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Stability of the antimicrobial capacity of human milk against *Cronobacter sakazakii* during handling

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Key Messages:

- The antimicrobial capacity of human milk has been studied against different microorganisms but not against *Cronobacter sakazakii;* however, recently, several cases of neonatal infection with *C. sakazakii*, due to consumption of expressed human milk, have been reported.
- Human milk showed antimicrobial capacity against *C. sakazakii* and antimicrobial capacity was affected to a greater or lesser degree depending on the treatment that is applied.
- If it is necessary to store human milk, the most advisable course is to refrigerate it for no longer than 72 hours or frozen for a short period of time.

Abstract

Background: Neonatal infections with *Cronobacter sakazakii* have been recently associated with the consumption of expressed human milk.

Study Aims: (1) To evaluate whether human milk has antimicrobial capacity against *C. sakazakii* and (2) to determine the stability of its capacity when it is subjected to various treatments.

Methods: The antimicrobial capacity of human milk against *C. sakazakii* was evaluated using an observational, cross-sectional, comparative design. Mature human milk samples (*N=29*) were subjected to different treatments. After incubation at 37 ºC for 72 hr samples were compared with fresh milk on the stability of their antimicrobial capacity. Two-way analysis of variance (ANOVA) was performed.

Results: In fresh milk, counts of *C. sakazakii* were reduced by 47.26% (SD = 6.74) compared to controls. In treated milk, reductions were: refrigeration at 4 ºC for 72 hr ($M = 33.84$, $SD = 13.84$), freezing at –20 ºC for one, two, and three months ($M = 40.31$, $SD = 9.10$; $M = 35.96$, $SD = 9.39$; $M = 26.20$, $SD = 13.55$), respectively, Holder pasteurization ($M = 23.56$, $SD = 15.61$), and human milk bank treatment with ($M = 14.37$, $SD = 18.02$) and without bovine fortifier ($M = 3.70$, $SD = 23.83$). There were significant differences ($p < .05$) between fresh and treated milk.

Conclusions: Human milk has antimicrobial capacity against *C. sakazakii* however, its capacity is negatively influenced by common preservation and hygienization methods. Milk should be stored refrigerated for a maximum of 72 hr or frozen for a short period of time.

Background

Human milk (HM) is the natural first food for infants. The current recommendation of the World Health Organization is that infants be exclusively breastfed until six months of age, with continued breastfeeding along with appropriate complementary diets up to two years of age or beyond (WHO, 2019). In addition to being an optimal source of nutrients, HM provides newborn infants with valuable antimicrobial substances. This is particularly important in the most vulnerable individuals, for example preterm and small for gestational age (Miller et al., 2018). Some of these substances include oligosaccharides (Ackerman et al., 2017, 2018; Bode, 2015; Thai & Gregory, 2020), lipids (e.g. glycerol monolaurate) (Schlievert, Kilgore, Seo, & Leung, 2019), proteins (e.g. lactoferrin and casein201) (Ochoa et al., 2020; Thai & Gregory, 2020; Zhang, 2017), and enzymes (e.g. lysozymes, peroxidases, and xanthine oxidase) (Thai & Gregory, 2020). However, it should be noted that breastfeeding is not possible in all cases. To circumvent this, it is becoming an increasingly common practice that lactating mothers express and store milk at home (refrigerated at 4 ºC or frozen at –18 ºC/–20 ºC). This, in turn, allows the use of milk at a later stage or its administration by a different person. Recently, the Academy of Breastfeeding Medicine (United States of America) has established a protocol for the treatment of milk at home (Eglash & Simon, 2017).

On the other hand, it is becoming increasingly recognized that more hospitals are incorporating operational human milk banks (HMBs) into their facilities. Although some countries (e.g. United States, Brazil, United Kingdom, and Spain) have developed their own hospital protocols with the aim of promoting a better practice, universally accepted guidelines are still lacking (Calvo et al., 2018). What is apparent when reviewing the literature is that some steps are common to all of them (Bharadva et al., 2014; Calvo et al., 2018; Committee on Nutrition, Section on Breastfeeding, Committee on Fetus and Newborn, 2017) including

freezing of HM expressed at home, storage frozen in HMBs (normally for a maximum of one month), thawing prior to pasteurization, Holder pasteurization (62.5 ºC for 30 min), refreezing (normally for a maximum of two months), and rethawing prior to consumption.

Researchers from recent studies have demonstrated that the type of treatment applied to milk, its duration, and its intensity can have a direct influence on the natural antimicrobial capacity (AC) of HM (Guerra, Mellinger-Silva, Rosenthal, & Luchese, 2018; Păduraru et al., 2018; Rodríguez-Camejo et al., 2020; Salcedo, Gormaz, Lopez, Nogarotto & Silvestre, 2015; Schlotterer, Parvez, & Perrin, 2019). In addition, milk can become contaminated during its processing, its administration, or both. The latter is particularly true when adequate and strict handling conditions are not followed. In line with this statement, Labiner-Wolfe and Fain (2013) showed that some of the most common practices performed at home significantly increase the risk for bacterial contamination and reduce the biological properties of HM.

Over the recent years, one of the emerging dangers in the feeding of infants has been the presence of *Cronobacter sakazakii* (Hunter & Bean, 2013; Odeyemi & Sani, 2016). Classically, this bacterium has been associated with neonatal infections caused by consumption of HM substitutes contaminated during the manufacturing process or handling. Because *C. sakazakii* is able to form biofilms in a wide range of surfaces (Gupta, Mowat, Brigthwell, & Flint, 2018), this microorganism has also been found on utensils used for its reconstitution (Siqueira Santos et al., 2013). More recently, some authors (Bowen et al., 2017; McMullan, Menon, Beukers, van Hal, & Davis, 2018; Sundararajan et al., 2018) have demonstrated that the incorrect handling of expressed HM can be also associated with its contamination with this bacterium causing disease in newborn infants. The aims of this study were (1) to evaluate whether HM has AC against *C. sakazakii* and (2) to determine the stability of its capacity when it is subjected to various treatments.

Methods

Design

This study was designed as an observational, prospective, and cross-sectional comparison of 8-treatment groups. This design was chosen to avoid the influence of the investigators on the AC of HM against *C. sakazakii*, evaluating the AC at a single point in time for each donor (Gilmartin-Thomas, Liew, & Hopper, 2018). This study was approved by the University CEU- Cardenal Herrera Ethics Committee.

Setting

This study was conducted using milk that was donated from lactating mothers residing in the Autonomous Community of Valencia and Murcia, Spain. These communities are located along the Mediterranean coast on the east side of the country. In these regions, the average socio-economic and cultural status are similar, with incomes per year about 25.000€ and high school dropout rates of 20%. Maternal age is around 32.20 years (Instituto Nacional de Estadística [INE], 2018) and breastfeeding rate at six weeks is 73.90%, dropping to 39% after 6 months (Ministerio de Sanidad, Consumo y Bienestar Social & INE, 2017). Of these two regions, only the Valencian Community has a HMB, where HM is donated by lactating mothers without receiving any type of economic compensation (Asociación Española de Bancos de Leche Humana [AEBLH], 2020).

Sample

Twenty-nine donors participated in this study. Criteria used to include the participants were: healthy lactating women of any age, with a lactation period longer than 30 days, residing in urban areas and with healthy habits (consuming mainly a Mediterranean-based diet and performing at least 30 minutes of daily exercise). Participants were excluded if any of the following criteria were met: receiving medical treatment, known addictions, restrictive diets, sleep disorders, or a lactation period shorter than 30 days. Based on the results of a previous study (Salcedo et al., 2015), sample size calculation was determined using the mean

and standard deviation (significant level = .05 and a power = 90%) (Suresh, & Chandrashekara, 2012).

Measurement

Demograhic Variables. Lactation period was defined as the postpartum time expressed in months at the time of milk expression.

AC of Milk against C. sakazakii. Based on previous studies (Silvestre, Lopez, March, Plaza, & Martínez-Costa, 2006) the method used was as follows: a strain of *C. sakazakii* (ATTC 29544, Spanish Type Culture Collection, Valencia, Spain) was used to determine the AC of HM. Bacteria were cultured overnight on Plate Count Agar (PCA) (Scharlab®, Barcelona, Spain). Some colonies were suspended in peptone water at 0.1% (Scharlab®, Barcelona, Spain) and adjusted to an absorbance of .145 at 546 nm (approximately 3 x 10^8 CFU/ml) using a Jenway 7200 spectrophotometer (reliability: ± .005A/h at .04A; validity: ± .01A/h at 1.0A). Each aliquot was prepared in duplicate by adding 0.2 ml of the bacterial solution and 0.8 ml of milk to Eppendorf tubes. For each analysis, a control sample was prepared in duplicate by mixing 0.2 ml of bacteria solution with 0.8 ml of peptone broth at 1% (Scharlab®, Barcelona, Spain). All tubes were thoroughly mixed before and after incubation at 37 ºC for 2 hr. After incubation, *C. sakazakii* counts were determined by inoculating appropriate dilutions of the contents of the Eppendorf tubes on PCA, in duplicate and incubating at 37 ºC for 48 hr, according to the standards for the enumeration of microorganisms established by the International Organization for Standardization (ISO, 2013) (reliability: ± .25 $\log_{10}$ microorganisms/ml; validity: ± .45 $\log_{10}$ microorganisms/ml).

Stability of the AC of Milk against C. sakazakii. The AC of milk samples subjected to different conservation and hygienization treatments was determined according to the previously described method for fresh HM.

Data Collection

Samples were collected between April and July 2018 and analyzed in batches of 5-8 samples. All experimental analyses were completed before October 2018. The principal investigator (SF) personally approached the eligible participants and invited them to complete a questionnaire. Written informed consent was obtained for all women chose to enroll in the study. Recruitment of milk donors, collection of milk samples and microbiological analysis were performed by the same author. To maintain the participants' confidentiality and anonymity, a reference code for each milk sample and its respective questionnaire was generated at the beginning of the study. To keep data secure, all information was stored in an external hard drive protected with password.

Milk expression was performed employing an electric breast pump attached to a sterile bag with a hermetic seal, ensuring complete milk expression from one breast. All milk samples were labeled indicating the date of expression and were transported refrigerated (temperature ≤ 4 ºC) until arrival to the laboratory (< 24 hr). Each sample was divided into eight aliquots containing no less than 2 ml of milk to ensure that analysis in duplicate was possible in all of them. To prevent microbial cross-contamination, sterile glass tubes were used for all aliquots.

The aliquots corresponding to fresh HM (A) were analyzed immediately and the aliquots F were analyzed after being treated with Holder pasteurization (heated at 62.5 ºC for 30 min). The rest of the aliquots were analyzed according to the treatment process: B) refrigeration at ≤ 4 ºC for 72 hr, C) frozen at –20 ºC for one month and thawing at 4 ºC during 24 hr, D) frozen at –20 ºC for two months and thawing at 4 ºC during 24 hr, E) frozen at –20 ºC for three months and thawing at 4 ºC during 24 hr, G) application of the global treatment that is performed in HMBs (frozen at –20 ºC for one month, thawing at 4 ºC during 24 hr, Holder pasteurization, re-frozen at –20 ºC for two months and thawing at 4 ºC during 24 hr), and H) global HMBs treatment with subsequent addition of bovine fortifier in

accordance with the manufacturer's specifications (PreNAM-FM 85, Nestlé®, Vevey, Switzerland). The overall treatment process applied to aliquots G and H can be seen in Figure 1.

Data Analysis

Demographic Variables. To evaluate the influence of the lactation period on the AC of HM subjected to different treatments, two specific postpartum time frames were established (1.5 to 6 months (n = 13) and 6 to 12 months (n = 16)).

AC of Milk against C. sakazakii. The AC was calculated as the difference between the average of *C. sakazakii* counts present in the controls (Ncontrol) and fresh milk (Nfresh milk) samples. The results were expressed as the percentage of reduction in the counts of *C. sakazakii* in fresh milk relative to those obtained in the controls.

$$\text{AC (\%)} = \frac{(\text{Ncontrol} - \text{Nfresh milk})}{\text{Ncontrol}} \text{x } 100$$

Stability of the AC of Milk against C. sakazakii. The AC of milk subjected to different treatments was calculated using the above formula and substituting the Nfresh milk value for the average counts of *C. sakazakii* in treated samples. To quantify the loss of AC in treated milk, the AC difference between fresh milk and treated milk was calculated. To express the results as the percentage loss of AC this difference was divided by the AC of fresh milk and multiplied by 100.

$$\text{\% Loss of AC} = \frac{(\text{AC of fresh milk} - \text{AC treated milks})}{\text{AC of fresh milk}} \text{x } 100$$

Two authors (MCL-M and DS) performed the statistical analysis. Two-way analysis of variance (ANOVA) was performed. The two factors considered were lactation period (between-subjects factor, with two levels (between 1.5 and 6 months postpartum or between 6 and 12 months postpartum)) and treatment (within-subjects factor, with eight levels (fresh milk, refrigeration for 72 hr, freezing for one month, freezing for two months, freezing for three

months, pasteurization, and global HMB treatment with and without added bovine fortifier)). Post hoc analysis with a Bonferroni adjustment test was used to determine the differences between groups.

Data were examined for normality using the Kolmogorov-Smirnov test ($p > .05$). The homogeneity of variance assumption between-subjects (lactation period) and within-subjects (treatment) was examined using Levene's test ($p > .05$) and Mauchly's test of sphericity ($p > .05$), respectively. Because the assumption of sphericity was violated, $\chi^2(2) = 82.240$, $p < .001$, a multivariate correction was applied.

All statistical analyses were performed using SPSS (IBM Corporation, Armonk, New York, United States of America) software. Statistical differences were considered significant at $p < .05$.

Results

Demographic Characteristics of the Sample

The following characteristics were common to all donors: nationality (Spanish), white race, residing in urban areas, medium level of education and socioeconomic status, ages (between 29 and 41 years), and number of children (between one and three). There was no significant association between the lactation period and the AC of HM subjected to treatment ($F(1, 14) = .52$, $p = .42$).

AC of Milk against *C. sakazakii*

All fresh milk samples had AC against *C. sakazakii* (mean, standard deviation, and maximum and minimum values are presented in Table 1).

Stability of the AC of Milk against *C. sakazakii*

The two-way ANOVA showed that there was a statistically significant difference in the AC values of milk subjected to different treatments ($F(7, 8) = 21.45$, $p < .001$), with a partial eta square = .949 and power = 1.000. The post hoc Bonferroni correction showed that

a statistically significant loss of AC in milk subjected to various treatments compared with fresh HM (Table 1). The quantification of loss of AC in HM per treatment compared with fresh milk are displayed in Table 2.

Refrigeration of milk at 4 ºC for 72 hr resulted in complete loss of AC in one of the samples. In reference to freezing treatment, with the exception of one sample that had been stored for three months, all samples showed AC. However, there were statistically significant differences in the AC amongst the different storage times used (one and two months (p = .038), one and three months (p = .002), and two and three months, (p = .022)). The Holder pasteurization caused a reduction in the AC in all samples. In four of them the AC was completely absent and in two of them the bacterial counts were higher than the control. The greatest AC reduction was observed in the milk subjected to HMB treatment, where twelve samples had no AC and in which nine of these bacterial growth was even promoted. Milk fortification after HMB treatment mildly improved the AC values in all samples, observing statistically significant differences between these samples and the unfortified ones (p = .048).

Discussion

Many researchers have shown that the HM has natural antimicrobial properties against several pathogenic microorganisms including *Staphylococcus aureus* (Ackerman et al., 2018); Reis et al., 2016; Schlievert et al., 2019; Zang et al., 2017), *Escherichia coli* (Jiang et al., 2016; Reis et al., 2016; Schlievert et al., 2019), *Candida* (Gunyakti & Asan-Ozusaglam, 2019), *Shigella* (Sharma et al., 2017), *Klebsiella pneumoniae* (Lorico, & Pérez, 2012), *Streptococcus* (Ackerman et al., 2017, 2018; Alamiri, Riesbeck, & Hakansson, 2019; Lin et al., 2017), and *Pseudomonas aeruginosa* (Takci et al., 2012). Although the contamination of HM with *C. sakazakii* has been previously reported (Bowen et al., 2017; McMullan et al., 2018; Sundararajan et al., 2018), to the best of the authors' knowledge this is the first study to specifically evaluate the AC against this microorganism. The results of the

present study indicated that fresh HM has natural AC against *C. sakazaki*. The authors of previous studies using the same methodology and evaluating the AC against *E. coli* showed a greater AC (> 70%) against this bacterium (Martínez-Costa et al., 2007; Silvestre et al., 2006; Silvestre, Ruiz, Martínez-Costa, Plaza, & López, 2008).

On the other hand, treatments that are commonly applied to HM may affect its antimicrobial properties to a greater or lesser degree (Guerra et al., 2018; Păduraru et al., 2018; Rodríguez-Camejo et al., 2020; Salcedo et al., 2015; Schlotterer et al., 2019). Martínez-Costa et al. (2007) and Silvestre et al. (2006) showed that refrigeration of HM for 72 hr results in significant reductions in the AC against *E. coli*, coinciding with the observation reported by the authors of the present study.

Takci et al. (2012) showed that HM has AC against *E. coli* and *P. aeruginosa* after being frozen for one and three months. In contrast to our results, the authors observed that there were no significant differences in the AC between fresh and frozen samples after one month however, the AC against *E. coli* was significantly decreased after three months. These findings suggest how the AC of HM depends on the length of time that it is frozen and the microorganism to which it is exposed. For these reasons, further studies replicating the present work with other pathogenic microorganisms (for example *S. aureus*, *Listeria monocytogenes,* or *Salmonella* spp.) or exploring other methods for conservation (e.g. lyophilization, freezing at -70 ºC) are necessary.

During the expression process, HM can be contaminated by microorganisms present on the skin or in the mother's milk ducts. Because some of them are potentially pathogenic, milk stored in HMBs is routinely subjected to heat treatment, with the most common being Holder pasteurization. While this hygienization process is necessary, the authors of a recent study (Rodriguez-Camejo et al., 2020) have shown that this treatment has a negative influence on the immunological properties of HM. This could explain the significant

reduction/complete loss of AC against *C. sakazaki* observed in this study, or even the multiplication of this microorganism in some of the samples. The authors of recent studies have compared the influence of milk pasteurization on some protective substances with that produced by other hygienization treatments, observing that the use of high temperature-short time (Aceti et al., 2019) or high hydrostatic pressures (Aceti et al., 2019; Pitino et al., 2019) preserves better these protective components. In view of this, it would be interesting determining which hygienization treatments are more convenient to maximally preserve the AC of HM.

To evaluate the influence of global treatment in HMBs (frozen at –20 ºC for one month, thawing at 4 ºC during 24 hr, Holder pasteurization, re-frozen at –20 ºC for two months and thawing at 4 ºC during 24 hr) on the AC against *C. sakazakii*, the authors adopted the methodology used in HMBs in which milk is pooled from single donors (e.g. National Institute for Health and Care Excellence [NICE], 2010). Interestingly, it was observed that the lowest values of AC occurred after applying this treatment. This finding might be explained by the summative effect caused by all the different steps involved in this treatment. It is worth mentioning that in some HMBs (e.g. Human Milk Banking Association of North America [HMBANA], 2018) milk is pooled from multiple donors prior to pasteurization. Thus, future studies evaluating the possible influence of this practice on the AC against *C. sakazakii* are warranted.

Lastly, fortification of HM is a common practice used to promote an optimum growth and development, especially in the case of premature infants (Moro et al., 2015; Steele, Czerwin, & Bixby, 2015). The authors of this study showed the positive impact of adding bovine fortifier on the AC against *C. sakazakii*. According to Schlotterer et al. (2019), the type of fortifier used influences the amount of some bioactive proteins, for example lysozyme

and IgA concentrations. In line with this, it would be also interesting evaluating the influence on the overall AC after using HM-derived fortifier.

To summarize, it is clear that future research evaluating the effect of other hygienizing, conservation and fortification treatments on the AC of HM against *C. sakazakii* is necessary. In addition, replicating this study using pooled milk from different lactating donors would be of interest. Lastly, the evaluation of the natural AC of HM against other pathogenic microorganisms represents an area of future research.

Limitations

This study has several limitations. Firstly, some of the criteria used to select the donors included in this study (e.g. diet, lifestyle, geographic region) might have had a positive or negative biological effect on milk properties and ultimately, on the AC against C. *sakazakii*. Secondly, only the AC of mature HM has been evaluated, precluding thus the possibility of drawing any conclusions regarding the longitudinal evolution of the AC against C. *sakazakii* during the early stages of lactation (colostrum and transition).

Conclusions

The authors of the present study show that HM has natural AC against *C. sakazakii* however, some of the methods that are commonly used for its preservation (refrigeration and freezing) and hygienization (Holder pasteurization) influence on its AC. If storage of HM is required it should be refrigerated for no longer than 72 hr or frozen for a short period of time. The global treatment applied in HMBs typically leads to an almost complete loss of the AC of HM, even when it is fortified.

Conflicts of Interest

All equipment used in this study was purchased with grant funds.

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All the authors of this study are affiliated to CEU-Cardenal Herrera University and all the equipment used belongs to the aforementioned institution.

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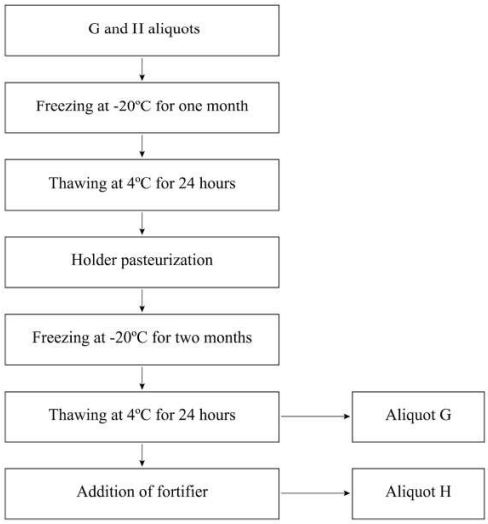

Figure 1. Treatments Applied to Aliquots G and H of Human Milk

Table 1

Comparison between the Antimicrobial Capacity against C. sakazakii of Fresh Human Milk and Treated Human Milk

Treatment	*M* (*SD*)	Minimum	Maximum	Test Statistic	*p-value*
A	47.26 (6.74)	33.48	61.03		
B	33.84 (13.84)	-2.86	58.60	15.66	**.015**
C	40.31 (9.10)	24.32	58.82	9.34	**.009**
D	35.96 (9.39)	18.63	55.82	12.59	**.000**
E	26.20 (13.55)	-4.08	54.55	25.52	**.000**
F	23.56 (15.61)	-11.17	48.88	19.21	**.000**
G	3.70 (23.83)	-61.22	37.50	49.74	**.000**
H	14.37 (18.02)	-19.38	39.40	35.58	**.000**

Note. Antimicrobial capacity (AC) was expressed as a percentage of reduction in the counts of the *C. sakazakii* with respect to the counts obtained in the controls (peptone broth); Bonferroni adjustment made at $p = .05$; A = fresh milk; B = refrigeration at ≤ 4º C for 72 hr; C = freezing at -20 ºC for one month; D = freezing at -20 ºC for two months; E = freezing at -20 ºC for three months; F = Holder pasteurization; G = human milk bank treatment; H = human milk bank treatment plus fortification; $p < .05$ are bolded and

indicates statistically significant differences between fresh milk and treated milk

Table 2

Loss of Antimicrobial Capacity against C. sakazakii of Treated Milk with Respect to Fresh Milk

Treatment	*M* (*SD*)
B	28.48 (28.68)
C	15.14 (15.27)
D	24.53 (15.55)
E	46.30 (25.17)
F	48.75 (33.76)
G	91.95 (53.56)
H	70.78 (38.34)

Note. The loss of antimicrobial capacity (AC) was expressed as loss percentage of AC against *C. sakazakii* of treated milk with respect to fresh milk. B = refrigeration at ≤ 4º C for 72 hr; C = freezing at -20 ºC for one month; D = freezing at -20 ºC for two months; E = freezing at -20 ºC for three months; F = Holder pasteurization; G = human milk bank treatment; H = human milk bank treatment plus fortification.