POPULATIONAL CHANGES TO *Cronobacter sakazakii* IN INFANT FORMULA ENRICHED WITH THE PROBIOTIC *Lacticaseibacillus rhamnosus* DURING TRANSITION OF AN INFANT GUT SIMULATION SUSTEM

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ABSTRACT
The objective of this study was to examine the potential benefits of adding the probiotic bacterium *Lacticaseibacillus rhamnosus* to a powdered infant formula (PIF) to enhance its safety against the opportunistic pathogen *Cronobacter sakazakii*. Commercially available PIF was appropriately reconstituted and inoculated with *C. sakazakii* (10^4 cfu/mL). The reconstituted infant formula (RIF) was divided into two equal parts (A, B) where the first (A) contained exclusively *C. sakazakii* (10^3 cfu/ mL) while the second was additionally inoculated with the probiotic bacterium *L. rha*mnosus (*C. sakazakii*: 10^3 cfu/ mL, *L. rhamnosus*: 10^7 cfu/mL). The two bottles (A, B) were incubated at room temperature (22ºC) and following 12 h of incubation, it was found that the population of *C. sakazakii* in the RIF where it was co-cultured with the probiotic bacterium was about 1 log cycle lower than the RIF where it was alone. After 12 hours of incubation, 15 mL were withdrawn from bottles A and B and filled to a final volume of 150 mL with new, uncontaminated, RIF. Infant gastrointestinal simulation was performed on samples A and B, and changes in *C. sakazakii* and *L. rhamnosus* populations, as well as pH variations, were measured. In both samples (A and B), a comparable decrease in the population of *C. sakazakii*, around 1 log cycle, was observed during the gastric phase's ending stages, when the pH level dropped below 4.3. Regarding the intestinal phase, it was found that the population of both bacteria showed a mild but steady upward trend. In conclusion, the enrichment of PIF with the probiotic bacterium enhanced, under certain conditions, the safety of the baby food in cases of neglecting the hygienic conditions during its use, while it had no effect on the survival of *C. sakazakii* when exposed to a simulation of the infant gastrointestinal system.

INTRODUCTION

Cronobacter sakazakii is a rod-shaped Gram-negative opportunistic bacterial pathogen, facultative anaerobe, motile, peritrichous, non-spore forming (Li et al, 2015). It belongs to the genus *Cronobacter* that is consisted of seven species (Iversen et al., 2007, Joseph et al., 2012, Iversen et al., 2003). The most pathogenic species include *C. sakazakii*, (Amalaradjou et al, 2009), *C. malonaticus*, and *C. turicensis* (Chauhan et al, 2020). *C. sakazakii* has been related to newborn meningitis, bacteremia, necrotizing colitis, and meningoencephalitis (Chauhan et al, 2020; Campion et al, 2017; Amalaradjou et al, 2009). Aside from a high death rate of 40-80% (Chauhan et al, 2020; Amalaradjou et al, 2009), infants who survive often suffer neurological sequelae such as hydrocephalus, quadriplegia, and delayed brain development (Holý and Forsythe, 2014).

Although, the primal reservoir of *C. sakazakii* is not definitely identified (Amalaradjou et al, 2009), it has been isolated from water and soil, factory equipment (Singh et al., 2015), utensils used for the preparation of powdered infant formula (PIF) (Kim et al., 2006) and food like milk, meat, cereals, and PIF. The presence of *C. sakazakii* in PIF represents a major public health concern due to the immature immune systems of neonates who merely consume RIF, making them much more susceptible to illness (Gurtler et al., 2205).

According to a NACMCF review (Anon., 2022) past surveys regarding PIF in the USA have documented elevated contamination rate, varying from 2 to 15% by *Cronobacter* spp., in this type of product. In the same report is mentioned the association observed between the use PIF and several cases of infection with *Cronobacter* spp. in neonates within the US from late 2021 to early 2022, highlighting that *C. sakazakii* occurrence in PIFs is clearly an ongoing issue.

The persistent occurrence of the *Cronobacter* spp. in PIFs has prompted, years now, the scientific community to investigate various ways of dealing with the risk of *C. sakazakii* infection and enhance the safety of PIF. Some of the biological methods include the use of probiotics, bacteriocins, organic acids, plant extracts in PIF. A relatively recent review of biological methods for managing the presence of *C. sakazakii* in food has been published by Chauhan et al., (2020) and Ke et al., (2021).

As regards probiotics, species like *Lactobacillus*, *Lacticaseibacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and *Streptococcus*, have been shown to be beneficial in treating a variety of clinical disorders, including necrotizing enterocolitis (Jamwal et al., 2019). Their capacity to suppress enteropathogens, including *C. sakazakii*, is based on either their derivatives, such as bacteriocins or organic acids, or on competition for nutrients and/or adhesion sites (Ruiz et al, 2020). More particularly, *Lacticaseibacillus rhamnosus* (formerly known as *Lactobacillus rhamnosus*) is a well-studied lactic acid bacterium that possesses desirable features of conventional probiotic strains like ability to endure gastrointestinal stresses, acid and bile tolerance, adhesion and protection of the epithelial barrier, enhancement of immune response (Mathipa-Mdakane and Thantsha, 2022).

However, in addition to the different risk management methodologies for *C. sakazakii* infection, it is important to investigate the effect of conditions prevailing in the gastrointestinal tract of infants against a *C. sakazakii* - contaminated infant formula.

The aim of this work was to investigate whether the enrichment of a commercially available powdered infant formula with the probiotic bacterium *Lacticaseibacillus rhamnosus* could enhance its microbiological safety both during its preparation and/ or after passing through an infant gut simulator system.

MATERIAL AND METHODs

Experimental Protocol

Commercially available powdered infant formula (PIF) was reconstituted according to the manufacturer's instructions. Overnight cultures of *C. sakazakii* and *Lacticaseibacillus rhamnosus* were also available. 200 mL of reconstituted infant formula were inoculated with approximately 10^4 cfu/mL *C. sakazakii* and then equally divided into two bottles (A and B). Bottle A was inoculated exclusively with *C. sakazakii*, while bottle B was also inoculated with an overnight culture of *Lacticaseibacillus rhamnosus* (co-inoculation) at approximately 10^7 cfu/mL. Both bottles were placed for 24 hours at 22ºC (room temperature), and at regular time intervals, samples were taken, microbiologically analyzed, and the colony-forming units (cfu) of *C. sakazakii* and *L. rhamnosus* were counted. Also, the pH of the reconstituted infant formula (RIF) was measured. In parallel, twelve hours after the beginning of the incubation at 22ºC, 15 mL of the RIF were taken from each bottle and mixed with 135 mL of fresh, uncontaminated, RIF (1:10 dilution). The available infant formula samples A (*C. sakazakii*) and B (*C. sakazakii + L. rhamnosus*) were subjected to digestion in a simulator of the infant gastrointestinal tract, which was based on the work of Ménard et al. 2004, with some modifications.

Bacterial strains and inoculla preparation

Cronobacter sakazakii (NTCT 9238) and *Lacticaseibacillus rhamnosus* (NCTC 10302) were available. Prior inoculation has been cultured twice in the reconstituted infant formula used for the experiments. Incubation of both cultures was carried out at 37ºC.

Solutions and enzyme preparations

The simulation of the Gastric (GIF) and Enteric fluids (SEF) was prepared according to Paramera et al., (2011). The gastric simulated fluid (GSF) consisted of the following ingredients (in g/l-final concentrations): glucose, 0.4; yeast extract, 3.0; bacto-peptone, 1.0; porcine mucin, 4.0; cysteine, 0.5; NaCl, 0.08; $NaHCO_3$, 0.4; K_2HPO_4, 0.04; KH_2PO_4, 0.04; $CaCl_2 \cdot 2H_2O$, 0.008; $MgSO_4 \cdot 7H_2O$, 0.008; xylan, 1.0; soluble starch, 3.0. Also, 2.0 USP of pectin/l and 1 ml of Tween 80/l were added to this mixture. Finally, the pH of the solution was adjusted to 3.0 and pepsin was gradually added. As for SEF, it consisted of gastric fluid adjusted to pH 6.5 where bile salts and pancreatin are gradually added though peristaltic pumps.

Simulation of infant gastrointestinal system

Two sterile bottles (G and E) with a volume of 500 mL were placed in a water bath at 37ºC. The bottles were interconnected with a peristaltic pump of adjustable volume. Bottle G contained 30 mL of GIF (fasted volume), to which 150 mL of reconstituted infant formula was instantly added. Immediately after, the gradual addition of pepsin as well as HCl 5N was commenced in bottle G. After 90 min (residence time) the transfer of the gastric contents to the second bottle (E), which contained 10 mL of SEF, began. Also, in bottle E, bile salts and pancreatin were gradually added through peristaltic pumps, while at the same time the pH value was monitored and adjusted to 6.5 by adding NaOH, 5N. The transfer lasted 30 min and the residence time in the intestinal fluid was 4 h. The parameters of the simulated infant gastrointestinal system described in Table 1.

Gastric conditions	
Infant formula ingested (mL)	150
Fasted volume (mL)	30
pH	3.0
Secretion/ Flows	
Pepsin – Flow	1250 U/mL – 0.25 mL/ min
Time in the stomach	90 min
Transient time	30 min
Intestinal conditions	
Fasted volume	5 mL bile (1%) + 5 mL pancreatin solution (10%)
pH	6.5 (steady)
Secretion/ Flows	
Bile salts – flow	1% w/v – 0.5 mL/ min
Pancreatin 10% w/v/ flow	10% w/v – 0.25 mL/ min
NaOH	5N

Table 1: Parameters of the *in vitro* gut/ intestinal conditions

Microbiological analysis

Samples of 1 mL were taken and serially diluted in Ringer solution and then 1 mL of each dilution was added in the appropriate selective media. *L. rhamnosus* was enumerated in MRS Agar (Merk, Darmstadt, Germany) plates, incubated under anaerobic conditions for 48h at 37ºC. *C. sakazakii* was enumerated on the selective medium Chromocult Enterobacter sakazakii agar (CESA, Merck, Darmstadt, Germany), following 48 h incubation at 37ºC.

pH

pH was measured in 4 mL samples using a pH meter (Metrohm AG, Switzerland) at constant temperature of 25 ± 0.1ºC

Statistical Analyses

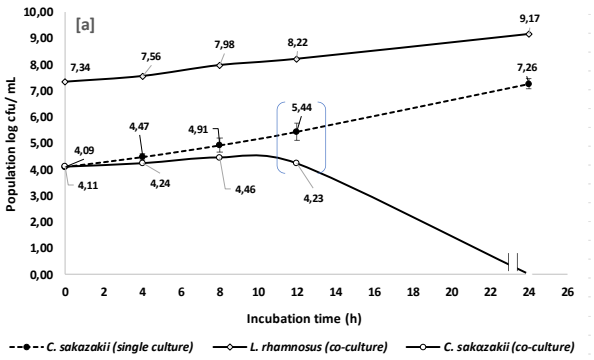
All the experiments were performed three times (extra-day) and the average values were used.

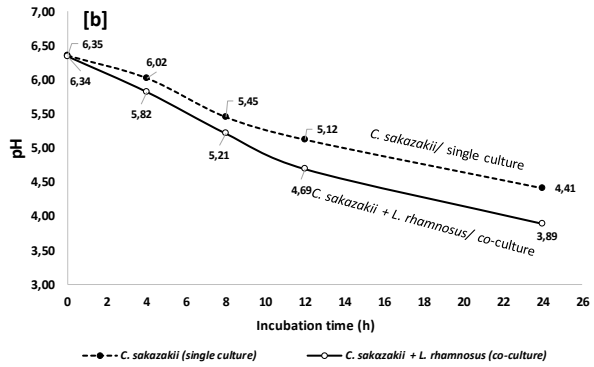
RESULTS

Figure 1 is shown the *C. sakazakii* cell count changes in the reconstituted infant formula incubated at 22ºC for 24 h both in a single-type culture and in co-culture with *L. rhamnosus*, while Figure 2 shows the corresponding changes in pH.

The initial population of *C. sakazakii* was about 10^4 cfu/mL, and in the single-type culture at 12 and 24 hours, it was approximately increased up to 10^5 and 10^7 cfu/ mL respectively. In RIF, on the other hand, where *C. sakazakii* was co-cultured with *L.* rhamnosus, the pathogenic bacteria's cell count started to drop after 12 h, and at the end of the incubation period (24 h) no colonies appeared on the plates.

Also, it is worth noting in Fig. 1, that after 12 hours of incubation at 22°C, the population of *C. sakazakii* in RIF, where it was co-cultured with *L. rhamnosus*, was approximately 1 log cycle lower compared to its population in RIF when cultured alone. As regards pH, in Figure 1b is

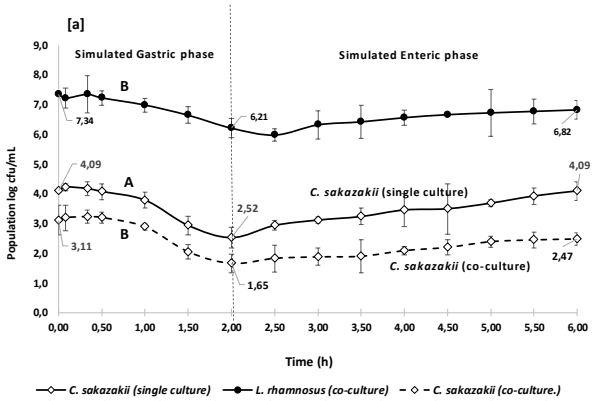




shown the change in the REF during incubation at room temperature (22ºC). In the sample containing only *C. sakazakii*, the decrease in pH was less pronounced compared to the co-culture sample, and the final value was 4.41. In contrast, in the REF in which the pathogen was co-cultured with *Lacticaseibacillus rhamnosus*, the decrease in pH was quite rapid, with a final value below 4.0 (3.89).

Figure 1: (a) Viable counts of *C. sakazakii* and *L. rhamnosus* in single and co-cultures during 24 h of incubation at 22ºC in Reconstituted Infant Formula and (b) pH profile.

In Figure 2 is shown the population changes of *C. sakazakii* and *L. rhamnosus* in the two samples A and B during their passage through a simulated infant gastrointestinal tract. In sample A the infant formula was contaminated with *C. sakazakii* while in the second one (B) *C. sakazakii* and *L. rhamnosus* coexisted. As shown in Figure 2, the cell counts (cfu/ mL) of both *C. sakazakii* and *L. rhamnosus* was found to have a downward trend in all samples (A and B) during the gastric phase. As far as *C. sakazakii* is concerned, the decrease in its population was more intense after 1 h of residence in SGF when the pH was lower than 4,5, which was caused by the continuous addition of HCl to that. It is noteworthy that the same profile of *C. sakazakii* population decline occurred both in the sample where *C. sakazakii* was alone and in the sample where it was co-cultured with *L. rhamnosus*. During the 1,5 h residence in the SGF and the additional 0.5 hour required for the transfer of approximately 150 mL from the gastric environment to the intestinal environment the total reduction of the pathogenic microorganism was the same in both samples (A and B) and



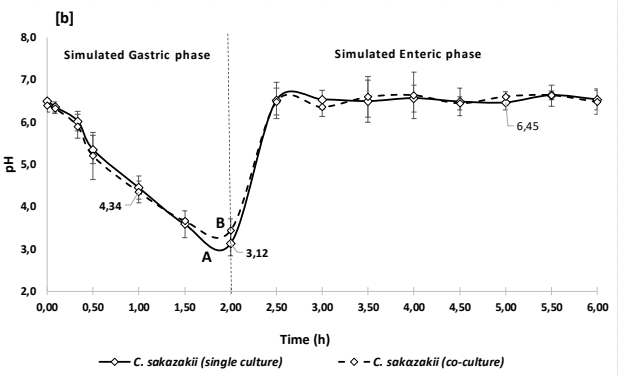
was approximately 1 log cycle.

During the gradual transfer of SGF to SEF where the pH was at 6.5, the population of *C. sakazakii* began to recover gradually, and after about 4 sojourns. The same trend was observed in both sample A and sample B.

Figure 2: (a) Changes of the bacterial populations in samples (A and B) of reconstituted infant formula during passage through simulating gastric (gastric phase) and intestinal fluid (intestinal phase) at 37ºC. Sample A had exclusively *C. sakazakii* while sample B contained a co-culture of *C. sakazakii* and *L. rhamnosus* and (b) changes of pH during the gastric and intestinal phase.

DISCUSSION

The experimental methodology followed in this work was



based on a hypothetical scenario ~~that~~ of what may occur ~~in~~ during the daily practice of infant feeding if the recommended equipment disinfection procedures are not followed (Henry et al., 2019). This hypothetical case concerns an infant formula that is contaminated with *C. sakazakii*. After the baby formula is reconstituted and consumed, a small amount of it remains in the baby's feeding bottle. The latter is not sanitized immediately but remains at room temperature for approximately 12 h. Then, again without sanitizing the baby's feeding bottle, a new amount of non-contaminated reconstituted infant formula is added to it and used to feed the baby.

The findings of this study show that the use of the *L. rhamnosus* in powdered infant formula has the potential, under certain circumstances, to decrease the presence of the

pathogenic *C. sakazakii* during infant food preparation if sanitation procedures have not been followed. In particular, the co-growth of the *L. rhamnosus* and *C. sakazakii* results in a gradual decline of the population of the latter due mainly to the production of lactic acid by the former. After 12 hours of the RIF being at room temperature (22ºC), the population of *C. sakazakii* had decreased by 1 log cycle.

In this work, for reasons related to the conduction of the experiment, the initial microbial load of *C. sakazakii* was chosen to be high (10^4 cfu/g). Under real conditions where the level of initial contamination is usually lower than 1 cfu per 100 g of PIF (Osaili, and Forsythe, 2009) the effect of the presence of lactic acid bacteria in the powdered infant formula could, perhaps, reduce the population level or even eliminate the *C. sakazakii*, always certainly under the conditions described in the experiment.

It is noteworthy that Kandhai et al. (2006) mentioned the rapid growth rate of *C. sakazakii* strains in infant formula; therefore, a request to slow this rate is of importance. According to the results of this work, it appears that the incorporation of appropriate probiotic bacteria into infant formulas recipe is a progression in this regard.

During the second phase of the experiments ~~that is,~~ -during the passage of the infant formula by simulating the gastric and intestinal system of the infant- it was found that the gradual drop in the pH of the gastric fluids to 3.0 reduces the population of the pathogenic bacterium, by about 1 log cycle. This reduction is mainly observed during the last stages of digestion, just before the transport to the intestinal system begins, because time is needed to reduce the pH in the stomach, the value of which rises immediately after taking food. However, it should be noted that the presence of *L. rhamnosus* in the sample did not improve the rate of pH decline in the simulating gastric fluid. The cause can be attributed to different causes among which is that there is not enough time for *L. rhamnosus* growth and production of lactic acid.

CONCLUSIONS

In certain instances, the use of probiotics in powdered infant formula might further impede the growth of *C. sakazakii* during the preparation phase, particularly when the sanitary standards of the equipment used are substandard.

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