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Original Article

In vitro antimicrobial activities of metabolites from vaginal *Lactobacillus* strains against *Clostridium perfringens* isolated from a woman's vagina

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Abstract

Background: More than 50 different species of bacteria may live in a woman's vagina, with lactobacilli being the predominant microorganism found in healthy adult females. Lactobacilli are relevant as a barrier to infection and are important in the impairment of colonization by pathogens, owing to competitive adherence to adhesion sites in the vaginal epithelium and their capacity to produce antimicrobial compounds.
Methods: The aim of the present study was to demonstrate the inhibitory capability of *Lactobacillus* metabolites against *Clostridium perfringens*, an anaerobic Gram-positive bacterium. These bacteria were isolated from vaginal swabs by using culture-dependent approaches, and the bacteriostatic effect of *Lactobacillus* metabolites, extracted from different isolates, was assessed using a modified E test.
Results: Among the 100 vaginal swabs, 59 (59%) samples showed the presence of *Lactobacillus* strains and only one sample contained *C. perfringens*. *Lactobacillus* metabolites demonstrated the significant potency of *in vitro* activity against *C. perfringens*, with minimal inhibitory concentration values ranging from 15.6 μg/mL to 31.2 μg/mL.
Conclusion: This study suggests that women without vaginal *Lactobacillus* strains may be susceptible to nonindigenous and potentially harmful microorganisms.

Keywords: antimicrobial activity; *Clostridium perfringens*; *Lactobacillus*; modified E test; vagina

1. Introduction

Lactobacillus strains are thought to play a major role in protecting the vaginal environment from nonindigenous and potentially harmful microorganisms. This is accomplished through their production of lactic acid, resulting in a low and protective pH (3.5–4.5). Vaginal *Lactobacillus* species are also known to produce other antimicrobial compounds besides lactic acid, including target-specific bacteriocins,[1] and broad-spectrum hydrogen peroxide.[2] *Clostridium perfringens* is an anaerobic Gram-positive bacterium known to be a common pathogen in humans, domestic animals, and wildlife, and is the primary cause of clostridial enteric disease in domestic animals. *C. perfringens* has 10 rRNA operons and 18 polymorphic sites among the 16S rRNA genes.[3–5] A common feature of *C. perfringens* is the production of 17 exotoxins and enterotoxin (CPE). *C. perfringens* is subdivided into five toxinotypes (A–E). Isolates originating from humans with gastrointestinal diseases carrying both cpb2 and cpe have

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recently been described.[6−8] Johansson et al[9] reported that a wide genetic diversity of *C. perfringens* from isolates causing enteric diseases in humans, based on pulsed field gel electrophoresis analysis. *C. perfringens* is part of the normal vaginal flora of 1−27% of healthy women. Therefore, ascending infection from the vagina to the uterus may occur.[6] Under appropriate conditions, the bacteria can cause endometritis that leads to sepsis.[10] Meanwhile, *C. perfringens* infection may occur in the uterus, especially after abortions via the use of contaminated surgical instruments.[10] However, the importance of *Lactobacillus* has been shown in preventing infections such as genital infections, urinary tract infections, and bacterial vaginitis, which are caused by reducing *Lactobacillus* and overgrowth of other microorganisms.[11,12] Accordingly, this study was conducted with the aim of assessing the inhibitory capability of vaginal *Lactobacillus* metabolites against *C. perfringens* isolated from a woman's vagina.

2. Methods

2.1. Design of study

In this research, 100 reproductive-age women who were referred to the women's department of Imam Khomeini Hospital in Ahvaz, Iran, were considered for this study. The inclusion criteria called for healthy married women who were 25−50 years of age. The exclusion criteria included cervix injuries, previous abortion, and divorced women.

In a 35-year-old nonpregnant woman, the only bacterium isolated from a cervicovaginal smear sample was *C. perfringens*. On physical examination, she had high fever (39°C), a small amount of necrotic tissue, and mild inflammation of the uterus. The patient appeared anemic and icteric, although her blood pressure and pulse rate were normal. On examination, her abdomen was normal and no free air was seen on plain chest X-ray films. Blood tests showed evidence of mild inflammation, with a white blood cell count of 14,150 μL and a C-reactive protein level of 8.0 mg/dL. The cervicovaginal smear samples were collected from vaginal swabs of women, and the swabs were immediately placed in thioglycollate transport medium (Hi Media, Laboratories, Mumbai, India) and transported in ice bags to the university's laboratory. The samples were incubated for 48 hours at 37°C.

2.2. Isolation of bacteria

The collected swabs were placed on the selective medium for lactobacilli, De Man−Rogosa−Sharpe (MRS) agar (Conda, Madrid in Spain). The plates were incubated at 37°C for 24−48 hours under anaerobic conditions using a candle jar. The *Lactobacillus* were presumptively identified by their ability to grow well on MRS medium[13] followed by differential biochemical tests including Gram staining, catalase test, fermentation of glucose, maltose, and sucrose in order to confirm the identification of *Lactobacilli*. *Lactobacilli* are rod-shaped, Gram-positive, fermentative, facultative anaerobic or microaerophilic organotrophs.

Isolation of *C. perfringens* from the vagina was accomplished by spreading the cervical swabs on a blood agar medium. Then plates were incubated anaerobically in a candle jar with gas packed for a period of 24−48 hours at 37°C.[14] The colonies that showed double zone hemolysis on blood agar plates were considered *C. perfringens*. Thereafter, other diagnostic tests including stormy clot, Nagler test, gelatin hydrolysis test, and indole test were performed in order to confirm the identification of *C. perfringens*.

2.3. Antimicrobial compound extraction from *Lactobacillus spp.*

The isolated colonies of *Lactobacillus* grown on MRS agar were transferred into 100 mL of MRS broth and incubated in an anaerobic chamber at 37°C for 5 days. Then, the antimicrobial compound was extracted using the three following methods.

2.3.1. Method 1

After incubation, a part of the culture medium was directly mixed with ethyl acetate (50:50) and then stirred using a magnetic stirrer for 6 hours. The upper organic layer was separated using a separating funnel and centrifuged at 6000 rpm for 15 minutes. Then the ethyl acetate layer was removed and transferred into a clean flask. The extract was pooled and dried in a rotary evaporator (Heidolph in Schwabach, Germany) at 45°C. The yield from the extract was dissolved in methanol for antimicrobial susceptibility testing.

2.3.2. Method 2

The second part of the medium was shocked by immersion in boiling water for 1 minute, and then placed in cold water for 3 minutes. Then, the extraction was followed by adding ethyl acetate similar to the first method.

2.3.3. Method 3

The third part of the culture medium was stressed using an ultrasonic device for 3 minutes (160 W); then, similar to the first method, extraction of the antimicrobial compound was performed.

2.4. Determination of minimum inhibitory concentration of *Lactobacillus spp.* extracts

The minimum inhibitory concentrations (MICs) of *Lactobacillus* extracts against *C perfringens* isolates were determined using an improved E test method (AB Biodisk, Solna, Sweden) in order to show the antimicrobial activity of isolated *Lactobacillus* spp. In the improved E test, several AB Biodisks impregnated with different dilutions of the extracts were used instead of strips. In fact, it was a simulated version of the standard E test.

The *C. perfringens* suspensions of freshly grown cultures were prepared in sterile saline and adjusted to a density of 10^6 cells/mL, corresponding to 68−82% transmittance at 530 nm. The plate of Mueller−Hinton agar (Hi Media India in

Mumbai, India) was inoculated by dipping a sterile cotton swab into the *C. perfringens* suspension and streaking it across the agar surface in three directions. The plates were dried at an ambient temperature for 15 minutes before applying the disks. Eight sterile disks (6 mm) were placed in a line on the agar surface. The *Lactobacillus* spp. extract was serially diluted in methanol, and 10 μL of each dilution was separately used to impregnate the disks. The plates were incubated for 24 hours at 37°C. The MIC values were read as the antimicrobial concentrations at the points where dense colonial growth intersected the disks. The test was performed in triplicate for each culture.[15]

3. Results

A total of 59 *Lactobacillus* spp. strains and one strain of *C. perfringens* were isolated from 100 vaginal swabs by using different selective media and biochemical tests. The morphological and biochemical characteristics of all bacterial isolates were tested according to *Bergey's Manual of Determinative Bacteriology*.[16]

The antimicrobial activity of the extracted compounds from 10 *Lactobacillus* strains showed that the first method of extraction using an ultrasonic device has more activity compared with others. The obtained MICs from all tested *Lactobacillus* strains based on the modified E test ranged from 15.6 μg/mL to 31.2 μg/mL (Figs. 1 and 2).

It should be mentioned that the inhibitory capability of *Lactobacillus* metabolites against some vaginal/uterine opportunistic bacteria were also assessed as an ancillary study

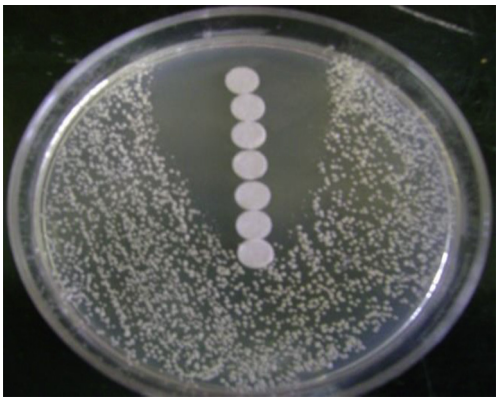
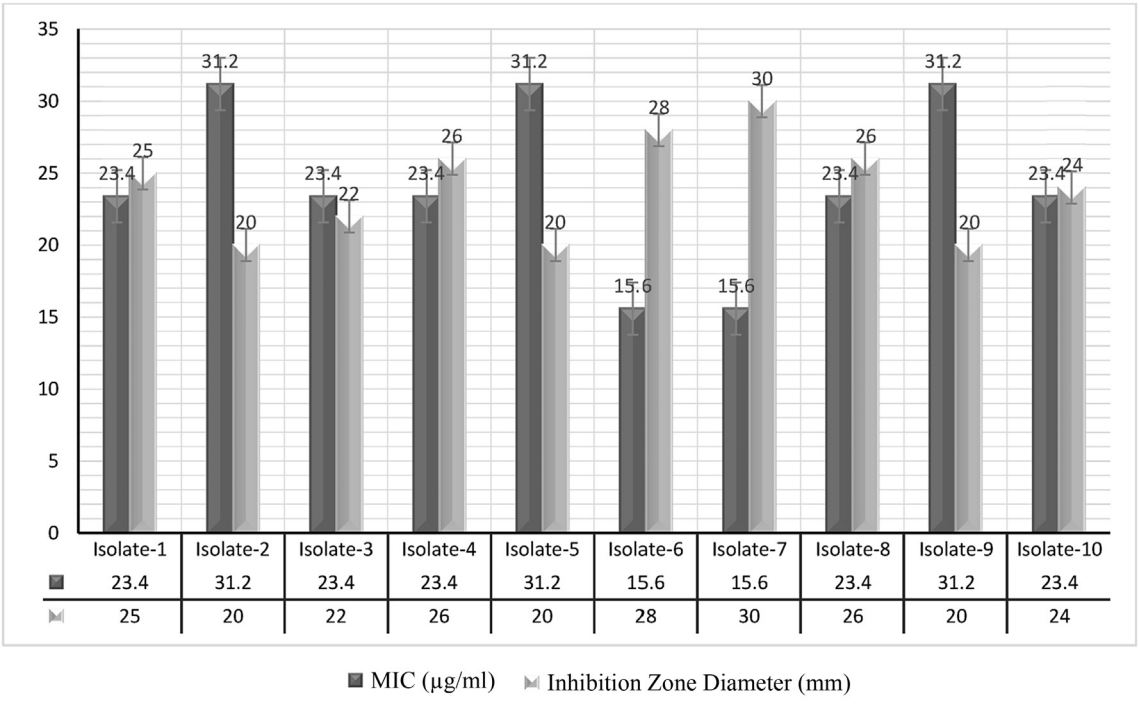


Fig. 2. Antimicrobial activity of *Lactobacillus* against *Clostridium perfringens* by modified E test.

(Table 1). These pathogens included *Candida albicans* NCIM 7102, *Staphylococcus aureus* PTCC 1113, and *Escherichia coli* PTCC 1533.

4. Discussion

C. perfringens, an anaerobic Gram-positive bacillus, is found in normal human intestinal and vaginal flora in approximately 25% of healthy women. The presence of *C. perfringens* in the vagina or uterus can be lethal because of the risk of developing sepsis and gas gangrene followed by genital sores or vaginal fissures that lead to additional gynecological examinations and even abortions. In cases involving gas gangrene, the mortality rate ranges from 50% to 85%.[17] Although few cases of clostridial gas gangrene associated



MIC = minimum inhibitory concentration.

Fig. 1. MIC and inhibition zone diameter of antimicrobial extract of *Lactobacillus* spp. against *Clostridium perfringens* using modified E test. MIC = minimum inhibitory concentration.

Table 1
MIC of antimicrobial extract of *Lactobacillus* spp. against *Candida albicans* NCIM 7102, *Staphylococcus aureus* PTCC 1113, *Escherichia coli* PTCC 1533, using the modified E test.

Lactobacillus spp.	MICs value (μg/mL)		
	Candida albicans	*Staphylococcus aureus*	*Escherichia coli*
Isolate-1	50.25	55.24	40.7
Isolate-2	100	90.8	80
Isolate-3	55	54	35
Isolate-4	65.77	70	55
Isolate-5	85	80.3	60.5
Isolate-6	35	40	30.3
Isolate-7	30	35.2	25
Isolate-8	55.47	55	40
Isolate-9	90.6	100	80
Isolate-10	55.32	55	45

MIC = minimum inhibitory concentration.

with uterine malignancy have been reported, further complication resulting in potentially fatal sepsis were possible outcomes in most of those cases.

The vaginal ecosystem is dynamic and contains microbiota that are protective against invading pathogens, including those causing urinary tract infections and sexually transmitted infections. *Lactobacilli* are the best-known bacteria of the normal vaginal microbiota as a probiotic.[18] Antonio et al[19] reported that women who were not colonized with H_2O_2-producing lactobacilli, such as *Lactobacilli crispatus*, *Lactobacilli iners*, *Lactobacilli jensenii*, *Lactobacilli gasseri* and *Lactobacilli vaginalis*, were 15 times more likely to have bacterial vaginosis than women who were colonized by these strains.[19] In fact, owing to the rise of bacterial resistance against antibiotics, scientists are looking to benefit from probiotics in order to both strengthen the immune system and infection prevention and control.[20,21] Previously, in many studies, the important role of vaginal lactobacilli as potential probiotics has been shown against such pathogens as *Candida* spp.,[22] and the most common opportunistic bacterial (*S. aureus*, *E. coli*, *S. agalactiae*, and *Klebsiella pneumoniae*).[23] In this study, the antimicrobial effect of the extracted compound of vaginal *Lactobacilli* was investigated on *C. perfringens*, in order to find the benefit of this probiotic to prevent the spread of clostridial infection in the uterus, reduce mortality, as well as reduce the use of antibiotics.

The results of this study showed that among the 100 vaginal samples, only one (1%) contained *C. perfringens*. Such frequency was reported to be 5% in the Jawetz book,[24] 1−27% of women in some published articles[10,17] and also 1−27% of those patients who had optional abortions.[25] Also, it was showed that 59 (59%) of the samples were *Lactobacillus* strains. These statistical results are consistent with other statistics that reported the prevalence of vaginal *lactobacilli* at a rate of 50−80%.[26] Amin et. al.[27] reported the prevalence of 49.5%. In the present study, all vaginal *Lactobacillus* strains showed significant antimicrobial activity (MIC ranged from 15.6 to 31.2 μg/ml) against *C. perfringens* like Amin's study (MIC against *S. aureus*: 4 μg/ml, MIC against *Candida albicans*: 1 μg/ml).[27] Coolborn[28] could isolate 8 Lactic acid bacteria from food sources and soil by using MRS medium and then evaluated their antimicrobial activity against some pathogenic bacteria with two methods of well-in agar and paper disc method. He defined that Lactic acid bacteria with inhibitory affinity over the bacterial indicators exhibited varying degrees of inhibitory zones.

In the present study, ethyl acetate was used to obtain the *Lactobacillus* extract from broth media similar to the antimicrobial extraction method of plants and also like Amin's study.[27] Extracts obtained from all three methods were able to make zones of inhibition. However, inhibition zone diameter was much less in the second and third method than in first method. The weight of *lactobacillus* extract obtained from the second and third methods was reduced, as compared to the first method. In particular, it was reduced considerably more in the second method. In other studies, researchers used the filtered supernatant fluids from MRS liquid medium containing *Lactobacillus* cells as antimicrobial extract. In the ethyl acetate extraction method, we tried to partially purify the extract, and therefore the inhibition zones were much wider than in other studies. In our study, Sodium hydroxide (NaOH) was added to the extract in order to neutralize its PH. Therefore, the antimicrobial activity of extract was not related to its acidic nature. Moreover, the use of boiling method to obtain the extract indicated that the antimicrobial activity of extract was not related to bacteriocin (antibacterial proteins), which confirmed the results of Rokka's study.[29]

This study suggested that women without vaginal *Lactobacillus* strains might be susceptible to non-indigenous and potentially harmful microorganisms. Moreover, if the normal vaginal flora is not disturbed by antibiotics, vaginal *lactobacilli* can prevent pathogen growth and *C. perfringens* infections.

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