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Evaluation of the Effect of the Technical Guidelines for Chilean Milk Dietary Service on the Microbiological and Nutritional Quality of a Powdered Infant Formula

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Abstract: The Chilean Technical Manual for Milk Dietary Service (SEDILE) was evaluated to determine its effectiveness in ensuring the microbiological safety and vitamin D adequacy of a commercially available powdered infant formula (PIF) used in SEDILE. The evaluation focused on whether adherence to the manual's guidelines positively influenced these factors. Both the PIF and the reconstituted PIF (RIF) were found to be free from *Cronobacter sakazakii* and *Salmonella enterica* contamination, with levels of total coliforms and Escherichia coli within acceptable limits. Moreover, the vitamin D content in the formula was within the expected range. These findings suggest that following the Chilean Technical Manual for SEDILE contributes to the microbiological safety and nutritional adequacy of RIF in dietary services.

Keywords: *Cronobacter sakazakii*; infant formula; *Salmonella enterica*; vitamin D; nutritional quality

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1. Introduction

Powdered infant formulas (PIF) are the best alternative to meet the nutritional needs of infants under 6 months of age who cannot be breastfed [1] because they contain vitamins, minerals, prebiotics, milk proteins, lactose, vegetable oils, among other nutrients [2]. However, PIF must be reconstituted for consumption, generating a risk of contamination. External factors such as utensils, handling, and reconstitution of PIF are vehicles of disease transmission.

Among the most well-known pathogenic microorganisms in powdered dairy foods is *Cronobacter sakazakii*, which belongs to the *Enterobacteriaceae family*. It is a facultative anaerobic, motile, gram-negative, rod-shaped bacterium that does not form spores [3,4]. This pathogen has caused fatal diseases in infants, with mortality between 40.0% and 80.0% in the USA and France [1,5–7].

The population at highest risk of *Cronobacter sakazakii* are infants under 1 year of age, immunosuppressed infants, newborns less than 28 days old, children with low birth weight less than 2500 g, as well as infants whose health is compromised for various reasons [8]. In this sense, today, several cases of meningitis, sepsis, bacteremia, and necrotizing enterocolitis caused by this pathogen have been reported in different countries such as the United Kingdom (2004), Belgium (late 1990s), France (1990s), the USA (2000s and the outbreak of 2011), and the Netherlands [4,9,10]. Another high-risk pathogen is *Salmonella enterica*. This microorganism in PIF has also been found to cause a serious infection called salmonellosis that causes diarrhea, fever, abdominal cramps, and, in some babies, bacteremia and meningitis, causing approximately 4.0% of mortality worldwide [11]. Both *Cronobacter sakazakii* and *Salmonella enterica* are category "A" microorganisms, meaning that there is clear evidence of causality that contamination of PIF and reconstituted PIF are,

epidemiologically and microbiologically, the vehicle and source of infections in infants [8]. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) requested scientific advice from the Codex Committee on Food Hygiene to have input for the revision of the Recommended International Code of Practice of Hygiene for PIF [8]. These recommendations have been accepted and implemented by the PIF industry, as well as by places where PIF are reconstituted and fed to infants.

In Chile, the Milk Dietary Services (SEDILE) are fundamental units within the structure of pediatric hospital centers, where the reception and storage of raw materials are carried out to later prepare, sterilize, and distribute infant formulas [12]. SEDILE is a physical space designed for the preparation of PIF that meets the nutritional requirements of each patient [13]. To ensure safe nutrition for hospitalized children, the Chilean Ministry of Health (MINSAL) developed the 2010 Technical Guidance for Dietary Milk Services (SEDILE) and Central Enteral Formulas (CEFE), which outlines the construction, organization, and operation of these services [13]. This guide provides a framework for public and private health establishments to create their own tailored "SEDILE Technical Manual" by adapting the general guidelines to their specific physical and economic conditions, ensuring consistent safety and nutrition standards for hospitalized children nationwide. In this way, the procedure for preparing the PIF is described step by step to ensure the microbiological quality of the products produced and distributed. These processes have supported the successful control of the preparation of PIF in the SEDILE of the country. However, ensuring adequate microbiological safety is usually associated with excessive heat treatment that could decrease the nutritional value of PIF. Among the different critical nutrients in infants, vitamin D deficiency has been detected as a cause for concern in Chile, as well as throughout the world [14]. Several factors influence vitamin D deficiency in the body, including lack of exposure to sunlight, diet, lifestyle, age, seasons, and latitude [15–17]. Its deficiency has been linked to obesity, cardiovascular diseases, depression, insulin resistance, autoimmune diseases, multiple sclerosis, and some cancers in adult life [14,15,17]. Due to these risks, food industries have chosen to fortify foods such as PIF with vitamin D [15,18,19]. The objective of this study was to evaluate the effectiveness of the Technical Manual for SEDILE on the microbiological and nutritional quality of a commercial PIF commonly used in Chilean hospital centers.

2. Materials and Methods

For this study, a commercial PIF designed specifically for premature infants and/or low-birth-weight infants was selected. This type of product contains an adequate caloric density, macronutrients, vitamins (e.g., vitamin D), and minerals according to the nutritional needs of this type of child. First, the microbiological quality and vitamin D content of the selected PIF were analyzed to obtain a baseline level of both variable responses. The same analyses were then carried out for the reconstituted product to evaluate the effect of the application of the SEDILE Technical Manual on both critical parameters.

2.1. Samples

To analyze the PIF, 4 kg of a widely consumed commercial product was used. The PIF corresponded to a single manufacturer, was derived from the same production batch, and was designed for premature infants. All these PIFs were reconstituted according to the "SEDILE Technical Manual" from a clinic in the Metropolitan Region of Santiago, Chile. For the preparation of reconstituted infant formulas (RIFs), a standard dilution of 14.5% was applied, as per the manufacturer's instructions.

2.2. Preparation of Reconstituted PIF

The PIFs were reconstituted in accordance with the "SEDILE Technical Manual" from a clinic in the Metropolitan Region of Santiago, Chile. The formulas were prepared in 200 mL bottles, with only initial sterilization performed on the bottles and utensils used in their preparation (Figure 1). It is important to note that no final sterilization of the reconstituted

PIF was conducted, as this decision was made to preserve the nutritional quality of the formulas, particularly their vitamin D content.

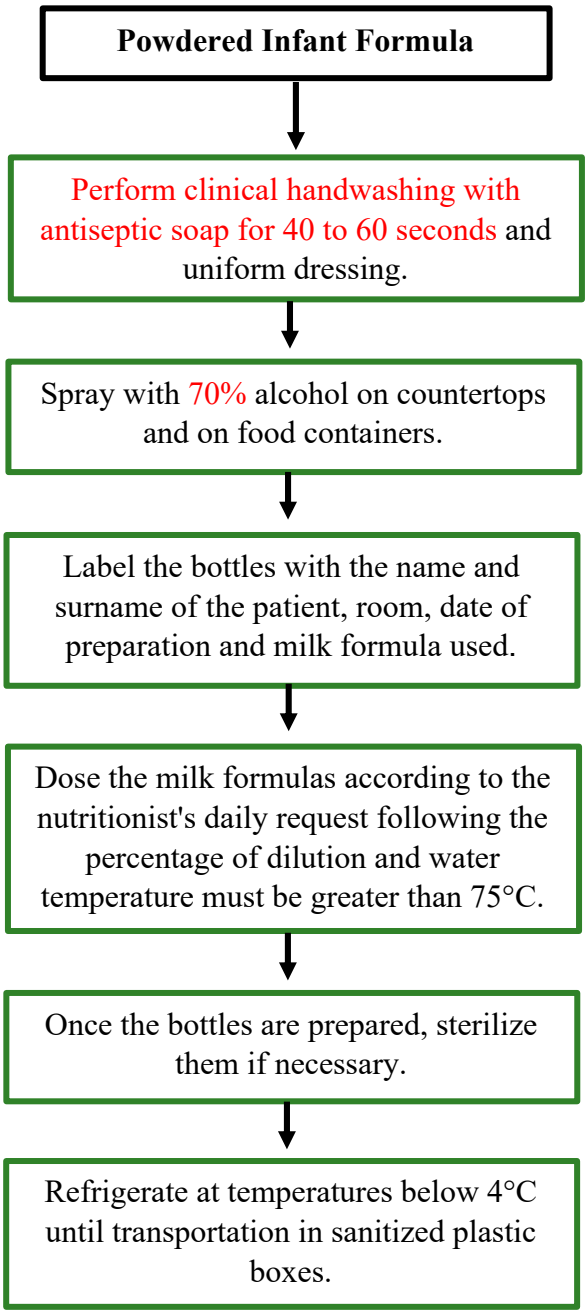

Figure 1. Preparation of reconstituted powdered infant formulas in Chile (SEDILE Technical Manual).

After preparation, the formulas were immediately stored in containers that maintained a temperature of 4 °C to prevent bacterial growth. These samples were then refrigerated until their subsequent microbiological and vitamin D analyses. They were transported by the study's technical team to the laboratory under these conditions within a period not exceeding 30 min.

2.3. Microbiological Analysis

2.3.1. Bacterial Strains and DNA Extraction

C. sakazakii strain ATCC 29544 was grown overnight (12–18 h) at 37 °C on chromogenic agar (Sigma-Aldrich, St. Louis, MO, USA). Bacterial genomic DNA was extracted using

a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The extracted DNA samples were stored at −20 °C until further use.

2.3.2. Primer's Design and qPCR Conditions

TaqMan primers and probes described by Seo & Brackett (Foster City, CA, USA) [20] were used. Real-time PCR was performed in an ABI 7500 real-time PCR cycler (Thermo Fisher Scientific, Foster City, CA, USA), according to Xie & Liu's methodology [21]. The total reaction mixture (25.0 µL) contained 12.5 µL 2 × Premix Ex Taq (Probe qPCR), 0.500 µL 50 × ROX Reference Dye II, 0.500 µL of each primer (10.0 µM), 0.500 µL of each probe (10.0 µM), and 2.00 µL of target bacterial genomic DNA. Sterile distilled water was used to replace genomic DNA for negative control. The thermal cycling conditions of q-PCR were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Experiments were performed in triplicate, and results were expressed as presence or absence.

2.3.3. Total Mesophilic Aerobe Count

Twenty-five grams of samples were mixed with 225 mL of Buffered Peptone Water (BPW) (Sigma-Aldrich, Darmstadt, Germany) after inoculation in petri dishes, which were subsequently seeded with Plate Count Agar (PCA) according to Chap et al. [22]. The plates were incubated overnight at 37 °C, then mesophilic aerobic colonies were counted, and the results were expressed as cfu/g.

2.3.4. Total Coliforms and *Escherichia coli*

Fifty grams of sample were mixed with 450 mL of Butterfield's phosphate-buffered water; decimal dilutions were prepared according to Feng et al. [23]. At least three dilutions were added to three tubes containing Lauryl Sulfate Tryptose (LST) broth (Sigma-Aldrich, USA), which were then incubated at 35 °C. The tubes were examined for gas production after 24 and 48 h. From each tube of LST containing gas, one loopful was transferred to Brilliant Green Lactose Bile (BGLB) broth (Sigma-Aldrich, USA). BGLB tubes were incubated at 35 °C and examined for gas production after 24 and 48 h. The results were expressed as MPN/g.

2.3.5. Bacterial Pathogens

For *Staphylococcus aureus*, 25 g of sample were mixed with 225 mL of BPW, and decimal dilutions were prepared according to S. Tallent et al. [24]. Inoculation was performed on Baird–Parker agar plates (Sigma-Aldrich, USA) at 35 °C for 45 h. Subsequently, plates containing colonies with typical *S. aureus* appearance were used for plate count. The results were expressed as cfu/g.

For *Clostridium perfringens*, 25 g of sample were mixed with 225 mL of BPW, and decimal dilutions were prepared according to Jeffery & Harmon [25]. Inoculation was performed on plates with Tryptose–Sulfite–Cycloserine (TSC) agar without egg yolk (Sigma-Aldrich, USA) at 35 °C for 24 h. After incubation, plates containing between 20.0 and 200 black colonies were selected for *C. perfringens* Enumeration. The results were expressed as cfu/g.

For the determination of *Bacillus cereus*, 25 g of sample were mixed with 225 mL of Butterfield's phosphate-buffered water, and decimal dilutions were prepared according to S. M. T. Tallent et al. [26]. The samples were inoculated on mannitol–egg yolk–polymyxin (MYP) agar plates (Sigma-Aldrich, USA) at 30 °C for 24 h. The number of cfu/g of *B. cereus* was calculated based on the percentage of morphologically consistent colonies.

For the determination of *Salmonella*, 25 g of sample was mixed with 225 mL of lactose broth, the pH was adjusted, and incubation was carried out at 35 °C for 24 h, according to Andrews et al. [27]. Samples were spiked with Rappaport–Vassiliadis (RV) medium and tetrathionate (TT) broth and incubated on bismuth sulfite (BS) agar, xylose lysine

deoxycholate (XLD) agar, and Hektoen enteric (HE) agar at 35 °C for 24 h. Plates were examined for the presence or absence of *Salmonella*.

2.4. Vitamin D Analysis

2.4.1. Chemical Reagents

Vitamin D_3 (purity ≥ 99.9 g/100 g), ethanol (purity ≥ 99.9 g/100 g), hexane (purity ≥ 99.9 g/100 g), and nitric acid (purity ≥ 99.9 g/100 g) (Sigma-Aldrich, MO, USA) were used.

2.4.2. Determination of Vitamin D_3

The vitamin D_3 content of PIF and RIF was determined by high-performance liquid chromatography (HPLC) coupled to a diode array (DAD) and ultraviolet (UV) detector [28]. The sample was saponified with aqueous potassium hydroxide and ethanol, followed by vitamin extraction with hexane and subsequent quantification by normal phase semi-preparative HPLC-DAD (Chromaster, Merck Hitachi Ltd., Tokyo, Japan), followed by reverse phase HPLC-UV (Chromaster, Merck Hitachi Ltd., Tokyo, Japan). The experiments were carried out in duplicate.

2.5. Statistical Analysis

Vitamin D data were analyzed using STATGRAPHICS Centurion (XVII software) to determine significant differences according to Tukey using one-way ANOVA and Multiple Range Test with a confidence level of 95.0%; significant differences between samples were considered a $p < 0.050$.

3. Results and Discussion

PIF are dehydrated products; due to this characteristic, they cannot be sterilized and, therefore, they may contain low levels of microorganisms. When pathogenic microorganisms are present, foodborne illnesses can develop after ingestion of contaminated products [29]. Infant formulas have been implicated in outbreaks of foodborne illness in infants [4,9,10,30]. Therefore, the microbiological safety of baby foods is important to reduce the risk to public health worldwide. Due to the risk, Chile considers carrying out microbiological controls on the PIF of the microorganisms listed in (Table 1). In the present study, both the PIF and RIF analyzed were found to the free from *Cronobacter Sakazakii* contamination. Using the q-PCR method. Chap et al. [22] found similar results. They evaluated and compared preparation instruction guides according to PIF labels from different countries considering this target pathogen. These authors found that out of seven countries evaluated, only one indicated in the food preparation instructions the use of water at a temperature higher than 70 °C for reconstitution, a recommendation given by the FAO/WHO [31]. In the case of Chile, the "Technical Guidance for Dietary Milk Services (SEDILE) and Central of Enteral Formulas (CEFE)" [13] suggests that water used for the preparation of RIF must be used at temperatures above 75 °C, which aligns with good hygiene practice (GHP).

For the total count of mesophilic aerobes in both PIF and RIF, counts lower than 10.0 cfu/g were found. These values were lower than those reported by Chap et al. [22], which range between $<10^2$ and $>10^5$ cfu/g. The observed variation could be attributed to the fact that PIFs from different countries were analyzed with no standardized water temperature for reconstitution, potentially promoting microbiological growth. Additionally, some of the PIFs contained probiotics, leading to elevated counts.

In total coliforms and *Escherichia coli*, the most probable number (MPN) technique is generally used. In this study, values lower than 3.00 MPN/g were found for both types of samples, complying with the limits established by the Chilean Food Health Regulations (RSA). Iversen and Forsythe [32] evaluated the general hygienic condition of several PIF samples. As part of the analysis, they determined the presence of Enterobacteriaceae, including *Escherichia coli*, using the plate count method. Although a different method was

used, the values obtained in both studies were below the established detection limit of 100 cfu/g.

Table 1. Microorganisms evaluated in powdered (PIF) and reconstituted (RIF) infant formula according to the requirements of the Chilean Food Sanitary Regulations (RSAs).

Type of Microorganisms	Example Type	
	Powdered Infant Formulas	Reconstituted Infant Formulas
Mesophilic aerobic count (cfu/g)	<10.0	<10.0
Total coliforms (MPN/g)	<3.00	Not required *
Staphylococcus aureus (cfu/g)	<10.0	<10.0
Salmonella in 25 g (P/A)	Absence	Absence
Bacillus cereus (cfu/g)	<10.0	<10.0
Cronobacter spp. (P/A)	Absence	Absence
Escherichia coli (MPN/g)	<3.00	<3.00
Clostridium perfringens (cfu/g)	<10.0	Not required *

* Not required by the Chilean Food Sanitary Regulations (RSAs).

In this study, it was found that the values of *Staphylococcus aureus* were <10.0 cfu/g for both powdered and reconstituted samples. Bogdanovicova et al. [33] evaluated the growth of *S. aureus* in reconstituted milk powder and found values < 10^2 cfu/g. The values in our study were lower than those reported by these authors, complying in both cases with the limits established by the European Union [34] of 10^2 cfu/g for milk powder.

Similarly, for both types of samples, the results for *Clostridium perfringens* and *Bacillus cereus* were <10.0 cfu/g. Pei et al. [35] found similar results when they evaluated the prevalence of these pathogens in PIF from China. Moreover, 92.5% of the PIFs evaluated contained less than 10.0 cfu/g of these pathogens. These results indicate that not only the manufacturers of these PIFs comply with hygienic practices but also those who reconstitute them. *Salmonella* and *Cronobacter sakazakii* also belong to category "A" since it has been demonstrated, epidemiologically and microbiologically, that they are responsible for infections in infants [8,29]. In our study, we report the absence of *Salmonella*, which agrees with the findings of Iversen & Forsythe [32], where *Salmonella* was below the detection limit of 100 cfu/g in the 82 PIFs evaluated in their study.

According to the one-way ANOVA and Multiple Range Test of Tukey applied in vitamin D values, it was found that there were no significant differences ($p > 0.05$) between the PIF and the RIF (Table 2).

Table 2. Effect of infant formula preparation on vitamin D content.

Samples	Powdered Infant Formula [mcg D_3/100 g]		Reconstituted Infant Formula [mcg D_3/100 mL]	
	PIF Sample	PIF Reference	Sample RIF	RIF Reference
Vitamin D content	9.08 ^{a} ± 0.080	9.00 ^{a} ± 0.020	1.89 ^{a} ± 0.100	1.80 ^{a} ± 0.020

PIF (powdered infant formula) and RIF (reconstituted infant formula). Values are shown as mean [mcg D_3/100 g] and [mcg D_3/100 mL] of two duplicate experiments ± SD (standard deviation). The same lowercase superscripts between columns of the same sample category indicate that there is no significant difference ($p > 0.050$) between sample and reference.

PIF reported values between 9.02 and 9.14 mcg D_3/100 g. Trenerry et al. [36] developed a simple and robust liquid chromatography–mass spectrometry method with a 2D ion trap (LC-MSn) to determine the levels of vitamin D_3 in infant formulas, obtaining values of 7.80 ± 0.200 µg/100 g, which are like those obtained in our study. Meanwhile, for the RIF

values between 1.82 and 1.96 mcg, D_3/100 mL were obtained. Perales et al. [37] reported similar values (1.13 ± 0.120 µg/100 mL) when they developed and validated a sensitive liquid chromatography method for determining vitamin D in infant formulas. Our data were not only like other studies but also like those reported on the nutritional labels of PIFs and RIFs, with values of 9.00 µg/100 mL and 1.80 µg/100 mL, respectively. RIF also meets the requirements established in the RSA of Chile [38], article 496, which establishes the basic composition of these formulas and the range of fortification with vitamin D (1.00–3.00 mcg/100 kcal).

4. Conclusions

Ensuring the microbiological safety and nutritional quality of vitamin D in foods intended for infants, especially powdered infant formula (PIF), is crucial, as this contributes to the growth and development of children. Although the food industry implements various guidelines, such as HACCP and GHP, to ensure food safety, cases of contamination in infant formulas caused by foodborne pathogens or illnesses resulting from contaminated infant formulas are still reported. Our findings confirm that SEDILE effectively meets the microbiological and nutritional requirements (vitamin D) in the preparation of reconstituted infant formulas. Therefore, the development and compliance of the SEDILE Technical Manual of this hospital, following the recommendations established in the Chilean SEDILE Technical Guidance, minimizes the risk of microbiological contamination and guarantees the nutritional quality of vitamin D in this analyzed milk formula.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Finis Terrae University, Santiago, Chile (Project identification code 11/2020).

Informed Consent Statement: Informed consent was not required.

Data Availability Statement: All data analyzed in this study are available upon request to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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