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DETECTION AND INACTIVATION OF *ENTEROBACTER SAKAZAKII* (CRONOBACTER) IN POWDERED INFANT MILK FORMULA

(With 5 Tables and 4 Figures)

By

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الكشف عن ميكروب الإنتيروباكتر ساكازاكى وإضعافه فى ألبان الأطفال الجافة

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يعد ميكروب الإنتيروباكتر ساكازاكى من الميكروبات الممرضة التى تهدد حياة الأطفال حديثى الولادة وحتى الشهر السادس وكذلك الأطفال ناقصى الوزن والمناعة لانه يسبب الإلتهاب السحائى وإلتهاب الأمعاء المتنكرز ولذلك أجريت هذه الدراسة للكشف عن هذا الميكروب فى ألبان الأطفال الجافة لأنها الغذاء البديل للبن الأم فى هذه المرحلة العمرية وكذلك استخدام انزيم الليسوزيم والليسوزيم المعدل بالحرارة لمعرفة مدى ملائمة استخدام هذا الإنزيم الطبيعى فى إضعاف الميكروب فى لبن الأطفال0 وقد أظهرت النتائج أن هذا الميكروب قد تم عزله بنسبة 5,1 ؛ 6,3 و 5 % من ألبان الأطفال ناقصى الوزن ، الألبان المخصصة للأطفال أقل من 6 شهور وتركيبة النمو للأطفال من عمر سنة إلى أربع سنوات بنسبة كلية 3,95% ؛ بينما لم يتم عزله من تركيبة حليب لمتابعة الرضاعة (من سن 6 أشهر)0 وقد أثبتت التجربة أن استخدام إنزيم الليسوزيم قد أثبت فاعلية لإضعاف الميكروب فى اللبن المعاد حله والمحفوظ فى الثلاجة لمدة ثلاث ساعات فقط؛ بينما إنزيم الليسوزيم المعدل بالحرارة قد أثبت فاعلية فى إضعاف الميكروب فى اللبن المعاد حله فى درجات حرارة 4، 25 و 37 درجة مئوية بنسب متفاوتة وكان أقوى تأثير مضعف للميكروب عند درجة حرارة 4 درجة مئوية0 ولهذا لابد من تضافر جهود منظمات الصحة مع مصانع انتاج ألبان الأطفال لتجنب الأخطار الناجمة عن وجود مثل هذا الميكروب وكذلك التوصية بإستخدام مثبطات الميكروبات الطبيعية مثل انزيم الليسوزيم المعدل بالحرارة لما له من فاعلية فى إضعاف ميكروب الإنتيروباكتر ساكازاكى0

SUMMARY

E. sakazakii is considered as an opportunistic bacterium in elderly people and infants. Epidemiological studies implicate dried infant formula as the primary source of transmission of this pathogen. A total of 177 powdered infant milk formula (PIMF) [39 low birth weight formula from 1 day: 6 months (LBWF), 63 infant milk formula for infant below 6 months (IMF), 55 follow-on formula from 6:12 months and 20 growing children formula

for age 1:4 years). The samples were collected from different pharmacies in Kafr El-Sheikh Governorate, Egypt, within the accurate shelf life period, and then transferred to the laboratory in their packages to be tested for detection of *E. sakazakii* and detection of the efficacy of Lysozyme and thermally modified lysozyme for inactivating *E. sakazakii* in reconstituted infant formula at different storage temperatures. The results revealed that *E. sakazakii* could be detected in 5.1, 6.3 and 5% of examined LBWF, IMF and growing children formula respectively with total percentage of 3.95%, but the organism could not be detected in follow-on formula. The results indicated that thermally modified lysozyme, was more effective than lysozyme in inhibition of *E. sakasakii* growth at 4 ℃ ($p < 0.001$), thermally modified lysozyme had more inhibitory effect on *E. sakasakii* at 4 ℃ than at 25and 37 ℃. In conclusion the combined efforts of public health and regulatory officials, as well as manufacturers, were considered important aspects of the management of risks associated with disease causing *E. sakazakii* in PIMF, also the uses of thermally modified lysozyme can exert a significant inhibitory activity against this organism in reconstituted milk formula specially when kept at refrigeration temperature.

Key words: *Powdered infant milk formula, E. sakazakii, lysozyme, thermally modified lysozyme*

INTRODUCTION

Powdered infant milk formula (PIMF) constitutes the majority of infant formula fed to infants' worldwide (Drudy *et al.*, 2006). This product is formulated to mimic the nutritional profile of human breast milk (Breeuwer *et al.*, 2003). PIMF is not a sterile product and can act as a potential source of harmful pathogens. In addition, infants and young children do not have a well developed immune system and hence are more vulnerable to food-borne infections. Therefore the microbiological safety of the infant and follow-up formula is critical. To assure the microbiological safety of PIMF, several microbiological tests are recommended and compared with the microbiological criteria set by the Codex Alimentarius Commission (CAC, 1979). The specific microbes commonly tested include *Staphylococcus aureus*, *Bacillus cereus*, *Enterobacter sakazakii (E. sakasakii)*, other Enterobacteriaceae and *Salmonella*e (Forsythe, 2005). Among the specific microbes tested for presence in infant milk formula, *E. sakazakii* is placed under category A by FAO–WHO (FAO/WHO, 2004) and is considered to be a potential agent for causing neonatal infections.

E. sakazakii, is a motile, non-sporeforming Gram-negative facultative anaerobe. It is considered as an opportunistic bacterium in elderly people and infants. While infections in adults are often underreported, in neonates and infants it is often occur as severe infection with a reported case fatality rate of 40–80% (Bowen and Braden, 2006). It is causing a rare, but life threatening form of neonatal meningitis, bacteremia, necrotizing colitis and meningo-encephalitis (Nazarowec-White and Farber, 1997a; Sanders and Sanders, 1997; Van Acker *et al.,* 2001). In addition to the high fatality rate of *E. sakazakii* infections, it may result in severe neurological sequelae such as hydrocephalus, quadriplegia and retarded neural development in survivors (Forsythe, 2005). Although the environmental source of *E. sakazakii* is not clearly understood, epidemiological studies implicate PIMF as the primary source of transmission (Van Acker *et al.,* 2001; Weir, 2002).

The bacterium has been isolated from PIMF by numerous investigators (Biering *et al.,* 1989; Simmons *et al.,* 1989; Muytjens and Kollee, 1990). Moreover; there were many recalls of *E. sakazakii*-contaminated infant formula in the United States. In November 2002, a nationwide recall of more than 1.5 million cans of dry infant formula contaminated with *E. sakazakii* was reported (FSNET, 2002). On April 12, 2002, the United States Food and Drug Administration (FDA) issued an alert to U.S. health care professionals regarding the risk associated with *E. sakazakii* infections among neonates fed PIMF (FDA, 2002). In addition, the International Commission on Microbiological Specification for Foods (ICMSF, 2002) has ranked *E. sakazakii* as 'Severe hazard for restricted populations, life-threatening or substantial chronic sequelae of long duration'. The FAO/WHO (2004; 2006 and 2008) recommended that research should be promoted to gain a better understanding of ecology, taxonomy, virulence and other characteristics of Cronobacter.

Being a nutrient-rich medium, reconstituted PIMF can support bacterial growth when favorable conditions of water availability, time and temperature are provided. Therefore once rehydrated the only limiting conditions for bacterial growth and infection are storage time and temperature. In this regard, *E. sakazakii* possesses several characteristics that enable it to be a successful infant formula-borne pathogen, Breeuwer *et al.* (2003) revealed that *E. sakazakii* has a high tolerance to osmotic stress and desiccation. *E. sakazakii* can grow at temperatures as low as 5.5 °C (Nazarowec-White and Farber, 1997b), which has been reported to be the temperature of many home refrigerators. Improper storage of reconstituted formula may permit its substantial growth. Therefore, incorporation of an effective antimicrobial barrier may potentially reduce

the likelihood of outbreaks of *E. sakazakii* infection in infants through ingestion of contaminated reconstituted infant formula.

In recent years, there has been an increasing interest in the use of natural antimicrobial substances due to concerns regarding the safety of synthetic compounds (Abee *et al.,* 1995). This is especially significant when selecting antimicrobials for use in infant foods. Lysozyme is part of the innate immune system, however, reduced lysozyme levels have been associated with broncho-pulmonary dysplasia in newborns (Revenis and Kaliner, 1992). Children fed infant formula lacking lysozyme in their diet have three times the rate of diarrheal disease. Since lysozyme is a natural form of protection from pathogens like *Salmonella*, *E.coli*, and *Pseudomonas*, a deficiency due to infant formula feeding can lead to increased incidence of disease (Lonnerdal, 2003). Lysozyme found in egg white, tears, and other secretions. It is responsible for breaking down the polysaccharide walls of many kinds of bacteria thus it provides some protection against infection. Investigations conducted by Ibrahim *et al.* (1996) indicated a possibility of extending the range of lysozyme activity to include Gram-negative bacteria, using thermal modification. It has also been found that heat denaturation of lysozyme caused by increasing temperatures results in the progressive loss of enzymatic activity, while its antimicrobial action against Gram-negative bacteria is greatly enhanced.

The aim of this work was planned to detect *E. sakazakii* in PIMF and the efficacy of lysozyme and thermally modified lysozyme for inactivating *E. sakazakii* in reconstituted infant formula at different storage temperatures.

MATERIALS and METHODS

1. Isolation and identification of *E. sakazakii*

1.1. Collection of samples

One hundred and seventy seven PIMF [39 low birth weight formula from 1 day: 6 months (LBWF), 63 IMF for infant below 6 months, 55 follow-on formula from 6:12 months and 20 growing children formula for age 1:4 years). The samples were collected from different pharmacies in Kafr El-Sheikh Governorate, Egypt, within the accurate shelf life period, and then transferred to the laboratory in their packages to be tested for detection of *E. sakazakii*.

1.2. Preparation of samples

Twenty-five grams of each sample after sterilization and opening of the cans were homogenized for 1 min at medium speed in a Seward

Stomacher (Seward, Thetford, UK) in 225 ml buffered peptone-water (CM 509 Oxoid Ltd.) and incubated overnight at 37°C (ISO 8261, 2001).

1.3. Isolation of *E. sakazakii* procedure (ISO/TS 22964, 2006)

To 10 ml Cronobacter Screening Broth (CSB) (CM1121 Oxoid Ltd.) 0.1 ml of pre-enrichment BPW was added and incubated at 41.5 °C for 24 h. From yellow colored broth tube 10 µl were streaked onto the surface of Brilliance *Enterobacter sakazakii* Agar (DFI) (CM 1055 Oxoid Ltd.) and colony morphology observed after incubation at 44°C for 24 h. Blue green colonies were picked off and streaked on Tryptone soy agar (TSA) (CM131 Oxoid Ltd.). Colonies that produced yellow pigment after incubation at 25°C for 48–72 h were termed presumptive *E. sakazakii*.

1.4. Identification of presumptive *E. sakazakii*

Biochemical identification of presumptive *E. sakazakii* was done according to Farmer and Kelly (1992).

2. Inactivation of *E. sakazakii*

2.1. Bacterial strain preparation

E. sakazakii isolate was cultured in 10 ml of sterile Tryptic soy broth (TSB) at 37°C for 20 h. The bacterial population was determined by pour plate technique after preparation of serial dilutions according to APHA (1992). Loopfuls from each dilution were streaked on previously prepared TSA plate and then the plates were incubated at 37℃ for 24 h, the colonies forming unite / ml was calculated.

2.2. Preparation of antimicrobials

Egg white lysozyme (BioShop, Canada Inc.) stock solution of 1mg/ml in potassium phosphate buffer (10 mM, pH 7.0) was prepared (Barbara *et al.,* 2000) and divided into two parts, one part was kept frozen at -20 ℃ and the other part modified thermally by heating at 80 ℃ for 20 min (Ibrahim *et al.,* 1996).

2.3. PIMF preparation

PIMF was reconstituted as per the manufacturer's instructions on the label. Briefly, 135 g of the formula were reconstituted in 900 ml of sterile distilled water; 100 ml volumes were dispensed into screw capped bottles (9) and pasteurized at 63℃ for 30 min.

2.4. Inoculation, incubation and determination of antibacterial activity

To the bottles 2, 5 and 8 lysozyme solution was added, and to bottles 3,6 and 9 thermally modified lysozyme (denaturated) solution was added in final concentration 50µg/ml (1000 U) of reconstituted IMF. Bottles 1, of 4 and 7 were control (devoid of any antibacterial agent). *E. sakazakii* was added to the nine bottles in final count $2x10^6$/ ml of reconstituted IMF.

Bottles 1, 2 and 3 were incubated at 4ºC, while bottles 4, 5 and 6 were incubated at 25ºC and bottles 7, 8 and 9 were incubated at 37ºC for 0, 3, 6, 9, 12 and 24 h. The surviving populations of the pathogen were enumerated by plating after serial dilutions (1:10) on triplicate TSA after incubation at 37ºC for 24 h.

3. Statistical analysis

Data were analyzed using General Linear Models procedures after log transformation. Least square means were computed for each treatment and group differences were tested using Bonferroni test. All experiments and analyses were replicated 3 times (SAS Institute, 1999).

RESULTS

Table 1: Incidence of *E. sakazakii* in examined powdered infant milk formula.

Types of samples	No. of examined samples	*E. sakazakii* positive samples	
		No	%
Low birth weight formula	39	2	5.1
PIMF for infant below 6 months	63	4	6.3
Follow-on formula from 6:12 months	55	0	0
Growing formula for age 1:4 years	20	1	5
Total	177	7	3.95

Table 2: Effect of lysozyme and thermally modified lysozyme on *E. sakazakii* count incubated at 4ºC.

Groups	Mean log count ±SE					
	0*	3	6	9	12	24
Control	14.26± 0.15^{a}	14.41±0.32 ^{a}	17.12±0.56^{a}	18.32±0.32^{a}	19.17±0.17^{a}	20.02±0.11^{a}
Lysozyme	13.60±0.51 ^{a}	11.74±0.46^{b}	13.73±0,26^{b}	14.12±0.31^{b}	15.40±0.17^{b}	16.52±0.07^{b}
Thermally modified lysozyme	13.76±0.43 ^{a}	2.95±0.37^{c}	5.90±0.32^{c}	6.58±0.65^{c}	9.08±0.39^{c}	11.51±0.06^{c}

Means in the same column without a common letter differ significantly (p<0.01).
*No significance difference p = 0.41

Table 3: Effect of lysozyme and thermally modified lysozyme on *E. sakazakii* count incubated at 25ºC.

Groups	Mean log count ±SE					
	0*	3	6	9	12	24
Control	14.11± 0.62[a]	15.91±0.51 [a]	17.70±0.43[a]	20.00±0.025[a]	24.93±0.15[a]	28.27±0.38[a]
Lysozyme	13.41±1.10 [a]	16.00±0.34[ab]	17.18±0.20[ab]	18.37±0.05[b]	20.49±0.46[b]	25.23±0.32[b]
Thermally modified lysozyme	13.78±0.77 [a]	9.11±0.32[c]	12.99±0.07[c]	13.57±0.15[c]	17.67±0.25[c]	22.81±0.06[c]

Means in the same column without a common letter differ significantly ($p<0.01$).
*No significance difference $p = 0.85$

Table 4: Effect of lysozyme and thermally modified lysozyme on *E. sakazakii* count incubated at 37ºC.

Groups	Mean log count ±SE					
	0*	3	6	9	12	24
Control	14.06±0.63[a]	16.65±1.13 [a]	18.13±0.55[a]	20.03±1.01[a]	23.85±1.60[a]	29.71±0.07[a]
Lysozyme	13.29±0.89 [a]	17.06±0.63[ab]	17.99±0,63[ab]	18.71±0.41[ab]	22.59±0.63[ab]	27.34±0.55[b]
Thermally modified lysozyme	13.34±0.99 [a]	11.65±0.52[c]	13.45±0.37[c]	13.95±0.52[c]	18.18±0.15[b]	22.81±0.33[c]

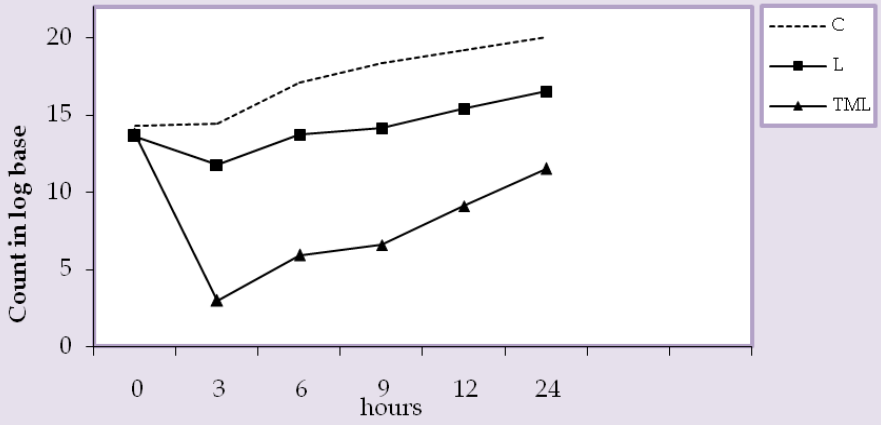
Means in the same column without a common letter differ significantly ($p<0.01$).
*No significance difference $p = 0.78$

Table 5: Effect of lysozyme and thermally modified lysozyme on *E. sakazakii* count at different incubation temperature throughout the experiment.

Groups	Mean log count ±SE		
	4º C	25º C	37º C
Lysozyme	14.19±0.38[a]	18.45±0.92[c]	19.50±1.10[c]
Thermally modified lysozyme	8.30±0.88[b]	14.99±1.05[d]	15.56±0.94[e d]

Means in the same column without a common letter differ significantly ($p = 0.03$).
Means in the same row without a common letter differ significantly ($p = 0.02$).

Fig. 1: Effect of lysozyme and thermally modified lysozyme on *E.sakasakii* count incubated at 4°C.



C: control
L: Lysozyme
TML: Thermally modified lysozyme

Fig. 2: Effect of lysozyme and thermally modified lysozyme on *E.sakasakii* count incubated at 25°C.

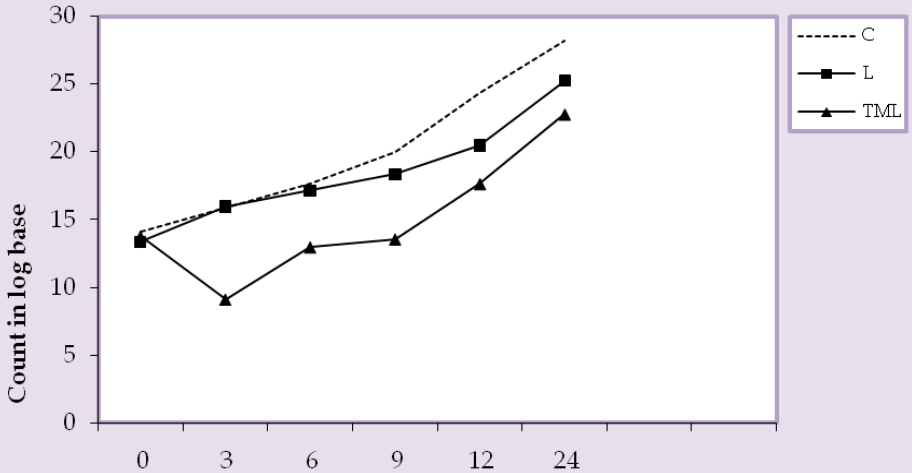


Fig. 3: Effect of lysozyme and thermally modified lysozyme on *E.sakasakii* count incubated at 37°C.

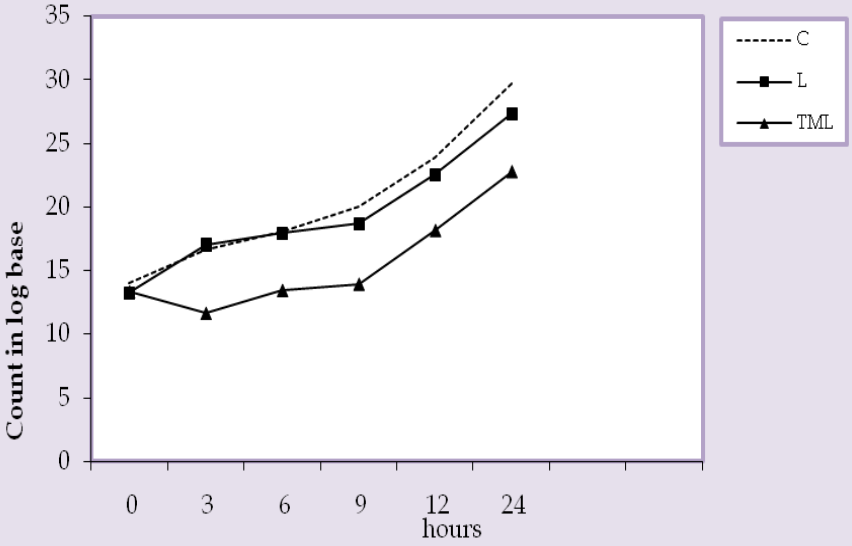
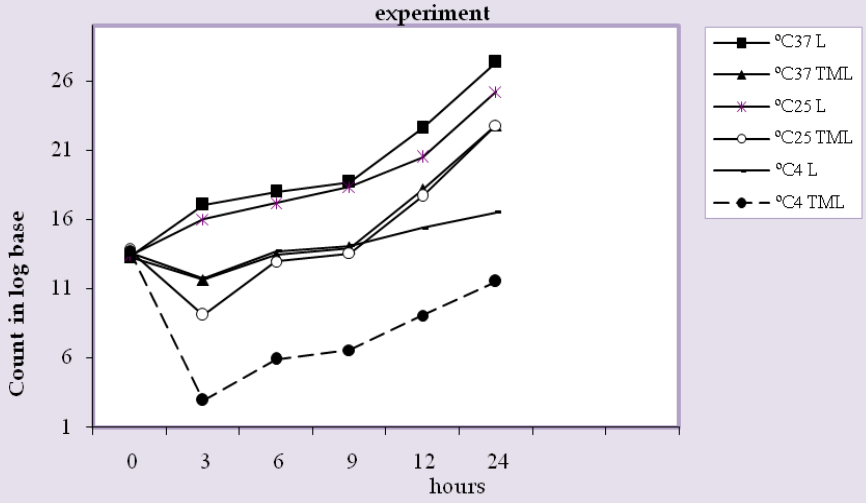


Fig. 4: Effect of lysozyme and thermally modified lysozyme on E. sakasakii count at different incubation temperature throughout the experiment



DISCUSSION

In Europe and the United State, several studies have reported cases of *E. sakazakii* infection in infants taking a PIMF, raising an important issue. This study was focused on PIMF in response to the FAO/WHO (2008) call for appropriate microbiological data. Results given in Table 1 pointed out that the rate of contamination with *E. sakazakii* was 5.1, 6.3 and 5% of the examined LBWF, IMF (for age < 6 months) and growing formula, respectively with a total percentage of 3.95%, while the organisms could not be detected in follow-on formula. Concerning prevalence in powdered formulae, some reported prevalence figures in positive batches were about 1 and 3% (FAO/WHO, 2008), and nearly similar results were reported by Yoo *et al*. (2005); Chap *et al*. (2009) and Oonaka *et al*. (2010). Presence of *E. sakazakii* in PIMF may be attributed to inadequate hygiene in factory or contamination of raw materials.

Cronobacter spp. infections have been reported for all age groups. Worldwide there have been 120 reported cases in infants and children < 3 years in age, of which 8 were cases aged between 6 and 35 months. In the UK between 1999 and 2007, 15/570 laboratory reported infections were from infants (< 12 months in age), and 16/570 were from children (1–4 years in age) (FAO/WHO, 2008). Risk for infection might depend on several factors, including the number of bacteria present in the product, handling after preparation, and underlying patient characteristics (e.g., immune-suppression, prematurity, or low birth weight). Because powdered formula is not sterile and can provide a good medium for growth, prolonged periods of storage or administration at room temperature might amplify the amount of bacteria already present.

FDA (2002) recommended that powdered infant formulas not be used in neonatal intensive care settings unless there is no alternative available. If the only option available to address the nutritional needs of a particular infant is a powdered formula, risks of infection can be reduced by preparing only a small amount of reconstituted formula for each feeding to reduce the quantity and time that formula is held at room temperature for consumption; minimizing the holding time, whether at room temperature or while under refrigeration, before a reconstituted formula is fed; and minimizing the "hang-time" (i.e., the amount of time a formula is at room temperature in the feeding bag and accompanying lines during enteral tube feeding), with no "hang-time" exceeding 4 hours. Longer times should be avoided because of the potential for significant microbial growth in reconstituted infant formula.

Natural antimicrobials have gained attention because of the demand for preservative-free food products (Payne *et al.,* 1990). Included as natural antimicrobial is lysozyme enzymes that can inhibit the growth of various intestinal pathogens and which can protect children against gastroenteritis (Lonnerdal, 2003).

The growth of *E. sakazakii* was evaluated after treatment with lysozyme and thermally modified lysozyme in reconstituted milk formula and incubated at 4, 25 and 37 ºC. The Effect of lysozyme and thermally modified lysozyme on *E. sakasakii* count in reconstituted milk formula incubated at 4ºC throughout the experiment was presented in Table 2 and Figure 1. The results declared that there was no significant difference between control, lysozyme and thermally modified lysozyme treated samples at zero time ($p = 0.41$), while there were significant difference between the three treatments from 3h to 24h ($p < 0.01$), at 3h of incubation the population of *E. sakazakii* was reduced to 11.74±0.46 log base CFU/ml by lysozyme and to 2.95±0.37 log base CFU/ml by thermally modified lysozyme. At the end of the 24 h, the count of *E. sakasakii* in the sample containing lysozyme reached to 16.52±0.07 log base CFU/ml which is 1.2 times than the initial count whereas that containing thermally modified lysozyme had 11.51±0.06 log base CFU/ml which is less than the initial count (13.76±0.43 log base CFU/ml). In the control sample devoid of neither lysozyme nor thermally modified lysozyme, the pathogen grew, reaching a final population of 20.02±0.11 log base CFU/ml. These results indicate that thermally modified lysozyme, was more effective than lysozyme in inhibition of *E. sakazakii* growth at 4 ºC ($p < 0.001$).

Table 3 and Figure 2 show the effect of lysozyme and thermally modified lysozyme on *E. sakasakii* count in reconstituted milk formula incubated at 25ºC throughout the experiment and revealed that there was significant difference between lysozyme and thermally modified lysozyme treated samples ($p < 0.01$), lysozyme had no antibacterial effect on *E. sakazakii* throughout the experiment. While at 3h and 6h thermally modified lysozyme reduced the pathogen count by 9.11±0.32 and 12.99±0.07 log base CFU/ml. At 9h of incubation the count reach approximately the initial count (13.57±0.15 log base CFU/ml), at 24 h of incubation the count reached 22.81±0.06 log base CFU/ml which is 1.7 times than the original count. In the control sample devoid of neither lysozyme nor thermally modified lysozyme, the pathogen grew, reaching a final population of 28.27±0.38 log base CFU/ml. These results indicate that

thermally modified lysozyme, was effective in inhibition of *E. sakazakii* growth at 25 ºC till 6 h of storage.

The Effect of lysozyme and thermally modified lysozyme on *E. sakazakii* count in reconstituted milk formula incubated at 37ºC throughout the experiment is presented in Table 4 and Figure 3 and revealed that there was significant difference between lysozyme and thermally modified lysozyme treated samples (p <0.01), lysozyme had no antibacterial effect on *E. sakazakii* throughout the experiment. While at 3h thermally modified lysozyme reduced the pathogen count by 11.65±0.52 log base CFU/ml. At 6h of incubation the count reach approximately the initial count (13.45±0.37 log base CFU/ml), at 24 h of incubation the count reached 22.81±0.33 log base CFU/ml which is 1.7 times than the original count. In the control sample devoid of neither lysozyme nor thermally modified lysozyme, the pathogen grew, reaching a final population of 29.71±0.07 log base CFU/ml. These results indicate that thermally modified lysozyme, was effective in inhibition of *E. sakazakii* growth at 37 ºC till 3 h of storage.

There were significant differences between lysozyme and thermally modified lysozyme treated samples at different incubation temperatures (p <0.03). There were significant differences between lysozyme treated samples incubated at 4 ºC and lysozyme treated samples incubated at both 25 and 37 ºC, also there were significant differences between thermally modified lysozyme treated samples incubated at 4 ºC and thermally modified lysozyme treated samples incubated at both 25 and 37 ºC (Table 5 and Figure 4). The results indicated that the thermally modified lysozyme had more inhibitory effect on *E. sakazakii* at 4 ºC than at 25 and 37 ºC.

Heat denaturation of lysozyme resulted in the progressive loss of enzymatic activity, but a greatly improved antimicrobial action towards Gram-negative bacteria through membrane perturbation (Ibrahim, 1998). The possibility to extend the range of lysozyme activity to include Gram-negative bacteria i.e. *E. coli*, is offered by the thermal and chemical-thermal modification, which leads to the formation of an enzyme preparation with increased content of polymeric forms (Lesnierowski *et al.*, 2004).

The antimicrobial action of lysozyme was due to structural factors. Specific bactericidal domain may be involved in the antimicrobial action of lysozyme (Düring *et al.*, 1999; Ibrahim, 1998 and Ibrahim, 2003).

The inhibitory effect of thermally modified lysozyme on *E. sakazakii* at 4 ℃ was more than at 25and 37 ℃ this may be attributed to the long generation time of the pathogen at refrigeration temperature (4.98h) than at room temperature (40 min.) and at 37 ℃ (24 min) (Nazarowec-White and Farber, 1997a; Pagotto and Farber, 2009).

The results of this study indicate that despite the fact that formulas are exposed to heat treatment during processing *E. sakazakii* was still isolated from these products. The combined efforts of public health and regulatory officials, as well as manufacturers, were considered important aspects of the management of risks associated with disease causing *E. sakazakii* in PIMF, also the uses of thermally modified lysozyme can exert a significant inhibitory activity against this organism in reconstituted milk formula specially when kept at refrigeration temperature.

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