Purification and Characterization of *Lactobacillus crispatus* Exopolysaccharide EPS and Its Antibacterial Activity against some Bacterial Vaginosis Isolates

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***Abstract*—** Five *Lactobacillus crispatus* isolates were obtained from vaginal swabs of 27 healthy premenopausal Iraqi volunteers women (aged 22–40 years old), who have no symptoms of vaginal or urinary tract infection. Exopolysaccharide extracted and purified by sephadex G-100 column (2×50 cm). *L.crispatus* isolate no. 4 produced the greatest amount of EPS 303.45 mg/l compared with other *L.crispatus* isolates in MRS medium. The Chemical Analysis of the Purified *L.crispatus* EPS was determined; soluble carbohydrate fractions of the EPS extracted from *L.crispatus* was assayed by the phenol- sulphuric acid method. The estimated value of carbohydrate content was 62.21 %, expressed as a percentage of the EPS dry weight*;* proteins 5.11%; uronic acid 31.22 %; hexoamines 0.15% and organic contents which represented by ester-linked acetyl groups recording 0.8% and Ketal-linked pyruvate groups recording 0.29% of EPS dry weight. The antibacterial activity of crude and purified EPS for bacterial vaginosis isolates was tested; *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Escherichia coli* and *Listeria monocytogenes* and *Acinetobacter baumanii* were isolated from vaginal discharge of women with symptoms of bacterial vaginosis. The crude and purified EPS at concentration of 100µg/ml had antibacterial activity against all the bacterial vaginosis isolates. The purified was much higher active against them and the most effected bacteria were *A. baumanii* and *S. epidermidis* then *S. agalactiae*, *L. monocytogenes*, *S. saprophyticus* and *E. coli.*

***Keywords*—** Antibacterial activity, bacterial vaginosis, chemical characterization, exopolysaccharide EPS, *Lactobacillus crispatus*

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I. Introduction

THE *Lactobacillus* group are the prevalent bacteria in healthful pre-menopausal women and play an effective preservation function by reducing growth of pathogenic bacteria[1], [2]. The equilibrium among the various bacteria accommodating the human vagina is effective for sustain of its homeostasis, acting directly the health situation of the woman. Among the habitant bacteria, the *Lactobacillus* isolates constituted at least 70% of the bacteria sampled [3] being the most prevalent *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus gasseri* and in less presence *Lactobacillus salivarius*, *Lactobacillus vaginalis*, and *Lactobacillus iners* [4], [5]. Because of their proportional predominance, lactobacilli have been suggested as probiotics to be used against the buildup and heavy growth of pathogenic bacteria in the vagina. This effectiveness would be expressed by two different ways: (i) contention for adherence locations on epithelial cells and pathogen co-aggregation and (ii) output of antimicrobial materials [6]. Vaginal probiotics are a rather new field of researching, therefore, not much is understood about the mechanisms, the circumstances or properties needed to evaluate their qualification [7] – [9]. *L. crispatus* can persevere in the gastrointestinal tract [10] and is among the most predominant species of the *Lactobacillus*-habituated human vaginal microbiota [11], and resistance to very low pH characteristic have also been studied [12]. So, we aimed in this study to investigate the antibacterial activity of the exopolysaccharide (EPS) from *L. crispatus* isolated from healthy vaginal woman against some multidrug resistant pathogenic bacteria isolated from women with vaginosis.

II. Materials and Methods

A.Isolation of Vaginal Lactobacilius crispatus from Healthy Women

Twenty seven pre-menopausal Iraqi volunteers women (aged 22–40 years old), who had no symptoms of vaginal or urinary tract infection, were enlisted for the present study. The women were non-menstruating and not had oral or local antimicrobial therapy within the two weeks prior to study.

Mid-vaginal secretions were self-collected by women with E-swabs (Copan, Brescia, Italy) and at once used for lactobacilli isolation. *Lactobacillus* isolates were isolated onto de Man, Rogosa and Sharpe (MRS) and Brain-Heart Infusion (BHI) agar plates (Difco, Detroit, MI). Both MRS and BHI agar plates were re-enforced with 0.05% L-cysteine. Plates were incubated anaerobically for 24 h at 37°C in anaerobic jars re-enforced with Anaerocult C (Merck, Milan, Italy) [13]. Colonies with various morphological characteristics yielding variable rod shaped bacteria by microscopic examination were chosen for glycerol stock preservation and identified by API 50 CHL kit.

B.Pathogenic Bacterial Isolates

Twenty four isolates including, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Escherichia coli* and *Listeria monocytogenes* and *Acinetobacter baumanii* were isolated from vaginal discharge of women with symptoms of bacterial vaginosis by a gynecologist with swab and inoculated on blood agar medium and kept in incubator. After 24 h, due to the growth of several types of colonies on blood agar medium, each of the colonies were cultured on blood agar and Eosin Methylene Blue (EMB) agar and identified by VITEK 2 system.

C.Antibiotic Susceptibility Test

The Antibiotic susceptibility test was done to the pathogenic bacterial isolates according to Kirby-Bauer (disk diffusion) technique [14], using Mueller-Hinton agar (Hi media/ India) and different antibiotic discs (Bioanalyse/Turkey and) including1 Vancomycin (VAN), Cephradine (CE), Amoxicillin-clavulanic acid (AMC) (20/10µg), Nalidixic acid (NA) (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) and, Netilmicin (NET) (30µg)). Inhibition zones were measured by a ruler and compared with the zones of inhibition determined by Clinical Laboratories Standards Institute (CLSI) [15].

D.Analysis of Bacterial Growth and EPS Production

Cultivation of each *L.crispatus* isolates for EPS production and bacterial growth was performed as batch cultures in 100 ml of MRS broth at 37°C in 250 ml Erlenmeyer flasks. Samples (5 ml of each isolate) were withdrawn after 24 h of incubation to measuring absorbance at 600 nm. Exopolysaccharide was isolated from samples according to the method described below.

E.Extraction and Detection of L.crispatus EPS

The ways used for extraction, purification, and detection of EPS are based on the method described in [16]. Briefly *L.crispatus* isolates were separately inoculated 1% in 500 mL of MRS and incubated at 37°C under anaerobic conditions for 24 h without pH control. Cells were removed after centrifugation (4,000 × *g*, 10 min, 24°C), and the supernatant was precipitated with same volume of ethanol overnight at 4°C followed by centrifugation (8,000 × *g*, 20 min, 4°C). The precipitates were collected and dissolved in distilled water, and lyophilized. Proteins were removed with one-quarter of the volume Sevage solution (chloroform:*n*-butanol; 4:1) followed by centrifugation (8,000 × *g*, 10 min, 24°C), and finally the collected EPS was dialyzed against distilled water for 1 d at 4°C and considered crude exopolysaccharide.

F.Purification of L.crispatus EPS Using Gel Filtration Chromatography

The sample containing the biomolecules and the salt is passed over a gel column whose exclusion limit is below the molecular weight of the biomolecules. Three milliliter samples solution (10 mg/ml) of the crude EPS produced from *L.crispatus* cultures was placed on a sephadex G–100 (Pharmacia fine chemicals) column (2 x 50 cm) with a total bed volume (Vt) of approximately 32 ml (the total volume occupied by the packed gel bed) and a void volume Vo of about 7 ml (the volume of the space surrounding and outside the gel particles). The column was eluted using sterilized phosphate buffer pH 7 and elution flow rate was run 12 drops / min. Fractions of 2 ml each were collected and then the total carbohydrates for each fraction were detected using the phenol–sulphonic acid method [17] to get the elution profile of the purified EPS samples. The highest carbohydrate fractions were then collected, dried and used for further physical and chemical characterizations of the EPS.

G.Chemical Analysis of the Purified L.crispatus EPS

a. Determination of total soluble carbohydrates in the EPS: To examine the existence of simple sugars oligosaccharides, polysaccharides and methyl derivatives of polysaccharide, the phenol – sulphuric acid method was used [17]. The free reducing groups of these compounds give an orange – yellow colour when treated with phenol and concentrated sulphuric acid

b. Determination of proteins in the EPS: To examine the existence and quantity of protein content in the EPS extracted from, *L.crispatus*, the ultraviolet absorption technique [18] was used.

c. Determination of uronic acids in the EPS: The amount of uronic acids constituents in the EPS was evaluated by using the carbazole method [19].

d. Determination of hexosamines in the EPS: For the determination of amino sugars in the EPS extracted from *L.crispatus*, the method described by [20] was used.

e. Determination of organic constituents in the EPS: 1) Ester – linked acetyl group; a semimicro detection technique was applied [21]. 2) Ketal – linked pyruvate group; the second organic constituents of *L.crispatus* EPS are the pyruvate groups which were detected using the chemical analysis method described by [22].

H.Antibacterial Activity of Crude and Purified EPS on Bacterial Vaginosis Isolates

Agar well diffusion assay was used to determine antibacterial activity of the crude and purified EPS produced by *L.crispatus* against the bacterial vaginosis isolates at concentration 100 µg /ml according to [23].

I. Statistical Analysis

Results were expressed as the mean ± standard deviation (SD). The intergroup variation was assessed by one way analysis of variance (ANOVA) $P<0.05$ using sigma state statistical software.

II. Results and Discussion

A.Isolation of Vaginal Lactobacilius crispatus from Healthy Women

Five *L.* crispatus isolates were obtained from vaginal swabs of 27 healthy premenopausal women. The *L. crispatus* isolates were identified to species level by API 50 CHL.

Seventeen *Lactobacillus* isolates were collected from vaginal swabs of 15 healthy premenopausal women; 8 isolates belonged to *L. crispatus*, 6 isolates to *L. gasseri* and 3 isolates to *L. vaginalis* [13]. Lactobacilli were the dominant bacteria of the vaginal flora of healthy women that plan the balance between the habitant microbiota and the vaginal environment [24]. *L. crispatus*, *L. jensenii*, and to a lesser prevalent *L. gasseri*, were dominant in the vagina of healthy women, whereas the prevalence of *L. iners* was associated with bacterial vaginosis [25]. Borgdorff and colleagues [26] identified six microbiota and revealed that *L. crispatus* prevalent cervicovaginal microbiota. Recent references described the isolation of *L. crispatus* as a member of the habitat useful flora of the vaginal mucosa [27] [28].

B.Antibiotic Susceptibility Test

The antibiotic sensitivity test revealed that the pathogenic bacterial isolates from the vaginosis samples were multidrug-resistant (see Table I).

TABLE I
Antibiotic Susceptibility Test of the Bacterial Vaginosis Isolates

Isolates	VAN	CE	AMC	NA	AT	CEC	CPZ	CTR	FEP	CTX	CAZ	CIP	GN	IM	NET
A. baumanii	3(100%)	1(33.3%)	0(0%)	2(66.6%)	0(0%)	0(0%)	1(33.3%)	2(66.6%)	2(66.6%)	2(66.6%)	2(66.6%)	2(66.6%)	0(0%)	2(66.6%)	3(100%)
E. coli	3(50%)	0(0%)	2(33.3%)	2(33.3%)	0(0%)	2(33.3%)	1(16.6%)	0(0%)	2(33.3%)	2(33.3%)	3(50%)	0(0%)	1(16.6%)	1(16.6%)	1(16.6%)
L. monocytogenes	2(50%)	2(50%)	0(0%)	1(25%)	0(0%)	3(75%)	0(0%)	2(50%)	0(0%)	1(25%)	1(25%)	2(50%)	1(25%)	1(25%)	1(25%)
S. epidermidis	4(50%)	3(75%)	1(25%)	2(50%)	2(50%)	1(25%)	1(25%)	2(50%)	0(0%)	3(75%)	2(50%)	1(25%)	2(50%)	3(75%)	0(0%)
S. saprophyticus	5(100%)	1(20%)	2(40%)	2(40%)	2(40%)	2(40%)	0(0%)	2(40%)	2(40%)	4(80%)	1(20%)	1(20%)	4(80%)	3(60%)	1(20%)
S. agalactiae,	0(0%)	0(0%)	1(50%)	1(50%)	0(0%)	0(0%)	1(50%)	1(50%)	0(0%)	0(0%)	1(50%)	0(0%)	0(0%)	0(0%)	2(100%)

C.Analysis of Bacterial Growth and EPS Production

The growth properties of bacteria and EPS yields of each isolate were investigated (see Table II). *L.crispatus* isolate no. 4 produced the greatest amount of EPS (303.45 mg/l) compared with other *L.crispatus* isolates in MRS medium. It also grew better in MRS (optical density at 600 nm was 2.01 than other isolates ($P < 0.05$). Based on these results *L.crispatus* isolate no. 4 was selected for EPS extraction and further studies.

TABLE II
Exopolysaccharide (EPS) production by *L.crispatus* isolates and their growth ability in MRS broth

L. crispatus isolates	Growth (OD) at 600nm (Mean ±SD)*	EPS Production mg/ml (Mean ±SD)*
L. crispatus isolate No. 1	0.29±0.26 a	149.55±3.12 a
L. crispatus isolate No. 2	1.75±0.11 b	243.26±4.99 b
L. crispatus isolate No. 3	1.22±0.07 c	201.05±3.46 b
L. crispatus isolate No. 4	2.01±0.89 d	303.45±7.29 c
L. crispatus isolate No. 5	0.74±0.18 a	203.89±6.12 b

[a-d] Data with different letters within a column are significantly different at $p<0.05$

[*] Each volume in the table is (mean±SD) of three trials

Another study led to the extraction of partially purified EPS from a LAB strain *Lactobacillus plantarum* YML009 with a maximum yield of 260 mg/l on modified MRS medium re-enforced with 10% glucose [29]. The maximum yield of EPS from *Paenibacillus polymyxa* EJS-3 was 22.82 g/l [30].

D.Purification of L.crispatus EPS Using Gel Filtration Chromatography

Purification of *L.crispatus* EPS was carried out for polysaccharide polymer using gel filtration chromatography on a sephadex G-100 column. Forty five fractions of carbohydrates, each of 2 ml, were eluted in the void volume of the column and tested for the amount of carbohydrates. The produced carbohydrate elution profile of *L.crispatus* EPS polymer was shown as a single peak; fraction number 22 recorded the highest amount of carbohydrates. The amount of total carbohydrates in the polymer was estimated and it was found to be 69.78% based on EPS dry weight. The EPS polymer was found to be conjugated with protein which was detected at 490 and 280 nm alternatively (see Fig. 1).

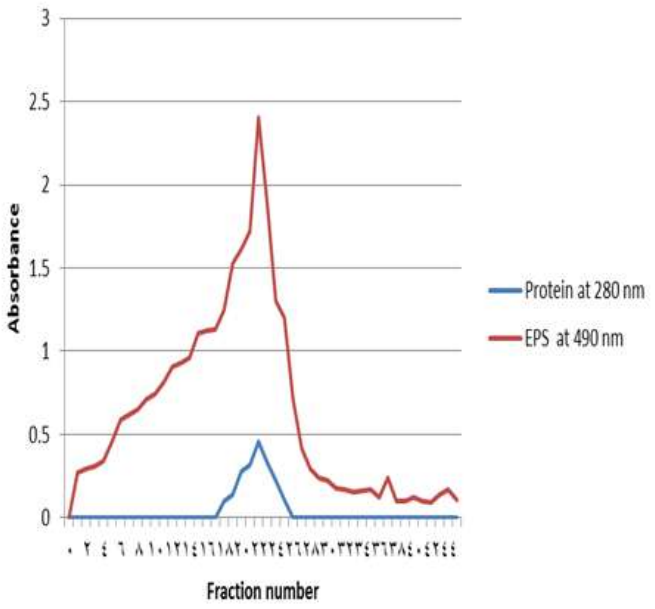

Fig. 1 Elution profile of *L.crispatus* EPS on sephadex G-100 columns chromatography

E.Chemical Analysis of the Purified L.crispatus EPS

a. Determination of total soluble carbohydrates in the EPS: Soluble carbohydrate fractions of the EPS extracted from *L.crispatus* was assayed by the phenol– sulphuric acid assay. The estimated value of carbohydrate content was 62.21 %, expressed as a percentage of the EPS dry weight, and was more than half of the whole polymer weight in the tested species. Reference [28] showed the estimated yield after membrane purification was on average 85%. The partially purified EPS from *L. plantarum* YML009 revealed 68.1% of total sugar content using phenol– sulphuric acid assay [29]. EPS extracted from *L. helveticus* MB2-1 showed 71.68% of total sugar constituent in crude EPS [31]. The total sugar constituent of *Bacillus* sp. I-450 EPS 450 was revealed to be 92%, revealing that the polymer was basically a polysaccharide [32]. The total carbohydrates of the EPS produced by *Vibrio harveyi* strain VB23 was 211.71µg/mg [33]. The analysis revealed that the exopolymer extracted from *Vibrio furnissii* was acidic in nature, and the constituent of the neutral sugars (total carbohydrates) was 166.17µg/mg [34].

b. Determination of proteins in the EPS: A protein peak was found to coincide with the carbohydrate peak in the elution profile of *L.crispatus* The protein content was estimated to be 5.11%. Reference [28] showed the purified EPSs solution had a protein constituent that was colse to 0.5%. The partially purified EPS isolated from *L.plantarum* YML009 showed 2.26% of total protein [29]. The crude EPS isolated from *L. helveticus* MB2-1 resulted in 3.08% of total protein constituent [31]. Also a lyophilized crude EPS from *L. plantarum* 301102S resulted with 1.6% protein constituent [35]. *Bacillus* sp. I-450 EPS450 protein content was found to be 8% [32]. The protein of EPS produced by *V. harveyi* strain VB23 was 186µg/mg [33], and 28.6µg/mg from *V. furnissii* [34]. The binding of divalent cations to biopolymer happens via electrostatic interactions with negatively charged functional groups such as (i) uronic acids, (ii) phosphoryl groups attached with membrane components and (iii) carboxyl groups of amino acids, proteins also display an effective role in emulsification activity [36].

c. Determination of uronic acids in the EPS: The uronic acids content of *L.crispatus* EPS materials was determined using carbazole assay because of their complexity of determination by HPLC technique after acid hydrolysis of the sample. Uronic acid was presented in the EPS of the examined isolate with a considerable percentage. It records 31.22% of the EPS dry weight of *L.crispatus* EPS. *Bacillus* sp. I-450 EPS 450 uronic acids were found to be 17.2% [32]. The uronic acids of EPS produced by *V. harveyi* strain VB23 was 114.62µg/mg [33], and 6.48µg/mg from *V. furnissii* [34]. Uronic acids express anionic properties upon the EPSs and are accountable for their biotechnological applications: their use in biodetoxification of heavy metals and waste waters [37]. Reference [38] had revealed that exopolymer from a marine bacterium contained huge amounts of galacturonic acid.

d. Determination of hexosamines in the EPS: The determined amounts of hexosamines were found to be traces in the *L.crispatus* EPS dry weight 0.15%.

e. Determination of organic constituents in the EPS: 1) Ester-linked acetyl groups; acetyl groups were estimated to be bind to some EPS polymers via an ester bond, and were considered as organic contents of the polymer that added to its characteristics. Acetyl groups were estimating to constitute a little percentage of *L.crispatus* EPS; recording 0.8% from its dry weight. 2) Ketal-linked pyruvate groups; the extracted EPS polymer of *L.crispatus* was carrying ketal–linked pyruvate groups at concentration 0.29% of *L.crispatus* EPS dry weight.

F.Antibacterial Activity of EPS on Bacterial Vaginosis Isolates

TABLE II

ANTIBACTERIAL ACTIVITY OF CRUDE AND PURIFIED EPS ON BACTERIAL VAGINOSIS ISOLATES

Isolates	Inhibition Zone Diameter (mm) Mean ± S.D		
	Control	Crude (100 µg /ml)	Purified (100 µg /ml)
A. baumanii	0 ± 0	14.27±1.43[c]	18.31±1.1[a c]
E. coli	0 ± 0	6.67±2.54[c]	11.44±1.17[a c]
L. monocytogenes	0 ± 0	8.25±1.66[c]	14.89±2.41[a c]
S. epidermidis	0 ± 0	11.55±2.08[c]	17.81±3.12[a c]
S. saprophyticus	0 ± 0	9.19±1.03[c]	13.51±1.22[a c]
S. agalactiae,	0 ± 0	12.01±1.19[c]	15.36±1.83[a c]

[c] Probability compared to control $p<0.05$
[a] Probability compared to control $p<0.05$

The crude and purified EPS at concentration of 100µg/ml had antibacterial activity against all the bacterial vaginosis isolates. The purified was much higher active against them and the

most effected bacteria were *A. baumanii* and *S. epidermidis* then *S. agalactiae, L. monocytogenes, S. saprophyticus* and *E. coli*.

Both EPS from *Bifidobacterium bifidum*WBIN03 and *L. plantarum* R315 showed antibacterial activities against tested pathogens such as *Cronobacter sakazakii*, *E. coli*, *L. monocytogenes*, *S. aureus*, *Candida albicans*, *Bacillus cereus*, *Salmonella typhimurium*, and *Shigella sonnei* at 300 µg/ml [39].

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