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INHIBITORY EFFECT OF LACTOFERRIN AGAINST *CRONOBACTER SAKASAKII* ISOLATED FROM INFANT FORMULA MILK POWDER

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ABSTRACT

In this study the antibacterial activity of lactoferrin against *Cronobacter sakasakii* (C. *sakasakii)* as a foodborne pathogen was investigated. The bovine lactoferrin (bLf) was isolated and purified from bovine colostrum by FPLC chromatography and visualized using (SDS-PAGE). Purification efficiency was 90%. *C. sakasakii* was isolated from a total of 100 samples of infant formula milk powder (IFMP) samples collected randomly from Qena city groceries and supermarkets using HiCrome *Enterobacter sakazakii*agar followed by biochemical testing. *C. sakasakii* was detected in 21% of the total samples examined. The antibacterial effect of bLF on *C. sakasakii* isolates was tested via performing disk diffusion method using Mueller–Hinton agar. The results revealed that bLF at concentration of 10mg/ml showed the maximum inhibitory effect whereas the least inhibitory effect was recorded at concentration of 1mg/ml. The results indicated that bLF may be useful for inhibition of *C. sakasakii* in infant formula through supplementation or fortification. More attention should be paid during manufacture and handling of IFMP.

Key words: *C. sakazakii*, Lactoferrin, Antibacterial activity, Infant formula milk powder.

INTRODUCTION

Lactoferrin is an 80 kDa iron binding glycoprotein of the transferring family. Lactoferrin is a major component of milk and presents in neutrophil granules or other exocrine secretions such as tears and saliva. It is found in concentration up to 1.5 mg/ml in bovine colostrum (Yekta *et al.,* 2010). Lactoferrin is an important host defence molecule and has diverse physiological functions such as antibacterial, antiviral and anticancer activities.

Many studies have demonstrated the bacteriostatic and bactericidal effect of Lactoferrin, against a wide range of Gram-positive and negative bacteria (Farnaudand Evans, 2003). Lactoferrin inhibits bacterial pathogens by a direct interaction mediated by binding of the lipid A portion of the lipopolysaccharide (LPS) of Gram-negative bacteria (Brandenburg *et al.,* 2001). The expanding demand for adding Lactoferrinto the products due to its nutritional values and physiological benefits has incentivized research workers to find much more simple and economic ways to isolate and purify Lactoferrin.

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C. sakazakii (formerly known as *Enterobacter sakazakii)* is a Gram negative rod, motile facultative anaerobic bacterium (Iversen *et al.,* 2008).*C. sakazakii* have associated with severe forms of necrotizing and meningitis especially in neonates with mortality rate varies from 40 – 80% (Healy *et al.,* 2010) and the infective dose is estimated to range from 10^3 to $\geq 10^8$ cells (Pagotto *et al.,* 2003).

*C. sakazakii*has been isolated from wide range of dairy products (Ye *et al.,* 2014). Moreover, powdered infant formula has been epidemiologically linked to *Cronobacter* infections in infants (Healy *et al.,* 2010 and Sani *et al.,* 2014). Much research has focused on the presence of *C. sakazakii* in baby foods. They have been isolated from various infant foods including; powdered infant formula (PIF), herbs and cereals (Sani *et al.,* 2014; Parra *et al.,* 2015 and Li *et al.,* 2016).

Due to immature immune system of infants, researcher have tried to prevent contamination of baby foods with *C. sakazakii* by irradiation (Osaili *et al.,* 2007), adding probiotic bacteria (Osaili *et al.,* 2008) and control it, if exist, in infant foods by plant essential oil (Al-Nabulsi *et al.,* 2015). Few attempts have been carried out to investigate the inhibition effects of bovine lactoferrin on the growth of this pathogen. Therefore, the objective of the current study was to isolate and purify lactoferrin from bovine colostrum and to investigate its inhibitory effects on the growth of *C. sakazakii* isolated from IFMP.

MATERIALS AND METHODS

Isolation and Purification of bovine Lactoferrin (bLF)

Bovine colostrum samples were collected within the first day after cow parturition from South Valley University dairy farm. Isolation of bLF was done as described by (Yoshida *et al.,* 2000). Lactoferrin was purified by carboxymethyl Sephadex-C50 chromatography (FPLC, Bio-RAD, USA). The stock solution of bLF was prepared with sterile distilled water to give a final concentration of 10 mg/ml and filter sterilized using 0.22 µm membrane filters. After filtration, the absorbance was monitored by ultraviolet absorption at 280 nm and the concentration of bLF was calculated as recommended by (Yoshida *et al.,* 1999). The final concentration of bLF solutions was adjusted to 1, 2, 5 and 10 mg/ml. The purity of bLF was compared with lactoferrin standard (Sigma-Aldrich, Beijing, China) using (SDS-PAGE) [sodium dodecyl sulphate-polyacrylamide gelelectrophoresis] as described by (Harouna *et al.,* 2015).

Isolation and identification of *C. sakasakii*

Collection of samples:

A total of 100 powdered infant formula milk (IFMP) (Recommended for infants from birth to 1 year old), were collected from different localities in Qena city then transferred to the laboratory to be examined. The samples were prepared according to FDA (2002) for *C. sakazakii* detection isolation.

IFMP were pre-enriched by reconstitution in sterilized, distilled water (10g sample/90 ml sterile distalled water) and incubated at 36°C for 24 h, 1 ml of the pre-enrichment culture was inoculated into 9 ml of the Enterobacteriaceae Enrichment Broth (EEB), which was then incubated for 24h at 36°C. 10 µl of the incubated broth was then streaked on the surface of chromogenic media (HiCrome *Enterobacter sakazakii*agar) (Oxoid M1577). Suspect blue-green colonies were observed after incubation at 36°C for 24h. Further identification of the isolated *C. sakasakii* was done according to FDA (2002) and Iversen *et al.* (2008).

***C. sakasakii* culture preparation (Harouna *et al.*, 2015)**

C. sakasakii isolated from IFMP in this study was kept in (EEB) then incubated for 24h at 36°C. One ml of the inoculated EEB was added to 9 ml of 1% peptone water and decimal dilutions in 1% peptone water were used to yield a suspension of 10^4 CFU/ml for antibacterial activity assays.

Antibacterial activity assay (CLSI, 2011)

Antibacterial activity of bLF was done by disc diffusion method using Mueller–Hinton agar (Oxoid) according to the recommendation of the Clinical Laboratory Standards Institute (CLSI, 2011). bLF discs were prepared according to Barry (1976) in which empty sterilized discs (What man no. 6 mm diameter) were impregnated with 50 µL per disc with different bLF concentrations (1, 2, 5 and 10 mg/ml). The discs were placed on and swabbed over the surface of the plates that inoculated with 50 µl of the previously prepared inoculum (10^4CFU/ml) then, were incubated for 24 h at 36°C. The susceptibility of *C. sakasakii* was determined by measuring the zone of growth inhibition around the discs. Inhibition of bacterial growth in the plates containing tested bLF was judged by comparison with growth in blank control plates without bLF discs (Harouna *et al.,* 2015).

DISCUSSION

Lactoferrin from bovine colostrum has become increasingly important because of its diverse range of biological activities, such as anti-infective activities toward a broad spectrum of species. Therefore it was important to isolate and purify lactoferrin from bovine colostrum. The purity of bLF in this study was checked by SDS-PAGE, which showed a single band corresponding to a protein of about 80 KDa and the purification efficiency was about 90% (photo 1). Moradian (2014) and Harouna *et al.* (2015) obtained the same purity. A higher purity of 91.3 % was recorded by Yafei *et al*. (2011) using SPEC 70 SLS cation exchange resin. Lower bLF purity of 87% by SP Sepharose Big Bead ion exchange column was obtained by Kong *et al.,* (2012). However the same authors recorded a purity estimated to be >95% using SDS-PAGE. From the above mentioned results it could be concluded that the stated method result in isolation of highly pure bLF indicating simple, low-cost and efficient method on preparation of bLF without loss its bioactivity, as compared with other previous methods of purification of lactoferrin.

Regarding the Incidence of *C. sakazakii* in IFMP, it was found that 21% of the total (100) IFMP samples examined were contaminated with *C. sakazakii* (Table 1) higher results of 24% and 23% were detected by El-Gamal *et al.* (2013) and Li *et al.* (2016), respectively as they use the same isolation media (HiCrome *Enterobacter sakazakii* agar) and using the FDA enrichment procedure.

The current results were higher than the previous studies by Iversen and Forsythe (2004), they isolated *C. sakazakii* from 2.4% out of 82 analyzed samples of IFMP. Oonaka *et al*. (2010); Shetty *et al*. (2011); Fu *et al*. (2011) could detect *C. sakazakii* in 6.6% (9/149), 5.4% (11/202) and 3.9% (3/77) IFMP samples, respectively. However, Sani and Yi (2011)

and Putthana *et al*. (2012) did not detect any positive samples in 390, 30 and 7 IFMP evaluated, respectively. The other *C. species* was not detected in the examined IFMP samples.

According to the current and previous studies, there was a direct relationship between IFMP and *C. sakazakii*, despite the fact that formulas are exposed to heat treatment during processing. That means post-pasteurization contamination of IFMP with *C. species* may occur via the addition of dry ingredients (as vitamins and minerals) or during packaging. However, the prevalence of the organism following the drying and survival in powdered foods for a long time may be partially due to the organism's ability to resist desiccation and osmotic stress (Arku, 2008). Therefore, hygienic measures and practices must be applied during the manufacture of formula to minimize entry of contaminants into the process.

Antibacterial activity is a biological function attributed to Lf (Farnaud and Evans, 2003). The mechanisms that account for the antibacterial properties have been reported to be iron dependent and iron independent (Orsi, 2004); the latter implies direct interaction of Lf with the bacterial cell surface (Brandenburg *et al*., 2001).

Regarding the antibacterial effect of bLf at different concentrations (1, 2, 5 and 10 mg/ml) on *C. sakazakii* growth, the results indicated that bLF at concentration of 1mg/ml had the least inhibitory effect whereas maximum inhibitory effect was recorded for 10 mg/ml against *C. sakazakii* (Table 2). These findings are in parallel to those reported by Wakabayashi *et al.* (2008), who found that Apo-LF at 0.5 mg/ml weakly suppressed the growth of *C. sakazakii* and Apo-LF at 2 to 8 mg/ml completely inhibited its growth. As well Maria *et al*. (2014) stated that bLF at a concentration of 10 mg/ml, inhibit adherence of *C. sakazakii* to intestinal epithelium.

It was noted that bLF at concentration of 2mg/ml was difficult to draw clear-cut conclusions about inhibition *versus* growth of the tested *C. sakazakii* (Table 2). This was contrary to the results of Moradian *et al.* (2014), they concluded that bLF above 1 mg/ml inhibited the bacterial growth especially for gram negative bacteria.

Bovine LF has been used as a supplement to some infant formulas (Wakabayashi *et al*., 2006). Bovine LF heated at 80°C showed similar anti-Enterobacter activity to non heated bLF at above 1 mg/ml. This observation suggests that bLF in the powdered infant formula may retain its antibacterial activity to some extent after reconstitution with hot water. In conclusion, bovine LF may have potential usefulness for the prevention of infection by *C. sakazakii* in foods such as infant formula.

RESULTS

Table 1: Incidence of *C. sakazakii* in powder infant formula milk (IFMP).

Type of sample	No. of analyzed samples	Positive samples	
		No.	%
IFMP	100	21	21

Table 2: The inhibitory effect of different bLF concentrations on growth of *C. sakasakii.*

Concentration of bLF	Diameters of inhibition zone (mm)	*C. sakasakii* growth
1mg/ml	0	+
2mg/ml	3	+/−
5mg/ml	6.8	−
10mg/ml	17	−

growth (+) no growth (−)

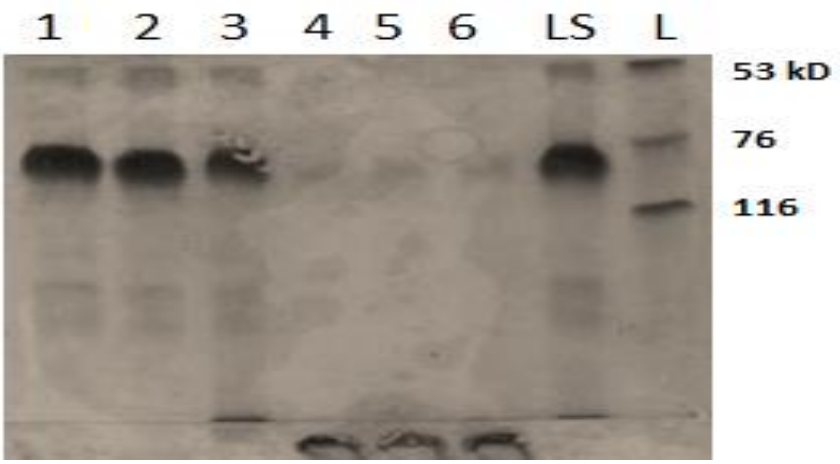

Photo 1: Bovine Lactoferrin (bLF) by SDS-PAGE

Lan LS: Lactoferrin Standard,
Lan L: Laddar (kDa) Molecular weight markers, transferrin (76 kDa),
Lan 1-3: bLF positive samples,
Lan 4-6: bLF negative samples.

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التأثير المثبط للاكتوفيرين ضد الكرونوباكتر ساكازاكي المعزولة من مسحوق حليب الرضع

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في هذه الدراسه تم التحقق من نشاط اللاكتوفيرين المضاد للميكروبات ضد ميكروب الكرونوباكتر ساكازاكي التى تنتقل عن طريق الأغذية. تم عزل وتنقية اللاكتوفيرين البقرى من اللبأ البقرى باستخدام جهاز الكروماتوجرافى. كفائة التنقيه سجلت ٩٠%. تم عزل الكرونوباكتر ساكازاكي من ١٠٠ عينه من مسحوق حليب الرضع التى جمعت عشوائيا من مدينه قنا. تم العزل على المستنبت الخاص بالكرونوباكتر ساكازاكي والتعرف على الميكروب بالتجارب البيوكيميائيه وقد وجد الميكروب بنسبة ٢١% من إجمالى العينات التى تم فحصها. وقد تم دراسة التاثير المثبط للاكتوفيرين البقرى على الكرونوباكتر ساكازاكي؛ وكشفت النتائج أن اللاكتوفيرين البقرى بتركيز ١٠ ملغ/مل أظهر أكبر قدر من التأثير المثبط على الميكروب بينما تركيز ١ ملغ/مل كان الأقل تثبيطا. وأشارت النتائج إلى أن اللاكتوفيرين البقرى مفيد لتثبيط الكرونوباكتر ساكازاكي المنتشر في حليب الأطفال الرضع. لذا يجب الانتباة أكثر أثناء تصنيع وتداول مسحوق حليب الأطفال الرضع.