IMMUNOMODULATORY OF *Bifidobacterium breve AND INHIBITORY EFFECT OF BIFIDOBREVICIN* -LHM ON *Streptococcus agalactiae* AND ITS β-HEMOLYSIN

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ABSTRACT

The objective of this research was production, purification and characterization of Bifidobrevicin-LHM from *Bifidobacterium breve* isolated from women breast Colostrum. The antibacterial activity of crude and purified Bifidobrevicin-LHM on *Streptococcus.agalactiae* were assayed, also investigated their effect on β-hemolysin activity which is purified from S.agalactiae in vitro .We developed an untried mouse model of acute S.agalactae pneumonia, biochemical, histopathological, microbiological, innate and specific immune response were determined. Purified and crude Bifidobrevicin-LHM exhibited bactericidal action against S.agalactiae isolates and its β-hemolysin activity in vitro. Oral and Intranasal administration of *B. breve* post infection leading to significantly increase *S.agalactae* clearance rates in lung and blood, reduce the BAL albumin concentration,lung injury and Lactate dehydrogenase activity, enhanced production of Interleukein 10,IL-4 and high level of BAL -anti *S.agalactiae* IGg compared with control P<0.05 .To our knowledge the present research is the first report on purification and characterization of bacteriocin produced by B.breve exhibiting inhibitory activity on *S.agalactiae* and its β-hemolysin. Bifidobrevicin-LHM can inhibit virulence in S.agalactiae is an alternative select for therapy, taking into consideration that orally and intranasally administered.B. breve stimulate both the specific and innate immune response in the respiratory tract. These end results recommend that specific immunomodulatory properties of B. breve should be characterized when developing clinical application

Key word: Bifidobacterium *Streptococcus agalactiae, bacteriocin, β-hemolysin immunomodulatory*

التحفيز المناعي بواسطة *Bifidobacterium breve* والتاثير التثبيطي للبيفيدوبريفيسين اتجاه *Streptococcus agalactiae* وما تنتجه من بيتاهيمولايسين

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المستخلص

هدف هذا البحث الى انتاج وتنقية وتوصيف بكتيريوسين من بكتريا *Bifidobacterium breve* المعزولة من لبا الانسان. تم اختبار التاثير التثبيطي للبكتيريوسين الخام والمنقى اتجاه بكتريا *Streptococcus.agalactiae* وانزيم البيتاهيمولايسين المنقى منها في الزجاج فضلا عن استحداث موديل جديد من الاصابات الرئوية للفئران ببكتريا S.agalactae اذ اجريت اختبارات كيموحيوية، نسيجية، ميكروبية اضافة الى الكشف عن الاستجابة المناعية الخاصة والمتاصلة .اظهرت النتائج ان للبكتيريوسين الخام والمنقى تاثيرا قاتلا تجاه بكتريا *S.agalactae* واظهر قدرته على تثبيط فعالية البيتاهيمولايسين المنج منها في الزجاج ،واثبتت النتائج قدرة بكتريا B.breve بعد اعطائها عن طريق الانف والفم للحيوانات المصابة ببكتريا *S.agalactae*في تصفية البكتريا وتقليل اعدادها في الدم والرئة محدثا تقليل الضرر النسيجي الحاصل في الرئة مؤكدة ذلك الفحوصات الكيموحيويةمتمثلةانخفاض تركيز الالبومين وفعالية انزيم lactodehydrogenase في السوائل الرئوية ورفع مستوى الاستجابة المناعية متمثلة بارتفاع تركيز Interleukin−10,4 و الكلوبيولين المناعي المضاد الخاص ببكتريا *S.agalactae-IGg* وبفروقات مهمة احصائيا مقارنة بالسيطرة. حسب الادبيات المتوفرة فان البحث الحالي هو اول بحث تناول تنقية وتوصيف بكتيريوسين من بكتريا بفدوبكتيريوم بريف ودراسة تاثيره المثبط تجاه بكتريا S.agalactae وانزيم البيتاهيمولايسين المنقى منهااي ان البكتيريوسين استطاع ان يثبط ضراوة البكتريا وهذا اسلوب بديل للعلاج ويجب ان يؤخذ بنظر الاعتبار ان اعطاء بكتريا *B.breve* للحيوانات عن طريق الانف والفم ادى الى تحفيز الاستجابة المناعية لذا يمكن التوصية بضرورة تطوير الفكرة بالتطبيقات السريرية .

كلمات مفتاحية : بفدوبكتيروم بريف ، المكورات المسبحية ، بكتيريوسين ، تحفيز مناعي ، بيتاهيمولايسين.

INTRODUCTION

Streptococcus agalactiae is most generally associated with neonatal disease such as sepsis, pneumonia and meningitis, since 1970, *S. agalactiae* has been thought a very significant cause of sever neonatal disease with high morbidity and mortality (1). The β-hemolysin of *S. agalactiae* is a main virulence factor, as a result non hemolytic strains rarely cause infection, β-hemolysin is considered as virulence factor very important for invasion of host tissue and electron microscopy revealed that the hemolysin acts as a pore forming cytolysin ,on the other hand, inhibiting bacterial virulence factor by normal compound is a new approach to overcome raised antimicrobial resistance in pathogenic bacteria(2).

Breast milk colostrum is a significant factor in the initiation of advancement and composition of the neonatal gut microbiota. Antimicrobial activity is one of the most significant selection criteria for probiotics, antimicrobial effect of probiotic is created by producing some substances such as organic acids, hydrogen peroxides, lantibiotic and bacteriocins (3,4,5). Although most studies concerning probiotic mediated improved immune defense is focused on gastrointestinal tract pathogen also,many Bifidobacterium bacteria effects are intervened through immune regulation, particularly by balance control of proinflammatory cytokines. A few recent studies experimented whether probiotic might sufficiently stimulate the general mucosal immune system and only limited data obtainable for *Bifidobacterium breve* bacteriocin purification and characterization and evaluate its effect on pathogenic bacteria. In our knowledge no studies exist on the capacity of orally and intranasal administered B. breve to stimulate respiratory immunity as a result, this work was carried out to examine some *B. breve* isolates that are isolated from Iraqian women breast colostrum for probable production of bacteriocin and to determine the physiochemical characteristics and antimicrobial spectra of this bacteriocin on *S. agalactiae* and its purified β-hemolysin activity in vitro, also to investigate whether the intranasal and oral administration of *B. breve* activates the respiratory immune system and whether it reduces the susceptibility to *S. agalactiae* lung infection.

MATERIALS AND METHOD

Isolation of *B. breve* from women breast colostrum:

The isolation material was human colostrum obtained from 11 healthy volunteers women without any pharmacological treatment, the study was conducted according to the ethical norms commendd by the Ministry of health, Government of Iraq. Two milliliter of colostrum were collected aseptically in duplicate by a registered nurse during the first day of the post-partum, the samples were stored at 4^{o}C until their processing in the laboratory (at the most 1 hour after their emissionEach sample was kept in a sterile bag containing peptone water (0.1%) added to 0.25% L-cysteine (Sigma, St. Louis, Mo., USA). Samples were then homogenized and diluted in peptone water with L-cysteine. 100µL of the dilution were spread on (MRS-Cys) agar (Biokar, Diagnostics, France) containing 0.25% L-cysteine and incubated at 37°C for 48h in an anaerobic chamber. The selected colonies were purified by repeated streaking on MRS agar and identified by the API 50 CH kit (Biomeriux- France) according to the guideline of the manufacturer.

Bacteriocin Detection and assay:

The isolates of B. breve were screened for bacteriocin production against *S. agalactiae* isolates using the spot on-lawn assay according to (6)

Bifidobrevicin-LHM production:

Fermentor conditions for production of bacteriocin by *B. breve* No. 2isolate in MRS broth containing 0.25% L-cysteine were described previously (7).

Bifidobrevicin-LHM purification:

crude bacteriocin was purified according to (8), concentrated protein at each step was determined by Lowry method [9].

Characterization of Bifidobrevicin-LHM

Sensitivity to heat, pH, and enzymes

After gel filtration, the effect of temperature on purified Bifidobrevicin-LHM activity was assayed by heating bacteriocin solution to(20, 30, 40, 50, 60, 70, 80, 90, 100)°C respectively. Bacteriocin activity was assayed after 30 minutes at each of these temperature.activity also assayed after 15

minutes at 121°C. Semipurified preparations of *Bifidobrevicin-LHM* were adjusted to various pH values in the range of 2 to 11. The pH-adjusted *Bifidobrevicin-LHM* samples were incubated at 37°C for 20 min and then neutralized to pH 6 and tested for bacteriocin activity. Susceptibility of *Bifidobrevicin-LHM* to various enzymes was performed by incubating *Bifidobrevicin - LHM* preparation in the presence of, trypsin, pepsine, ,lipase and amylase (1 mg/ml) at 37°C for 1 h. After incubation, the enzymes were inactivated by heat treatment at 70C for 30 min and tested for bacteriocin activity.(10)

Evaluation of the molecular weight of *Bifidobrevicin-LHM*:

The molecular weight of *Bifidobrevicin - LHM* was determind by gel filtration chromatography according to Andrews (11)

***Streptococcus agalactiae* ISOLATES:**

Seven isolates of *S. agalactiae* obtained from Department of Biology –College of Science-University of Al-Mustansiriya , were used. all isolates were confirmed with VITEK 2 system (Biomerieux-France), all isolates had been previously serotyped by Lancefield group streptococcal antisera (Oxoid)

Determination of β-hemolysin activity:

S. agalactiae β-hemolysin activity was assayed by microtiter plate assay as described by Marchlewicz and Duncan (12).

Extraction and purification of *S. agalactiae* β-hemolysin:

The β-hemolysin of clinical isolate S. *agalactiae* isolate No. 4 was extracted and purified by a modification of the method described by Marchlewicz and Duncan (12), protein at each step determined by Lowry method [9]. The purity of purified β-hemolysin was estimated by SDS-PAGE was done according to the method of Laemmli described by Garfin (13)

Antibacterial activity of *Bifidobrevicin-LHM* on *S. agalactiae* isolates in vitro:

, Agar well diffusion method described by [12] was followed to detect inhibitors effect of crude and purified *Bifidobrevicin-LHM* on *S. agalactiae* isolates. The titers of crude and purified *Bifidobrevicin-LHM* were quantified by two fold serial dilutions of bacteriocin in saline solution. The antimicrobial activity of *Bifidobrevicin-LHM* was defined as the reciprocal of the highest dilution showing inhibition of *S. agalactiae* isolates and was expressed by activity units per milliliter (Au/ml) [14].

Effect of Bifidobrevicin - LHM on *S. agalactiae* cell viability

The effect of Bifidobrevicin-LHM on S. agalactiae cell lysis was detected according to(kelly etal 96). Bifidobrevicin-LHM solution was added to each culture of S. agalactiae to obtain final concentrations(80and 160)AUlml of Bifidobrevicin-LHM and the cultures were incubated at 37^{o}Cfor different incubation period(0,8 and 24) hours.

Effect of *B. breve* bacteriocin on *S. agalactiae* β-hemolysin activity:

B. breve bacteriocins crude and purified by gel filtration tested for their ability to inhibit *S. agalactiae* β-hemolysin activity, various concentrations (160 and 80)AUlml were incubated with 100 H of hemolysin for 10 minutes at 37^{o}C, the samples were chilled at 4^{o}C and the remaining hemolytic activity was determined according to 15 Immunomodulatory activity and antagonistic effect of *B. breve* against *S. agalactiae* in vivo

A –microorganisms *S. agalactiae* isolate No.4 used to prepare bacterial suspention containing10^{8}cfu/ml (spectrophotometrically controlled) was instilled

B. breve isolate No.3 culture for 8h at 37c in MRS broth,and the bacteria were harvested through centrifugation at 5000 rpmfor 10 min then washed three times with steril 0.01Mphosphate buffer saline (PBS)pH7.2

B-animals:

BALB/c mice aged 4-5 weeks old (19-20 gm) obtained from Animal House, medicine college- Baghdad University, were housed under standard conditions.The mice were anesthetized by i.p. injection of 0.2 ml of 0.65% sodium pentobarbital before bacterial inoculation. Animals were infected by intranasal. The study was performed according to the ethical norms commended by the Ministry of health, Government of Iraq and by the animal ethics committee guidelines of our institution

EXPERIMENTAL DESIGN:

Animals were divided into (3) groups,each containing (10) mice

GroupI: control

Animal challenged with 50µl of *S. agalactiae*, fed with 0.1 ml a single dose of PBS via orogastic gavage along the period

GroupII: Thes animal were challenged with 50µl of *S. agalactiae,*after24h mice were fed orally (0.1)ml of *B. breve* 1×10^8cfu/ml along the period

GroupIII : Mice were infected with 50µl of S. agalactiae, after24h, mice were administred intranasal 20 µl of *B. breve* 1×10^8cfu/ml along the period.

Animal were observed and evaluated, mice by exposure to co2 on day 3 and 9 post infection blood samples were obtained through cardiac puncture, the lungs were then removed for quantitative bacteriological and histopathological studies Blood and lung bacteriological examination

One lung for each animal was dissected under aseptic conditios and suspended in saline (1)ml.they were homogenized, blood and lung homogenates were quantitatively inoculated onto (Todd-Hewitt broth)Difco by serial dilution followed by incubation for 37 h.

Albumin content and Lactate dehydrogenase activity assays in Bronchoalveolar lavage (BAL):

BAL samples were obtained according to the technique explaind previously (16). A measure to quantitate raised permeability of the bronchoalveolar-capillarity barrier was determined colorimetrically based on albumin binding to bromocresol green using an albumin BCG diagnostic kit (Roche Diagnostics, Indianapolis, USA). The results were expressed as mg/mL.LDH activity was determined according to Roche Diagnostic reagents and procedures (Roche Diagnostics, Indianapolis, USA). The results were expressed as U/L of BAL fluid

Cytokine assay: The levels of interleukin-10(IL-10)and IL-4 in the BAL fluid were determined using Duo Set ELISA Kits (R&D Systems, Minneapolis, MN, USA).

Statistical analysis: Differences between groups were tested for significance using unpaired *t*-test. Values with $P < 0.05$ were considered statistically significant and analysed using the SAS software R 9.1

RESULTS AND DISCUSSION

***B. breve* isolation from colostrum:** A total of 31 *Bifidobacterium* isolates were obtained from 11Iraqian colostrum samples in this study, there were 1-3 isolated *Bifidobacterium* species per sample, results corresponding to the *Bifidobacterium* identification were: the predominant species were *B. bifidum 11* isolates (35.48)% followed by B longum 8 isolates (25.8)% and *B. adolescentis* 7 isolates (22.58)% while *B. breve* was found in the proportions of (16.12)% 5 isolates. Our results verify that breast colostrum is a source of *Bifidobacterium breve* which may probably impact the newborns' gut colonization progression health promoting effects. Some studie suggest that these bacteria might be brought to the lactating breast tissue channel the endogenous trafficking of bacteria-loaded dendritic cells created from the intestinal mucosa (17). Our results indicate that *B. bifidum and B.longum* were the predominant species found in Iraqian colostrum samples, while *B. pseudocatenulatum* was not found and *B. breve* were found in lower proportion.

Screening for bacteriocinogenic *B. breve*:

About 5 Bifidobacterium breve isolates are isolated from Iraqian mothers colostrum examined for bacteriocin activity using the spot-on-Lawn assay. The five isolates *B. breve* (No. 1, No. 2, No. 3, No. 4, No. 5) showed the ability to produce bacteriocin but isolate No. 2 which showed the strongest bacteriocin activity against indicator bacteria, depending on the results above the isolate *B. breve* No. 2was chosen for further study, because of its interesting inhibition spectrum.

The purification of *Bifidobrevicin-LHM*:

B. breve crude bacteriocin obtained by ammonium sulfate flocculation also fractionated by sephacryl S-300 gel filtration chromatography. Each eluted 5 ml fraction read at 280 nm and the curve was plotted between the absorbance and fraction number which gave 2 protein peaks as shown in figure .1, the maximum activity of purified bacteriocin was observed in the fractions (31-34) the specific activity and purification folds, summarized in Table 1.

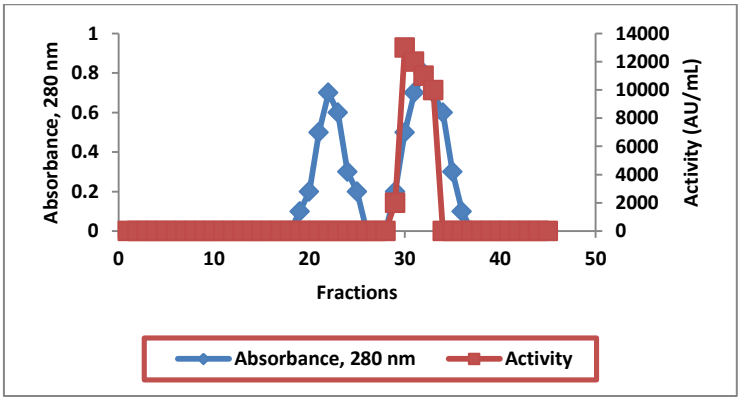

Figure 1. Elution of *Bifidobrevicin-LHM* for sephacry, S – 300 gel filtration

Table 1. purification of *Bifidobrevicin-LHM* produced by *B. breve* .

Fractions	Volume (mL)	Total Protien (mg)	Total Activity (AU)	Protien Concentration (mg/mL)	Activity (AU/mL)	Specific Activity (AU/mg)	Fold Purification
Cell Free Supernatant (CFS)	2900.0	102.249.0	56,500,000	35.258	19,482	552,5	1
Precipitation (NH4)2SO4	31.0	211.7	37,795,000	6.82	1,154,677	169,083	306
Gel Filtration Sephacryl S-300	8.4	120.7	24,681,850	2.938	293,831	204,489	370
HPLC	0.92	0.1	24,070	0.108	26,161	240,700	473

Characterization of *Bifidobrevicin-LHM*:

Results in Table 2. show that *Bifidobrevicin-LHM* was stable at pH values ranging from 3 to 8, at these values, bacteriocin remained active while at pH values 2, 9 and 10, the bacteriocin lost 60% of its activity, and whole activity of *Bifidobrevicin-LHM* was lost at the pH values 11 indicating its sensitivity to alkali treatment.

Thermo stability of *Bifidobrevicin-LHM* was assayed at different temperatures as shown in Table 2., the bacteriocin was resistant to treatment (20, 30, 40, 50, 60, 70, 80, 90 and 100)^{o}C for 30 minutes. However 50% of activity was lost after autoclaving 121^{o}C/15min, this thermo stability feature might be related to the molecular structure of *Bifidobrevicin-LHM*.

As shown in Table 2. complete in activation in antibacterial activity was observed after treatment of *Bifidobrevicin-LHM* with trypsin and pepsin, confirming its proteinaceous nature whereas treatment with Lipase and α-amylase did not affect the activity suggesting that structure of *Bifidobrevicin-LHM* did not contain lipid or carbohydrate moiety) (18 and 19).

Table 2. characterization of *Bifidobrevicin-LHM*

Treatment	Activity (%)
Cntrol	100
PH Treatment	
2	40
3	100
4	100
5	100
6	100
7	100
8	100
9	40
Heat Treatment	
20°C/30min	100
30°C/30min	100
40°C/30min	100
50°C/30min	100
60°C/30min	100
70°C/30min	100
80°C/30min	100
90°C/30min	100
100°C/30min	100
121°C/15min	50
Enzyme Tretment	
Trypsin	0
Pepsin	0
Lipase	100
a-amylase	100

Estimation of *Bifidobrevicin-LHM* molecular weight:

The molecular weight of purified *Bifidobrevicin-LHM* was determined by gel filtration chromatography as shown in Figure 2.as 11700 Dalton.

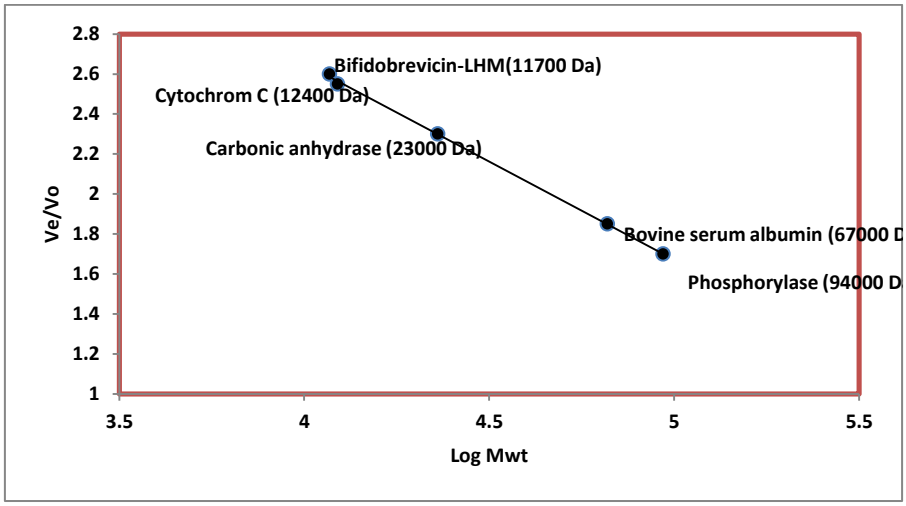

Figure2. Molecular weight of *Bifidobrevicin-LHM* produced by *B.breve*

Production of *S. agalactiae β*-hemolysin:

All of *S. agalactiae* isolates tested indicated extracellular β-hemolysin, the production by isolates increased considerably, β-hemolysin was purified from one of the isolates *S. agalactiae* No. 4, which produce β-hemolysin in a much larger quantity than other isolates.

Purification of *S. agalactiae* β-hemolysin:

S. agalactiae β-hemolysin was purified by column chromatography on a sephadex G-100 column. After elution from the column, the fractions were assayed for hemolytic activity and absorbance at 280 nm. Three distinct peaks of 280 absorbing material were detected. The second peak possessed the majority of the hemolytic activity, the peak fractions with hemolytic activity (25-29), were pooled lyophilized, the recovery and the specific activity at each step of purification are shown in Table 3. . Appearance only one band of protein reflects the purity of *S. agalactiae* β-hemolysin Figure 3..

Figure 3. Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE).for the purified β-haemolysin produced from *S.agalactiae*

Table 3.Steps in Purification of *β*- hemolysin from *S.agalactiae* isolates no,4

Purification Steps	Volume (mL)	Protein (mg/mL)	Total Protien (mg)	Hemolytic Activity (HU/mL)	Total Hemolytic Activity (HU)	Specific Activity Protein (HU/mg)	Recovery Hemolytic Activity (%)
Crude Extract	1110	0.108	120	100	$111*10^3$	$0.925*10^3$	100
Pricipitation (NH4)2SO4	115	0.434	50	939.13	$108*10^3$	$2.16*10^3$	97.321
Sephadex G-100	18	0.444	8	3333	$60*10^3$	$7.5*10^3$	54.05

Antibacterial activity of *Bifidobrevicin-LHM* on *S. agalactiae* in vitro:

Table 4. show the antibacterial activity of purified and crude *Bifidobrevicin-LHM* against *S. agalactiae* isolates. The results indicate that purif *Bifidobrevicin-LHM* possesses significant antibacterial activity against all S. agalactiae isolates contrast with control and the antibacterial activity of purified *Bifidobrevicin-LHM* was significantly higher than crude $P < 0.01$=ied and crude.

Table 4. Antibacterial activity of *Bifidobrevicin-LHM on S. agalactiae in vitro*

Titer	Zone of inhibition (mm) mean ± SD *		
	crude bacteriocin	purified bacterioion	Control
NEAT	22.11±0.82 P1	29.51±0.87 P2 P1	0±0
1/2	17.33±1.02 P1	22.11±0.41 P2 P1	0±0
1/4	12.91±1.14 P1	15.92±0.57 P2 P1	0±0
1/8	5.89±1.63 P1	10.32±1.31 P2 P1	0±0

***: 7 isolates of S. agalactiae**
P1: probability compared to control $P < 0.05$
P2: probability compared to crude at a same concentration $P < 0.05$

Effect of *Bifidobrevicin-LHM* on cell viability

After 8 hour of incubation the complete killing of S. agalactiae cells was observed at 160AUlml Bifidobrevicin-LHM concentration , wherease at 80AUlml the complet killing of S. agalactiae cells were occurred after 24 hours of incubation indicating bactericidal mode of action of Bifidobrevicin-LHM taking into consideration Bifidobrevicin-LHM bactericidal activity ,protienaceous nature,heat thermostability and molecular weight Bifidobrevicin - LHM can be classified as heat stable small bacteriocin apparently belonging to class II according to (20)

Effect of *Bifidobrevicin-LHM* on *β* hemolysin activity of *S. agalactiae* :

As shown in Table 5. complete inactivation of hemolytic activity were observed after treatment, *S. agalactiae* purified hemolysin with 160*AUlml* of crude and purified Bifidobrevicin-LHM, however, 50% activity was lost after treatment with *80AUlml* crude bacteriocin, also 30% activity was lost after treatment with *80AUlml* of purified bacteriocin. The purified and crude *Bifidobrevicin-LHM* showed the inhibitory spectrum and it is important to highlight the activity against S. agalactiae isolates which is important human pathogens,, purified and crude Bifidobrevicin-LHM had inhibition effect to β-hemolysin activity, it has decreased β-hemolysin activity to a very low level while crude Bifidobrevicin-LHM appeared to be the stronger inhibitor to β-hemolysin activity in vitro. A possible reason for the observed strong inhibiting of β-hemolysin activity by crude *Bifidobrevicin-LHM* may be the fact that this crude Bifidobrevicin-LHM contain bacteriocins and other substances such as bio surfactant, especially bio surfactant have the ability to inhibit a number of enzymes such as streptococcus mutanglucosyltransferase [21]. Several investigators have attempted to correlate the production of β-hemolysin from *S. agalactiae* is associated with injury in the lung epithelial cells which explan for pulmonary damage and electron microscopy showed that the β-hemolysin acts as a pore-forming cytolysis (22). Hemolysin also motivated cytolysis and apoptosis of the phagocytes (23). The mechanisms by which bacteriocin influence bacterial growth can be explained by influencing on cellular membranes instability and permeability According to (24), it is possible that classII use the similar mechanisms of action. Bacteriocin link to the plasmatic membrane through electrostatic interfaces with phospholipids charged negatively. The monomers of bacteriocin cause in the pore development with the consequent depart of ions (mainly potassium and magnesium), loss of the proton-motriz power, and exit of ATP and amino-acids. The proton motriz-force has a essential role in ATP production, in active transport and in the bacterial movement; for that reason, the production of macromolecules is inhibitedin addition to the production of energy, causing in cell death (25). Some authors have documented that the antimicrobial activity of probiotic possibly due to a number of factors including reduced pH levels, production of materials with a bactericidal or bacteriostatic activity including bacteriocins, hydrogen peroxide, bio surfactant (26,27).

Table 5. Effect of *Bifidobrevicin-LHM* on *S. agalactiae* β-hemolysin activity in vitro.

Treatment	Bacteriocin concentration *AUlml*	Residual hemolytic activity %
Crude	160	0
	80	50
Purified	160	0
	80	70
Control	0	100

Antagonistic effect of *B. breve* against *S. agalactiae* in vivo:

Bacterial count were determined in lung and blood during 3rd and 9th days post infection shown in Table 6., results indicated that *S. agalactiae* was able to establish a lung infection, the number of *S. agalactiae* in lung tissue for group 1 (control) had significantly higher level than group 2 and 3 whom treated with L. oris P<0.05, however, mice infected with *S. agalactiae* and administered orally *B. breve* exhibited significant delay in the clearance of S. agalactiae from the lung compared with mice in group 3 which were infected with *S. agalactiae* and administered intranasal L. oris at day 3 and 9 , P<0.05. Also mice in group 3 had negative hemocultures in 3rdand 9th days but group 2 had significantly lower number of S. agalactiae in blood culture at 3rd and 9th days compared with control P<0.05. These results demonstrate that oral and intranasal administration of *B. breve* reduces the number of *S. agalactiae* in lungs of mice after infection. It can be conclude that oral and intranasal administration of *B. breve* was able to increase *S. agalactiae* clearance rates in lung and blood, enhanced infected mice survival and decreased lung injuries.

Table 6. Effect of *B. breve orally and intranasally administered on S. agalactiae in vivo*

Groups	mean ± SD log Cfu/g lung		mean ± SD Log cfu/m/ Blood culture	
	after 3 day	after 9 day	after 3 day	after 9 day
control	9.86 ±0.95	8.74 ±0.91	5.39 ±0.84	3.81 ±0.54
group II administered *B. breve* orall	7.89 ±1.43 *	$<5*10^2$ *	3.78 ±1.32 *	0 ±0 *
group III intranasal administered	6.21 ±1.52 * P2	0 ±0 *P2	0 ±0 * P2	0 ±0 *

*: probability compared to control, $P < 0.05$

P2: probability compared to group II, $P < 0.05$

Albumin content and Lactate dehydrogenase activity assays in BAL fluid:

Albumin content and LDH activity were used as indicator of lung injury. Infection with *S. agalactiae* caused raise in BAL albumin concentration and the activity of lactate dehydrogenase in all groups, but the factors were significantly lower in *B. breve* intranasal administered and mice administered orally with *B. breve at 3rdand 9th days* compared with the control, also the difference between group II and group III reach statistical significance P<0.05. as shown in Table 7. we observed decrease concentration of albumin concentration and LDH activity in all groups at ninth day , however, these alterations were significantly smaller in *B. breve orally and intranasally* treated mice compared to control group, these results indicate lower tissue damage.

Table 7. Effect of B. breve on lung injuries induced by S. agalactiae infection

Groups	albumin concentration mg/ml (mean ± SD)		LDH activity U/ml (mean ± SD)	
	3 rd day	ninth day	3 rd day	ninth day
control	0.81 ±1.63	0.66 ±2.5	59.11 ±2.69	100 ±0.11
group II orall administered *B. breve*	0.53 ±1.01 d	0.42 ±1.23 d	41.82± 3.42 d	52 ±2.67 d
group III intranasal administered *B. breve*	0.38 ±0.78 d a	0.25 ±0.19 d a	28.4 ±1.57 d a	16.5±2.03 d a

d: probability compared to control$P < 0.05$

a: probability compared to group II $P < 0.05$

The immunomodulatory effect of oral and intranasal administration of *B. breve* in the respiratory tract of mice infected with *S. agalactiac*:

In the present study the level of BAL IL-10 and IL-4 were determined at 3rd and 9th days post infection. Mice treated intranasal with *B. breve* showed higher values of IL-10 and IL-4 than mice treated orally with *B. breve* and control Fig4., and results showed that IL-10 AND IL-4 WERE significantly increased in orally and intranasal *B. breve* treated mice compared with those in control P<0.05.Further more BAL-anti *S. agalactiae* IgG antibodies were determined at 3rd and 9th days post infection Fig4. . Levels of anti *S. agalactiae* IgG in BAL of mice intranasal treatment with *B. breve* were higher than in mice that were orally treated with *B. breve* and control, however, mice groups that were treated with *B. breve* presented higher values than those in the control animals. *B. breve* treatment induced a significant increase in specific *S. agalactiae* BAL IgG antibodies compared with control P<0.05. The beneficial effect of *B. breve* treatment was related to an up regulation of the specific and innate immune responses in mice infected with S. in this study IL-10 and IL-4, significantly raised in the BAL of animals that received *B. breve* .This difference could be responsible for the protective effect of B.breve because IL-10 and IL-4 inhibit the synthesis of pro-inflammatory cytokines such as TNF-α (28). which in accordance with the raise in the levels of specific *S. agalactiae* IgG in BAL, moreover, specific IgG during infectious process is important because this antibody production coincided with the development of a chronic process and was related with a reduction in the number of *S. agalactiae* in the lung, therefore, mice that are treated with *B. breve* showed an improved respiratory humoral immune response which was an evidence by the higher levels of BAL *S. agalactiae* IgG. This agree with other studies that suggest IL-4, and IL-10 contribute to stimulate B cells to proliferate and mature into polymeric IgA- producing cells to develop specific antibodies (29). Other studies reported that together with innate immune response, the production of IgG specific antibodies is important to decrease colonization of the respiratory epithelium and to evade dissemination into blood (30).

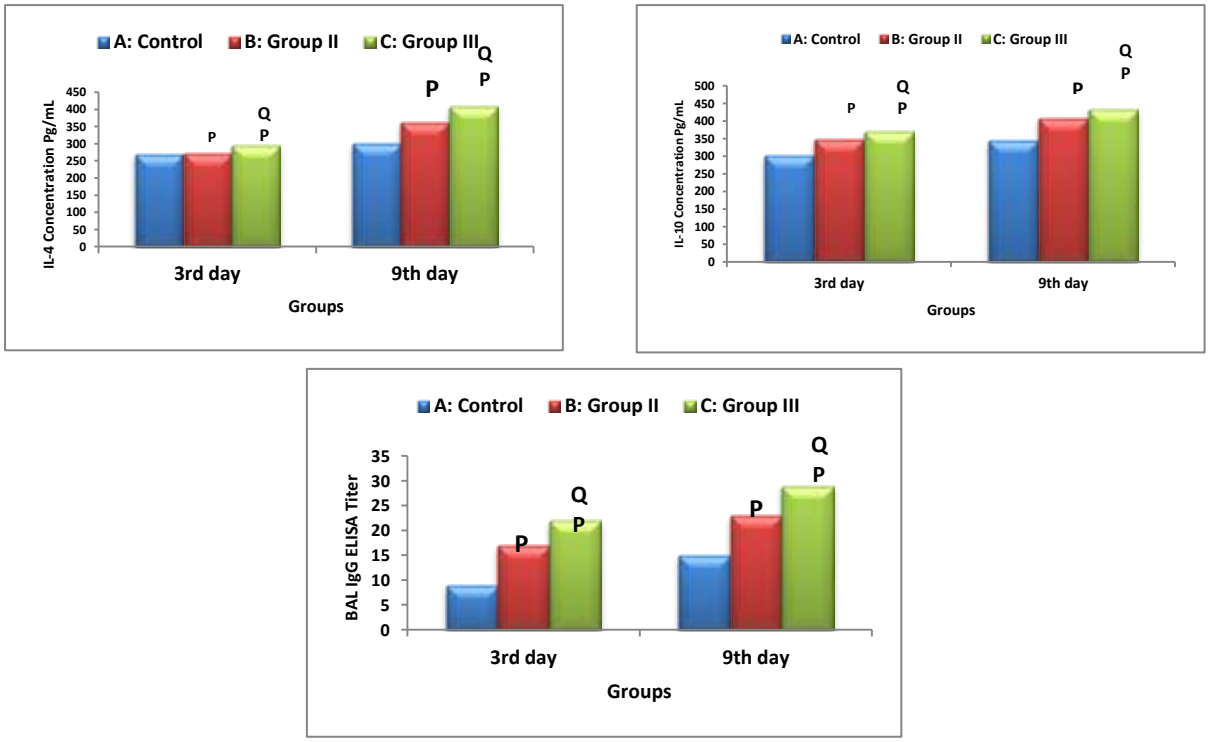

Figure 4. The immunomodulatory effect of oral and intranasal administration of *B. breve* in the respiratory tract of mice infected with *S. agalactiac*

Q: Significant differences compared with other groups, P<0.05

P: Significant differences compared with control, P<0.05

Pathological examination:

Pathological examination of the lungs of mice infected with *S. agalactiae* and treated with *B. breve* orally and intranasal exhibited only mild to moderate pneumonia, less interstitial edema and inflammatory cell infiltration around the bronchi as showon in Fig 5 (A)10X and chronic inflammatory around the bronchi (B)40X. whereas, mice infected with *S. agalactiae* whom non-treated showed sever pneumonia , these differences are verified in terms of pathological features such as air space filled with inflammatory cells (arrow) and epithelial hyperplasia, heavy chronic inflammatory cell with necrosis, damage of the lung parenchyma all alveoli damaged (C), destruction of alveoli even inside small bronchi (D), destruction to alveoli (E), chronic inflammatory cells with thickening in alveoli septae (F) and high number of macrophages (G AND H). Animals that were treated with live *B. breve* showed decrease of lung injury with less interstitial edema and inflammatory cell infiltrated. These data collectively suggest that *B. breve* treatment was more efficient using intranasal administration than oral administered as a therapy strategy against *S. agalactiae*.

From the current study we can conclude that human colostrum may be used as a potential normal source to isolate *B. breve*. The existence of *B. breve* greatest probably offers the beneficial effect of this liquid on the newborn health and showed a major anti-bacterial activity of crude and purified *Bifidobrevicin-LHM* against *S. agalactiae* and can prevent and decrease activity of its β-hemolysin in vitro.Also,our recent data propose that through *S. agalactiae* infection, intranasal and oral management with *B. breve* avoid detrimental lung inflammation by triggering humoral and innate immune response detecting of bacteria, thus inhibiting bacterial virulence factor by normal compounds is a new method to overcome increased antimicrobial resistance in pathogenic bacteria, therefore, metabolites synthesized by *B. breve* may assist as valuable drugs to control infectious disease, *B. breve* treatment possibly promising strategy to develop clinical lung injury subsequent *S. agalactiae* infection and this new line of studies open novel possibilities for the application of B. breve and *Bifidobrevicin-LHM* in the prevention of disese.

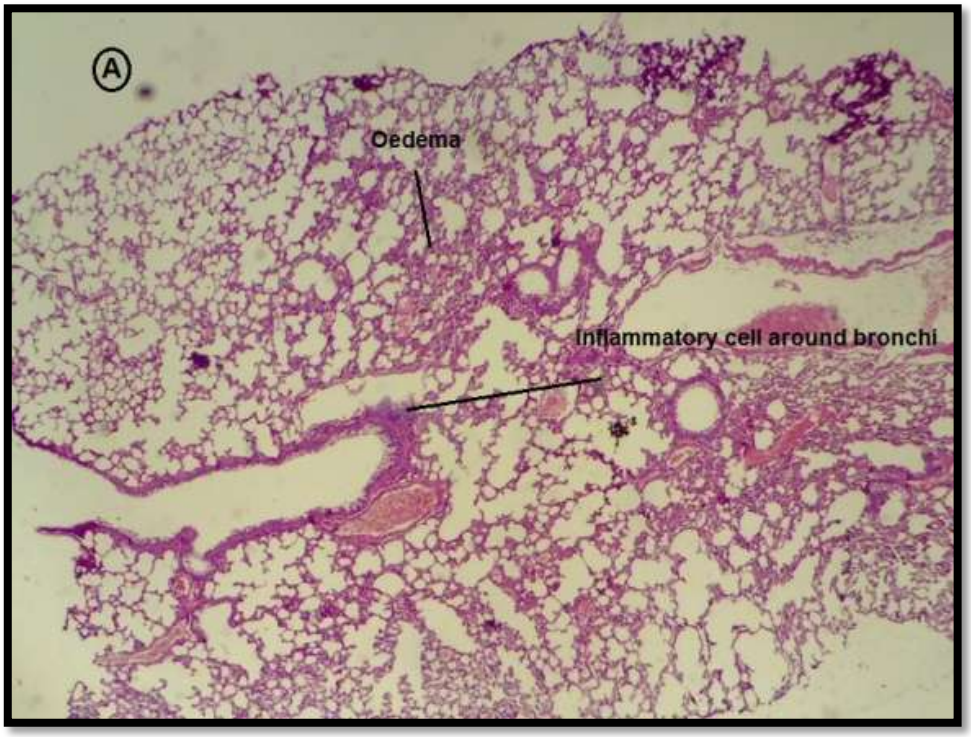

Fig 5A. *Histopathologic analysis. H&E-stained lung tissue* from mice infected with *S. agalactiae* and treated with *B. breve* orally at ninth day exhibited only mild to moderate pneumonia original magnification(X10)

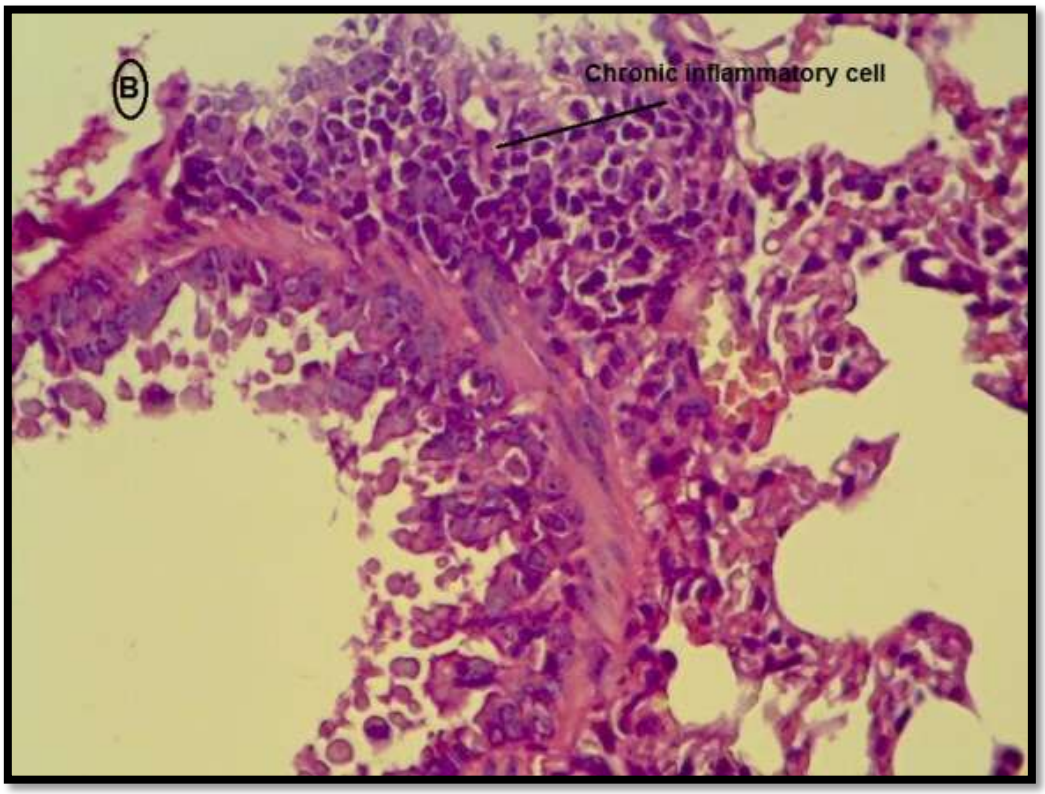

Fig5B. Histopathologic analysis. H&E-stained lung tissue from mice infected with S. agalactiae and treated with B. breve intranasal at ninth day exhibited only mild to moderate pneumonia original magnification 40X

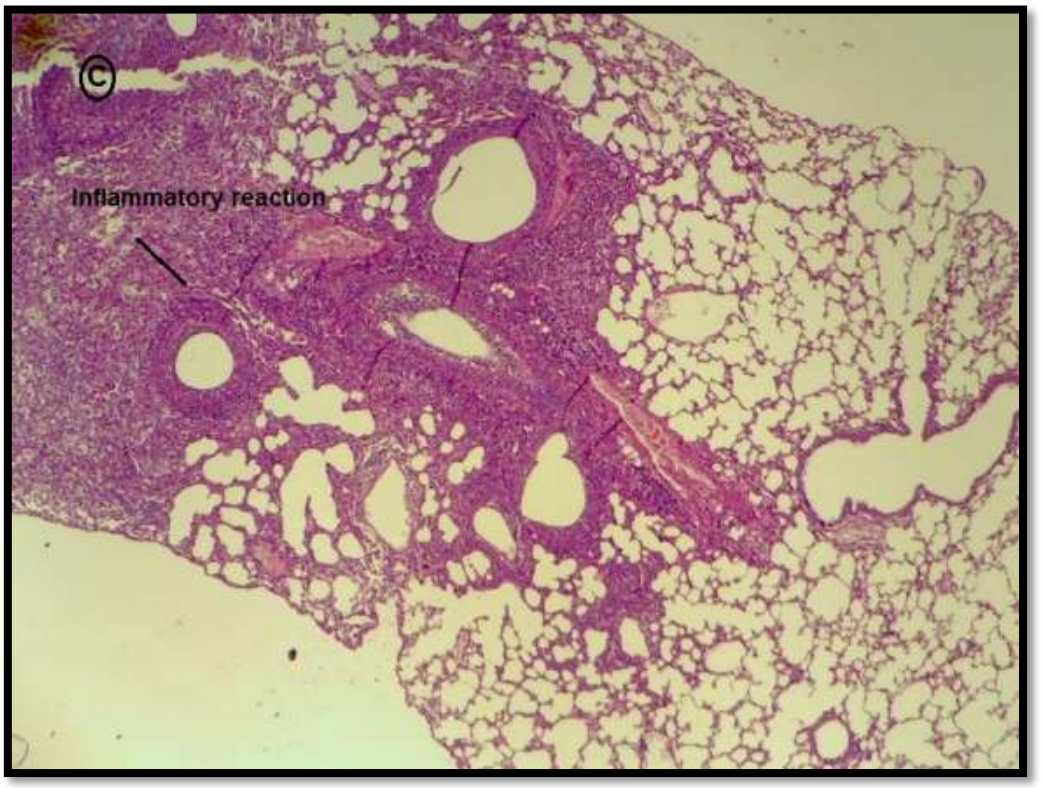

Fig5C. Histopathologic analysis. H&E-stained lung tissue from mice infected with S. agalactiae and whom non-treated control group 10X

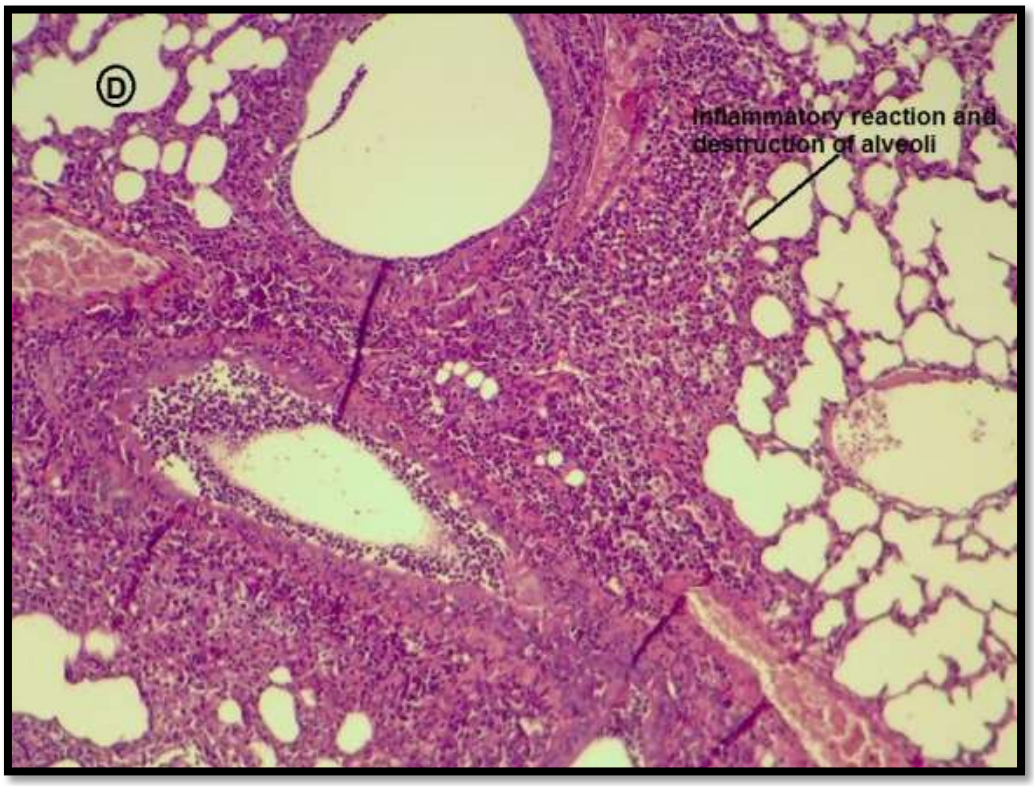

Fig5D. Histopathologic analysis. H&E-stained lung tissue from mice infected with S. agalactiae and whom non-treated control group 40X

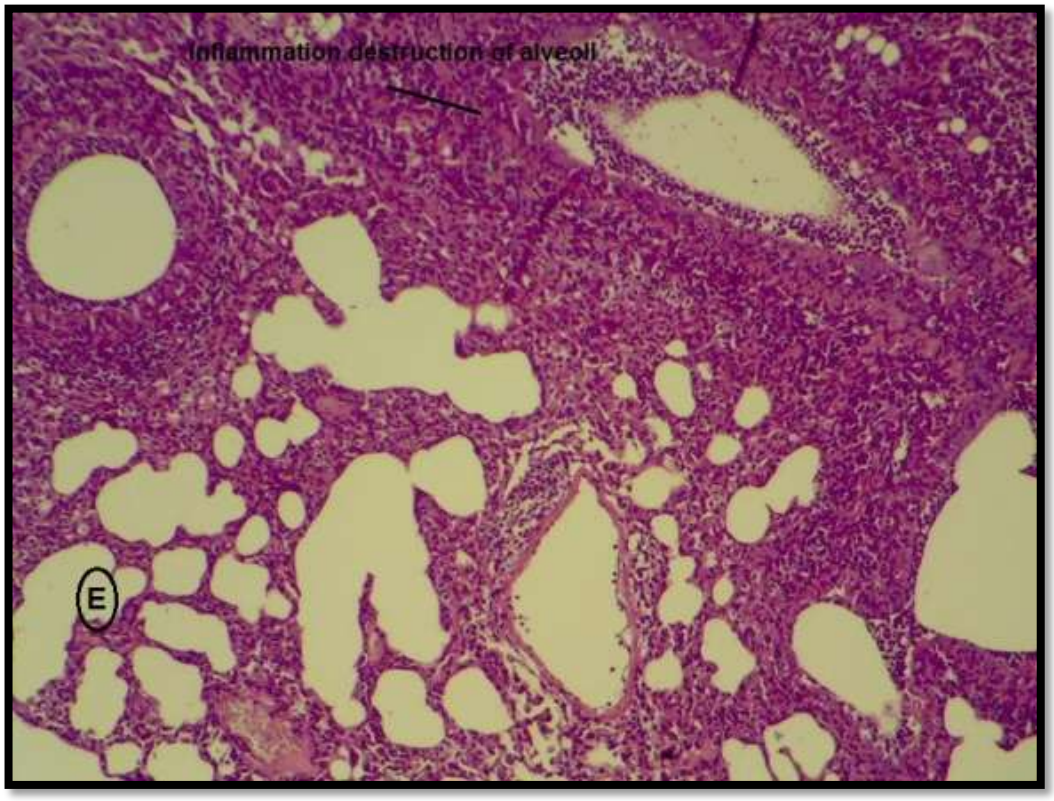

Fig5E. Histopathologic analysis. H&E-stained lung tissue from mice infected with S. agalactiae and whom non-treated control group 40X

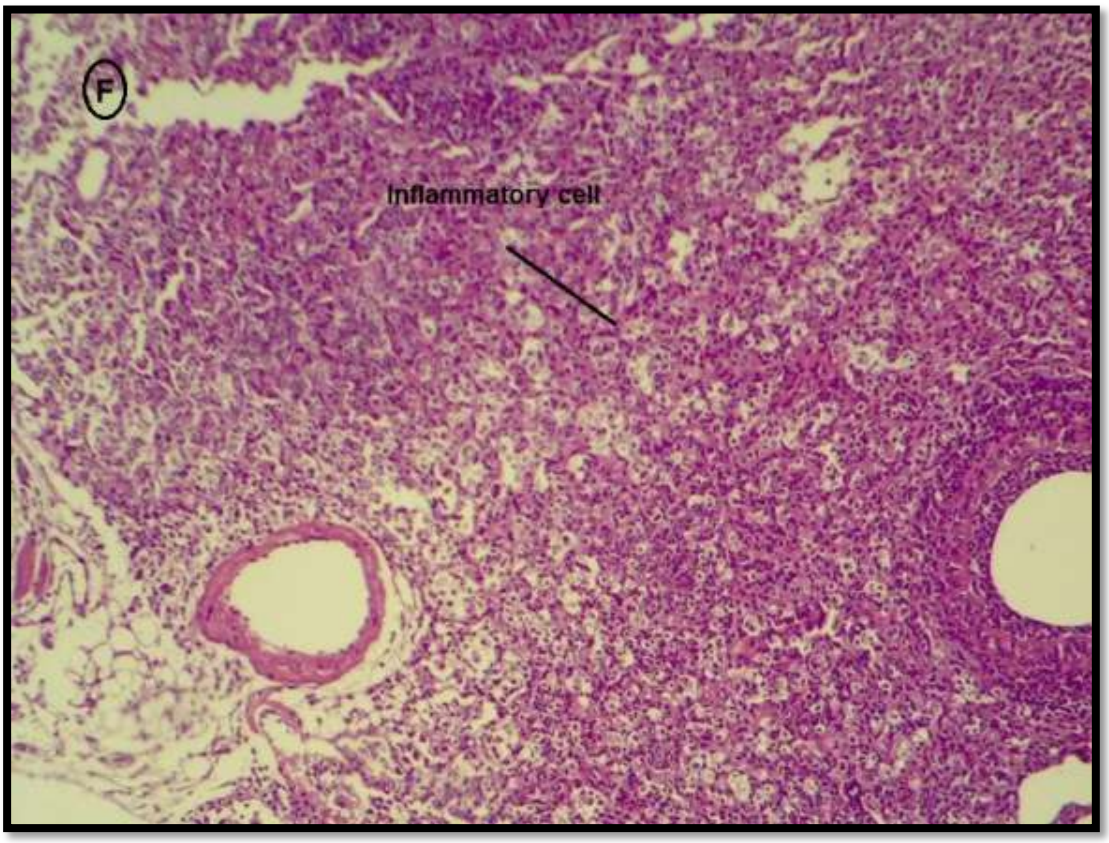

Fig5F. Histopathologic analysis. H&E-stained lung tissue from mice infected with S. agalactiae and whom non-treated control group 40X.

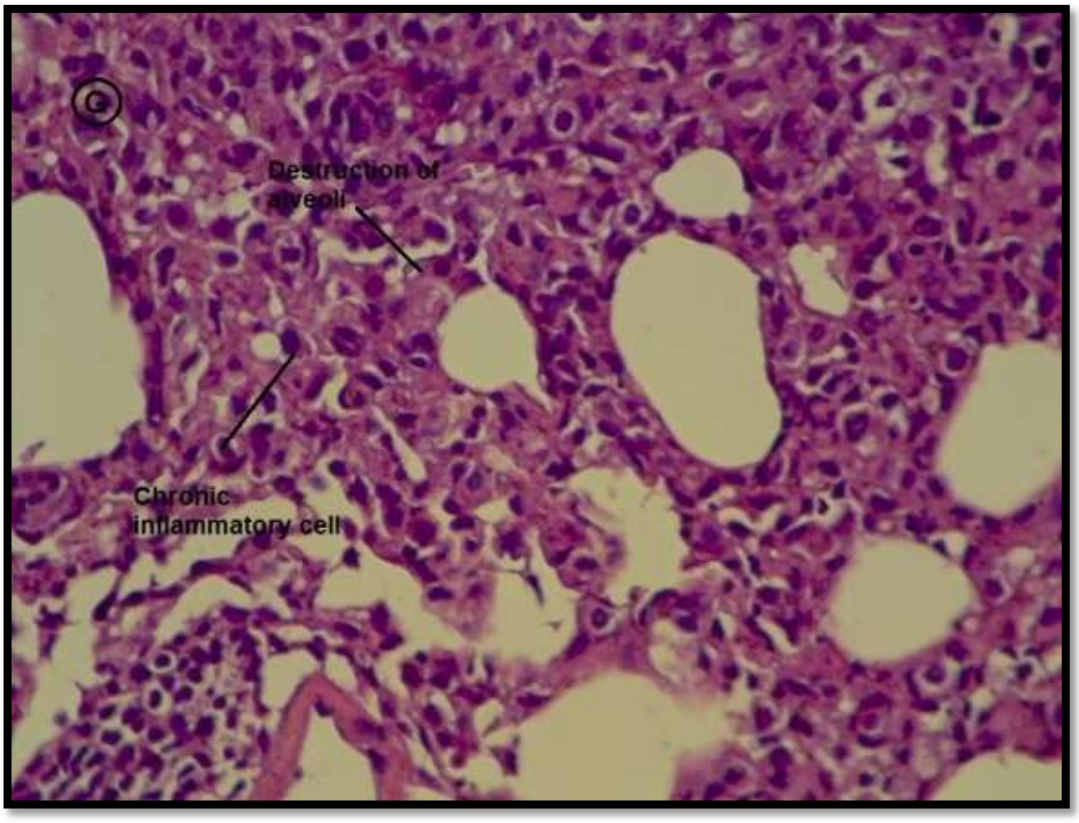

Fig5G. Histopathologic analysis. H&E-stained lung tissue from mice infected with S. agalactiae and whom non-treated control group 40X

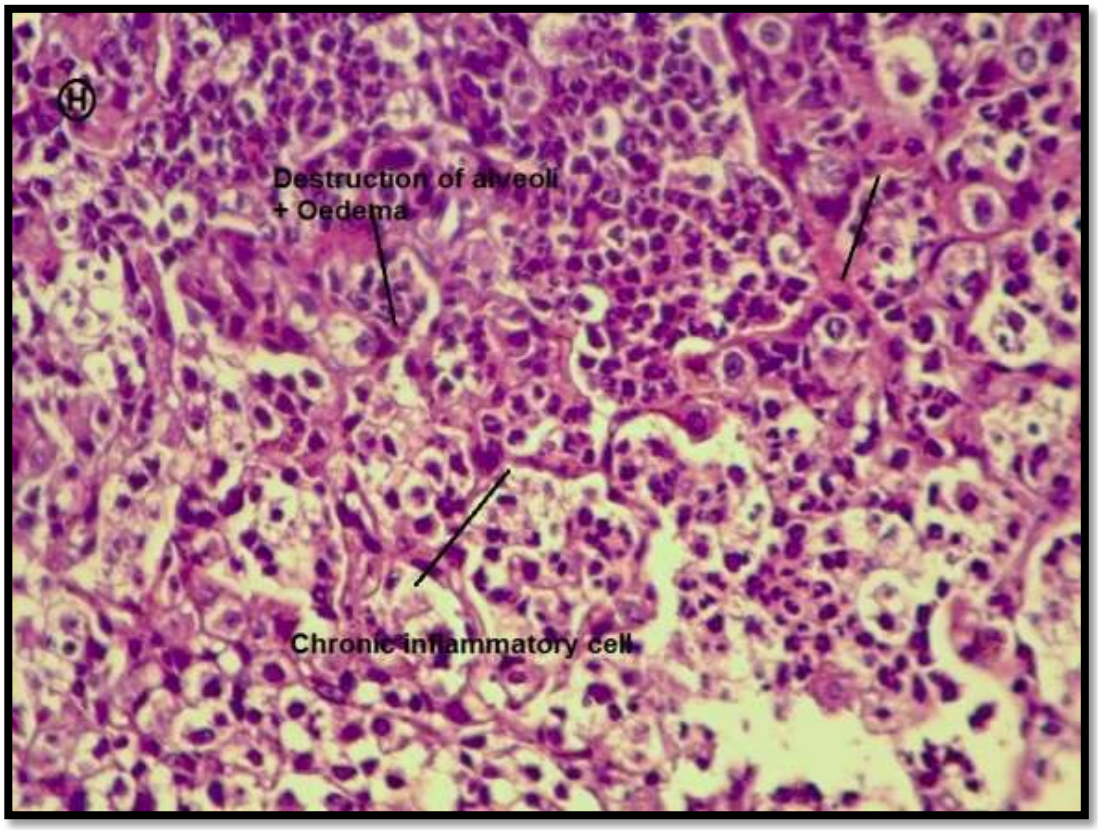

Fig5H. Histopathologic analysis. H&E-stained lung tissue from mice infected with S. agalactiae and whom non-treated control group 40X

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