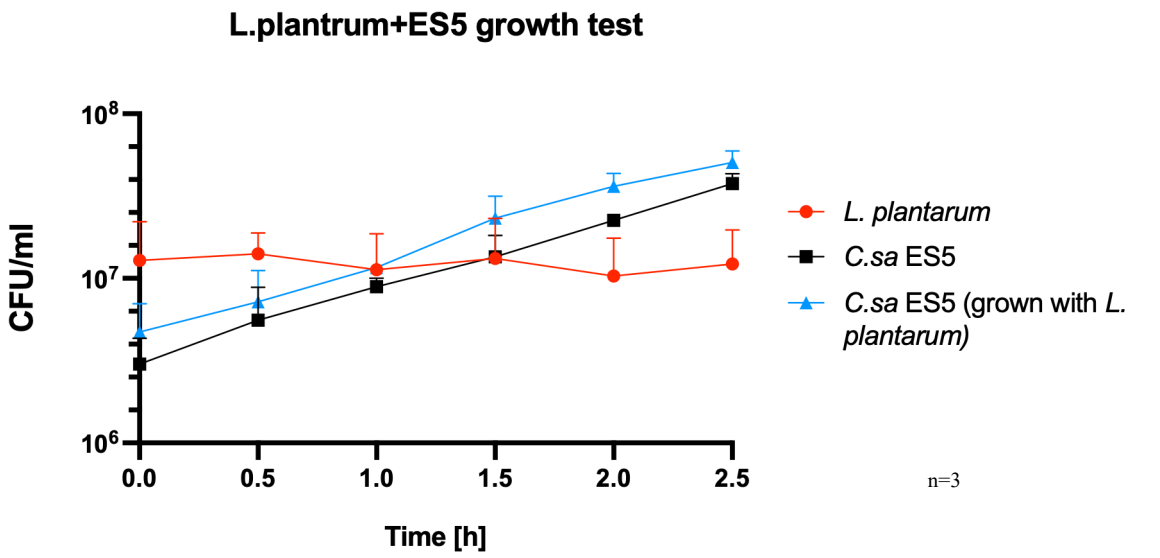


Fig. 12. *C. sa* ES5 and *L. plantarum* cells were grown together in RPMI for 2.5 h.

L. plantarum cells proved to be neither bacteriostatic nor bactericidal against *C. sa* ES5 growth. The rate of growth between the *C. sa* ES5 with *L. plantarum* and the *C. sa* ES5 grown on its own remained similar throughout the 2.5 h incubation period.

Determining the Minimum Inhibitory Concentration and the Minimum Bactericidal Concentration

The MIC against *C. sakazakii* ES5 for all strains of LAB used in this assay was determined to be approx. 10 mg/ml of lyophilised CFS dissolved in Mueller-Hinton broth. The results determined by simply looking at the 96-well plate for visible growth and the results taken from a microplate reader at OD_{600} were the same. The MBC was determined to also be approx. 10 mg/ml for all LAB strains (table 7).

Table 7: The MIC and MBC of the CFS for all LAB strains against *C. sa* ES5.

CFS	**Autoclaved**			**Sterile filtered**		
	MIC (eye)	**MIC (reader)**	**MBC**	**MIC (eye)**	**MIC (reader)**	**MBC**
	[mg/ml]			**[mg/ml]**		
L. plantarum	10	10	12.5 - 15	10	10	12.5 - 15
L rhamnosus	10	10	12.5 - 15	10	10	12.5 - 15
L. casei	10	10	12.5 - 15	10	10	12.5 - 15
L. helveticus	10	10	12.5 - 15	10	10	12.5 - 15

Cell-Free Supernatants of *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* Exhibited Reduced Antimicrobial Activity at Higher pH

In a 96-well plate, *C. sakazakii* ES5 was grown in L-broth with 25 mg/ml of *L. plantarum*, *L. rhamnosus*, *L. casei*, or *L. helveticus* CFS, and the pH of the culture changed to 3, 5, 7 and 9. A culture with no pH change was used as control. The pH of the control cultures were determined as: pH 4.27 for *L. plantarum* CFS, pH 4.23 for *L. rhamnosus* CFS, pH 4.24 for *L. casei* CFS and pH 4.23 for or *L. helveticus* CFS. After 18 hours, the control and cultures with adjusted pH to 3 and 5 had no visible *C. sakazakii* ES5 growth when measured both by eye and by a microplate reader at OD_{600}. Cultures with adjusted pH to 7 and 9 showed visible *C. sakazakii* ES5 growth despite the higher concentration of CFS in solution.

Table 8: Summary table of how pH affected the antimicrobial activity of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS

CFS	Control	pH 3	pH 5	pH 7	pH 9
L. plantarum	X	X	X	✓	✓
L rhamnosus	X	X	X	✓	✓
L. casei	X	X	X	✓	✓
L. helveticus	X	X	X	✓	✓

A similar experiment, to examine the effects of pH on the antimicrobial activity of the *L. plantarum*, *L. rhamnosus*, *L. casei*, or *L. helveticus* CFS, was done on solid media, melted L-agar mixed with sterile-filtered CFS from a 48 h culture. The pH of the CFS from the 48 h cultures were determined to be: pH 3.74 for *L. plantarum* CFS, pH 4.0 for *L. rhamnosus* CFS, pH 3.69 for *L. casei* CFS and pH 3.72 for *L. helveticus* CFS. The pH of all the agar plates were measured using pH strips both at t=0 and t=18. At t=0 the pH of the agar plates were confirmed as: pH 3-4 for the control plates, pH 3 for the pH 3-adjusted plates, pH 5 for the pH 5-adjusted plates, pH 7 for the pH 7-adjusted plates, and pH 8-9 for the pH 9-adjusted plates. At t=18, the pH of the plates were measured again: the control plates had pH 3-4, the pH 3-adjusted plates had a pH of 3, the pH 5-adjusted plates had a pH of 6-7, the pH 7-adjusted had a pH of 8, and the pH 9-adjusted plates had a pH of 8-9.

There was no visible *C. sakazakii* ES5 growth in both the control and pH 3 adjusted agar plates. While the plates which were adjusted to pH 5, 7 and 9 all had *C. sakazakii* ES5 growth (fig. 13).

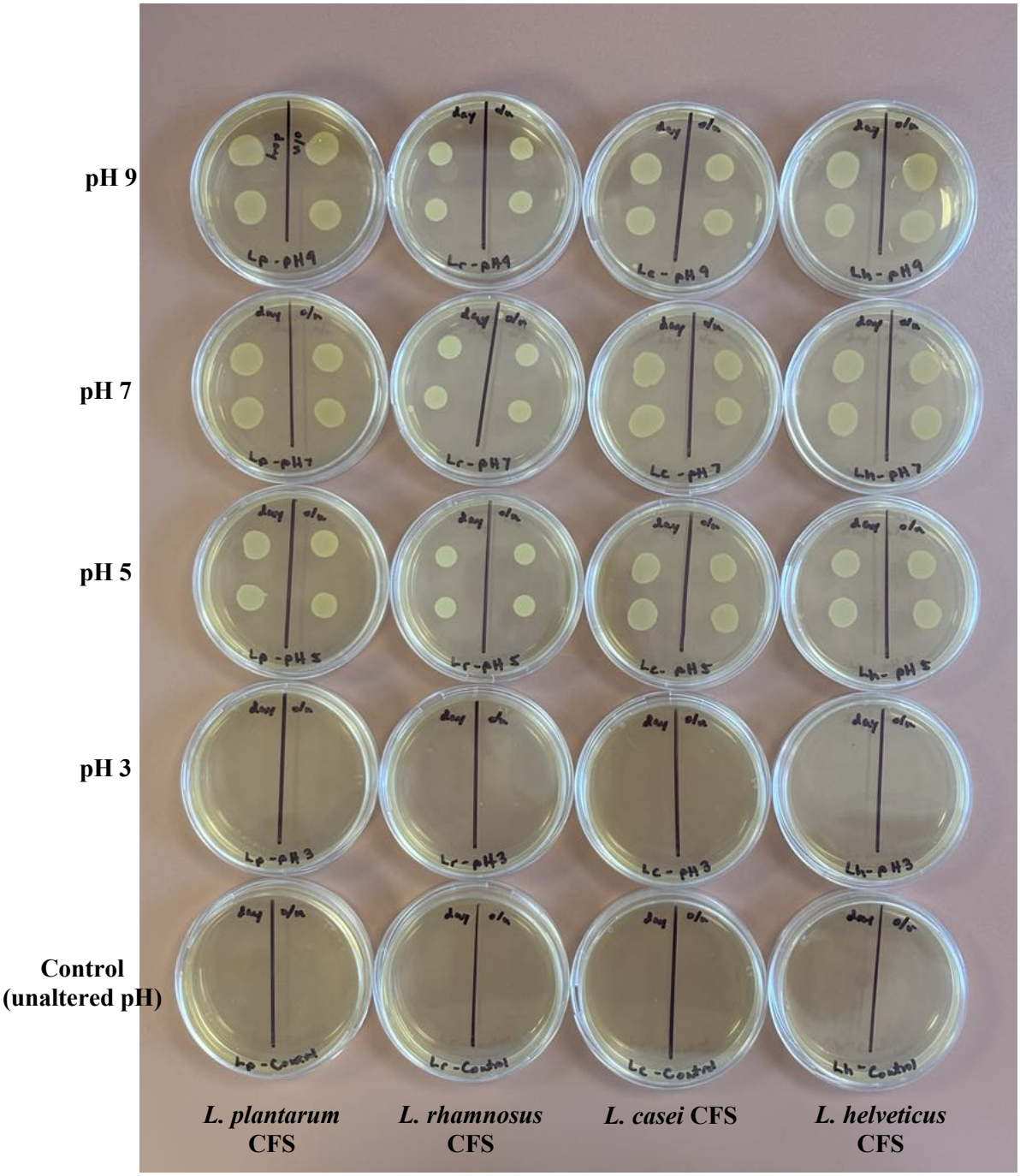


Fig. 13. The melted L agar mixed with CFS agar plates were spotted with 5 µl from both overnight and exponential phase cultures of *C. sa* ES5. On the left side of the line drawn on the agar plates, *C. sa* ES5 were spotted, while on the right side overnight cultures of *C. sa* ES5 were spotted. Both overnight and exponential phase cultures of *C. sa* ES5 did not show visible growth on all control and pH-adjusted plates. All the plates with higher pH, however, all showed visible *C. sa* ES5 growth.

Protease Treatment Did Not Affect Antimicrobial Activity of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS Against *C. sakazakii* ES5

L. plantarum, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS, the CFSs were treated with either trypsin or Proteinase K and then examined for any changes in antimicrobial activity against *C. sakazakii* ES5. After 24 h of incubation at 37℃, the absorbance readings in the wells that contained 25 mg/ml of CFS showed no visible or very little increase despite protease treatment of the CFS (fig. 14). The concentrations of trypsin and Proteinase K used appeared to have no significant effect on all the CFS' capability to inhibit *C. sakazakii* ES5 growth.

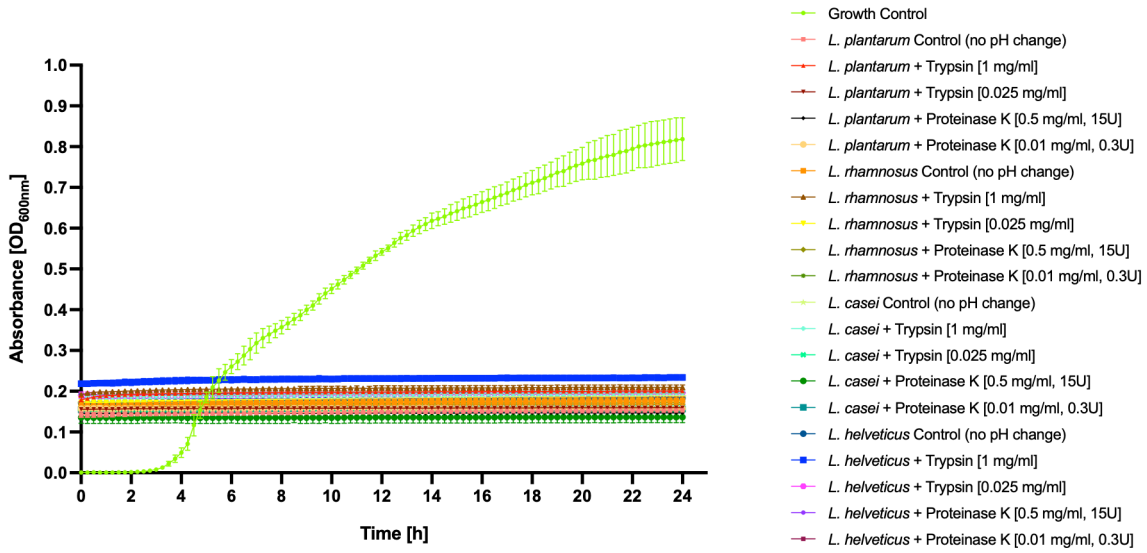


Fig. 14. *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFSs were treated with either trypsin or proteinase K, and then checked for changes antimicrobial activity against *C. sa* ES5.

All cultures treated with CFS inhibited *C. sa* ES5 growth despite protease treatment. Only the growth control showed *C. sa* ES5 growth.

A Concentration of 10 mg/ml of Cell Free Supernatant is Enough to Inhibit *C. sakazakii* ES5 Growth in L-Broth

A *C. sakazakii* ES5 growth test in L broth was conducted using varying CFS concentrations to determine the minimum amount of CFS needed for use in the following in vitro simulated gastrointestinal conditions experiment. In this experiment, it was confirmed that the MIC (10 mg/ml) was enough to inhibit *C. sakazakii* ES5 growth for 24 h at 37℃ (fig. 15).

It was also determined how much CFS additions to PIF reconstituted in 0.5% (w/v) NaCl affected the pH of the solution. The 0.5% (w/v) NaCl was used to imitate a younger baby's stomach pH, and a pH 3 adjusted 0.5% (w/v) NaCl was used to imitate the stomach pH of an older baby. All of the concentrations added, 10, 15, 20 and 25 mg/ml, all did not decrease the pH of solution below pH 4 (table 9 A and B). The decrease in pH were all in acceptable ranges considering that the pH normally ranged from 3.55 to 5.24 in commercial baby foods (4).

It was decided that a concentration of 10 mg/ml was to be used to treat reconstituted dried baby food with for the following simulated gastrointestinal conditions experiment.

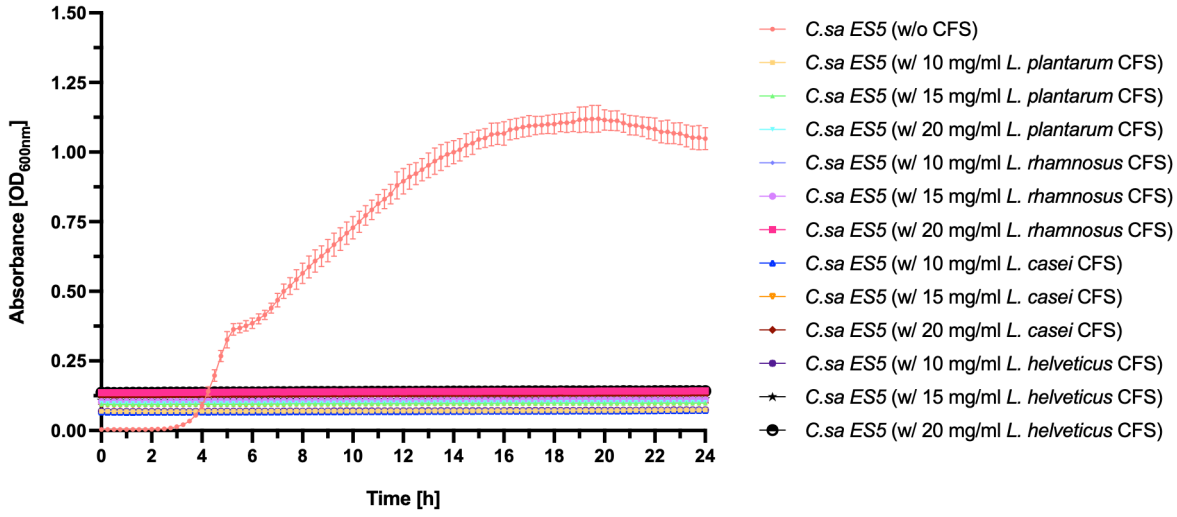


Fig. 15. A *C. sa* ES5 growth test using different concentrations of CFS from all four LAB strains.

The growth curve of *C. sa* ES5 over 24 hours. Except for the control (w/o CFS), all cultures of *C. sa* ES5 treated with differing concentrations (from 10 mg/ml to 20 mg/ml) of CFS had their growth inhibited.

Table 9 A and B: A summary table of the pH changes in PIF + 0.5% (w/v) NaCl solution when CFS is added.

A

PIF + 0.5% NaCl					
	0 mg/ml	**10 mg/ml**	**15 mg/ml**	**20 mg/ml**	**25 mg/ml**
Control	6.03				
L. plantarum		4.95	4.51	4.31	4.11
L. rhamnosus		4.88	4.57	4.37	4.20
L. casei		4.88	4.54	4.36	4.25
L. helveticus		4.93	4.54	4.35	4.20

B

PIF + 0.5% NaCl (pH 3.5)					
	0 mg/ml	**10 mg/ml**	**15 mg/ml**	**20 mg/ml**	**25 mg/ml**
Control	5.88				
L. plantarum		4.74	4.41	4.26	4.18
L. rhamnosus		4.70	4.40	4.25	4.14
L. casei		4.70	4.62	4.36	4.23
L. helveticus		4.83	4.48	4.28	4.20

L. plantarum, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS Effective as Prophylactic Additions to Baby Food

Ready-to-feed infant formula was treated with *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFSs (final concentration 10 mg/ml) and subsequently inoculated with approx. 5 CFU/10 mL of *Cronobacter sakazakii* ES5. The mixture was then subjected to simulated gastrointestinal conditions to assess whether the antimicrobial activity of the LAB CFS was retained. Figure 16 show that *C. sakazakii* ES5 growth could not be detected throughout the entire simulated gastric and enteric phase conditions time-frame (6 hours) at 37℃ in ready-to-feed infant formula solutions treated with *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFSs. Samples taken at t=24, however, show *C. sakazakii* ES5

growth for all samples despite CFS treatment. At t=24, the *L. plantarum* CFS treated baby formula had a *C. sakazakii* ES5 CFU/ml of 1.20×10^9, 1.1×10^9 CFU/ml for the *L. rhamnosus* CFS treated, 1.23×10^9 CFU/ml for the *L. casei* CFS treated, 1.21×10^9 CFU/ml for the *L. helveticus* CFS treated, and 1.55×10^9 CFU/ml for the Control. Antimicrobial effect of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS against *C. sakazakii* ES5 appeared to have decreased between t=6 and t=24.

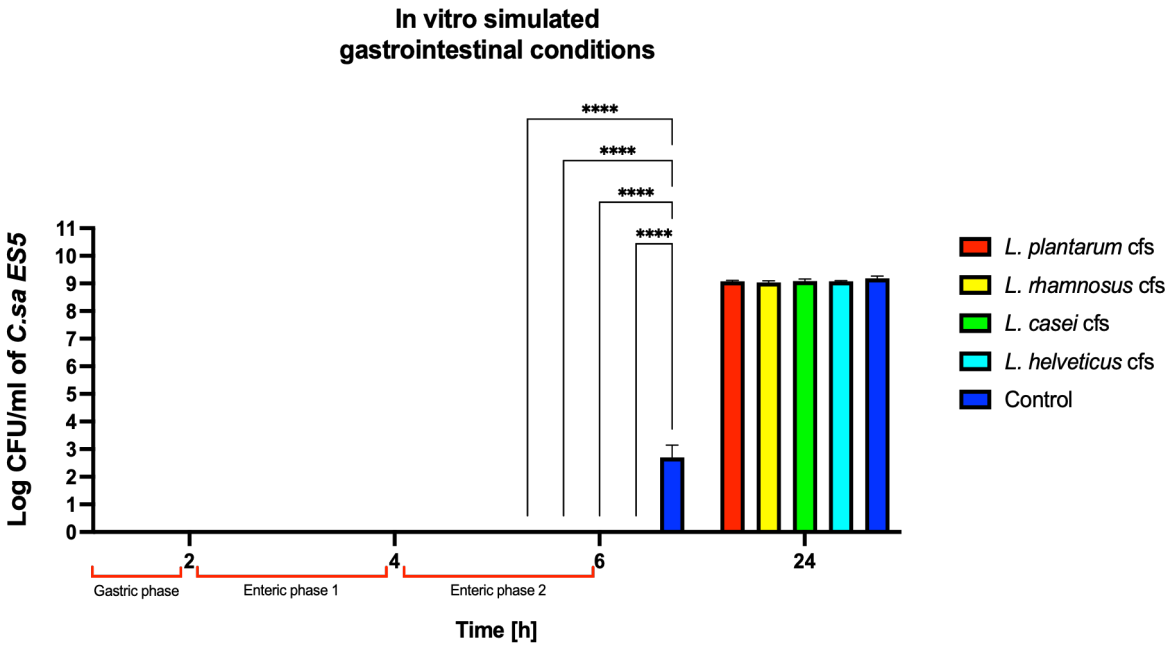


Fig. 16. Ready to feed baby formula was treated with LAB CFS was inoculated with approx. 5 cfu/10 ml of *C. sa* ES5. It was then tested if the antimicrobial activity of the LAB CFS could be retained under simulated gastrointestinal conditions.

L. plantarum, L. rhamnosus, L. casei, and *L. helveticus* CFS inhibited *C. sa* ES5 growth for a minimum 6 hours at 37℃, but effectivity seemed to have dropped between t=6 and t=24.

CFU values were $\log_{10}$-transformed prior to statistical analysis (2way ANOVA) to normalize data distribution and meet assumptions of homogeneity of variance. Only results at t=6 were found statistically significant.

The antimicrobial effects of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS (10 mg/ml) against *C. sakazakii* ES5 were also tested on sterile ready to feed baby formula at different storage temperatures: at 5℃, at room temperature (22℃) and at 37℃. *C. sakazakii* ES5 CFU counts for all samples, including the control, could not be determined for storage at 5℃ and room temperature due to the absence of distinguishable colonies on the CCI agar plate even after 24 hours. For storage at 37℃, CFU counts could also not be determined for t=2 and t=4. At t=6, the CFU/ml of *C. sakazakii* ES5 for the control was

66.67/ml, and the *C. sakazakii* ES5 CFU counts could not be determined for the baby formula treated with CFS. At t=24, the *C. sakazakii* ES5 CFU/ml was determined to be 7.03×10^8, 3.8×10^5 for the *L. rhamnosus* CFS treated baby formula, 1.06×10^7 for the *L. casei* CFS treated baby formula, and the CFU counts for the baby formula treated with *L. plantarum* and *L. helveticus* CFS could not be determined due to the absence of distinguishable colonies on the CCI agar plate (fig. 17).

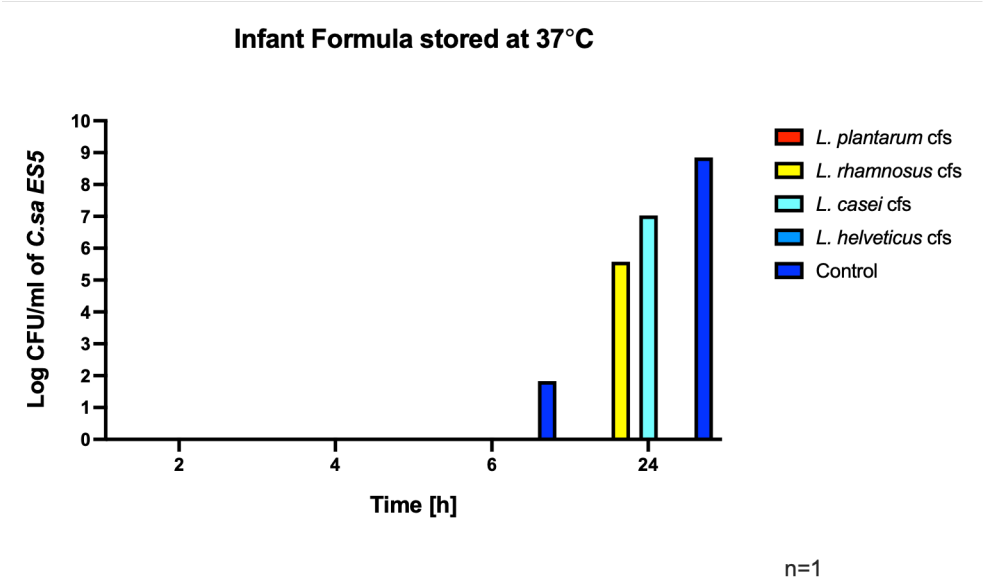


Fig. 17. The antimicrobial activity of *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* CFS were tested against *C. sa* ES5 inoculated in ready to feed formula stored at 37°C.

C. sa ES5 CFU/ml could not be determined at t=2 and t=4. Antimicrobial effect of CFS from *L. plantarum, L. rhamnosus, L. casei,* and *L. helveticus* could be seen at t=6 and t=24, by inhibiting/slowing *C. sa* ES5 growth.

CFU values were $\log_{10}$-transformed.

Biofilm Morphology Varied Across Different *Cronobacter* Strains

Food isolated *Cronobacter* strains (highlighted in table 1) *C. sakazakii* MHI 996, *C. sakazakii* MHI 21038, *C. sakazakii* MHI 977, *C. sakazakii* MHI 995, *C. sakazakii* MHI 21006, *C. sakazakii* MHI 21014, *C. sakazakii* MHI 21051, *C. sakazakii* MHI 21170, *C. sakazakii* MHI 21066, *C. sakazakii* MHI 21171, *C. condimenti* MHI 21097, *C. dublinensis* MHI 21093, *C. dublinensis* MHI 21095, *C. malonaticus* MHI 21091, *C. muytjensii* MHI 21096, *C. muytjensii* MHI 21213, *C. turicensis* MHI 21049 and *C. universalis* MHI 981 were streaked and grown on both overexpression and milk agar plates and then incubated for 2 weeks. Biofilm colours on the plates were either amber, yellow or ivory and textures

ranging from rough, slimy or smooth. The biofilm colours and textures changed with time as detailed in table 10. Biofilm colours in the milk agar only varied between yellow and ivory, textures varied between rough, slimy or smooth, and after 2 weeks of incubation at room temperature, some strains had bubbles visible on the biofilms (table 10). Figures 18A and 18B shows the data results as percentages in a pie chart.

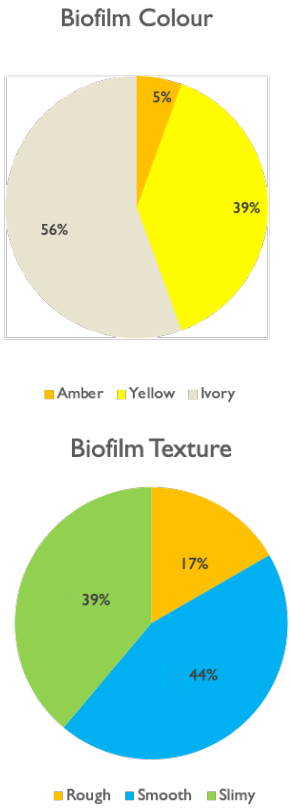
Table 10: Changes in *Cronobacter* spp. biofilm colour and texture with time. The overexpression agar plates were incubated at 37℃ for two overnights and then incubated at room temperature for 12 more days. The milk agar plates were incubated at 37℃ for one overnight and then incubated at room temperature for 4 more days.

Overexpression Agar	**Colour**			**Texture**		
Strain	**1 o/n**	**2 o/n**	**2 weeks**	**1 o/n**	**2 o/n**	**2 weeks**
***C. sa* 996**	Yellow	Yellow	Yellow	Smooth	Slimy	Slimy
***C. sa* 21038**	Ivory	Ivory	Ivory	Smooth	Smooth	Smooth
***C. sa* 977**	Ivory	Ivory	Yellow	Rough	Smooth	Smooth
***C. sa* 995**	Ivory	Yellow	Yellow	Smooth	Slimy	Slimy
***C. sa* 21006**	Ivory	Yellow	Yellow	Slimy	Slimy	Slimy
***C. sa* 21014**	Ivory	Yellow	Yellow	Smooth	Slimy	Slimy
***C. sa* 21051**	Ivory	Ivory	Ivory	Smooth	Smooth	Smooth
***C. sa* 21170**	Ivory	Ivory	Ivory	Smooth	Smooth	Smooth
***C. sa* 21066**	Yellow	Yellow	Yellow	Rough	Rough	Rough
***C. sa* 21171**	Yellow	Yellow	Yellow	Rough	Smooth	Slimy
***C. tu* 21049**	Ivory	Ivory	Ivory	Smooth	Smooth	Smooth
***C. co* 21097**	Amber	Amber	Amber	Slimy	Slimy	Slimy
***C. du* 21093**	Yellow	Yellow	Yellow	Slimy	Slimy	Slimy
***C. du* 21095**	Ivory	Yellow	Yellow	Slimy	Slimy	Slimy
***C. ma* 21091**	Ivory	Yellow	Yellow	Slimy	Slimy	Slimy
***C. mu* 21096**	Yellow	Yellow	Yellow	Slimy	Slimy	Slimy
***C. mu* 21213**	Yellow	Yellow	Yellow	Slimy	Slimy	Slimy
***C. un* 981**	Yellow	Yellow	Yellow	Slimy	Slimy	Slimy

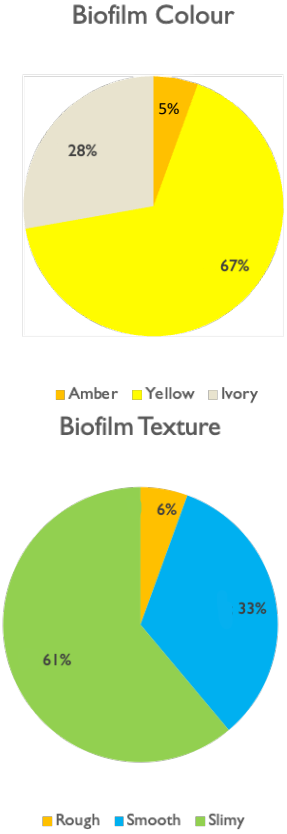
Milk Agar	**Colour**		**Texture**		**Bubbles present**	
Strain	**1 o/n**	**1 week**	**1 o/n**	**1 week**	**1 o/n**	**1 week**
***C. sa* 996**	Yellow	Yellow	Slimy	Slimy	No	Yes
***C. sa* 21038**	Ivory	Ivory	Smooth	Smooth	No	No
***C. sa* 977**	Ivory	Ivory	Slimy	Slimy	No	No
***C. sa* 995**	Yellow	Yellow	Slimy	Slimy	No	No
***C. sa* 21006**	Yellow	Yellow	Slimy	Slimy	No	Yes
***C. sa* 21014**	Ivory	Ivory	Slimy	Slimy	No	Yes
***C. sa* 21051**	Ivory	Ivory	Rough	Rough	No	No
***C. sa* 21170**	Ivory	Ivory	Rough	Rough	No	No
***C. sa* 21066**	Ivory	Ivory	Rough	Slimy	No	No
***C. sa* 21171**	Ivory	Ivory	Slimy	Slimy	No	Yes
***C. tu* 21049**	Yellow	Yellow	Smooth	Smooth	No	No
***C. co* 21097**	Yellow	Yellow	Slimy	Slimy	No	Yes

A

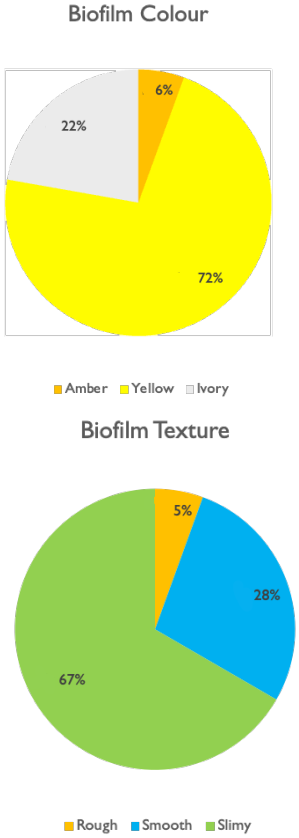
Overexpression agar
1 overnight
37°C



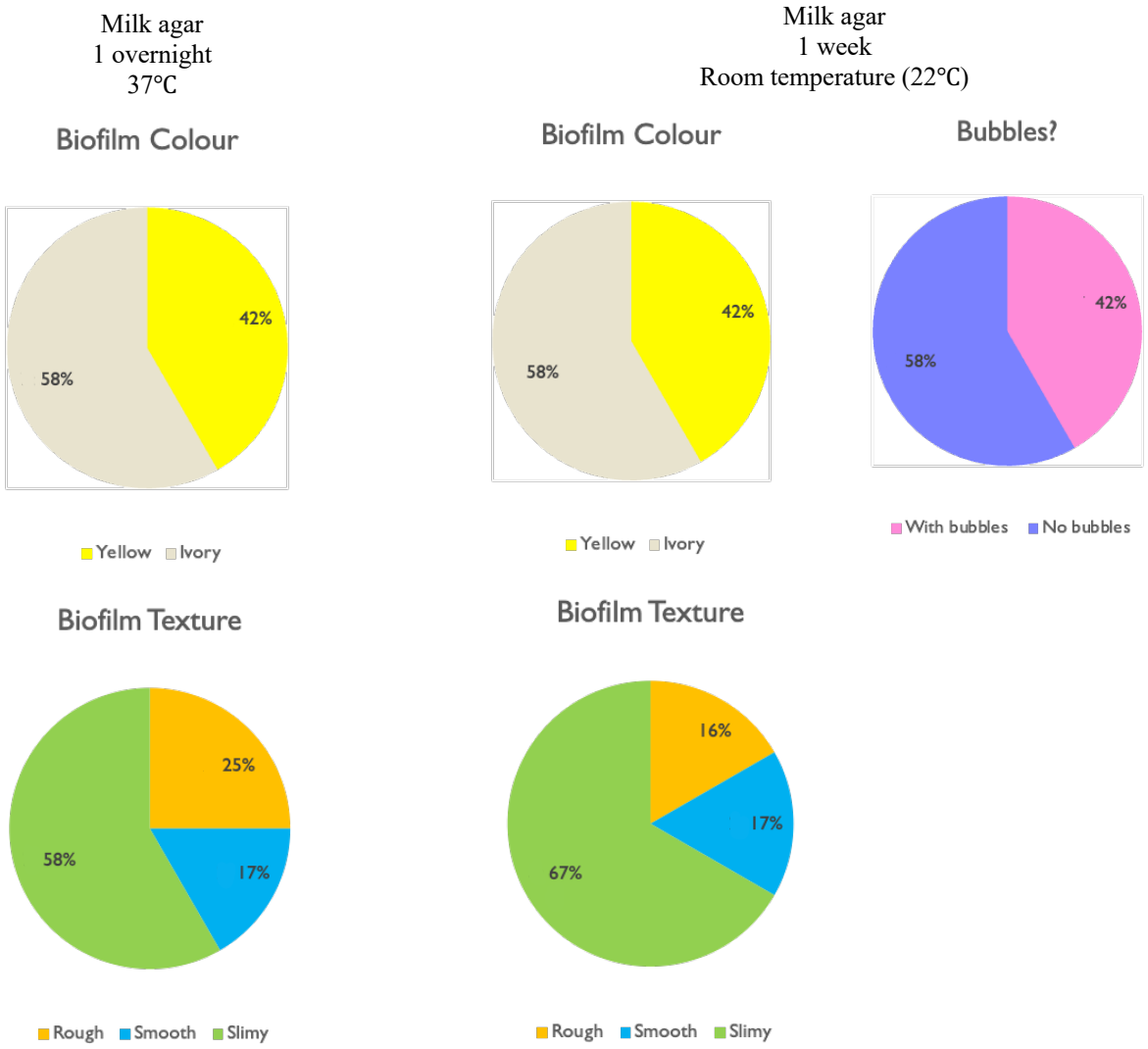
Overexpression agar
2 overnights
37°C



Overexpression agar
2 weeks
Room temperature (22°C)



B



C

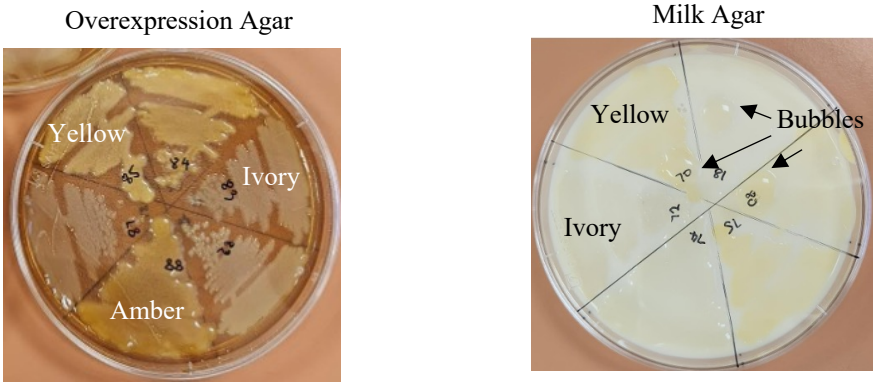
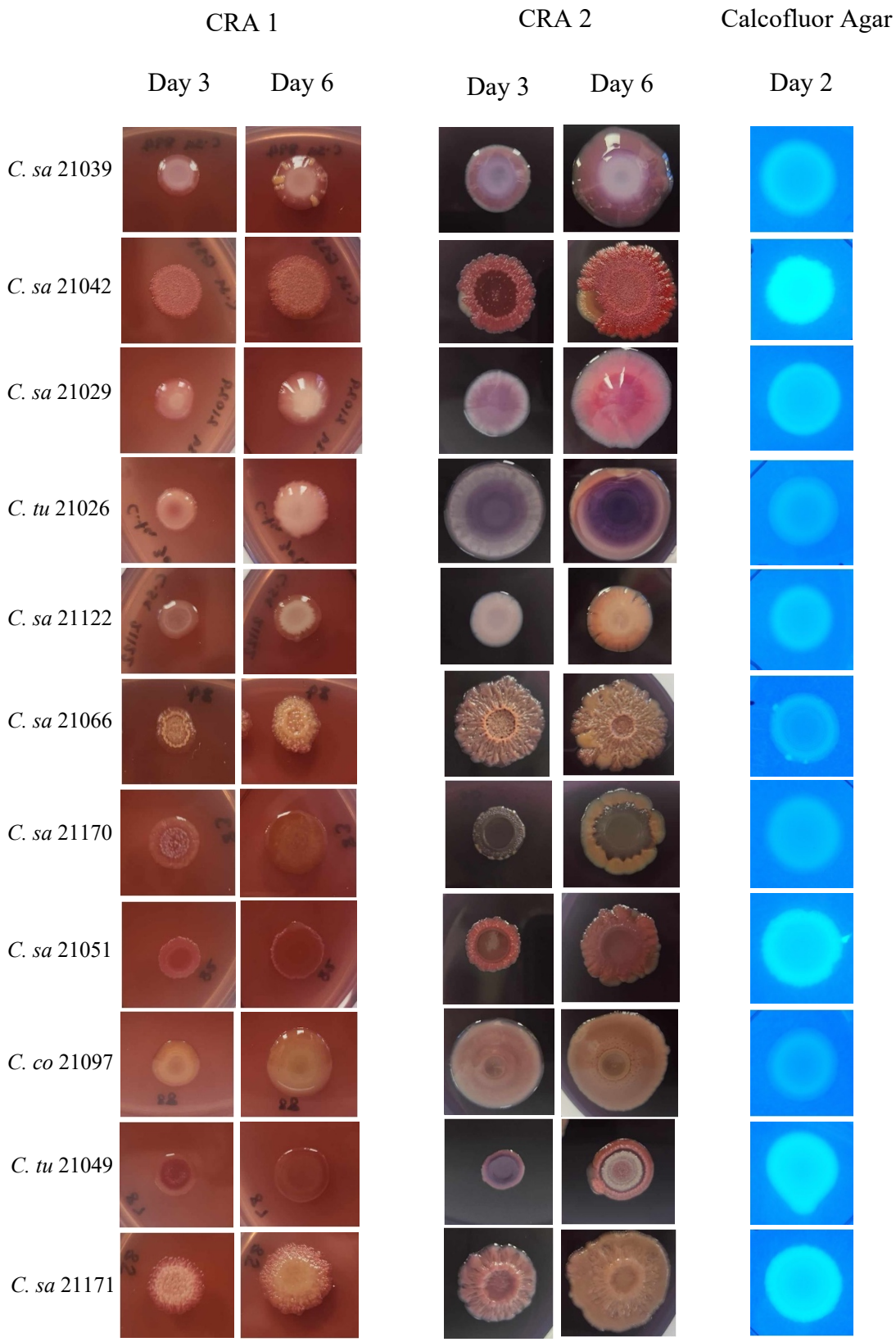


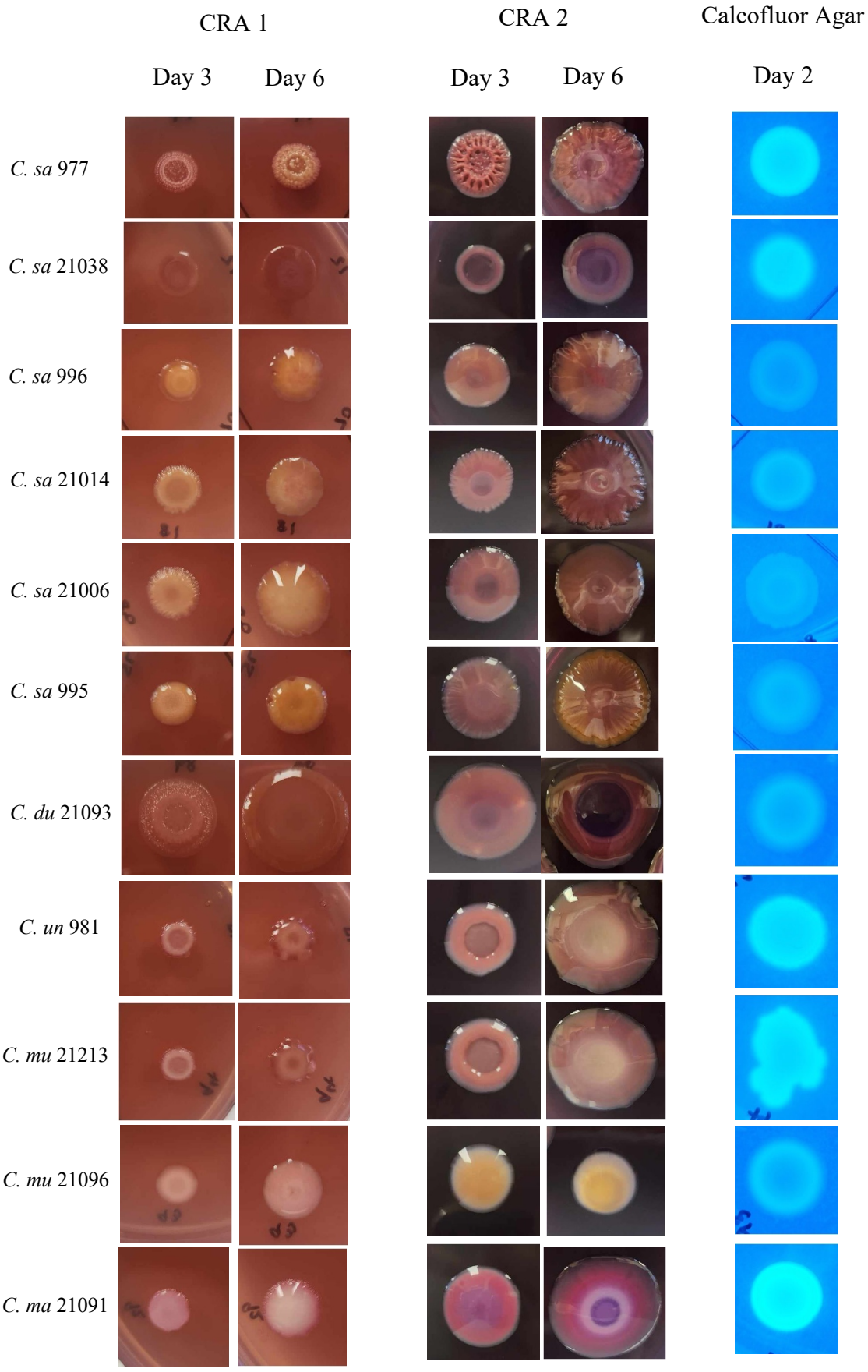
Fig. 18. *Cronobacter* strains were streaked onto overexpression and milk agar plates to analyse biofilm morphology.
In the overexpression agar, biofilm colour and texture skewed towards yellow and slimy with time. In the milk agar, biofilm colour percentage did not change between day 1 and day 7. Biofilm texture, however, became more slimy for certain strains and bubbles became to form on some biofilms. Figure 18C shows the different colours of biofilms and the bubbles present on biofilms on the milk agar. Bubbles indicate trapped gases beneath the biofilms due to lactose fermentation by *Cronobacter*.

Cronobacter strains *C. sakazakii* MHI 996, *C. sakazakii* MHI 21038, *C. sakazakii* MHI 977, *C. sakazakii* MHI 995, *C. sakazakii* MHI 21006, *C. sakazakii* MHI 21014, *C. sakazakii* MHI 21051, *C. sakazakii* MHI 21170, *C. sakazakii* MHI 21066, *C. sakazakii* MHI 21171, *C. condimenti* MHI 21097, *C. dublinensis* MHI 21093, *C. dublinensis* MHI 21095, *C. malonaticus* MHI 21091, *C. muytjensii* MHI 21096, *C. muytjensii* MHI 21213, *C. turicensis* MHI 21049, *C. universalis* MHI 981, *C. sakazakii* MHI 21039, *C. sakazakii* ES5, *C. sakazakii* MHI 21029, *C. sakazakii* MHI 21122 and *C. turicensis* MHI 21026 were spotted onto Congo Red Agar plates (CRA) 1 and 2, as well as on Calcofluor agar plates to detect production of cellulose, the aggregative fimbriae Curli, and poly-*N*-acetylglucosamine (PNAG). Evaluation of the plates were done according to Römling et al. (5,6) (table 11).

Table 11: Biofilm morphology interpretation.

CRA 1		**CRA 2**		**Calcofluor agar**	
Biofilm Morphology	**Components Present**	**Biofilm Morphology**	**Components Present**	**Biofilm Morphology**	**Components Present**
Red	PNAG and curli production	Red, dry, and rough (rdar)	Curli and cellulose production	Fluorescent	Cellulose production
		Pink, dry, and rough (pdar)	Cellulose production only		
		Brown, dry, and rough (bdar)	Curli production only		
White	No PNAG, no curli production	Smooth and white (saw)	Neither curli nor cellulose production	Not Fluorescent	No cellulose production
		Red and smooth (ras)	Cellulose production only		
		Pink and smooth (pas)	Cellulose production only		


CRA 1
CRA 2
Calcofluor Agar
Day 3
Day 6
Day 3
Day 6
Day 2
C. sa 21039
C. sa 21042
C. sa 21029
C. tu 21026
C. sa 21122
C. sa 21066
C. sa 21170
C. sa 21051
C. co 21097
C. tu 21049
C. sa 21171


CRA 1
CRA 2
Calcofluor Agar
Day 3
Day 6
Day 3
Day 6
Day 2
C. sa 977
C. sa 21038
C. sa 996
C. sa 21014
C. sa 21006
C. sa 995
C. du 21093
C. un 981
C. mu 21213
C. mu 21096
C. ma 21091

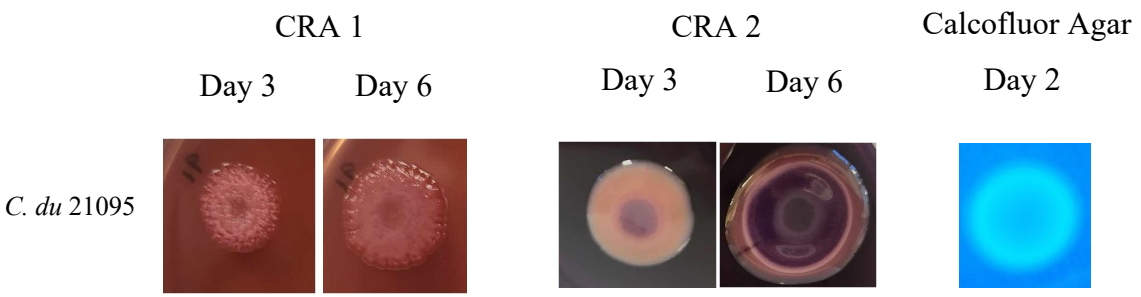


Fig. 19. Images of the different *Cronobacter* strains were spotted on CRA 1 and 2 plates as well as on Calcofluor plates and then incubated at room temperature. Pictures were taken at different days and on different agars. The images were all taken at the same distance and then laid out for comparison. The calcofluor agar plates were viewed and the images taken under uv light to detect for cellulose production.

Protein Concentration in the Cell Free Supernatants

A BCA assay kit was used to determine the protein concentration of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFSs from 20 h, 24 h and 48 h cultures. Results for this assay are to be viewed with caution as the *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* CFS used were derived from bacterial cultures grown in MRS broth, which also contained proteins. These proteins were also measured. According to data interpolated from a BSA standard prepared beforehand and multiplied by 32 as the CFS were diluted 1:32 before measurement, a 20 h culture of *L. plantarum* CFS had approx. a protein concentration of 5.11 mg/ml, *L. rhamnosus* CFS had 4.94 mg/ml, *L. casei* CFS had 4.85 mg/ml, and the *L. helveticus* CFS had 5.06 mg/ml. From the 24 h culture, *L. plantarum* CFS had approx. a protein concentration of 5.46 mg/ml, *L. rhamnosus* CFS had 4.95 mg/ml, *L. casei* CFS had 5.59 mg/ml, and *L. helveticus* CFS had 5.74 mg/ml. From the 48 h culture, *L. plantarum* CFS had approx. a protein concentration of 6.06 mg/ml, *L. rhamnosus* CFS had 5.15 mg/ml, , *L. casei* CFS had 5.49 mg/ml, and *L. helveticus* CFS had 5.72 mg/ml.

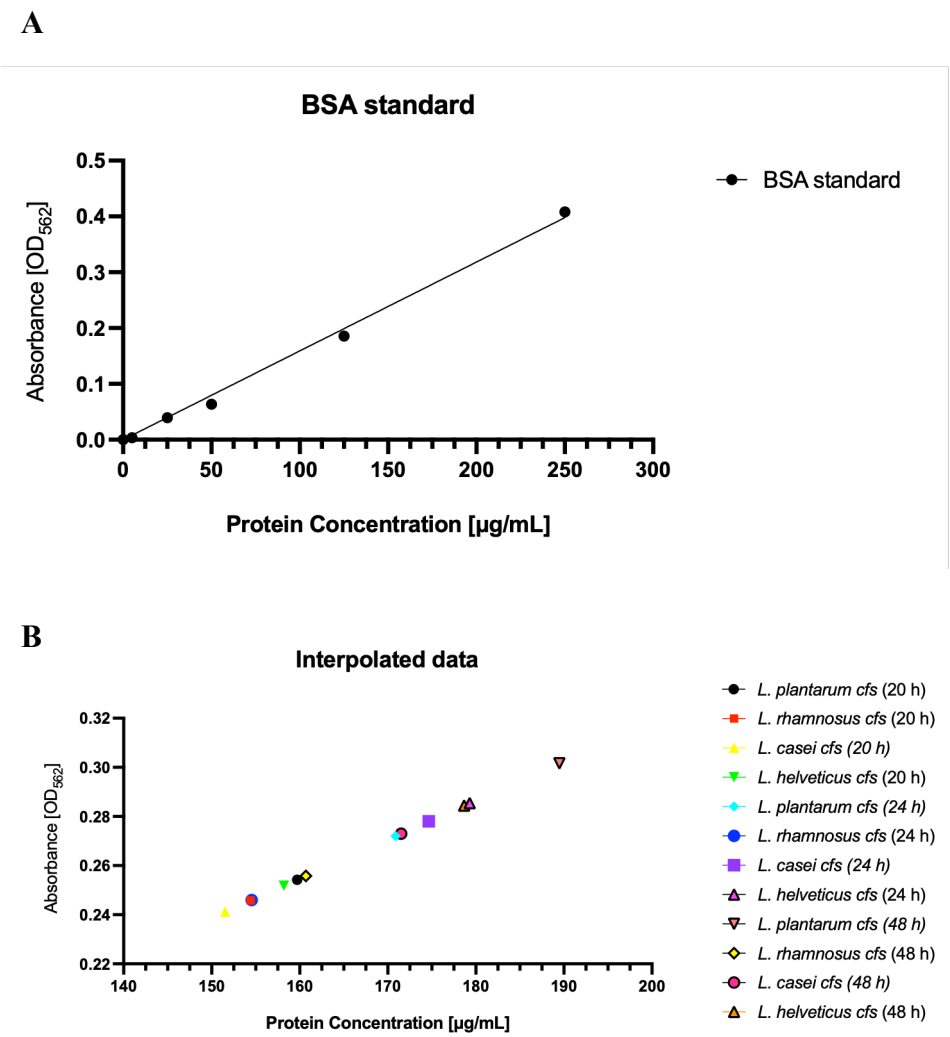


Fig. 20. Protein concentration of *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* were determined using a BCA kit.

Data from figure 20B were interpolated from a linear regression of the BSA standard from 20A and zoomed in for a clearer image. Of the four LAB strains tested, both *L. plantarum* and *L. helveticus* had the highest protein concentration in CFS for all three time points.

Kirby-Bauer Test on L. plantarum, L. rhamnosus, L. casei and L. helveticus

A Kirby-Bauer test was performed on *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* using both MH and MH mixed with lysed erythrocytes agar and then incubated for 48 h. The lysed erythrocytes added onto MH agar did not affect the sensitivity of *L. casei* and *L. helveticus* to the antibiotics used in the Kirby-Bauer test. The diameters for the zone of inhibition of each individual antibiotics showed normal fluctuations for both strains when plated on both MH agar and MH agar with lysed erythrocytes, and so results from both plates were used as biological replicates. *L. casei* and *L. helveticus* have two

biological replicates, while the assay was only done once for *L. plantarum* and *L. rhamnosus*.

The diameters of the zones of inhibition of each antibiotics (fig. 21) used for all four LAB strains are presented in Table 12. As shown, all four strains have an intrinsic resistance to vancomycin and appear to be rather sensitive to meropenem.

Table 12: Antibiotics used and the diameters of their zones of inhibition against the four LAB strains.

Antibiotic Group	**Antibiotics**	***L. helveticus***	***L. plantarum***	***L. rhamnosus***	***L. casei***
		Mean diameter of zone of inhibition [mm] ± SD			
Aminoglycosides	Gentamicin [10 µg]	23.25±0.35	33	30	37.5±3.54
Glycopeptides	Vancomycin [30 µg]	0±0	0	0	0±0
Sulfonamides and Trimethoprim	Sulfamethoxazole/Trimethoprim [25 µg]	15.5±1.4	41	12	20.5±4.24
Phenicols	Chloramphenicol [30 µg]	27.5±0.71	32	32	35.5±0.71
Oxazolidinones	Linezolid [10 µg]	34	34	32	58
Penicillins	Piperacillin/ Tazobactam [110 µg]	40.5±0.71	42	38	53±1.41
	Ticarcillin [75 µg]	43±2.8	28	38	54.5±3.54
Carbapenems	Doripenem [10 µg]	0±0	30	11	0±0
	Imipenem [10 µg]	27.75±2.48	58	34	33±1.41
	Meropenem [10 µg]	16.5±3.54	11	0	20±1.41

Macrolides	Erythromycin [15 µg]	41±1.41	40	38	48.5±2.12
Tetracyclines	Tetracycline [30 µg]	39.5±2.12	18	38	47±1.41

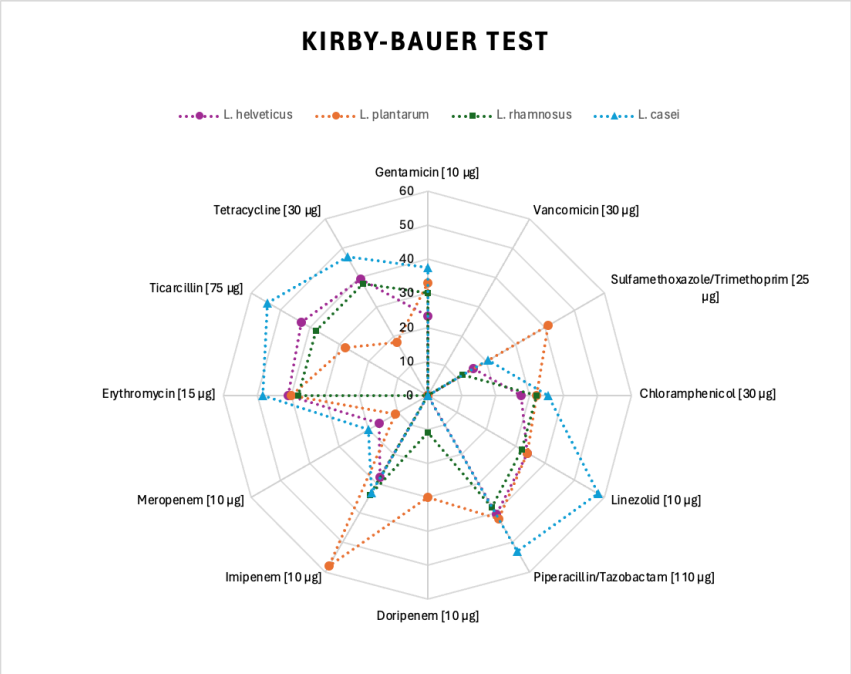


Fig. 21. Radar chart of the Kirby-Bauer test results from both the MH and MH+lysed blood agars. The radius (from 0-60) represents the diameter of the inhibition zones, the individual axes represent the antibiotics tested, and the coloured line show the LAB strain.

General Features of the L. plantarum, L. rhamnosus, L. casei and L. helveticus Genome

The genomic DNA of *L. plantarum*, *L. rhamnosus*, *L. casei* and *L. helveticus* were extracted and then sequenced by Nanopore sequencing. The genome of *L. plantarum* consisted of a circular chromosome of 3,000,916 bp with a total of 2,955 predicted genes by Bakta and an average GC content of 44.8%. 2,825 out of 2,955 were found to be coding sequences (CDSs), 65 tRNAa, 16 rRNAs, 1 tmRNA, 8 ncRNAs, 9 pseudogenes, 143 hypothetical genes and 0 CRISPR arrays. 4 putative oriCs were detected by Bakta across 3 contigs: two on contig 1 (positions 2,600,573–2,600,749 and 2,602,118–2,602,622), and one each on contigs 2 (position 6,048–6,766) 3 (position 10,240–10,919). Given the proximity of the two predictions on contig 1, they likely represent the same chromosomal origin. The oriC on contig 1 was located near the dnaA gene and coincides with a GC

skew inversion, supporting its identity as the functional origin of replication. The oriC-like sequences on contigs 2 and 3 were likely non-functional and may represent mobile or horizontally acquired elements. The *L. rhamnosus* genome had a circular chromosome with 3,045,436 bp and an average GC content of 46.7%. A total of 2,923 genes predicted, 2,806 were CDSs, 61 tRNAs, 1 tmRNA, 15 rRNAs, 12, ncRNAs, 23, 4 pseudogenes, 182 hypothetical genes and 0 CRISPR arrays. The *L. casei* genome had a circular chromosome with a length of 3,024,536 bp and an average GC content of 46.4%. It had a total of 3,033 predicted genes with 2,922 CDSs, 59 tRNAs, 1 tmRNA, 16 rRNAs, 7 ncRNAs, 11 pseudo genes, 171 hypothetical genes and 1 CRISPR array. The sequenced *L. helveticus* genome was found to have a circular genome with 3,157,715 bp and an average GC content of 46.4%. 3,122 predicted genes were annotated with 3,012 CDSs, 59 tRNAs, 1 tmRNA, 15 rRNAs, 5 ncRNAs, 20 pseudogenes, 222 hypothetical genes and 1 CRISPR array. Two oriC regions were detected on contig 6 at positions 1,237,758–1,237,927 and 1,239,280–1,240,209. Given their close proximity (~1.3 kb apart), these are likely components of a single origin of replication region.

In the sequenced *L. plantarum* genomic DNA, homologs of the pediocin operon genes *pedA* (producing bacteriocin pediocin PA-1) and *pedC* (which produces PedC/BrcD family bacteriocin maturation disulfide isomerase) were detected. A gene homologous to *vanZ* was also detected in the annotated genome of *L. plantarum*, but no additional vancomycin resistance genes (e.g., *vanA*, *vanH*, *vanX*) were identified. Interestingly, 60 CDS out of 2,955 CDS in the *L. plantarum* genomic DNA were annotated as prophages by Bakta. Four prophages were annotated in the *L. rhamnosus* genome, two in the *L. casei* genome, and one in the *L. helveticus* genome.

Table 13: Annotation of the sequenced gDNA by Bakta.

	L. plantarum	***L. rhamnosus***	***L. casei***	***L. helveticus***
Genome length	3,000,916	3,045,436	3,024,536	3,157,715
GC [%]	44.8	46.7	46.4	46.4
Coding density [%]	84.9	85.8	86.2	86.0
tRNAs	65	61	59	59
tmRNAs	1	1	1	1
rRNAs	16	15	15	15
ncRNAs	8	12	7	5
ncRNA regions	34	23	22	22

CISPR arrays	0	0	1	1
CDSs	2825	2806	2922	3012
Pseudogenes	9	4	11	20
Hypotheticals	143	182	171	222
sORFs	2	4	5	5
oriCs	4	1	1	2
oriVs	0	0	0	0
oriTs	0	0	0	0

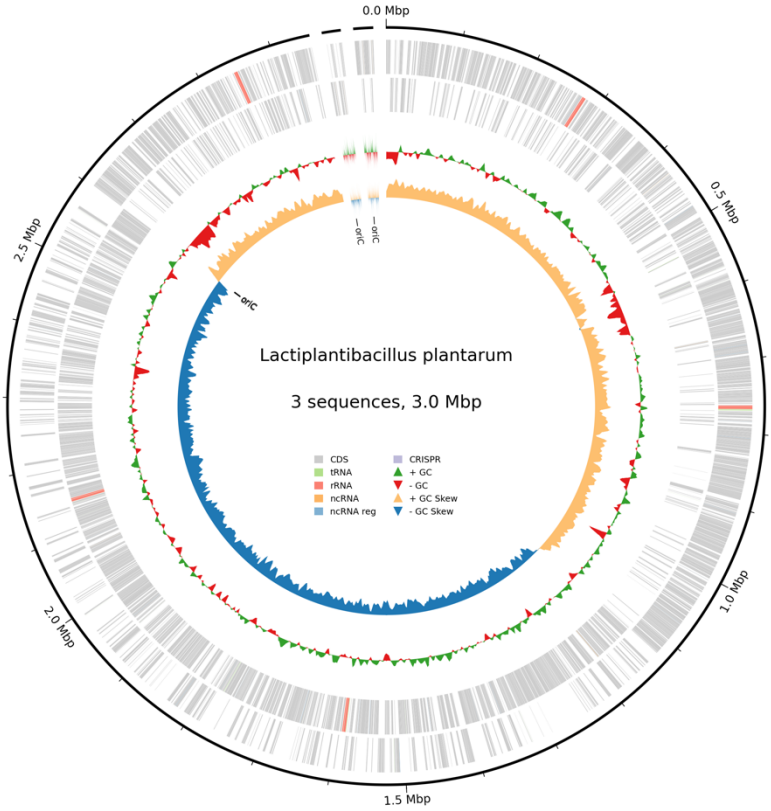


Fig. 22. Genomic DNA from *L. plantarum, L. rhamnosus, L. casei* and *L. helveticus* were extracted and sequenced by nanopore sequencing.

A circular plot of the sequenced *L. plantarum* gDNA downloaded from Bakta, which annotated the genome.

DISCUSSION

Cronobacter spp. are opportunistic, Gram-negative pathogens associated with life-threatening infections in neonates such as meningitis, necrotizing enterocolitis, and sepsis. Contaminated powdered infant formula (PIF), which is not a sterile product, has been determined as a primary vehicle for transmission. Postbiotics, non-viable microbial products or metabolic by-products with health-promoting effects (Salminen, 2021), carry many benefits similar to that of probiotics but with the advantage of fewer risks, such as immunocompromised infants, and often are more stable, and thus easier to preserve and transport.

In this study, the cell-free supernatants (CFS) from *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* were examined to determine if they can serve as prophylactic additions to dried baby foods such as PIF to prevent *Cronobacter* spp. infection in neonates. CFS from *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* was mixed into reconstituted PIF inoculated with approx. 1 cfu/10 ml of a *Cronobacter* strain. In this experiment, CFU counts for all *Cronobacter* strains tested in the reconstituted PIF could not be determined even after 24 h of incubation at 37℃ due to the lack of countable colonies on the CCI agar plates. However, *Cronobacter* strains *C. sakazakii* MHI 21006, *C. sakazakii* MHI 21014, *C. sakazakii* MHI 21051, *C. sakazakii* MHI 21170, *C. dublinensis* MHI 21095, *C. malonaticus* MHI 21091, *C. muytjensii* MHI 21096, and *C. muytjensii* MHI 21213 all had 1-5 colonies isolated from the CCI agar plates for PIF solutions treated with all four CFS from the LAB strains. CFS from *L. casei*, in particular, also had all those *Cronobacter* strains isolated from the treated PIF plus *C. sakazakii* MHI 996, *C. sakazakii* MHI 21038, *C. sakazakii* MHI 995, and *C. sakazakii* MHI 21171 (fig. 8B). There could be several possible reasons for this: 1) The CFS from the four LAB strains are only potentially bactericidal for the *Cronobacter* strains where colonies could be not isolated while only bacteriostatic for the strains where some strains were isolated, 2) It's possible that some of the undissolved PIF clumps in solution acted as a protective barrier for some *Cronobacter* cells to hide in to avoid the antimicrobial activity of the CFS from the LAB strains, and 3) The PIF buffered the drop in pH the CFS from *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* enough to have rendered its antimicrobial effects less effective against certain *Cronobacter* strains. While the first and second points require further testing, the third

point is a very likely reason for the survivability of *Cronobacter* in treated PIF. Lactic acid produced by LAB is a common antimicrobial component in postbiotics. Organic acids, such as lactic and acetic acid, exist in their protonated (uncharged) form at lower pH values allowing them to penetrate bacterial membranes, acidify the cytoplasm and disrupt enzyme function, which can be toxic to pathogens such as *Cronobacter*. However, at neutral or near-neutral pH levels, such as reconstituted PIF, their antimicrobial effect is reduced due to ionisation, making them less membrane permeable (Russell & Diez-Gonzalez, 1998; Alakomi et al., 2000). Similarly, protein-based postbiotic components such as bacteriocins may also lose structure and bioactivity outside their optimal pH range. Indeed, the effect of pH on the antimicrobial activity of *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* CFS were noticed in this study. In another assay, CFS derived from *L. plantarum* was tested against *C. sakazakii* ES5 using multiple dried baby foods from different manufacturers. Out of the six dried baby foods tested, two allowed *C. sakazakii* ES5 growth despite the presence of *L. plantarum* CFS in solution, while *C. sakazakii* ES5 could not be detected for the other four (fig. 10A). After determining the buffering capacities of the six dried baby foods, it was confirmed that the two dried baby foods which allowed *C. sakazakii* ES5 growth, while not having the greatest acid buffering capacity of the six, in fact were the least acidic of the six with a pH of approx. 6.25 (fig. 10B) when reconstituted with no additives. Again, in another experiment where the pH of *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* CFS at 25 mg/ml had been adjusted to pH 3, 5, 7 and 9, *C. sakazakii* ES5 growth was detected in the wells of a 96-well plate where the CFS pH had been adjusted to pH 7 and 9 (table 8). This was despite the MIC having been determined as approx. 10 mg/ml. On L agar mixed with crude CFS from a 48 h culture diluted 1:5, *C. sakazakii* ES5 growth was detected on plates mixed with CFS with pH adjusted to 5, 7 and 9 whereas pH 3 and the unaltered pH CFS plates inhibited *C. sakazakii* ES5 growth (fig. 13). This confirmed that if *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* CFS were to be used as postbiotics to be added to dried baby foods, both the pH and acid buffering capacity of the food must be considered first to ensure effectiveness.

Results from the gentamicin protection assay conducted in this study showed that while both *L. plantarum* and its CFS had no significant effect against *C. sakazakii* ES5 adhering onto Caco-2 cells, it did, however, affect *C. sakazakii* ES5 invasion of the epithelial cells albeit at varying levels with treatment using *L. plantarum* CFS pre-incubated with the

Caco-2 cells for 2 h especially having displayed the greatest reduction of *C. sakazakii* ES5 invasion into the Caco-2 cells, followed by treatment using *L. plantarum* with a 2 h pre-incubation with the Caco-2 cells, and then treatment by *L. plantarum* cells with no pre-incubation (fig. 11). While the exact mechanisms could not be determined in the assay, a study by Qin et al. in 2009 suggests that *L. plantarum* can prevent damage to tight junction proteins (claudin-1, occluding-1, ZO-1, JAM-1) induced by enteroinvasive *E. coli*. Pre-treatment of the Caco-2 cells with *L. plantarum* reduced macromolecular permeability, maintained transepithelial electrical resistance and prevented redistribution of TJ proteins. And while the study was between enteroinvasive *E. coli* and *L. plantarum*, *C. sakazakii* employs similar tactics as enteroinvasive *E. coli* to invade intestinal epithelial cells (Liu et al., 2012). It is then likely that the reduction of *C. sakazakii* ES5 invasion into the Caco-2 cells were prevented by *L. plantarum* similarly to what was described above in the study by Qin et al. in 2009. In this assay it is clear, however, that the *L. plantarum* CFS with pre-incubation treatment was far more effective at preventing *C. sakazakii* ES5 invasion than using the live *L. plantarum* cells, likely due to the higher concentration of antimicrobial products found in the CFS than what live *L. plantarum* cells could produce during the assay period. Results from the *C. sakazakii* ES5 growth test with *L. plantarum* cells (fig. 12) supports the notion that *L. plantarum* cells, at an amount approx. 10× more than *C. sakazakii* ES5, could not produce enough antimicrobials to inhibit *C. sakazakii* ES5 growth, and that perhaps a higher concentration, such as in CFS or to increase the amount of *L. plantarum* cells, would be more effective.

A study from Awaisheh et al. in 2013 showed that the CFS from *L. casei* was heat-labile as its antimicrobial activity was abolished when heated at 60℃ or 80℃ for either 10 min or 2 h as opposed to the *L. casei* CFS used in this study. Most of the *L. casei* CFS, and *L. plantarum*, *L. rhamnosus*, and *L. helveticus* CFS, used in this study were sterile filtered and then autoclaved (120℃) before use for assays and yet, its antimicrobial properties were retained as can be seen in results from figures 8 and 14 where the CFS were all autoclaved before use. The *L. casei* CFS in the study by Awaisheh et al. in 2013 also had its antimicrobial activity diminished against *C. sakazakii* when treated with trypsin at a final concentration of 1 mg/ml. Again, the *L. casei* CFS, as well as *L. plantarum*, *L. rhamnosus*, and *L. helveticus* CFS, in this study did not show diminished antimicrobial activity against *C. sakazakii* ES5 when treated with trypsin (final concentration 1 mg/ml)

or proteinase K (final concentration 0.5 mg/ml, 15 U). This is not to say that the antimicrobials in the CFSs used in this study did not contain proteinaceous substances, it is possible that there was simply an excess of protein in the CFS to saturate the proteases used. Of course, another reason that the CFSs used in this study retained antimicrobial activity against *C. sakazakii* ES5 despite proteinase treatment is that other non-protein antimicrobial compounds, such as lactic acid, are present in the CFSs. This highlights the importance of strain specificity when it comes to postbiotics as their compositions depends on what a specific strain produces as well as the amount it produces.

As mentioned above, it is possible that the *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* CFSs used this study contained a high concentration of proteinaceous substances in solution. Results from the BCA assay conducted in this study are to be viewed with caution as the LAB CFSs used were derived from bacterial cultures grown in MRS broth, which also contained proteins. These proteins were also measured in the BCA assay. For future iterations of this assay, to improve accuracy of protein concentration readings, the LAB CFSs could be lyophilised and then resuspended in PBS, where only proteins produced and secreted by the LAB strains and other unused proteins from the initial MRS broth remain. Most of the proteins from the initial MRS broth would be metabolised by the LAB inoculated during the 48 h incubation, minimizing the amount of those proteins measured in future BCA assays. Afterwards, the proteins in the CFS could be precipitated using trichloroacetic acid (TCA), concentrated before being resolved by SDS-PAGE, and then identified by tandem mass spectrometry according to a procedure by Sánchez et al. in 2012. A *Cronobacter* growth inhibition test could also be performed using the resolved proteins. The protein bands from the SDS gel could be cut and the pieces placed on an L-agar plate on which *Cronobacter* had already been spread, similar to a Kirby-Bauer procedure. After incubation of the plates, possible inhibition zones could be observed and measured, providing that the antimicrobial properties of the proteins were not destroyed by the SDS conditions.

From the sequenced *L. plantarum* genomic DNA, homologs of the pediocin operon genes *pedA* (producing bacteriocin pediocin PA-1) and *pedC* (producing pediocin PA-1 biosynthesis protein PedC) were detected (Marugg et al., 1992). *Pediococcus acidilactici* is a typical producer of pediocin PA-1, not *L. plantarum*, and the presence of these genes suggest that its capacity to produce pediocin-like bacteriocin could possibly have been

acquired via horizontal gene transfer (HGT) either through transduction as both *Pediococcus acidilactici* and *L. plantarum* are LAB and inhabit similar environments (Olajugbagbe et al., 2020), or transduction is also a possibility as 60 CDS in the *L. plantarum* genomic DNA were annotated as prophages by Bakta. A gene homologous to *vanZ* was also detected in the genomes of *L. plantarum* genome. Additional resistance genes such as *vanA*, *vanH* and *vanX*, however, were not detected. After using BLAST to align the protein sequence of the *vanZ* homologue from *L. plantarum* with the protein sequence of the *vanZ* gene from the Tn1546 of *Enterococcus faecium*, known to confer low level resistance to teicoplanin (Arthur et al., 1995), percentage identity was low at only 26.55%. This suggests that the *L. plantarum* genome may naturally have *vanZ*-like genes, but it is not likely to be functional or mobile. Further BLAST results show that the protein sequence of the *vanZ* homologue has a 93.57% identity, a 100% query cover and an E-value of 1×10^{-101} (1e-101) with another protein sequence producing a VanZ family protein from *Lactiplantibacillus* sp. DA1, although its functional role remain unclear. Further experimental validation would be required to confirm functional expression .

While this study demonstrated that CFS derived from *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* exhibited inhibitory effects against several *Cronobacter* spp., important questions remain that warrant further investigation. The antimicrobial activity observed was shown to be pH-dependent (table 8 and figure 13) and retained activity despite autoclaving and proteinase treatment (figure 14), suggesting that the active components in the CFS are likely to contain non-proteinaceous and heat-stable metabolites, such as organic acids or other small molecular weight compounds. However, the precise active components responsible for inhibition of *Cronobacter* were not identified in this study. Future work should therefore focus on chemical characterisation of the CFS using techniques such as high-performance liquid chromatography (HPLC) (Abera et al, 2025), mass spectrometry-based metabolomics (Alseekh et al, 2021), and fractionation approaches to determine whether the inhibitory effect is attributable solely to organic acid production or to additional antimicrobial metabolites. Identifying the active compounds would help clarify the mechanism of action of *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* CFS's inhibitory effects against *Cronobacter* spp.. Additional work should also examine the long-term stability of the CFS under storage conditions

relevant to powdered and ready-to-feed infant formulas, including assessment of shelf-life and compatibility with commercial manufacturing processes.

The findings in this study suggest potential commercial merit in the development of postbiotic-enriched infant formula formulations aimed at inhibiting *Cronobacter* growth in reconstituted products. Unlike probiotics, postbiotics could offer advantages in terms of safety, stability with little to no interaction with components of the food matrix, and regulatory feasibility, particularly for vulnerable populations such as premature or immunocompromised infants although safety should be demonstrated on a strain-specific basis (Vázquez-Frias et al., 2025; Taşkoparan et al., 2025). The reduction in *Cronobacter* growth (fig. 8B) and epithelial cell invasion (fig. 11A) by *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. helveticus* CFS shown in this study supports the concept of using postbiotics as a prophylactic safety enhancement rather than as a therapeutic intervention. However, in the EU, postbiotics are generally subject to novel food and health claim regulations which require comprehensive safety and toxicological assessment prior to approval, and the variability across different infant formulas (fig. 10A and 10B) highlights the need for formulation-specific validation (Taşkoparan et al., 2025). Overall, while the data in the study provide certain proof-of-concept evidence, further mechanistic, formulation, and safety studies are necessary to determine whether incorporation of LAB-derived postbiotics into infant nutrition products is both technically feasible and commercially viable.

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