Anticariogenic and antibiofilm of purified curvatcin LHM and immunomodulatory effect of *Lactobacillus curvatus* in streptococcal bacteremia

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Streptococcus mutans and *Streptococcus sanguinis* have been associated with the development of caries, oral infections and bacteremia. In fact, there are only a few case reports in the literature describing bacteremia in relation to *S. mutans* and *S. sanguine*. The objective of this study was purification and characterization of curvatcin LHM from *Lactobacillus curvatus*, evaluation of its antibacterial activity against *S. mutans* and *S. sanguinis* and evaluation the effect of minimum inhibitory concentration of curvatcin LHM and chlorohexidine on biofilm formation as well as, study the effect of *L. curvatus* as immunomodulator. Curvatcin LHM was isolated and purified from *L. curvatus* culture. Purified and crude curvatcin LHM exhibited bactericidal action against *S. mutans* and *S. sanguinis* isolates *in vitro*. Significant differences ($P < 0.05$) were found in viable count between pre and posttreatment of *S. mutans* and *S. sanguinis* biofilms with curvatcin LHM and chlorohexidine. Unlike chlorohexidine, curvatcin LHM left no viable bacterial cells in biofilm of *S. mutans* and *S. sanguinis*. Bagg Albino laboratory breed (BALB/c) mice were orally administered with *L. curvatus* for 2 weeks and then intravenously injected with *S. mutans* and *S. sanguinis*. Four days before inoculation, microbiological and immune response were determined, serum proinflammatory cytokine, TNF-α, IL-10 and IL-6 were evaluated by ELISA. The *L. curvatus* treatment significantly decreased *S. mutans* and *S. sanguinis* in the organs and blood of mice with bacteremia as compared with the non-*L. curvatus*-treated mice ($P < 0.05$). Furthermore, proinflammatory cytokine, TNF-α, IL-10 and IL-6, were significantly higher in groups pretreated with *L. curvatus* ($P < 0.05$) prior streptococcal infection. These data suggest that curvatcin LHM may be a good alternative to chlorhexidine as an additive for teeth-protective materials. Curvatcin LHM cotreatment with chlorohexidine might help to increase the anticariogenic efficacy of chlorohexidine. On the other hand, *L. curvatus* can be serving as direct modulator of proinflammatory responses.

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Introduction

In caries, there is an increases in acid-tolerating and acidogenic bacteria like lactobacilli and some species of streptococci, even though additional bacterial agents with related properties can be found such as nonmutans streptococci, *Bifidobacteria*, *Propionibacterium* spp., *Actinomyces* spp., *Atopobium* spp. and *Veillonella* spp. [1]. The use

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of a chlorhexidine (CHX)-based mouthwash in combination with normal tooth care can help reduce the build-up of plaque and improve mild gingivitis [2]. However, the retention of CHX on tooth surface also leads to it's an undesirable side-effect which is tooth staining and calculus formation [3,4]. Bacteriotherapy is a fascinating approach in oral infectious disease management [5,6], whereas lactic acid bacteria (LAB) play such role. The most significant LAB predictable for their capability to minimized toxigenic mold growth were *Lactococcu*s and *Lactobacillus* and to a less significant *Leuconostoc* and *Pediococcus* [7]. *Enterococcus faecium* and *Enterococcus faecalis* isolates were employed to study the antagonistic effect of *Lactobacillus fermentum* against them. Preparation of *L. fermentum* cell-free supernatants showed significant activity against both *Enterococcus* isolates and showed closely related results [8]. Bacteriocin produce by many species of bacteria [9,10] including LAB [11–13] may involve in protection role and management of caries and oral infections. Recent studies reveal that this bacteriocin is mediating competition between some strains and others. Recently, a greater attention towards bacteriocin-producing bacteria was increased as bioprotective and probiotics [12–14].

Streptococcus sanguinis and *Streptococcus mutans* involved in predicting caries formation in children and adults, and they result in diseases of the oral cavity [15,16]. However, many conventional antibacterial drug have been used for treatment of such infections [17] as well as some disinfectants like CHX [2]. Recent studies focused on the effect of bacteriocin-producing LAB and their own bacteriocin on other pathogenic bacteria [12,13,18]. The objective of this study was the purification and partial characterization of a bacteriocin formed by *Lactobacillus curvatus* isolated from local raw goats milk to study its effect on *S. mutans* and *S. sanguinis* isolates and comparison its effect with CHX *in vitro*. As well as in-vivo determination the prophylactic and immunomodulatory effect of *L. curvatus* on *bacteremia* caused by *S. mutans* and *S. sanguinis*.

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Subjects and methods

Isolation of *Lactobacillus curvatus*

Five isolates of *L. curvatus* isolated from raw milk of Iraqi goats from Abu-Ghraib and Al-Radwaniyah farms diagnosed by biochemical test and confirmed by the API 50 CHL system (BioMerieux, Craponne, France).

Streptococcus mutans *and* ***Streptococcus sanguinis*** *isolates*

A total of seven and five isolates of both *S. mutans* and *S. sanguinis*, respectively, obtained from Biology Department, College of Science, University of Mustansiriyah were identified by Vitek 2 according to the manufacturer's instruction. AQ10

Preparation of crude curvatcin

L. curvatus (LHM 5) isolate was grown in Man, Rogosa and Sharpe broth (pH 6) inoculated with overnight culture (5%) and incubated anaerobically at 30 °C for 48 h. The cells were removed by centrifugation at 10 000 × *g* (15 min, 4 °C). The pH of supernatant was adjusted to 6 then lyophilized and it was used as crude curvatcin LHM [8].

Curvatcin LHM activity assay

The antibacterial activity of the bacteriocin was determined by the well diffusion method according to Mahdi [13].

Purification of curvatcin LHM

One isolate of *L. curvatus* was chosen for bacteriocin production according to its potent ability. The purified *bacteriocin* was called *curvatcin* LHM according to the producer isolate the crude curvatcin was precipitated with 35–65% ammonium sulphate (Ranboxy, New Delhi, India) saturation. The precipitate was collected by centrifugation at 10 000 × *g* (20 min, 4 °C), dissolved in 0.05 mol/l phosphate buffer (pH 6.8) and then dialyzed. The antimicrobial activity of reconstitute protein was checked. Sephadex G-75 (Sigma, St. Louis, Missouri, USA) was used for molecular exclusion chromatography and equilibrated with phosphate buffer. Determination of void volume was by passing blue dextran 2000-kDa through the column, and 1 ml of concentrated bacteriocin preparation obtained from 35 to 65% ammonium sulphate saturation was chromatographed at a time. Bacteriocin activity of obtained fractions was determined by measuring the absorbance at 280 nm. The fractions that showed antibacterial activity were concentrated by dialysis against sucrose. The active antimicrobial fractions were collected and checked for purity [19].

Determination of protein concentration

Protein concentration of curvatcin LHM was determined by protein assay kit (Bio Rad, Hercules, California, USA) was used according to the manufacture's manual.

Characterization of curvatcin LHM

Determination of molecular weight

Molecular weight of curvatcin LHM was determined by gel filtration chromatography as described elsewhere [20].

Sensitivity of curvatcin LHM to enzymes, pH, temperature and detergents

Purified curvatcin LHM was incubated for 2 h at 37 °C in the presence of proteinase K, trypsin, pepsin, α-amylase and lipase (Sigma, Ronkonkoma, New York, USA) at concentration of 1 mg/ml and the control was untreated sample. The pH of curvatcin LHM containing supernatant was adjusted to 2–10 to test the effect of pH on

curvatcin LHM activity. The supernatant was incubated for 1 h at 37 °C then the pH was adjusted to pH 6 [18,21]. The temperature effect on the curvatcin LHM was tested by exposure the purified bacteriocin to different temperature, 30, 45, 60, 75 and 100 °C for 1 h, sample at room temperature was used as a control [22]. The effect of EDTA, Tween 80, SDS and urea at different concentrations, 0.1, 1, 2 and 5%. Control consisted of active supernatant. All samples and control were incubated at 37 °C for 5 h. The residual activity was estimated after each treatment as described previously [18].

Chlorhexidine gluconate

Stock solution 10 mg/ml of CHX gluconate were prepared in deionized distilled water and sterilized by filteration through cellulose acetate filter 0.2 μm pore size Millipore (Whatford, UK).

Determination of curvatcin LHM and chlorhexidine minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of curvatcin LHM and CHX were determined according to Batdorj *et al.* [23] in concentrations (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024) μg/ml.

Streptococcus mutans *and* ***Streptococcus sanguinis*** *biofilm formation*

Method described by Maldonado *et al.* [24] was followed to achieve *S. mutans* and *S. sanguinis* biofilm formation. The highest biofilm producing isolates of *S. mutans* and *S. sanguinis* (S2, S5, S7 and S8 isolates) were selected to inhibit their biofilm by pyocin. Same protocol described Maldonado *et al.* [24] was followed to produce a biofilm. Then, before the staining step, the previously prepared CHX and purified curvatcin LHM containing media with MIC as it presented in Table 1 were added to the biofilm containing wells.

Subsequently, the tray was incubated for another 24 h at 37 °C, after incubation period all wells were washed and stained as the same procedure described above. Also viable count was carried out depending on the procedure described Harley and Prescott [25].

Table 1. Minimum inhibitory concentration of chlorhexidine and curvatcin LHM used to inhibit *Streptococcus mutans* and *Streptococcus sanguinis* biofilm.

isolates	Chlorhexidine MIC (μg/ml)	Curvatcin LHM MIC (μg/ml)
S. mutans 1	16	256
S. mutans 6	128	128
S. sanguinis 3	32	64
S. sanguinis 4	8	512

MIC, minimum inhibitory concentration.

Prophylactic effect of *Lactobacillus curvatus* against streptococcal bacteremia *in vivo*

Few colonies of *S. mutans* and *S. sanguinis* isolates from an overnight culture on Columbia sheep blood agar were allowed to grown in Todd-Hewitt broth at 37 °C in the presence of 5% CO_2 which were adjusted spectrophotometrically to scale, 1×10^5 colony forming unit per millilitres (CFU/ml) then it was injected intravenously. All injection volumes were adjusted to 100 μl [13]. Each day a stock solution of *L. curvatus* was prepared using a sterile technique by suspension of freeze dried powder in sterile distilled water to give 1×10^8 CFU/ml. The suspension was stored at 4 °C for up to 12 h.

All animal experiments were performed in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Bagg Albino laboratory breed (BALB/c) mice their age was between 3 and 4 weeks (17–19 g) obtained from animal house of college of medicine – Baghdad University. Before bacteria been injected, the mice were injected with 0.65% sodium pentobarbital as anaesthetic intrapertonially. The ethical norms of Ministry of Health, Iraq were considered. Four mice groups were formed, six mice each as follow: AQ11

(1) Mice orally administered with PBS for 14 days followed by *S. mutans* intravenous injection later.
(2) Mice orally administered 1×10^8 CFU *L. curvatus* a single feed for 14 days followed by *S. mutans* intravenous injection later.
(3) Mice orally administered with PBS for 14 days followed by *S. sanguine* intravenous injection later.
(4) Mice orally administered 1×10^8 CFU *L. curvatus* in a single feed for 14 days followed by *S. sanguinis* intravenous injection later.

Mice ($n = 6$) in each groups, were euthanized at 96 h following injection of either *S. mutans* or *S. sanguinis* then blood samples were drawn.

Determination of the viable bacterial levels

To determine the *S. mutans* and *S. sanguinis* clearance upon *L. curvatus* prophylaxis, samples of kidney, heart, spleen, liver and lungs were excised in sterile conditions and processed. Serially dilutions of drawn blood samples were performed and diluted blood were plated on the Todd-Hewitt agar (Difco Laboratories, Grand Island, New York, USA) plates. The excised organs were homogenized in tissue homogenizer (Tyco Healthcare Group, Mansfield, Massachusetts, USA). Serially diluted tissues were inoculated on Todd-Hewitt agar (Difco Laboratories) plates. After 24 h of incubation at 37 °C under anaerobic conditions, colonies were enumerated as CFU/g for tissues and CFU/ml for blood. Three independent experiments representing three biological replicates were performed.

Table 2. Purification steps of curvatcin LHM.

Purification stage	Volume (ml)	Total activity (Au/ml)	Total protein (mg)	Specific activity (Au/mg)	Purification (fold)	Recovery (%)
Culture supernatant (crude)	100	7350	225.2	32.64	0.0	100
Ammonium sulphate precipitation & dialysis	25	2680	28.7	105.92	3.25	41.36
Sephadex G-75	5	800	0.68	1176.47	11.11	26.32

ELISA

Concentrations of TNF-α, IL-10 and IL-6 in serum were assayed by ELISA using mouse Quantikine kits (R&D Systems, Inc., Minneapolis, Minnesota, USA).

Statistical analysis

One way analysis of variance (ANOVA) test was performed to assess the intergroup variation and *P* less than 0.05 was considered as significant value. One way ANOVA was performed using sigma state statistical software.

Results

Purification of curvatcin LHM

The purification of curvatcin LHM bacteriocin was reached up to 11.11-fold from supernatant of culture. Table 2 summarized the overall activity and yield.

Characterization of curvatcin LHM

Determination of molecular weight: when used gel filtration chromatography found that, the molecular weight of curvatcin LHM was 9500 Da as shown in Fig. 1.

Sensitivity of bacteriocin to enzymes, pH, temperature and detergents: The antimicrobial activity of the curvatcin LHM was estimated for its tolerability to enzymes, temperature, pH and detergents and are summarized these results (Table 3). Results of enzymes inactivation showed that, antimicrobial activity was lost once treated with proteolytic enzymes (pepsin, trypsin and proteinase K), whereas treatment with α-amylase, catalase and lipase make no-difference in activity of curvatcin LHM. The curvatcin LHM activity sustained after incubation for 1 h. At pH value from 2 to 8, but it was decreased with pH 10 and best activity was when the pH reached to 6. The curvatcin LHM revealed thermal stability as long as it conserved 95.8% of its initial activity after 1 h incubation

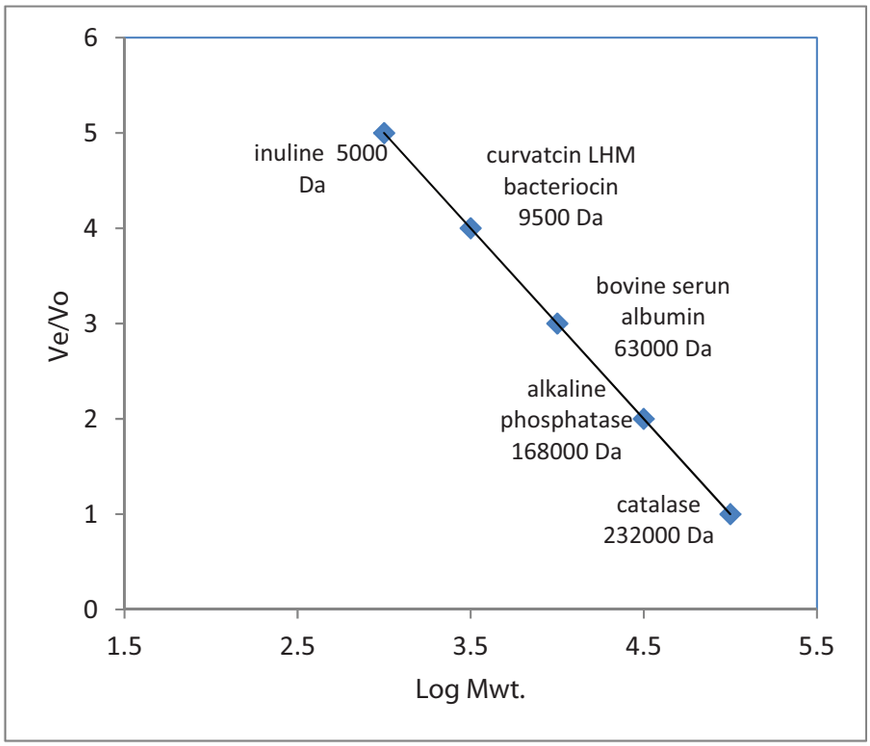

Fig. 1. Determination of molecular weight of curvatcin LHM by gel filtration chromatography. Da, Dalto; LogMwt, logarithm of molecular weight; Ve, velocity; Vo, volume. AQ16

Table 3. Effect of enzymes, pH, temperature and detergents on the activity of curvatcin LHM.

Treatment	Residual antimicrobial activity (%)
Enzymes	
Trypsin	0
Proteinase K	0
Pepsin	0
α-Amylase	100
Lipase	100
Catalase	98.6 ± 0.41
pH	
2	75.6 ± 0.41
4	81.42 ± 0.50
6	97.15 ± 0.21
8	67.9 ± 0.18
10	40.71 ± 0.22
Temperature	
30	100
45	100
60	95.8 ± 0.15
75	89.6 ± 0.34
100	71.8 ± 0.53
Detergents	
SDS	81 ± 0.21
Tween-80	61 ± 0.33
Others	
EDTA	0
Urea	0

at 60 °C, whereas it retained only 71.8% activity at 100 °C. Detergents, SDS and Tween 80, stimulated curvatcin LHM antimicrobial activity, whereas urea and EDTA inhibit antimicrobial activity completely.

Streptococcus mutans and *Streptococcus sanguinis* isolates biofilm formation

All *S. mutans* and *S. sanguinis* isolates assayed for the production of biofilm, and the results obtained are presented in Table 4 where the capability of different *S. mutans* and *S. sanguinis* isolates from dental caries to produce biofilm is summarized. The results indicated that, isolates were varied in the capability of biofilm formation under the same conditions of experimentation. Four isolates were high producers ($P < 0.05$), the thickest biofilm; 0.922, 1.743, 1.192 and 1.822, respectively. Obviously, *S. sanguinis* 4 achieved the highest biofilm thickness.

Table 4. Absorbance of *Streptococcus mutans* and *Streptococcus sanguinis* isolates biofilm at 540 nm and statistical analysis.

Isolate number	Absorbance ± SD
S. mutans1	0.922 ± 0.851*
S. mutans2	0.862 ± 0.195
S. mutans3	0.512 ± 0.654
S. mutans4	0.807 ± 0.228
S. mutans5	0.591 ± 0.178
S. mutans6	1.743 ± 0.654*
S. mutans7	0.637 ± 0.459
S. sanguinis1	0.546 ± 0.956
S. sanguinis2	0.805 ± 0.382
S. sanguinis3	1.192 ± 0.419**
S. sanguinis4	1.822 ± 0.572**
S. sanguinis5	0.535 ± 0.118

ANOVA, analysis of variance.
*The averages of three experiments represent SD with three replicates per experiment. The ANOVA test was used for data analysis.
*Probability compared with *S. mutans* isolates number 2, 3,4,5,7; $P < 0.05$.
**Probability compared with *S. sanguins* isolates number 1, 2, 5; $P < 0.05$.

Purified bacteriocin have antimicrobial activity significantly higher ($P < 0.05$) than the control and crude bacteriocin when tested against both *S. mutans and S. sanguinis*. On the other hand, no significant difference in antimicrobial activity between purified bacteriocin and CHX, whereas antimicrobial activity of CHX is significantly higher ($P < 0.05$) than antimicrobial activity of crude bacteriocin (Table 5).

Inhibitory effect of chlorhexidine and curvatcin LHM on *Streptococcus mutans* and *Streptococcus sanguinis* biofilm

The effect of MIC of CHX and curvatcin LHM were assessed on biofilm for *S. mutans and S. sanguinis* and the results revealed that, the absorbance and viable count for these isolates declined in comparison with pretreatment data ($P < 0.05$). These isolates showed significant differences ($P < 0.05$) after treatment with CHX and curvatcin LHM according to optical density parameter, and showed significant reduction ($P < 0.05$) in comparison with control when treated with CHX and curvatcin LHM; however, viable count showed that, curvatcin LHM as well as CHX had a significant inhibitory effect ($P < 0.05$) on biofilm of *S. mutans and S. sanguinis* (Table 6). All tested biofilms even those which showed a decline in absorbance or in viable count, when cultured on plate count agar. The result assured that, all CHX tested biofilms revealed a presence of viable cells, whereas curvatcin LHM left no viable bacterial cells.

The *Lactobacillus curvatus* prophylaxis against streptococcal bacteremia

Prophylactic effect of *L. curvatus* against *S. mutans* and *S. sanguinis* bacteremia was summarized in Table 7. The mice treated with *L. curvatus* exhibited reduced load of *S. mutans* and *S. sanguinis* CFU/ml of blood compared with the *L. curvatus* nontreated group (groups 1 and 3). Analysis of the bacterial decreasing data in blood culture revealed that the mice orally administered with *L. curvatus* followed by *S. mutans* and *S. sanguinis* intravenous injection showed significantly decreasing at 96 h ($P < 0.05$), whereas a significantly higher bacterial burden was observed in the mice intravenously injected with *S. mutans* and *S. sanguinis* and nontreated with *L. curvatus*. A significant decreasing of *S. mutans* and *S. sanguinis* bacteremia was found at 96 h in *L. curvatus* orally administered mice' tissues of different organs namely liver, spleen, kidney and heart ($P < 0.05$) when compared with the non-*L. curvatus*-treated infected groups of mice.

AQ17 **Table 5. Antimicrobial activity of the curvatcin LHM in compared with chlorhexidine against *Streptococcus mutans and Streptococcus sanguinis* isolates *in vitro*.**

Bacterial Isolates	Inhibition zone (mm) mean ± SD												
	Concentration (μg/ml)												
S. mutans	Crude bacteriocin				Purified bacteriocin				Chlorhexidine				Control (D.W.)
	1000	500	250	125	1000	500	250	125	1000	500	250	125	
	26 ± 0.66 p1 p2	17 ± 0.19 p1 p2	11 ± 0.29 p1 p2	7 ± 0.38 p1 p2	33 ± 0.89 p1	24 ± 0.11 p1	18 ± 0.73 p1 p2	14 ± 0.41 p1 p2	35 ± 0.02 p1	28 ± 0.71 p1	23 ± 0.51 p1	19 ± 0.33 p1	0 ± 0
S. sanguinis	18.55 ± 3.05 P1 p2	15.68 ± 1.98 p1 p2	8.09 ± 2.79 p1 p2	0 ± 0 p2	29.67 ± 2.89 p1	25.44 ± 1.73 p1	13.62 ± 2.08 p1 p2	9.43 ± 1.09 p1 p2	30.21 ± 0.25 p1	24.91 ± 2.58 p1	20.11 ± 2.09 p1	14.58 ± 2.6 p1	0 ± 0

P1, curvatcin LHM compared with control ($P < 0.05$); P2, curvatcin LHM compared with chlorhexidine ($P < 0.05$).

AQ18 **Table 6. Optical density and viable count for *Streptococcus mutans and Streptococcus sanguinis* biofilm after treatment with curvatcin LHM and chlorohexidine.**

Isolates	Parameter	Before treatment	After treatment with	
			Chlorohexidine	Curvatcin LHM
S. mutans 1	OD	0.922 ± 0.851	0.103 ± 0.04[a]	0.214 ± 0.023[a]
	VC (CFU/ml)	**324 41.09 ± 1167.53**	**ND**	**ND**
S. mutans 6	OD	1.743 ± 0.654	0.123 ± 0.34[a]	0.308 ± 0.051[a]
	VC (CFU/ml)	**100 089.3 ± 3488.02**	**ND**	**ND**
S. sanguinis 3	OD	1.192 ± 0.419	0.256 ± 0.039[a]	0.298 ± 0.75[a]
	VC (CFU/ml)	**998 421.7 ± 72 134.13**	**ND**	**ND**
S. sanguinis 4	OD	1.822 ± 0.572	0.195 ± 0.103[a]	0.307 ± 0.144[a]
	VC (CFU/ml)	**90 667.7 ± 44 968.14**	**ND**	**ND**

Each datum is the mean of triplicate. ND, no detected data; OD, optical density; VC, viable count.
[a]No significant differences between the treatment with chlorohexidine and curvatcin LHM, both suppress the viable count of *S. mutans and S. sanguinis* isolates in biofilms.

Determination of TNF-α, IL-10 and IL-6 concentrations in serum

The statistical analysis results of the average of proinflammatory cytokines concentration, TNF-α, IL-10 and IL-6, in serum of mice groups was as follows: the average TNF-α concentration of group 2 is significantly higher than TNF-α concentration of group 1. Both groups were infected with *S. mutans* but group 1 treated with PBS, whereas group 2 was *L. curvatus* administrated for 14 days (19.15, $P < 0.05$). On the other hand, the average of TNF-α concentration of group 4 is significantly higher than TNF-α concentration of group 3. The two groups were infected with *S. sanguinis* but group 3 pretreated with PBS, whereas group 4 pretreated with *L. curvatus* (21.23, $P < 0.05$). The average of serum IL-10 concentration of groups 2 and 4 is significantly higher than IL-10 concentration of groups 1 and 3, respectively (332.26, $P < 0.05$ and 391.59, $P < 0.05$). The same results were obtained in the average of IL-6 concentrations, groups 2 and 4 is appear to be significantly higher than IL-6 concentration of groups 1 and 3, respectively (325.15, $P < 0.05$ and 349.58, $P < 0.05$). Figures 2–4 summarize these results.

Table 7. Prophylactic effect of *Lactobacillus curvatus* against *Streptococcus mutans* and *Streptococcus sanguinis* bacteremia.

Groups	(Mean ± SD)Log_{10} CFU/ml of blood and CFU/g of tissue					
	Spleen	Lung	Liver	Kidney	Heart	Blood
Group (1) ***S. mutans*** + PBS	0.988 ± 0.22	2.05 ± 0.91	1.037 ± 0.81	0.95 ± 0.48	0.62 ± 0.29	2.41 ± 1.01
Group (2): ***S. mutans*** + *L. curvatus*	0 0.38 ± 0 0.091	0 0.53 ± 0.89	0.27 ± 0 0.08	0 ± 0	0 ± 0	0.86 ± 0.16
	a	**a**	**a**	a	a	a
Group (3): ***S. sanguinis*** + PBS	1.04 ± 0.91	1.85 ± 0.92	1.07 ± 0.84	0.44 ± 0.79	0.51 ± 0.186	1.96 ± 1.01
Group (4): ***S. sanguinis*** + ***L. curvatus***	0.73 ± 0.45	0.93 ± 0.41	0.53 ± 0.08	0 ± 0	0.11 ± 0.08	0.62 ± 0.19
	b	**b**	**b**	b	b	b

AQ19

a: probability compared with group 1, $P < 0.05$. b: probability compared with group 3, P less than 0.05.

AQ1

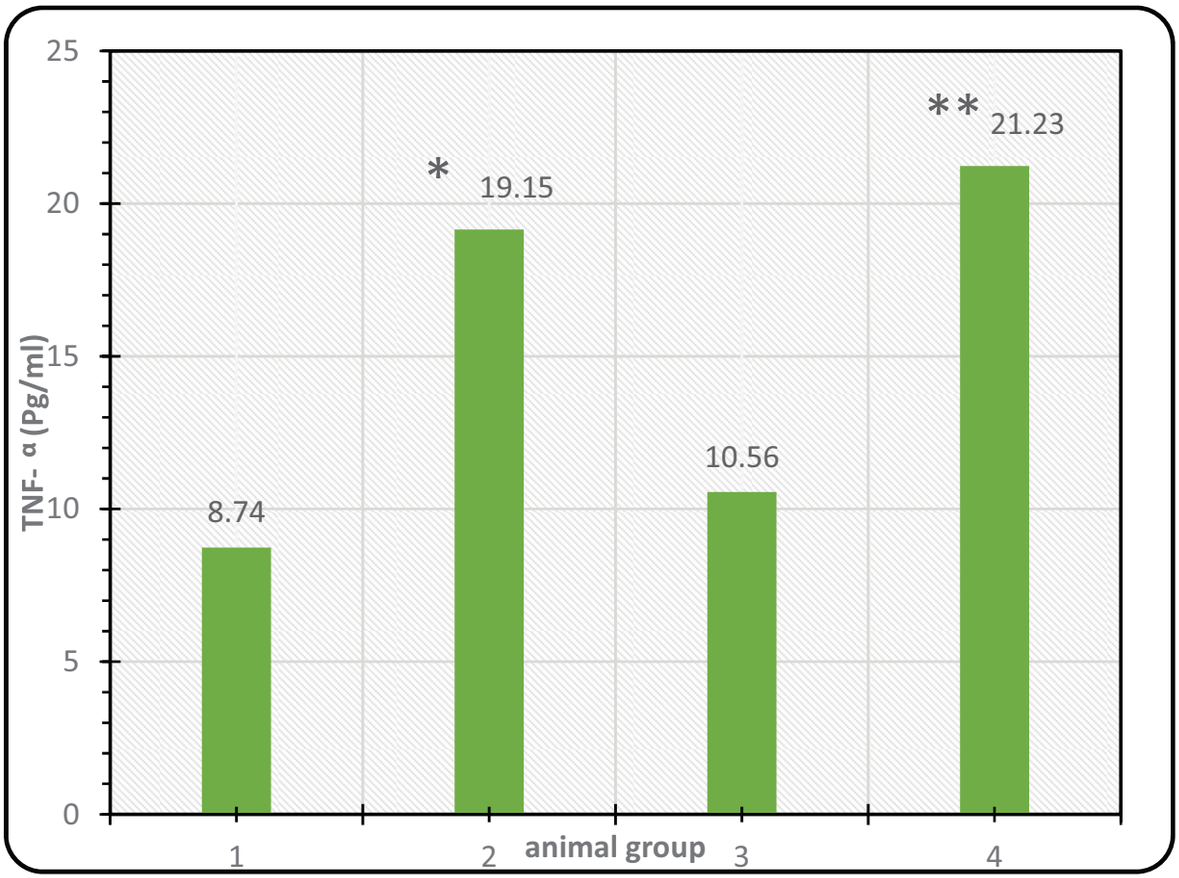

Fig. 2. Level of TNF-α concentration of four challenged animal groups. Groups 1 and 2 were infected with *Streptococcus mutans* while groups 3 and 4 were infected with *Streptococcus sanguinis*. Groups 2 and 4 were *Lactobacillus curvatus* orally administrated for 14 days. Groups 1 and 3 are PBS-treated mice. *Significant difference between groups 1 and 2 ($P < 0.05$), **significant difference between groups 3 and 4 ($P < 0.05$).

Discussion

The characterization of purified bacteriocin of *L. curvatus*, curvatcin LHM, suggested proteinaceous nature of curvatcin LHM, hence it is inactivated after digestion with protein digested enzymes, proteinase K, trypsin and Pepsin. As reported by Kaktcham *et al.* [26] who found that, the bacteriocin produced by *L. curvatus* was affected by both proteinase K and trypsin and therefore can be classified as a bacteriocin. The antimicrobial activity of curvatcin LHM was conserved when treated with carbohydrate and lipids – digesting enzymes suggesting the absence of lipid and carbohydrate moieties or it may indicate to carbohydrate and lipid moiety unimplication in inhibitory action of curvatcin LHM [27]. As reported previously, different pH values do not affect curvatcin LHM activity (pH 2.0–10.0) [26], whereas the results of current study showed reducing in activity of curvatcin LHM at pH 10. In addition, antimicrobial activity was not due to acidity or hydrogen peroxide as antimicrobial activity was not lost after readjustment of pH to 6 or treatment with catalase. Furthermore, curvatcin LHM was resistant to heat. It sustained a remarkable antibacterial activity (89%) at relatively high temperature reached to 75 °C and stable in boiling temperature with good antibacterial activity. Tween 80 and SDS, reduce the antimicrobial activity of curvatcin LHM to 81 and 61%, respectively. Although EDTA and urea are completely inactivate curvatcin LHM. Tween 80 induces protein aggregation and oxidation. The aggregation is linked by disulfide and nondisulfide bonds in temperature-dependent manner [28]. Although SDS and urea are protein folding and protein denaturing agents and these agents are potent enzyme inactivators [29].

Purified curvatcin LHM exhibit antimicrobial activity significantly better than crude curvatcin LHM, this may be attributed to the increase the concentration of purified curvatcin LHM as compare with concentration of crude curvatcin LHM and the contamination of crude curvatcin LHM with other substances during production of the bacteriocin. Significantly, CHX and purified curvatcin LHM showed no difference in antimicrobial activity making the used of purified curvatcin LHM is promising

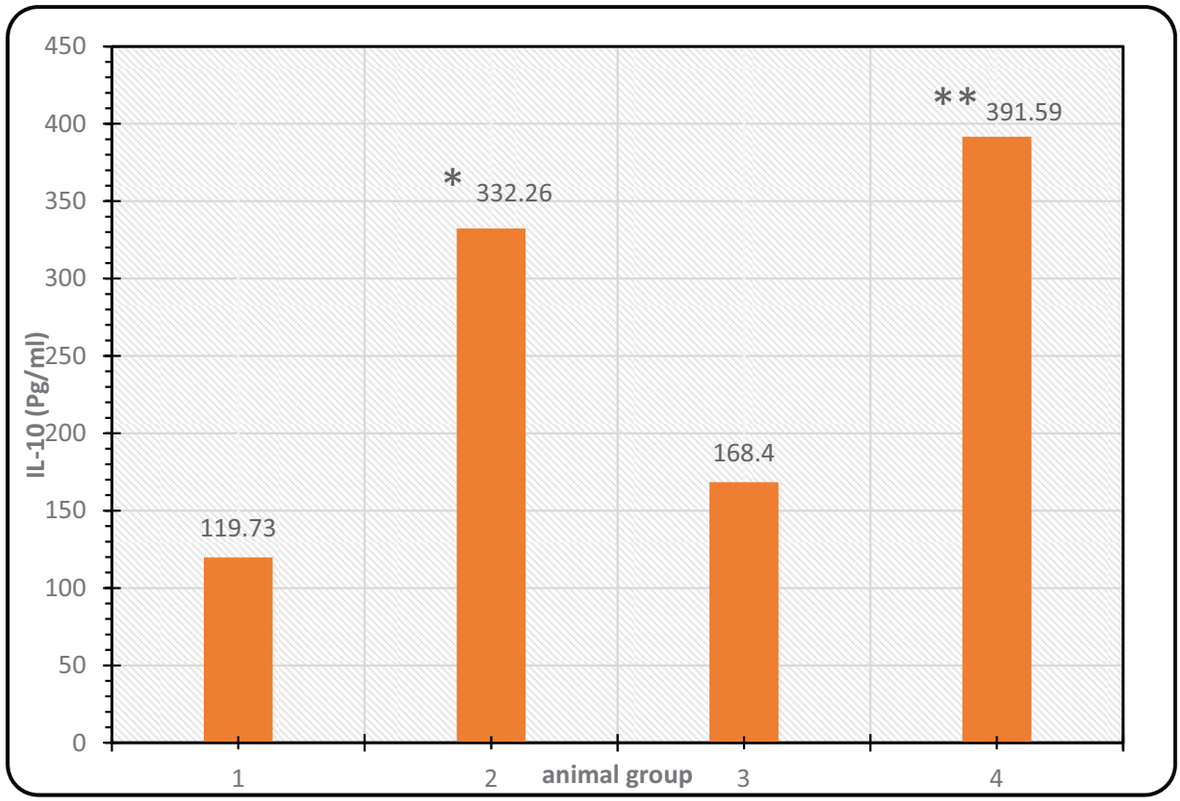

Fig. 3. Level of IL-10 concentration of four challenged animal groups. Groups 1 and 2 were infected with *Streptococcus mutans*, whereas groups 3 and 4 were infected with *Streptococcus sanguinis*. Groups 2 and 4 were *Lactobacillus curvatus* orally administrated for 14 days. Groups 1 and 3 are PBS-treated mice. *Significant difference between groups 1 and 2 ($P < 0.05$), **significant difference between groups 3 and 4 ($P < 0.05$).

in medicine specially caries prevention and oral cavity diseases management. Toxicity tests must be performed before setting in clinical trials specially the tests related to immune system evokes hence curvatcin LHM is protein in nature [30].

S. mutans and *S. sanguinis* aptitude to produce biofilm is varied because of the natural differences in isolates capacity to form biofilm [31]. Both curvatcin LHM and CHX had a significant inhibitory effect on biofilm of *S. mutans and S. sanguinis*. Despite of these results, CHX tested biofilms revealed a presence of viable cells. Dependently, these finding strongly suggest that, chlorohexidine which succeeded in killing the planktonic cells of *S. mutans and S. sanguinis,* failed to effectively kill all bacterial cells within the biofilm which will be able to establish a new biofilm. Curvatcin LHM, unlike the chlorohexidine, left no viable bacterial cells. The cognate O.D. reading perhaps referred to the remaining of exopolysaccharids. The capacity of curvatcin LHM to completely eradication of *Streptococcus* spp. from biofilms gives an advantage on chlorohexidine making it an excellent alternative to it.

Ingestion of *L. curvatus* significantly decreasing *S. mutans* and *S. sanguinis* load in both blood and tissues. The recent results was proceeded by Jo *et al*. [32] who found that, oral administration of *L. curvatus* WiKim38 lowered the incidence of deaths of mice. Similarly, immunomodulatory effects were observed in this study after administration of *L. curvatus* orally for 2 weeks. The cytokines profile in the experimental mice groups was modified as a consequence and significant rising was observed in the mice groups systemically infected with *S. mutans* and *S. sanguine* as compared with control. Taken together, *L. curvatus* have protective effects via immunomodulatory effects, and *L. curvatus* may be useful for various diseases prevention and therapy. In-vivo study using a mixture of *Lactobacillus* strains observed an improvement of animal infected with *Salmonella enterica* [33]. The immunomodulatory effects of probiotics are applied either by probiotics own products or interaction of probiotics with immune cells [34].

It was previously found that, probiotics induce expression of epithelia's IL-6 and IL-10 which potentiate immunoglobulin production by B cells [35]. Other studies have been shown over expression of such immunomodulatory cytokines, induced by *Lactobacillus* strains that increase TGF-β and IL-10 levels [36,37]. Like some *Lactobacillus* species, another lactic acid bacterium, *Bifidobacterium* spp.,

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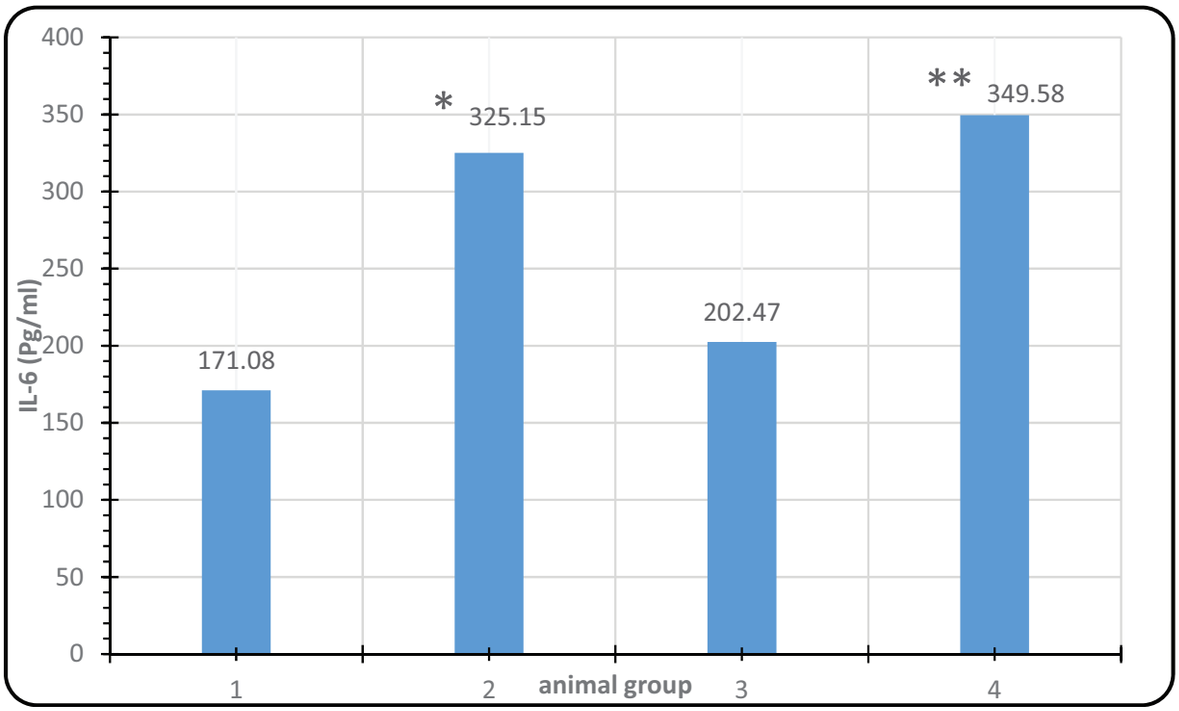

Fig. 4. Level of IL-6 concentration of four challenged animal groups. Groups 1 and 2 were infected with *Streptococcus mutans*, whereas groups 3 and 4 were infected with *Streptococcus sanguinis*. Groups 2 and 4 were *Lactobacillus curvatus* orally administrated for 14 days. Groups 1 and 3 are PBS-treated mice. *Significant difference between groups 1 and 2 ($P < 0.05$), **significant difference between groups 3 and 4 ($P < 0.05$).

augments TNF-α and IL-6 secretion by antigen presenting cells. Consequently, induced proinflammatory cytokines, TNF-α and IFNγ, activate the antigen-presenting cells and induce cell-mediated immunity [38]. Controversially, some *Lactobacillus* species, namely *L. acidophilus* and *L. salivarius*, decrease IL-10 and TGF-β levels in mice [39]. Finally, lactobacillus exoproteosome is likely to introduce numerous immunomodulatory molecules by which this bacterium modulates cytokines profile for the benefit of the host [40].

As a conclusion, *L. curvatus* may has prophylactic effects and its product, curvatcin LHM, have antibacterial and antibiofilm. Unlike chlorohexidine, curvatcin LHM can eradicate streptococci from the biofilm making curvatcin LHM may be a good alternative to CHX as an additive for teeth-protective materials. *L. curvatus* can reduce bacterial load in blood and internal organs and can be serve as direct immunomodulator of proinflammatory responses that improve the status and eradicate the bacteria *in vivo*.

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Conflicts of interest

There are no conflicts of interest.

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