

UTILIZATION OF ISOLATED THERAPEUTIC BACTERIA IN TOPICAL OINTMENT FOR INFECTION TREATMENT

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ABSTRACT

This study includes the using therapeutic bacteria isolate in topical ointment for bacterial vaginosis treatment and to compare it with metronidazole ointment that use in a clinical trial under medical supervision. Four active *Lactobacillus* probiotics bacteria were cultured in %12 skim milk , three of these bacteria were commercial types and one Iraqi local isolate , the total bacterial count of probiotics bacteria were around $\geq 10^{12}$ cfu/ml . %10 of individually culture free cells filtrate were added to Muller Hinton broth with secretions of patients infected with bacterial vaginosis "BV", %50 of natural organic components and %50 of local isolate *Lactobacillus plantarum* cultured in skim milk were mixed to prepare the natural ointment. Eighty-nine BV volunteers were divided into two group, first was 43 volunteers used probiotic ointment, the second group was 46 volunteers used metronidazole ointment "as control group", both groups were under medical observation for two weeks. The results shown that Iraqi local isolate *Lb . plantarum* gave best inhibition in vitro, according to Amsel criteria the rate of healing with *Lb . plantarum* ointment group was %97.87, it was higher than metronidazole ointment %91.30 and the healed cases numbers were increased at the day 7 and 14, it has been noticed that the results were positive based on vaginal secretions, and whiff test, clue cell and the pH was above 4.5 shown significant progress. According to Nugent criteria *Lb . plantarum* ointment gave higher healing percentage which was %95.35.

Keywords: probiotic, vaginitis, *Lb. plantarum*, amsel criteria, therapeutic ointment, metronidazole.

الزيادي وآخرون

مجلة العلوم الزراعية العراقية - 2020: 51(6): 1661-1669

استخدام عزلة من البكتيريا العلاجية في مرهم موضعي لعلاج العدوى البكتيرية

نور علي لفته العيساوي

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باحث

استاذ

استاذ مساعد

المستخلص

استعملت اربعة انواع من المعززات الحيوية البكتيرية ونميت في وسط الحليب الفرز المسترجع %12 ثلاثة منها كانت تجارية والعزلة الرابعة محلية *Lactobacillus plantarum* ، الاعداد الحية للمعززات كانت $\leq 10^{12}$ و.و.م/مل . استعمل المستخلص الخالي من الخلايا بنسبة %10 من هذه المعززات لتنشيط مسحات السوائل المهبلية المرضية في وسط مولر- هنتون واطهرت النتائج ان *Lactobacillus plantarum* كانت الاكثر مقدرة في تثبيط البكتيريا الهوائية والبكتيريا اللاهوائية. حضر مرهم من مكونات طبيعية مكون من %50 من المخمر اللبني للعزلة المحلية. 89 متطوعة ممن يعانون من التهاب المهبل البكتيري تم تقسيمهم الى مجموعتين، ضمت الاولى 43 متطوعة استعملن مرهم المعزز الحيوي فيما استعملت المجموعة الثانية مرهم المضاد الحيوي Metronidazole . كلتا المجموعتين تمت مراقبتهما طبييا كل ثلاثة ايام لمدة اسبوعين مدة التجربة ، وطبقا لمعيار امسل اظهرت النتائج ان نسبة الشفاء لمجموعة المعزز الحيوي كانت %97.87 مقارنة بمجموع المقارنة %91.3، كما لوحظ ان سرعة الشفاء كانت اعلى وبالترتيب نفسه من الايام 7 و14 يوم وكانت النتائج ايجابية في كلا من مؤشرات الافرازات المهبلية و خلايا Clue و Whiff Test (الرائحة السمكية) ودالة الحموضة للمهبل ($pH > 4.5$). وطبقا لمعيار نانت اظهر استعمال مرهم المعزز الحيوي اعلى نسبة شفاء وكانت %93.35 .

كلمات مفتاحية: معزز حيوي، معيار امسل، مرهم علاجي، التهاب المهبل البكتيري، معيار نانت

*Received:20/12/2019, Accepted:9/3/2020

INTRODUCTION

Therapeutic or bacteria probiotics defined as living microorganisms that help, encourage and support the restoration of microbial balance and granting of health benefits in host's body (15). Most of the therapeutic bacteria belong to *Lactobacillus* genus, which constitutes the vast majority of vaginal flora in healthy women, and play an important role in protection, enhancement immunity and inhibition of opportunistic bacteria within the vaginal ecosystem (20). The results of scientific research indicate that some types of therapeutic bacteria can protect bacterial infection of the vagina through a number of factors such as pH decreasing, producing bacteriocins and releasing H_2O_2 (18). More recently, global marketing trends suggest that therapeutic bacteria can be a promising alternative to traditional drugs because of their constants prevention and healing actions against many diseases and bacterial pathogens (12). vaginal bacteria infections considered as one of important global health problems, WHO reports that one out three of adults females suffering from vaginal bacteria infections at, adulthood an abnormal condition caused by the microbial imbalance of vaginal flora, caused by an excessive growth of some bacterial strains led to vaginal infections (13), which is one of the most common diseases in adults females, especially in the fertility age, the incidence rate of the infection is more than 70%. Bohbot (2) mention the Inflammation caused by the low production of hydrogen peroxide mostly released by *Lactobacillus*, which led to an increasing in the number of anaerobic bacteria such as *Gardenerella vaginalis*, *Mycoplasma huminis* and *Prevotellaspecies* (27) Also *Bacteroides* species, *Peptostreptococcus* species, *Ureaplasma urealyticum*, *Mobiluncus*, *Megaspaera*, *Sneathia* and *Clostridiales* species as well as *Fusobacterium* species and *Atopobium vaginae* (3). Bacterial vaginosis can led to many health complicated, vaginal infections are associated with many pathological conditions confirmed by many studies, especially during pregnancy period, as the risk of early rupture of the embryonic or Prematur Rupture of Membrane (PROM) Premature birth, abortion, chronic pelvic

infections, endometrial and cervical infections, urinary tract infections, increased susceptibility to HIV infection, and ectopic pregnancy (17) Common symptoms of the disease include increased vaginal secretions, which are often similar to the smell of fish like, due to the presence of amine compounds, and white to yellowish white color accompanied sometime with burning of urine and the appearance of Clue-cell in the secretions which are the cells of the vagina epithelium tornd and raise pH up to 4.5, which is known as Amsel Criteria (7). The aim of this study was to utilize therapeutic bacteria isolate in topical ointment for bacterial vaginosis treatment and to compare it with metronidazole ointment.

MATERIALS AND METHODS

Samples collection

Twenty two vaginal swabs were collected from patients women suffering from bacterial vaginosis visiting the "women's obstetrics and gynecology hospital in Samawah city in Muthanna province– Iraq" these samples were collected by a specialist physician. all cases were diagnosed as a bacterial infection based on symptoms and clinical signs according to approved diagnostic criteria. swabs were taken from the vagina by using Sims' vaginal speculum (A double-blade surgical instrument used to examine the vagina and cervix) sterile cotton swabs were inserted at a depth of 2 cm in gentle with rotation on the wall of the vagina (6) The swabs then placed in stuart transport media and transferred to the laboratory. A microscopic examination of the vaginal secretions was performed. Then Blood Agar (Himedia company – India) was inoculated by samples and incubated in aerobic and anaerobic conditions using anaerobic Jar (Manufactured locally) at 35-37 °C for 24 hrs.

Therapeutic bacteria culture

Lactobacillus rhamnosus GG (North Hollywood US) and two commercial isolates of *Lactobacillus plantarum*, *Lactobacillus casei* (Quest Nutra Pharma UK) and local isolates of *Lactobacillus plantarum* were obtained from the Department of Food Science / College of Agricultural Engineering Sciences / University of Baghdad, activated in sterilized (121 °C for 5 min) skim milk

(Vitamilk Skimmed Milk 0%fat , Regilait Company. France), incubated at 37°C until coagulation. The activation was repeated three times for each type of therapeutic bacteria separately (4) Total numbers count were estimated in Pour Plate Count by using MRS-L.Cysteine.HCL agar (Himedia company – India) with anaerobic conditions (5% CO₂ ,37°C ,48hrs.).

Culture cells filtrate preparing

Culture cells Filtrate of *Lb. rhamnosus* GG , *Lb. plantarum* , *Lb. casei* and local isolate *Lb. plantarum* live cells were prepared by transfer a single colony to sterilized skim milk 12% and incubated at 37 ° C until coagulation, The numbers of each type of therapeutic bacteria were estimated ($\geq 10^{12}$) then centrifuged using a high-speed refrigerant centrifuge (Huttich, Germany) 4000 rpm for 20 min at 20°C. separately, then the top clear layers were separated carefully and filtrated using millipore filter unit 0.22 μ (Millipore corp, Spain) (24).=

Antimicrobial activity- in vitro

The test was done according to Balouiri (1) with some modification , aerobic and anaerobic bacteria from BV infection were cultured on Muller Hinton medium and Muller Hinton medium with 0.1% L-cysteine Hcl (Oxide) individually , 10% (V/V) of therapeutic bacteria culture supernatant was added, then kept at 37 °C for 24 hrs. Aerobic and anaerobic bacteria from BV infection were evaluated as a logarithm of viable numbers before and after 24 hrs. of the addition by using Pour plate count method.

Therapeutic topical ointment compounds

Therapeutic topical ointment compounds formula made from mixture of %50 organic Shea butter (Ghana, West Africa) plus organic Coconut Oil (Barleans Company -Philippine , %50 of therapeutic bacteria skim milk culture (which shown highest inhibition activity).

Clinical trial design– in vivo

This study carried out from 1/10/2017 to 30/5/2018. Eighty nine volunteers from the Women 's Medical Clinic , Supervised by specialist obstetrician gynecologist in Samawah city , All females suffering from vaginal and genital tract infections in varying degrees. The ages were from 17 to 48 years, with the written document consent from

volunteers. These documents included that the volunteers were in healthy mental conditions , non-pregnant, did not have oral contraceptives, did not suffering from bleeding or menopause, did not had intercourse in the past 48 hours, did not suffering from chronic diseases, and they will not take any antibiotics or other drugs during the last two weeks of this trial or any vaginal medicine and must not participated in other trials in the last two months, As well as special criteria which included that no antibiotics should be used during the trial period except usual detergents and compliance with the instructions of the physician supervising (25). B.V infection was diagnosed by a specialist physician according to the approved diagnostic criteria. The volunteers were monitored and supervised during the two-week trial period, trials were randomized double-blinded so volunteers were randomly divided into two groups, the first group 43 volunteers used the ointment containing local isolate *Lactobacillus plantarum*. the second group 46 volunteers used the antibiotic containing Metronidazole ointment as the standard treatment for comparison purposes. Volunteers in the two groups were subjected to a thorough examination by a specialist before starting the trial (each group separately) , volunteers used ointment of therapeutic bacteria and standard treatment twice daily for two weeks under supervision and inspection from specialist every three days, The criteria of the study were elected very carefully, which included a Amsel's Criteria and Nugent's Criteria (16).

RESULTS AND DISCUSSION

Total count of therapeutic bacteria in skim milk: Results in Table 1 show the total viable count of the four therapeutic bacteria, which were *Lactobacillus rhamnosus* GG, *Lactobacillus plantarum*, *Lactobacillus casei* and local isolates, *Lactobacillus plantarum*, were 71×10^{12} , 83×10^{12} , 68×10^{12} , 88×10^{12} cfu / ml respectively, Vitamilk Skimmed Milk 0%fat was used in the preparation of the therapeutic bacteria as it was rich nutrient medium fortified by eight types of vitamins in addition to calcium, that led to promote the growth of therapeutic bacteria as the *lactobacillus spp* fastidious bacteria (29), and that may explains the higher number of

therapeutic bacteria in this type of skim milk compared to other skim milk not fortified. (5). It was noticed that the bacterial numbers were variant in the growth and that variety in the study, may due to use different species and strain and have different metabolic activities,

the high total count they shown because of ideal conditions used for the growth during incubation enabled to obtain high initial numbers, a requirement for the use of therapeutic bacteria in the field of medical treatment and prevention (28).

Table 1. viable count of the therapeutic bacteria cfu/ml

<u>Probiotics Bacteria</u>	<u>CUF\MI</u>
<u><i>Lactobacillus rhamnosus GG</i></u>	71×10^{12}
<u><i>Lactobacillus plantarum</i></u>	83×10^{12}
<u><i>Lactobacillus casei</i></u>	68×10^{12}
<u><i>Lactobacillus plantarum</i></u>	88×10^{12}

Antibacterial activity of therapeutic culture cells filtrate in vitro:====The results in Fig. 1 show the antibacterial activity of therapeutic cells filtrate used in inhibition growth of BV aerobic bacteria, the initial number of BV aerobic bacteria was 5.12 cfu / mL in the zero time, the decline level as log cycles were between 1.23 to 2.08 cycles after 24 hr. , *Lactobacillus plantarum* local isolate cell filtrate showed a higher efficiency. The results in Fig. 2 show the antibacterial activity of the therapeutic bacteria filtrates used in inhibition the growth of BV anaerobic bacteria, the initial number of BV anaerobic bacteria was 7.48 cfu / ml in the zero time. The decline measured as log cycles were between 1.72 to 2.5 cycles after 24 hr., also local isolate *Lactobacillus plantarum* showed a higher efficiency. *Lb. plantarum* has a relatively large genome compared to other *Lactobacillus* species, which explains their ability to adapt to some extreme conditions and the wide range of their metabolic products (14,30) . *Lb. plantarum* produces metabolic compounds considered as antimicrobial agents play an important role in maintaining health, this effect is due to its presence in the natural flora and its competition with pathogenic bacteria on adhesion sites and provide protection and prevention from pathogenic bacteria, in

addition, it produces a number of bacteriocins , most notably is Plantaricin, which is a heat-resistant protein and a inhabits some gram positive and negative -bacteria such as *Listeria* spp. , *Staphylococcus* spp. , *Pediococcus* spp. (30). *Lb.plantarum* promote IL-10 , the regulatory cytokine that inhibits IL-12 in a way that interferes with the action of T-helper cells , organize T cells and stimulates the production of high levels of interleukins (24) . In addition, *Lb.plantarum* stimulates the increase of Neutrophils numbers and Monocytes, thus enhancing innate immunity (22). Glycogen, which is collected in epithelial cells by the activity of estrogen, is converted by *Lb. plantarum* to organic acids that maintain a pH level to 4.5 that inhibits growth of most pathogenic organisms by creating unsuitable environment for many pathogenic microbes (19), As well as the production of biosurfactants, which are linked to the surfaces of pathogenic bacteria to disallow them from adhering to host cell surfaces (9), which explains their restoration to the natural balance of vaginal flora, especially in healthy women. Based on the inhibition efficacy of the local isolation *Lb.plantarum* to aerobic and anaerobic bacteria causing vaginal infections, it was nominated to use in the preparation of therapeutic ointment.

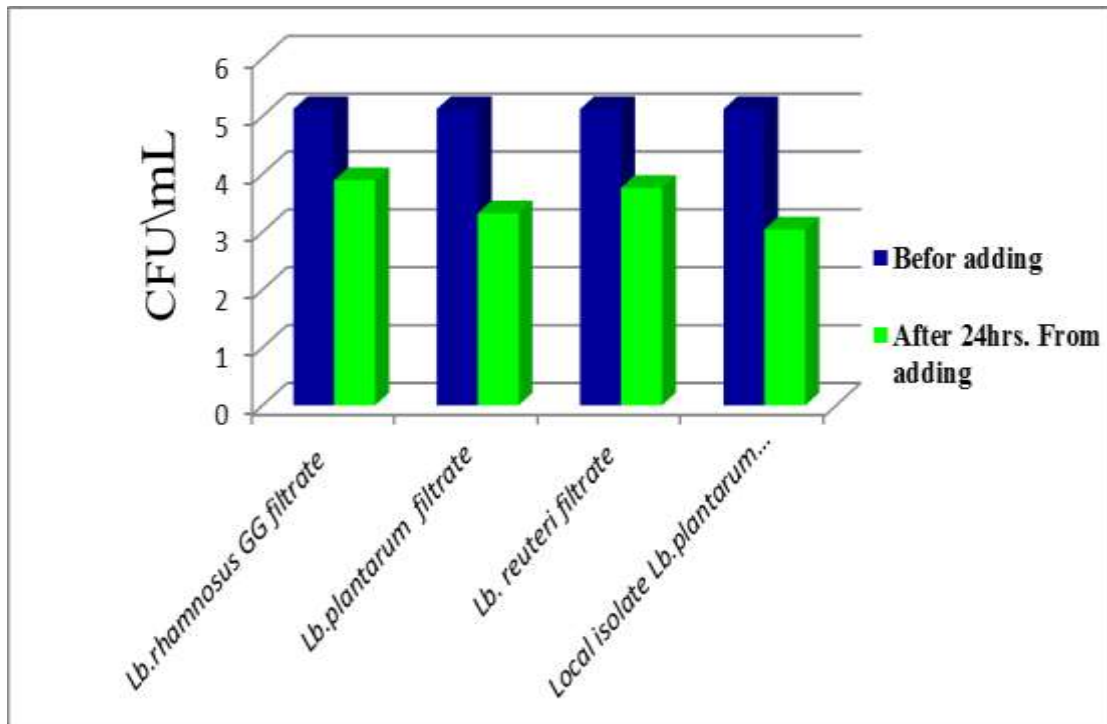


Figure 1. Logarithmic numbers of aerobic bacteria before and after 24 hours of addition of the culture cells filtrate

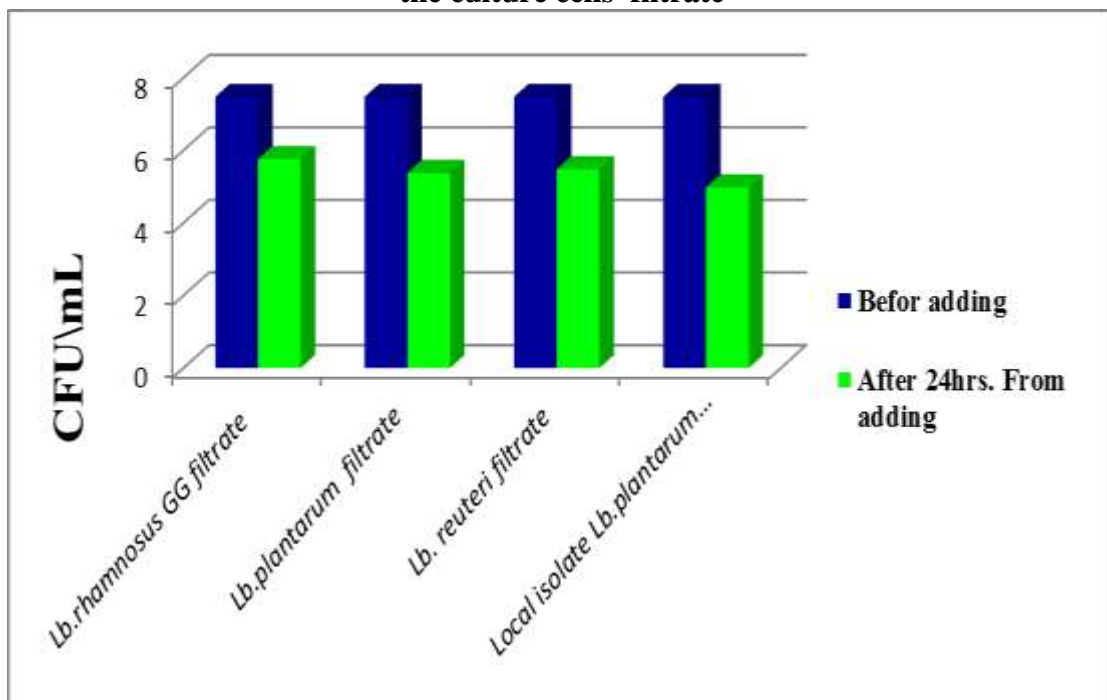


Figure 2. Logarithmic numbers of anaerobic bacteria before and after 24 hours of addition of the therapeutic culture cells filtrate

Clinical trial

Several criteria are used to diagnosis bacterial vaginosis, Amsel’s Criteria is the standard one it is established by Amsel 1983. This Criteria depends on four indicators. According to these indicators, women are considered to have bacterial vaginitis if three of these indicators are show positive results. This Criteria is

sensitive and positive predictive exceeding %90 (8) In addition to the Nugent criteria, both effective and affordable, as well as reliability and high accuracy of results (21). Other criteria such as Spiegel, similar to the Nugent criteria, are used as they are based on the assessment and estimation of natural flora (16).

Table 2. examination of bacterial vaginosis according to the Amsel criteria for ointment of therapeutic bacteria *Lb. plantarum* and metronidazole ointment group after 7 and 14 days

Amsel criteria		ointment group <i>Lb. plantarum</i>	(standard treatment) Metronidazole group
Vaginal Discharge	Baseline	43(100%)	46(100%)
	7 DAY	18(41.68)	20(43.47)
	14 DAY	6(13.95%)	9(19.56%)
Clue cells	Baseline	43(100%)	46(100%)
	7 DAY	20(46.5%)	23(50%)
	14 DAY	4(9.30%)	7(15.21%)
Whiff test	Baseline	43(100%)	46(100%)
	7 DAY	12(27.90%)	15(32.60%)
	14 DAY	4(9.30%)	6(13.04%)
Vaginal pH > 4.5	Baseline	43(100%)	46(100%)
	7 DAY	10(23.25)	36(78.26%)
	14 DAY	3(6.97%)	29(63.04%)
No. of patients		1(2.32%)	4(8.69%)
Healing rate		97.67%	91.30%

*If 3 of the 4 criteria are positive, the patient has bacterial vaginosis

Table 2 shows the results of bacterial vaginosis examination according to the Amsel criteria for the study groups, which included the therapeutic ointment group and the metronidazole ointment group after two week treatment under the supervision of specialized physician. It was noted that the ointment of therapeutic bacteria *Lb. plantarum* gave a higher cure rate than the metronidazole group (2 weeks after treatment), reached 97.87% compared with standard treatment group (91.30%), the result shown in table 2 all the parameters of Amsel criteria were decline for both groups, ointment group shown more effectiveness in all these four parameters after 7 and 14 days compering with standard treatment. The vaginal discharge decreased to 18 , 6 and 20,9 cases after 7and 14 days for ointment group and standard group respectively, Clue cells after 1and 2 weeks for ointment group and standard group respectively were 20 , 4 and 23 ,7 cases , Whiff test after 1and 2 weeks for ointment

group and standard group respectively were 12 , 4 and 15 , 6 cases . Vaginal pH > 4.5 after 1and 2 weeks respectively for ointment group and standard group respectively were 10,3 and 36,29 Table 3 shows the results of bacterial vaginosis examination according to the Nugent criteria. *Lb. plantarum* ointment group gave a higher cure rate for patients than metronidazole standard group (2 weeks of trial), reached 95.35% compared to standard metronidazole standard group 91.30%. It was noted from the results that the number of the cases in the ointment group of therapeutic bacteria shown an increase in the gram-positive rods (*Lactobacillus* morphotypes) after 7 and 14 days of the trial compared with the standard treatment group and decreased in the number of small gram-negative to gram-variable rods (*G.vaginalis* and *Bacteroides* morphotypes), and curved gram variable rods (*Mobiluncus* spp morphotypes) compared to the metronidazole group.

Table 3. examination of bacterial vaginosis according to the Nugent's criteria for ointment of therapeutic bacteria *Lb. plantarum* and metronidazole ointment group after 7 and 14 days

Nugent's Criteria/ SCORE		<i>Lb. plantarum</i> ointment group	Metronidazole group (standard treatment)
0 -3	Baseline	0(100%)	0(100%)
	7 DAY	25(58.13%)	11(23.91)
	14 DAY	38(88.37%)	31(67.40%)
4 -6	Baseline	0(100%)	0(100%)
	7 DAY	12(27.09)	27(58.69)
	14 DAY	3(6.97%)	11(23.91%)
7 -10	Baseline	43(100%)	46(100%)
	7 DAY	6(13.95)	8(17.39)
	14 DAY	2(4.65%)	4(8.70%)
No. of patients		2	4
Healing rate		95.35%	91.30%

*Total score:-0-3 normal; 4-6 intermediate, repeat test later; 7-10 bacterial vaginosis

The results in Table 4 show the side effects associated with the trial during the treatment period, *Lb. plantarum* ointment group was shown no side effects or complications compared with the metronidazole group, which included six cases were suffered from itching and burning, three cases showed swelling and skin rash, while a fungal infection occurred in two other cases also the Table 4 shown the reason of the side effects

Table 4. Side effects associated with the experiment

Describe the side effect	<i>Lb. plantarum</i> ointment group	Metronidazole group (standard treatment)
Pharmaceutical Allergy	no found	no found
Itching and burning	no found	6(13.04%)
Swelling and rash	no found	3(6.52%)
Pain during urination	no found	no found
Fungal infection	no found	2(4.34)
Approximate numbers of <i>Lactobacillus</i> species CfU/mL	$1 \times 10^9 \pm 2.7$	$1 \times 10^4 \pm 3.7$

In this study, the use of an ointment mixture of *Lb. plantarum* treatment has contributed to raising the numbers of *Lactobacillus* bacteria which represents the normal flora that provides protection for the vaginal environment. this study didn't need to use statistical analysis as a result of absence of these side effects compared to traditional treatment gave the advantage to *Lb. plantarum* ointment as well as the recovery rate that exceeded the traditional treatment. Also Metronidazole has been associated with neurotoxicity and genotoxicity (10). *Lb. plantarum* ointment maybe had no side effects compared to Metronidazole antibiotic ointment and it could be used by pregnant women with bacterial Vaginosis infections, for long-term safety use and the therapeutic bacteria *Lb. plantarum* may give it the ability to restore the natural healthy balance in vaginal flora, as well as it could be no more worry of fungal infection, which is associated with antibiotics treatment. Moreover *Lb. plantarum* ointment avoids the transmission of antibiotic resistance genes to BV bacteria as a results of treatment with antibiotics side effect(11).

Ethical approvals

The study and Clinical Trial were conducted with the approval of the Muthanna Governorate Health Directorate/ Ministry of Health /IRAQ with its approval number 484 dated 7/3/2019.

absence which was due to the presence of high numbers of *Lactobacillus* species CfU/ml in ointment group compared with standard ointment 1×10^9 and 1×10^4 respectively that's caused healthy vagina natural balanced flora environment, *Lactobacilli* bacteria in the vagina considered as biomarkers for vaginal health, its secreted lactic acid and hydrogen peroxide, which give the vagina its acidic pH level (17)

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