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SYNERGISTIC EFFECT OF COLOSTRUMS LACTOFERRIN AND PURIFIED CHARACTERIZED BREVICIN ON ESBL PRODUCING *PANTOEA AGGLOMERANS* ISOLATED FROM BLOOD CULTURES OF INFANTS IN BAGHDAD.

Likaa Hamied Mahdi Al Musawii*[1], Nadheema Hammood Hussein and Buthainah Mohammed Taha

Department of Biology, College of Science, Al-Mustansiryah University.

Corresponding Author: Likaa Hamied Mahdi Al Musawii

Department of Biology, College of Science, Al-Mustansiryah University.

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ABSTRACT

In the present study, seven *Lactobacillus brevis* (*L. brevis*) strains isolated from human breast colostrums identified by API 50WERE USED. To evaluate the potential use of these strains as probiotics, the effect of gastric juice, pH and bile salts tolerance were conducted. Cell tolerancey to acidity and bile salt are important factor that affect the probiotics to remain and exert their potential functionalities in a host. Brevicin produced from *L. brevis* strain no.5 purified by ammonium sulfate precipitation, Sep-pack C18 cartridge and reverse-phase HPLC chromatography on C18 Nucleosil column and characterized. Lactoferrin had been isolated from human colostrums purified by gel filtration and ion-exchange chromatography. Antibacterial activity of crude and purified brevicin against sixteen *Pantoea agglomerans* strains was determined moreover synergestic effect of lactoferrin and brevicin against these strains was conducted. All *L. brevis* strains showed acid resistance which survive at pH 3 and 2.5, bile salt concentration until 0.5% and all the strains had the ability of aggregation. Brevicin was considered to be heat stable in different temperature degrees and also it was stable at pH 6 and 7 which gave 100% activity. Brevicin was insensitive to lipase and α-amylase but sensitive to pronase E, pepsine and proteinase K, the chromatogram by HPLC showed a single symmetric peak by absorbance at 280 nm and the specific activity of the purified brevicin was increased $1.68*10^4$ fold with a final yield of 0.4%. Also the molecular weight of purified brevicin by gel filteration was estimated at approximately (3.8) K Daltons. Our results showed that *Pantoea agglomerans* strains were had high resistant rates for most antibiotics under study and the most effective antibiotics against these bacteria were imipenem and netilmicin. Also 12(75%) of these bacteria were positive ESBL producing strains by screening test. Out of 12 positive ESBL producing strains, 8(66.67%) harboring *bla*CTX-M gene by genotypic detection while all strains don't harboring *bla*SHV gene. Detection of ESBL bacteria is importance for infection control and prevention of dissemination of ESBL producers by cross-transmission and for epidemiological purposes. Also increasing in number of ESBL resistant bacteria have been reported worldwide and alternative compounds should be used against these bacteria. Our results showed that the purified brevicin was more effective than crud brevicin in all concentrations ($p \leq 0.05$) and there was a synergic antimicrobial effect of brevicin and lactoferrin when they were tested together, even in the concentration of 30 µg/mL brevicin and 30 µg/mL of lactoferrin (the highest values evaluated). *Pantoea agglomerans* strains were affected by lactoferrin and significantly more affected by the synergic use of both proteins (brevicin and lactoferrin) ($P<0.05$).

KEYWORDS: *Lactobacillus brevis*, brevicin, lactoferrin, *Pantoea agglomerans*, ESBL producing bacteria and antimicrobial activity.

INTRODUCTION

Blood stream infection is a serious problem that it needs direct attention and treatment. It is represent a cause of high mortality especially if caused by multi-drug resistant bacteria.[1]

Bacteremia caused by *Pantoea agglomerans* has been described in association with the contamination of intravenous fluid.[2] Outbreak of septicemia caused by contaminated closures on bottles of infusion fluids. Since then, *P. agglomerans* bacteremia has been also association with the contamination of intravenous fluid, parenteral nutrition, blood products, the anesthetic agent propofol and transference tubes used for intravenous hydration.[3]

During the past decades, an increasing number of Extended-Spectrum β-Lactamase **(**ESBL) resistant Gram-negative bacteria have been reported worldwide.[4] Nowadays ESBL producing bacteria are emerging threat

and the main mechanism for this ESBL resistance is by production of enzymes called extended spectrum β-lactamases (ESBLs).[5]

Breastfeeding is the most effective interference for protecting infants from all causes of mortality.[6] Breast (colostrum) secreted within the first 3 days after childbirth is one of the first essential factors for neonates. While acting as multifunctional nutrition source, it provides immunological benefits and a gut colonizing benefits bacteria for the infant's gastrointestinal tract.[7] The first most abundant protein in human breast colostrum is lactoferrin. It is a glycoprotein consisting of a single polypeptide chain considered to be an important host defence molecule and has a diverse range of physiological functions such as antimicrobial, antiviral and anticancer.[8] Breast colostrum has been shown to be a continuous source of probiotic bacteria to the infant gut and these bacteria may play an important role in the reduction of the incidence and severity of infections in the infants.[9]

Lactobacillus is an important bacterium with a wide variety of applications, both in the food industry and as a probiotic agent for the improvement of human health and it consists of a number of different species, *Lactobacillus brevis* is one species of *Lactobacillus*.[10] These bacteria have the ability to inhibit the growth of certain gut pathogens.[11]

Bacteriocins can inhibit closely related and sometimes more distantly related strains of bacteria.[12] Bacteriocins have the ability to inhibit the growth of Gram-positive pathogenic bacteria, spoilage bacteria and yeasts.[13&14] Also it can inhibit the growth of some Gram-negative species of bacteria.[15]

The purpose of this study was to evaluate the potential of *Lactobacillus brevis* strains isolated from colostrum as a probiotic by characterizing the aggregation activity , acid and bile tolerance as well as purification and characterization of brevicin produced by *L. brevis* moreover evaluate in vitro antibacterial activity of crude and purified brevicin in addition purification of lactoferrin and determine the synergestic effect of brevicin and lactoferrin against ESBL producing *Pantoea agglomerans* strains isolated from infant`s blood cultures in Baghdad.

MATERIALS AND METHODS

Isolation and Identification of *Lactobacillus brevis* strains from colostrum samples

Seventy one[71] colostrum samples were collected from human mothers in small sterile bottles and then were transferred in sterile conditions to the laboratory for the isolation of *Lactobacillus brevis* strains**.** Colostrum samples inoculated on MRS (Man de Rogosa Sharpe) agar, supplemented with (0.05%) w/v L-cystein, incubation under anaerobic conditions at 37 ºC for 48 h. using GEN box anaerobic kit (Bio-Merieux, France). The selected colonies were purified by repeated streaking on MRS agar and then the isolated bacteria were identified by using API 50 CH (Biomerieux, France) according to the guide of the manufacturer.

Aggregation test

Aggregation test for *L. brevis* strains was performed as described by Jankovic *et al*.[16] The aggregation phenotype was scored positive if the overnight cultures were clear with cells clumped at the bottom of the tube. The strains were considered non aggregating if the overnight cultures were turbid.

Tolerance to low pH

Tolerance to low pH for *L. brevis* strains were performed according to Gusils *et al*.[17] and Ehrmann *et al*.[18] *L. brevis* strains were tested at pH 2.0, 2.5, 3.0, 3.5 and 4. After 3 h at 37°C, the appropriate dilutions were cultured on MRS agar and incubated at 37°C for 48 h.

Bile Tolerance

Bile tolerance for *L. brevis* strains was determined in MRS broth containing 0.3,0.5% bile acids (oxgall; Difco). Before testing for bile tolerance, all strains were incubated at 37°C for 18 h in MRS broth without bile. After centrifugation (4,000 × *g* for 10 min, 4°C), the collected cells were resuspended in sterile saline (0.85% NaCl) and then inoculated at 106 cfu/ml into MRS broth containing 0.3,0.5% bile acids. Cultures were incubated at 37°C. The bacteria were plated and enumerated after 24 h of incubation, viable cell count was performed in duplicate.

Production of crude brevicin

Lactobacillus breves strain No.5 was grown in MRS broth (Hi Media Laboratory Pvt Ltd. India) (pH-6.0) seeded with 5% inoculum of overnight culture and maintained anaerobically at 30ºC for 48 h. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4ºC). The cell-free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as crude brevicin.[19]

Determination of brevicin activity

The antibacterial spectrum of the brevicin from *Lactobacillus breves* strain No.5 was determined by using the well diffusion method. The supernatant from a 48 h. culture of *Lactobacillus breves* strain No.5 was sterilized and filtered by passage through a 0.45mM pore size membrane filter (PALL Corporation, Mumbai). Aliquots (50:l) of the sterile supernatant were placed in 4-mm-diameter wells that had been cut in Mueller-Hinton agar plates previously seeded with the indicator bacteria (*E. coli* ATCC 25922). After 12-18 h of incubation, the diameters of the zones of growth inhibition were measured. Antimicrobial activity was expressed in arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition (i.e.

turbidity <50% of the turbidity of control culture grown without *L. brevis* supernatant).[20]

PURIFICATION OF BREVICIN

Ammonium Sulphate Precipitation

Method of precipitation with ammonium sulfate, Sep-pack C18 cartridge and reverse-phase HPLC on a C18 Nucleosil column. A 24-h-old culture (250 ml) of *L. brevis* No.5 centrifuged for 15 min at 20,000 x g, 4ºC.

The active supernatant was treated for 10 min at 80ºC to prevent brevicin proteolysis. Ammonium sulfate (Kimax) was gently added to the cell supernatant (maintained at 4ºC) to obtain 60% saturation and stirred for 4h. After centrifugation (1h. at 20,000 x g, 4ºC), the pellet was resuspended in 25 mM ammonium acetate (pH 6.5) and loaded on a Sep-Pack C18 cartridge (Waters Millipore, MA, USA). The cartridge was washed with 20% i-propanol in 25 mM ammonium acetate (pH 6.5) and the brevicin was eluted with 40% i-propanol in 25 mM ammonium acetate (pH 6.5). After drying under reduced pressure (Speed-Vac; Savant, France), the fractions were concentrated in lyophilzer and used to prepare different concentrations (60, 30, 15, 7.5 and 3.75) µg/ml and tested for antimicrobial activity.

This active fraction was further purified by reverse-phase HPLC on a C18 Nucleosil column (250 x 4.6 mm). Elution was performed by applying a linear gradient from 0.1% TFA (solvent A) to 90% acetonitrile in 0.1% TFA (solvent B) in 65 min. Polypeptides, detected by A 220, were collected manually. After drying under reduced pressure and resuspension in 1 ml of de-ionised water, the aqueous polypeptide solutions were stored at -20ºC and concentrated in lyophilizer.

Estimation of protein

Protein concentration of the brevicin was determined by the method of Lowry *et al.*[21]

Characterization of brevicin

Lactobacillus brevis crude bacteriocin was characterized with the sensitivity to heat, pH and enzymes.

Sensitivity to heat: Aliquots of the crude brevicin were exposed to heat treatments of (25, 30, 40, 50, 60, 70, 80, 90, 100 and 121°C for 15 and then were tested for remaining antimicrobial activity.

Ph

Also crude brevicin was adjusted to various pH values in the range of 2 to 10. The pH-adjusted brevicin samples were incubated at 37°C for 20 min and then neutralized to pH 6 and tested for brevicin activity.

Enzyme treatments

Susceptibility of brevicin to various enzymes was performed by incubating the brevicin preparation in the presence of pronase E (1 mg/ml), lipase (1mg/ml), α-amylase (1mg/ml), 1mg/ml pepsine and proteinase K (1mg/ml) at 37°C for 1 h. After incubation, the enzymes were inactivated by heat treatment at 65°C for 30 min and tested for brevicin activity.

Evaluation of brevicin molecular weight

The molecular weight of the brevicin from *L. brevis* was evaluated by gel filtration according to principles described by[22]

Purfication of lactoferrin.

Lactoferrin was isolated and purified from pooled human colostrum by combined gel filtration and ion-exchange chromatography as previously described.[23]

Isolation and Identification of *Pantoea agglomerans* strains

Blood specimens were collected from infants inpatients lying in one children's hospitals in Baghdad that suspected with blood stream infections. All blood specimens were cultured and incubated by used BacT/ALERT 3D device (Bio-Merieux, France), the pathogenic bacteria were isolated from blood specimens and then isolated bacteria were identified by using VITEK 2 system (Bio-Merieux, France), according to the manufacturer's instructions.

Antibiotic Susceptibility Test

The Antibiotic susceptibility test was done according to Kirby-Bauer (disk diffusion) technique (24), using Muller-Hinton agar (Hi media/ India) and different antibiotic discs (Bioanalyse/Turkey and) including: Ampicillin (AMP) (30µg), Amoxicillin-clavulanic acid (AMC) (20/10µg), Amikacin (AK) (30µg), Aztreonam (AT) (30µg), Cefador (CEC) (30µg), Cefoperazone (CPZ) (75µg), Ceftriaxone (CTR) (30µg), Cefepime (FEP) (30µg), Cefotaxime (CTX) (30µg), Ceftazidime (CAZ) (30µg), Ciprofloxacin (CIP) (5µg), Gentamicin (GN) (10µg), Imipenem (IMP) (10µg), Netilmicin (NET) (30µg) and Tetracycline (TE) (30µg). Inhibition zones were measured by a ruler and compared with the zones of inhibition determined by Clinical Laboratories Standards Institute (CLSI).[25]

Phenotypic Detection of ESBL producing *Pantoea agglomerans* strains

Screening test was used to detect the ESBL producing *Pantoea agglomerans* strains, the strains were swabbed on to a Mueller-Hinton agar plates. Antibiotic discs cefotaxime (30 µg) (Bioanalyse/Turkey) and ceftazidime (30 µg) (Bioanalyse/ Turkey) were placed on the surface of the agar and incubated at 37 ºC for 24h. If a zone diameter was ≤22 mm for ceftazidime and was ≤ 27 mm for cefotaxime recorded, then the bacterial strain was considered as ESBL producer.[26]

Genotypic Detection of ESBL genes by PCR.

Polymerase chain reaction (PCR) assay was performed in order to detect the presence of ESBL genes in *Pantoea agglomerans* strains.

- **Extraction of genomic DNA**

Genomic DNA was obtained by suspending 2-3 colonies of each *Pantoea agglomerans* strain in 500 µl of distilled water and heating by using a water bath at 90°C for 10 min. Samples were spun at 10000 rpm for 10 min. These DNA samples were used as the bacterial DNA template for PCR assay.[27]

- **Polymerase chain reaction (PCR) to detection of ESBL genes**

The sequences of primers used in this study were listed in table-1, including: *bla*CTX-M and *bla*SHV (Alpha DNA, Canada) were provided in lyophilized form, dissolved in sterile deionized distilled water to give a final concentration of 100 picomole/ µl as recommended by provider and stored in a deep freezer until use.

Table: 1 The sequences of primers used in this study.[28]

Primers	Sequences	Length (bp)
SHV-F	5-ATTTGTCGCTTCTTTACTCGC-3	1051
SHV-R	5-TTTATGGCGTTACCTTTGACC-3	
CTX-M-F	5-TTTGCGATGTGCAGCACCAGTAA-3	544
CTX-M-R	5-CGATATCGTTGGTGGTGCCATA-3	

Uniplex PCR mixture was set up in a total volume of 25 µl included: 12.5µl of PCR master mix 2X (Kapa, India), 1.5µl Forwared primer (from each primer) and 1.5µl Reverse primer (from each primer), 4 µl of template DNA and the rest volume was completed with sterile D.W. PCR reaction tubes were vortexed and then putted into thermocycler PCR instrument (Thermal Cycler Agilent Sure Cycler 8800). The programs of amplification that used in the thermocyler PCR were listed in table -2.

Table: 2 The programs that used in this study.[29]

Primers	Stage	Temperature (time)	
For all primers	Initial denaturation	96°C (5 min)	
	Denaturation	96°C (30 sec)	30 cycles
SHV primer	Annealing	60°C (30 sec)	
CTX-M primer	Annealing	51°C (30 sec)	
For all primers	Extension	72°C (1 min)	
	Final extension	72°C (5 min)	

- **Agarose gel electrophoresis**

Detection of PCR products was determined by gel electrophoresis in which the visualized was done with UV transilluminator documentation system and the aid of ethidium bromide.[30]

Antimicrobial Activity of brevicin against ESBL producing *Pantoea agglomerans* strains

Agar well diffusion method was used to detect antimicrobial activity of crud and purified brevicin produced *L. brevis* strain No.5 against *Pantoea agglomerans* strains at different concentrations[60,30,15,7.5 and3.75] µg/ml for each crud and purified brevicin according to.[31]

Synergistic effect of lactoferrin and brevicin against ESBL producing *Pantoea agglomerans* strains

To study the synergistic effect of lactoferrin and brevicin, agar well diffusion method was used to detect antimicrobial activity of lactoferrin alone and in combination with crud and purified brevicin against *Pantoea agglomerans* strains according to method described by.[31] The concentrations used in this test were; lactoferrin alone (60µg/ml), lactoferrin + crude brevicin (30 µg/ml+30 µg/ml) and lactoferrin + purified brevicin (30µg/ml+30 µg/ml). The zone diameter of inhibition (ZDI) of different concentrations has been determined against *Pantoea agglomerans* strains.[32]

Statistical analysis

All statistical analyses were performed by ANOVA with the Tukey test for multiple comparisons. A P-value of< 0.05 was considered statistically significant.

RESULTS

Isolation of *Lactobacillus brevis*

Out of 71 colostrum samples collected, 33 *Lactobacillus* strains were isolated and. Out of those 33 *Lactobacillus* 7 strains were *Lactobacillus brevis*.

Aggregation test

The process of aggregation is the process of reversible accumulation of cells, that causing them to spontaneously precipitate in the medium that in which they are suspended.[33] As shown in table-3, all *L. brevis* strains isolated from human mothers colostrum, were had aggregation activity.

Table: 3 Aggregation activity of *L. brevis* strains under study.

Strains	Aggregation
***L. brevis* no.1**	+
***L. brevis* no.2**	+
***L. brevis* no.3**	+
***L. brevis* no.4**	+
***L. brevis* no.5**	+
***L. brevis* no.6**	+
***L. brevis* no.7**	+

Also the tolerance of *L. brevis* strains to low pH values was tested and the result showed that all strains grown at 3, 3.5 and 4 pH values, while all strains don't grow at 2 pH value as shown in table -4.

Table: 4 The effect of Low pH values on *L. brevis* strains.

Strains of *L. brevis*	Low pH Values				
	2	**2.5**	**3**	**3.5**	**4**
L. brevis no.1	-	+	+	+	+
L. brevis no.2	-	+	+	+	+
L. brevis no.3	-	+	+	+	+
L. brevis no.4	-	-	+	+	+
L. brevis no.5	-	+	+	+	+
L. brevis no.6	-	+	+	+	+
L. brevis no.7	-	+	+	+	+

+: indicates growth, - : indicates no growth

Bile Tolerance

All *L. brevis strains* isolated from human colostrum were able to grow on the medium with the addition of 0.3% and 0.5% bile salts that indicated *L. brevis strains* isolated from human colostrum were able to survive and grow in the upper intestinal tract where bile salts secreted. All *L. brevis strains* were able grew more in bile salt concentration of 0.3% compared to 0.5% but did not show significant differences and the results were summarized in table-5.

Table: 5 The bile tolerance of *L. brevis* strains.

Strains	(mean ±SD) Log cfu/ml	
	Bile concentration %	
	0.5	**0.3**
L. brevis no.1	7.25 ± 1.04	7.66 ± 0.73
L. brevis no.2	6.77 ± 3.15	6.99 ± 0.58
L. brevis no.3	6.91 ± 0.97	7.49 ± 0.47
L. brevis no.4	6.9 ± 0.15	7.09 ± 0.48
L. brevis no.5	8.11 ± 0.41*	8.34 ± 0.26*
L. brevis no.6	6.75±0.55	7.89 ± 0.62
L. brevis no.7	7.06 ± 0.57	7.74± 1.02

*** significant differences p<0.05 according to other strains at the same concentration**

Screening for bacteriocinogenic *Lactobacillus brevis*

All 7 *L. brevis* strains that isolated from colostrums were examined for the bacteriocin like activity against indicator bacteria by agar well diffusion method, and the strain no. 5 showed the highest bacteriocin activity against indicator bacteria.

Purification of brevicin

Purification of *L. brevis* bacteriocin by precipitation with ammonium sulfate, sep – pack C18 cartridge and reverse phase HPLC on a C18 Nucleocil column was done and the results were listed in table-6.

The chromatogram by HPLC showed a single symmetric peak by absorbance at 280 nm, after these steps the specific activity of the purified brevicin was increased $1.68*10^4$ fold with a final yield of 0.4 %.

Table: 6 Results of purification of *L. brevis* brevicin.

Sample type	Volume (ml)	Total Activity (Au)	Protein (mg)	Specific Activity (Au/mg)	Yield (%)	Purification factor
Crude Brevicin	250	$7.1*10^5$	1923	$3.69*10^2$	100	1
A ammonium sulfate precipitation	24	$2.9*10^5$	11.8	$2.457*10^4$	9.6	$6.63*10^1$
sep – pack 40% isopropanol	3	$2.8*10^4$	0.094	$2.978*10^5$	1.2	$8.04*10^3$
HPLC C18	0.4	$6.2*10^3$	0.001	$6.2*10^6$	0.4	$1.68*10^4$

Characterization of brevicin

Effect of enzymes on brevicin activity

Crude brevicin produced by *L. brevis* no. 5 was tested for their sensitivity to various enzymes under study and the result showed that crude brevicin was insensitive to α-amylase and lipase but sensitive to proteolytic enzymes such as pronase E, pepsine and proteinase K as shown in table -7.

Table: 7 The effect of various enzymes on brevicin activity by *L. brevis* strains

Enzyme	Activity%
Pronase	0
Lipase	100
α-amylase	100
pepsine	0
Protinase K	0

Effects of temperature on brevicin activity

The effects of temperature on brevicin activity were determined and the result showed that brevicin produced by *L. brevis* was considered to be the most heat stable in different temperature degrees after heating at 40 to 80ºC which gave 100% activity, while brevicin gave 95% activity at 100ºC as shown in table -8.

Table: 8 Effect of temperature on brevicin activity produced by *L. brevis*

Temperatures	Activity%
25	0
30	80
40	100
50	100
60	100
70	100
80	100
90	100
100	95
121	0

Effect of pH on the activity of brevicin activity

The effect of pH on the activity of brevicin was carried out and the result showed that brevicin produced by *L. brevis* was stable at pH 6 and 7 gave 100% activity as shown in table-9.

Table: 9 Effect of pH on brevicin activity produced by *L. brevis*

pH	Brevicin activity %
2	0
3	0
4	55
5	78
6	100
7	100
8	0
9	0
10	0

Evaluation of brevicin molecular weight

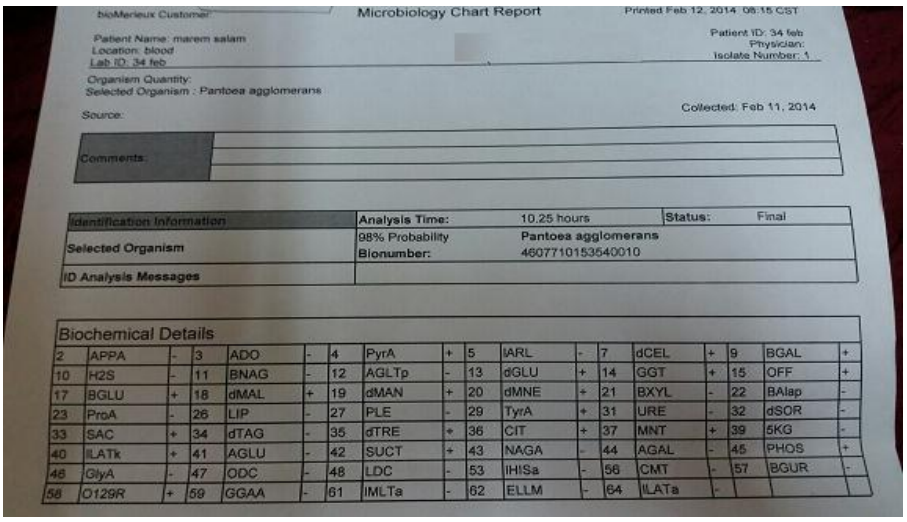
The molecular weight of purified brevicin by gel filtration Sephadex G-100 was estimated at approximately (3.8) K Daltons.

Purification of human colostrum Lactoferrin

Human colostrums Lactoferrin (Lf) was released from 0.4 to 0.5 M of NaCl linear gradient. A strong peak was observed between 0.4 to 0.5 M. The single band of purified Lf has been observed in SDS-PAGE electerophoresis. The concentration of Lf determined by lowry assay and it was about 2.9 mg/ml

Isolation and Identification of *Pantoea agglomerans* strains

A total of 16 *Pantoea agglomerans* strains were isolated from blood cultures of infants inpatients at one children´s hospital in Baghdad during 2014-2015. All *Pantoea agglomerans* strains were identified depending on Vitek-2 system (Bio-Merieux, France) by using ID-GNB card as shown in figure-1.



Microbiology Chart Report

Printed Feb 12, 2014 06:15 CST

Patient Name: marem salam
Location: blood
Lab ID: 34 feb
Organism Quantity:
Selected Organism : Pantoea agglomerans
Source:
Collected: Feb 11, 2014
Comments:

Patient ID: 34 feb
Physician:
Isolate Number: 1

Identification Information	Analysis Time:	10.25 hours	Status:	Final
Selected Organism	98% Probability Bionumber:	Pantoea agglomerans 4607710153540010		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	+	5	lARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	+
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Figure: 1 Identification of *Pantoea agglomerans* strains by Vitek-2 system.

Antibiotic Susceptibility Test

Antibiotic susceptibility test was performed by using Bauer-Kirby method to show the effect of some antibiotics under the test on *P. agglomerans* strains which isolated from infant`s blood cultures.

The results of antibiotic susceptibility test were summarized in figure -2. Data presented in this figure shows that the highest resistant antibiotics are ampicillin, cefoperazone and cefador which gave 16(100%) resistance rate followed by aztreonam and amoxicillin-clavulanic acid 14(87.5%) and 13(81.25%) respectively. Resistant rate for cefotaxime, ceftazidime and ceftriaxone was 12(75%). On the other hand the results shows that the most effective antibiotics were imipenem and netilmicin which gave the highest sensitivity rate 16(100%) and 15(93.75%) respectively, and the other antibiotics showed different degrees of resistance as shown in figure -2.

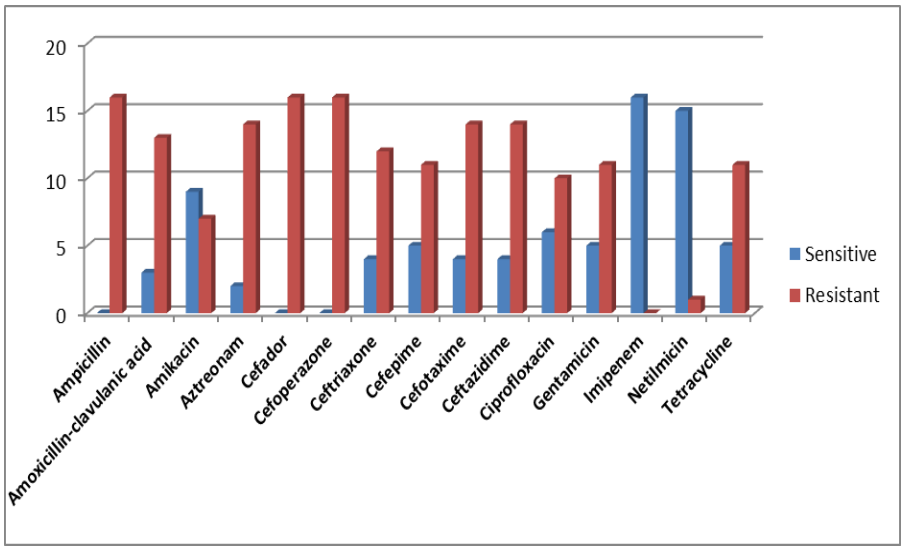


Figure: 2 Antibiotic resistance of 16 *Pantoea agglomerans* strains

Phenotypic Detection of ESBL producing *Pantoea agglomerans* strains

Phenotypic detection of ESBL producing strains was done by screening test. The result showed that out of 16 *P. agglomerans* strains, 12(75%) were positive ESBL producing strains by screening test (figure-3).

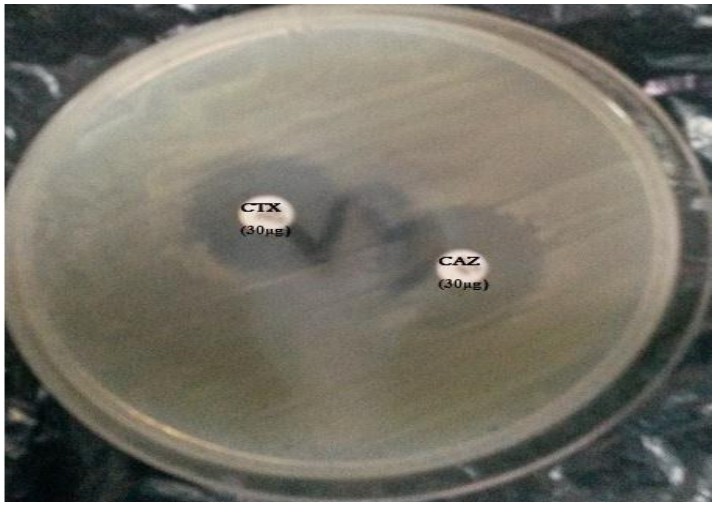


Figure: 3 Positive ESBL producing strains by screening test.

Genotypic Detection of ESBL producing *Pantoea agglomerans* strains by PCR

All 12 positive ESBL producing *P. agglomerans* strains by phenotypic detection were subjected to PCR assay in order to detect the presence of *bla*CTX-M and *bla*SHV genes in those strains. The results presented in figure -4A showed the presence of *bla*CTX-M gene in 8(66.67%) out of 12 positive ESBL producing strains while the results in figure -4B showed that all 12 positive ESBL producing strains don't have *bla*SHV gene.

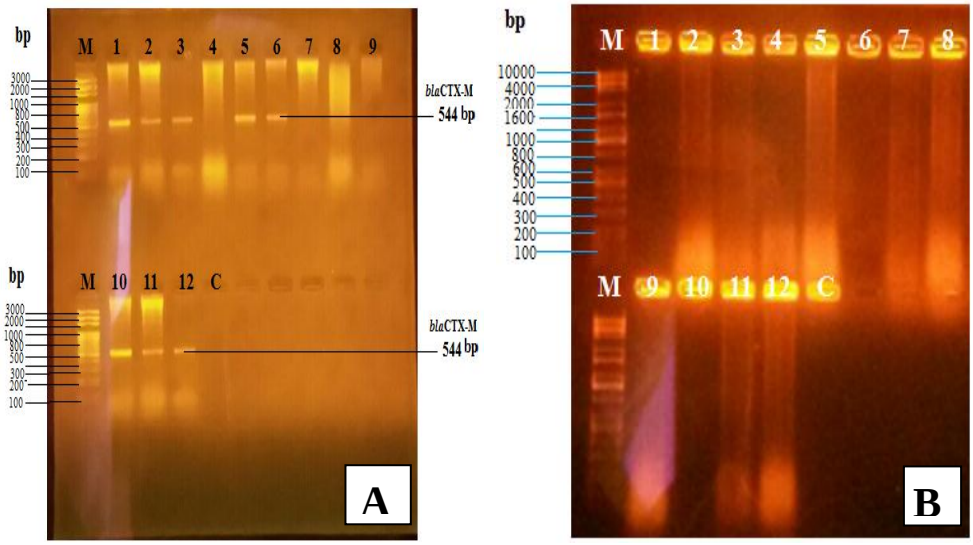


Figure-4: Represent agarose gel electrophoresis (1%) for the products of ESBL genes by PCR assay at 5V/cm for 1.5 hr. stained with ethidium bromide and visualized on a UV transiluminator documentation system. Lane M: DNA ladder (BioDyne, Swede), lane C: Negative control (contain all PCR mixture including water instead of DNA template) and lanes (1-12): *Pantoea agglomerans* strains.
A: Presence of *bla*CTX-M gene in 8 *Pantoea agglomerans* strains (1,2,3,5,6,10,11 and 12).
B: All *Pantoea agglomerans* strains don't have *bla*SHV gene.

Antimicrobial Activity of brevicin against *Pantoea agglomerans* strains

Crud and purified brevicins from *Lactobacillus brevis* strain No.5 were tested for their antimicrobial activity against all *P. agglomerans* strains. Results showed that crud brevicin was effective against *P. agglomerans* strains under the first two concentrations (60 and 30) µg/ml, while the purified brevicin was more effective than crud brevicin in all concentrations ($p<0.05$) as shown in table -10 and figure -5.

Table: 10 Inhibition zone (mm) of crude and purified brevicins against all 16 *P. agglomerans* strains.

Brevicin concentration µg/ml	control	Zone of inhibition(mm) mean ± SD *	
		Crude brevicin	Purified brevicin
60	0±0	21.56 ± 0.84 p	30.52 ± 0.91 a p
30	0±0	16.86 ± 0.44 p	29.21 ± 1.02 a p
15	0±0	1.32 ± 0.01	22.36 ± 1.34 a p
7.5	0±0	0±0	19.46 ± 0.98 a p
3.75	0±0	0±0	15.39 ± 1.17 a p

P: probability compared to control p≤ 0.005
a: probability compared to Crude brevicin at the same concentration p≤ 0.005
: 16 strains of *Pantoea agglomerans

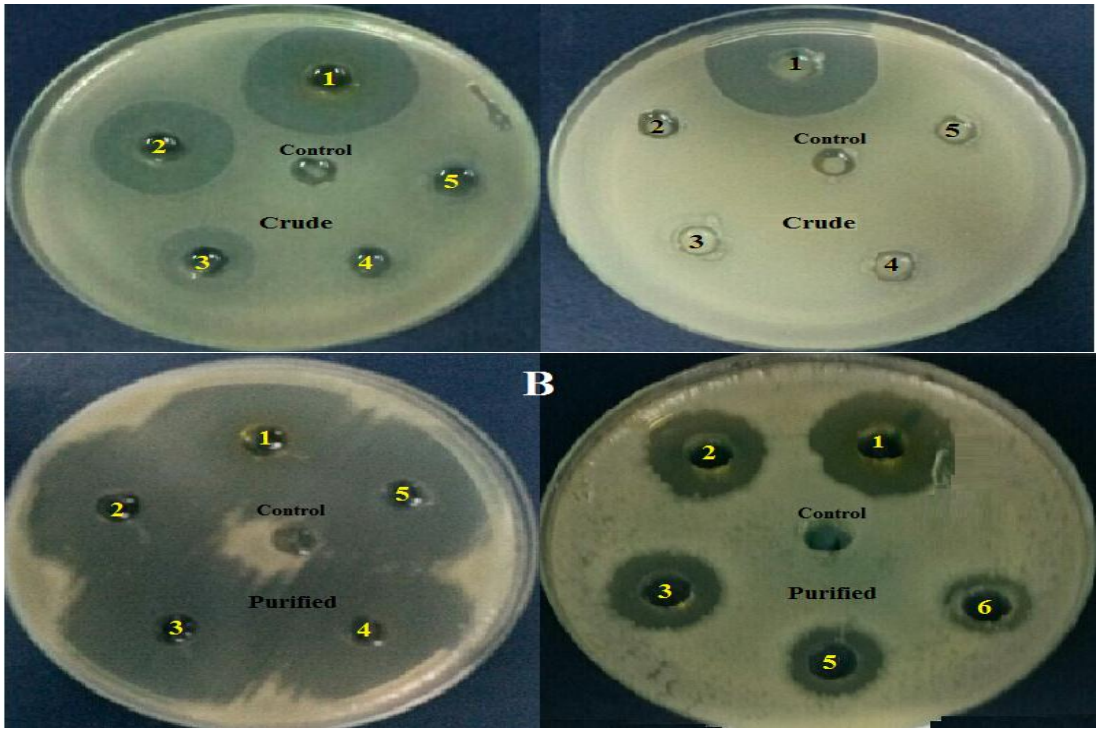


Figure -5: Antibacterial activity of crude and purified brevicins from *L. brevis* strain No.5 on *P. agglomerans* strains in concentrations (1= 60, 2= 30 3= 15 4= 7.5 and 5= 3.75) µg/ml.
A= Crude brevicins and **B=** purified brevicins

Synergistic effect of lactoferrin and brevicin against ESBL producing *P. agglomerans* strains

The antibacterial activity of lactoferrin alone and in combination with brevicin against *P. agglomerans* strains was observed using agar well diffusion method by measuring the diameter of the growth inhibition zone. The lactoferrin in combination with crude and purified brevicins showed a positive synergistic effect and good antibacterial activity against *P. agglomerans* strains (figure- 6). As shown in table-11 the synergistic effect between combination of lactoferrin and brevicins (crude and purified) was with significant differences ($p<0.05$) compare to lactoferrin and brevicins alone.

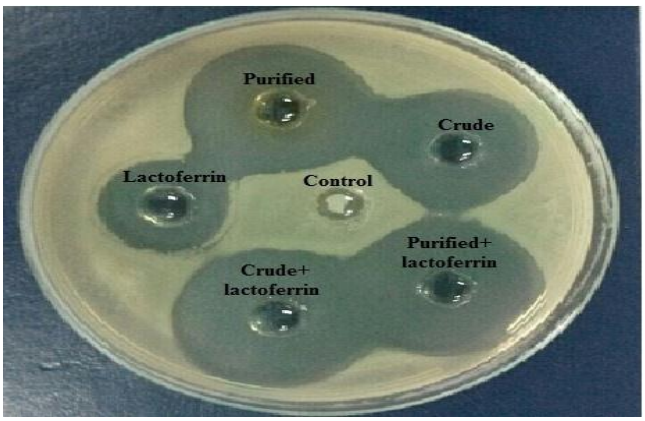


Figure -6: Antibacterial activity of Lactoferrin alone and in synergisim with crude and purified brevicins

from *L. brevis* strain No.5 against *P. agglomerans* strains in concentrations (lactoferrin1= 60 µg/ml, 2= purified brevicin60 µg/ml, 3= crude brevicin 60 µg/ml, 4= crude brevicin30 µg/ml +lactoferrin30 µg/ml and 5= purified brevicin30 µg/ml +lactoferrin30 µg/ml).

Table: 11 Inhibition zone (mm) of lactoferrin alone and in combination with crude and purified brevicins against all 16 *P. agglomerans* strains.

Concentration µg/ml	(mean ± SD) inhibition zone (mm)
Lactoferrine 60	20.93 ± 2.14 a
Crude brevicin 60	21.56 ± 0.84 a
Purified brevicin 60	30.52 ± 0.91 b
Lactoferrine + Crude brevicin (30+30)	33.43 ± 1.98 c
Lactoferrine + Purified brevicin (30+30)	32.16 ± 2.07 c

[1]Each datum is the mean of 16 isolates.
[2]Similar letters in the same column refer to insignificant differences

DISCUSSION

Our results confirm that human breast colostrum is a source of lactoferrin and *L. brevis* strains which may possibly impact the new born's gut colonization process and exerts health promoting effects moreover we observed that *L. brevis* was not the predominant species found in the colostrum samples. The results indicate that *L. brevis* may be detected early after delivery as the colostrum samples we used were obtained during the first two days post partum Cabrera-Rubio *et al*. (34) reported bacterial diversity to be higher in colostrum than in transitional milk or in mature milk.

The seven strains of *L. brevis* tested in this study have been isolated from human colostrum showed acid resistance, all strains have been shown to survive at pH 3 and 2.5 (were able to pass through the stomach without losing viability), and bile salt concentration until 0.5% both characters were main characters that must be owend by probiotics bacteria, therefore *L. brevis* strains have probiotic characterstics.

In this study *L. brevis* strain no.5 exhibited excellent bile tolerance compared with other strains ($p \leq 0.001$), the heat stability of the antimicrobial compounds present in the culture supernatants and their susceptibility to protease enzymes suggests that the antimicrobial compounds are perhaps bacteriocin.

The presence of bile salts may be limiting factor for such colonization but our results show that all the strains isolated from colostrum samples were resistant to the bile salt and low pH also all strains isolated had the ability of aggregation These characteristics may be advantageous for a probiotic culture to be successful in colonizing and to compete with pathogens in the gastrointestinal environment. may be important for the new born's health contributing to reduce the risk of infection by pathogenic bacteria and to modulate the immune response.

The *L. brevis* bacteriocin was active over a wide range of pH, and was stable to various heat treatments. The loss of antimicrobial activity following treatment with proteinase K, pepsin and pronase E indicated that the active component secreted extracellullarly by *L. brevis* strain no.5 was proteinaceous in nature. Similar properties have been reported for other bacteriocins including lactacin, lactacin 27, acidolin, pediocin A, and pediocin PA-1 (35, 36). These bacteriocins were also stable over a wide range of pH. This heat and pH stability may be useful if the bacteriocin is to be used as an antimicrobial agent in fermented foods or thermally processed baby foods.

However there have been no reports regarding the molecular weight of brevicin produced by *L. brevis* isolated from colostrum in this study, the molecular weight of purified bacteriocin was (3.8) K Dalton as determined by Gel filtration.

Lactoferrin isolated from human breast colostrums was able to inhibit growth of all *P. agglomerans* strains at concentration 60. Antimicrobial activity of Lf against *P. agglomerans* may be explain by several mechanism. The first mechanism is that Lf is an iron-binding protein which scavenger free iron and reduce in the environment of microorganism. Thus deficiency of iron prevents biofilm formation by *P. agglomerans.* Biofilm formation which was proposed as a colonial organization adhesion method. Through biofilm formation bacteria become highly resistant to host cell defense mechanism and antibiotic treatment.[37] It is well known that some bacteria stains require high level of iron to form biofilms. Thus Lf's function as an iron chelator has been hypothesized to effectively inhibit biofilm formation through iron sequestration.[38]

The second mechanism is suggested that lactoferrin with binding to the lipid A causes discontinuity membrane of gram-negative bacteria resulting destabilizing the outer membrane of the bacteria and release of lipopolysaccharide (LPS) and ultimately can be lead to changes permeability in the membrane (39). Rceptors for the N-terminal region of Lf have been discovered on the surface of some microorganisms. The binding of Lf to

these receptors induces cell-death in Gram negative bacteria due to disruption in the cell wall.

Sixteen *P. agglomerans* strains were isolated in our study from blood cultures of infants with high resistant rate for most antibiotics under study. Liberto *et al*.[40] reported that within three months 6 *P. agglomerans* strain were isolated from blood cultures of 1 patient from ICU and 5 patients from oncology departments.

Also 12(75%) of these bacteria were positive ESBL producing strains by screening test and 8(66.67%) out of 12 harboring *bla*CTX-M gene by genotypic detection while all strains don't harboring *bla*SHV genes. One study found that a strain of *P. agglomerans* was ESBL-producers that have blaCTX-M-15 gene of ESBL genes and this strain was isolated from the blood culture of an adult male.[41]

Lactoferrin and brevicin (crude and purified) were tested alone and in combination with each other for their antibacterial activity against *P. agglomerans* strains and our results showed that purified brevicin was more effective compare with crude brevicin ($p \leq 0.05$), and on other hand lactoferrin in combination with brevicin showed significant differences ($p \leq 0.05$) compare to lactoferrin and brevicins alone.

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