University of Babylon
College of Medicine

A Study of the Relationship Between Lactobacilli and Opportunistic Bacterial Pathogens Associated with Vaginitis in Babylon Province

A Thesis

Submitted to the Council of the College of Medicine-University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology

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دراسة العلاقة بين العُصيات اللبنية والمُممّرِضات البكتيرية الانتهازية المتعلقة بالتهاب المهبل في محافظة بابل

رسالة
مقدمة الى مجلس كلية الطب/ جامعة بابل كجزء من متطلبات نيل درجة الماجستير في علم الاحياء المجهرية الطبية

من قبل

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بسم الله الرحمن الرحيم

﴿ وَمَا أَنزَلْنَا عَلَيْكَ الْكِتَابَ إِلَّا لِتُبَيِّنَ

لَهُمُ الَّذِي اخْتَلَفُوا فِيهِ وَهُدًى

وَرَحْمَةً لِّقَوْمٍ يُؤْمِنُونَ ﴾

صدق الله العلي العظيم

﴿ سورة النحل / الآية 64 ﴾

Abstract

In this study (110) vaginal swabs were obtained from women suffering from vaginitis and admitted to Babylon Hospital of Maternity and Paediatrics in Babylon province,swab samples from private clinics were also included.These swabs were distributed between married women(100) and unmarried women(10), pregnant(20) and non pregnant(80), with intrauterine device(70) and without intrauterine device(30). The period of sample collection was from November 2008 to April 2009.

The study involved the role of intrauterine device among married women with vaginitis and involved also the isolation of opportunistic bacterial isolates among pregnant and non pregnant women.

This study also involved studying the cultural characteristics of Lactobacilli, their probiotic role in maintaining vaginal health, in addition, the ability of Lactobacilli to produce some defense factors like hydrogen peroxide (H_2O_2), bacteriocin and lactic acid were also included.

The results showed that a total of 130 bacterial isolates were obtained,10 from unmarried women and 120 from married women.

The results revealed that intrauterine device (IUD) was a predisposing factor for vaginitis giving a percentage of (71%) among women used intrauterine device versus(29%) among women without it .

The most common species of opportunistic bacterial isolates were *Staphylococcus aureus* (44.7%) followed by *Escherichia coli(42%), Streptococcus agalactiae* (26.9%) , *Klebsiella pneumoniae (21%),*while other types of bacterial isolates differ between pregnant and non pregnant women.

The results showed that all Lactobacilli isolates were unable to produce hydrogen peroxide while, some isolates were able to produce bacteriocins that inhibit some isolates of opportunistic pathogens (*S.aureus, E.coli*).

This study also investigated the effects of antibiotics on Lactobacilli and on opportunistic bacterial isolates. The results revealed that 93.4% of Lactobacilli isolates were sensitive to erythromycin while 93.3% of Lactobacilli isolates were resistant to ciprofloxacin .The results also showed (40%, 53.3%) resistance of Lactobacilli toward amoxicillin and gentamycin respectively. These results were studied in relation to the effects of antibiotics on opportunistic bacterial isolates and it was found that most of these opportunistic isolates were sensitive to ciprofloxacin in contrast to erythromycin to which most of them were resistant except for *Listeria monocytogenes* and *Streptococcus agalactiae*. So, the study concluded that the types of antibiotics used to treat vaginitis must be very selective in order not to kill the beneficial bacteria(Lactobacilli) that help to preserve the vaginal health and ecosystem as being one of probiotic bacteria.

الخلاصة

تـم فـي هـذه الدراسـة اخضـاع 110 مسـحة مهبليـة للعـزل وللتشـخيص وتـم استحصـالها مـن النسـاء اللـواتي يعـانين مـن التهـاب المهبـل ويـراجعن مستشـفى بابـل للولادة والاطفال في محافظة بابل بالاضـافة الـى المسحات التـي اخذت مـن العيـادات الخاصـــة .ووزعـــت هـــذه المســـحات بـــين النســـاء المتزوجـــات(100) وغيـــر المتزوجـات(10) , الحوامـل(20) وغيـر الحوامـل(80) , اللـواتي يسـتعملن موانـع الحمـل داخـل الـرحم (70) وغيـر المسـتعملات لـه (30). للفتـرة مـن تشـرين الثـاني 2008 الى نيسان 2009.

تضمنت هذه الدراسة دور مانع الحمل داخل الرحم لدى النساء المتزوجات المصابات بالتهاب المهبل كما شملت ايضاً عزل البكتريـا الانتهازيـة مـن النسـاء الحوامـل وغيـر الحوامل.

كمـا تضـمنت الدراسـة الكشـف عـن الخصـائص الزرعيـة للعصـيات اللبنيـة ودورهـا كمعـزز حيـوي فـي الحفـاظ علـى صـحة المهبـل اضـافة الـى قـدرة العصـيات اللبنيـة علـى انتـاج بعـض العوامـل الدفاعيـة مثـل بيروكسـيد الهيـدروجين (H_2O_2)، والبكتروسين وحامض اللبنيك(lactic acid)

اظهرت نتائج الزرع 130عزلة بكتيرية حيث استحصلت120 عزلـة بكتيريـة من النساء المتزوجات و 10 عزلات من النساء الغير متزوجات

كمـا اشـارت النتـائج أن مـانع الحمـل داخـل الـرحم كـان مـن العوامـل المسـببة لإلتهاب المهبل معطيا نسبة(71%) للنساء اللاتـي يسـتعملن مـانع الحمـل داخـل الـرحم مقابل(29%) للنساء اللاتي لايستعملنه.

كانت اكثر انـواع البكتيريـا الانتهازيـة التـي عزلـت هـي *S.aureus*(44.7%) جـاءت بعـدها *E.coli* (42%) ثـم*Streptococcus agalactiae* (26.9%) ثم *K.pneumoniae*(21%) بينمـا اختلفت الانـواع الاخـرى مـن العـزلات البكتيريـة بين النساء الحوامل وغير الحوامل.

اظهرت النتائج إن جميع عزلات العصيات اللبنية *كانت غير قادرة* على انتاج بيروكسيد الهيدروجين بينما اظهرت بعض العزلات القابلية على انتاج البكتروسين الذي يمتلك القدرة على تثبيط بعض عزلات الممرضات الانتهازية مثل (*S.aureus* و *E.coli*)

شملت هذه الدراسة ايضاً تأثير المضادات الحيوية على عزل ونمو العصيات اللبنية وعلى البكتريا الانتهازية حيث كانت 93,4% من عزلات الـ Lactobacilli حساسة للأرثرومايسين بينما كانت 93,3% من هذه العزلات مقاومة للسبروفلوكساسين واظهرت النتائج بإن هناك(40%,53,3%) مقاومة بالنسبة للاموكسيلين والجنتامايسين من قبل Lactobacilli *على التوالي*.تم دراسة وربط هذه النتائج مع تأثير المضادات الحيوية على البكتريا الانتهازية وتبين أن معظم عزلات البكتريا الانتهازية كانت حساسة للسبروفلوكساسين عكس الأرثرومايسين الذي كانت مقاومة له بأستثناء بكتريا *Streptococcus agalactiae* و *L.monocytogenes*.*يستنتج من ذلك* أن نوع المضاد الحيوي الذي يستعمل لعلاج التهاب المهبل يجب ان يختار بشكل جيد كي لا يقتل البكتريا المفيدة(العصيات اللبنية) وللحفاظ على صحة المهبل وبيئته كون هذه البكتريا تمتلك دور المعززات الحيوية.

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Bara'

Appendix (1)

Morphological and Biochemical Features for Identification of Lactobacilli.

Test	Lactobacilli
-gram stain	Gram positive
-appearance	Single or pairs or short chains
Oxidase	-ve
Catalase	-ve
Coagulase	-ve
Indole	-ve
Glucose	+ve
Lactose	+ve
Spore	-ve
Mannitol	-ve
Incubation	Anaerobically
Motility	-ve
Selective media	mRS broth, mSR agar

Appendix (2)

Morphological and Biochemical Features for Identification of Gram Positive Isolates.

Test	GBS	*S.aureus*	*S.epidermidis*
Gram stain	Gram +ve cocci (pairs or chains)	Gram +ve cocci (clusters)	Gram +ve cocci (clusters)
Catalase	-ve	+ve	+ve
Oxidase	-ve	-ve	-ve
Hemolysin	Beta (narrow zone)	Beta	Gama
Coagulase	-ve	+ve	-ve
Growth on mannitol	-ve	Golden	White
Motility	-ve	-ve	-ve

Appendix (3)

Morphological and Biochemical Features for Identification of Gram Negative Isolates.

Test	*E.coli*	*K.pneumoniae*	*Actinobacter*	*P.aeruginosa*
Gram stain	Gram -ve short rods	Gram -ve short rods	Gram –ve coccobacilli (Dipplococci)	Gram -ve short rods
Catalase	+ve	+ve	+ve	+ve
Oxidase	-ve	-ve	+ve	+ve
Capsule	-ve	+ve	-ve	+ve
Hemolysin	-ve	-ve	-ve	+ve
Indole	+ve	-ve	-ve	-ve
M.R	+ve	-ve	-ve	-ve
Citrate	-ve	+ve	+ve	+ve
V.P	-ve	+ve	-ve	-ve
Urease	-ve	+ve	-ve	-ve
Growth on KIA	A/a	A/a	k/k	K/K
H_2S production	-ve	-ve	-ve	-ve
Motility	+ve	-ve	-ve	+ve
EMB	Metallic sheen	Centrally dark	Pale	Pale

Certification

We certify that this thesis was prepared under our supervision at the Department of Microbiology, College of Medicine, University of Babylon as partial fulfillment of the requirements for the degree of Master of Science in Medical Microbiology.

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In view of the available recommendation, I present this thesis for evaluation by the Examining Committee.

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1.1. Introduction

Vaginitis, is an infectious or non infectious inflammation of the vaginal mucosa, sometimes with inflammation of the vulva (external genitals). This inflammation often causes itching, burning, irritation, discharge and discomfort. It is one of the most common reasons for women to seek medical care (Egan & Lipsky, 1999).

Vaginitis falls into many forms: irritant, hormonal, foreign body, sexual transmitted diseases and infective. All types can cause great discomfort to a woman (Joesoef *et al*.,1999).

Irritant vaginitis can be due to allergic reactions to spermicides, condomes, soaps, douches, perfumes, medications and hot tubes in addition to abrasion, tampons and sanitary napkins (Hudson, 2007).

Hormonal vaginitis, is usually due to low levels of circulating estrogens in the body. This causes the lining of the vaginal canal to be thin and become atrophic. A thin vaginal lining may predispose a women to secondary infections. Typically a woman will complain of discharge, dryness, itching or burning. Estrogen is necessary to maintain the homeostasis of the vaginal flora and the proper pH of 4.5, The natural acidic environment of the vagina limits the growth of abnormal bacteria and maintains the presence of healthy bacteria (Fang *et al*., 2001).

Infective vaginitis may be caused by bacteria, fungi, parasites, viruses. The bacteriological agents associated with vaginitis include a wide variety of bacteria that are dominated by overgrowth and marked by deficiency of hydrogen peroxide-producing Lactobacilli. The most bacterial agents causing vaginitis include *S.aureus*, *E.coli*, *Group B streptococcus*, *L.monocytogenes*, *K.pneumoniae*, *Acinetobacter* spp., *N.gonorrhoea*, and the bacteria that cause bacterial vaginosis in addition to Chlamydia (Stenchever, 2001).

Other types of vaginitis due to sexual transmitted disease include *N.gonorrhoea*, Chlamydia, in addition to *Trichomonas vaginalis* which is one of the most common protozoan infection. Normally the acidic nature of the vagina renders the environment resistant to *Trichomonas* infection, symptoms present as profuse frothy discharge often bloody, green, yellow or grey, the discharge has unpleasant odor and vaginal itching, burning and pain may be present. The pH is between 5 and 7 (Soper, 2004).

The most important factor is to maintain the balance of the vaginal flora by the Lactobacilli and its protective and probiotic role in treating and preventing vaginal infection by producing antagonizing compounds which are hydrogen peroxide, lactic acid and bacteriocin (Hawes *et al*., 1996).

These properties of Lactobacilli lead to the use of this bacteria as probiotic in addition to being member of normal flora and are generally regarded as safe for use in humans. So that, in treatment of vaginitis, the normal flora disturbances must be restored by avoiding the use of wrong antibiotic that disturbs the normal vaginal flora (Andreu, 2004)

This study aims to:-

1. Isolate the Lactobacilli and evaluate their protective role against vaginitis.
2. Isolate and identify the common bacterial opportunistic pathogens in vaginitis.
3. Investigate some defense factors of Lactobacilli like hydrogen peroxide (H_2O_2), bacteriocins and lactic acid.
4. Study the effects of antibiotics on the bacterial isolates and compare their effect on the presence or absence of Lactobacilli.

1.2. Literatures Review

1.2.1. Female Genital Tract Anatomy

The mucosa of the vagina and outer portion of the cervix (ectocervix) are made up of a highly vascularized submucosa and a superficial non keratinized, stratified sequamous epithelium. This sequamous epithelium abruptly changes to simple stratified columnar epithelium at the transitional zone, which marks the beginning of the inner portion of the cervix (or endocervix), this is the site of hormonally regulated secretion of specialized mucous that facilitates sperm transport. The endocervix ends in the uterine cavity, the lining of which is referred to as either endometrium in the non pregnant state or decidua during pregnancy. Depending on hormonal stimulation, the endometrium varies from 1 to 6 mm in thickness and is made of several muscular layers. If pregnancy and implantation occurs, it hypertrophies further to become the nutrient-rich, intensely glandular decidua, if pregnancy does not occur, this layer is shed at the time of menses (Beckerman & Dudley, 2001).

The site of fertilization is the fallobian tube, a muscculary membranes structure lined by a highly vascular mucosa (endosalpinx), which consists of ciliated and secretory cells (Beckerman & Dudley, 2001).

2.2.1. Development of the Normal Vaginal Flora

At birth, the vagina of the female infant is sterile, after only a few days, when the glycogen content of the vaginal epithelial cells has been elevated under the influence of the mother's oestrogen, the infant's vagina is colonized by the Lactobacilli migrating from the mother. This is in line with generally recognized fact that the normal bacterial flora in humans originates from the mother. In most instances, this flora continues to dominate throughout the individual's lifetime, and this is also the case with the vaginal flora though it is in the vagina's physiological environment during the life cycle (Cauci *et al*.,2002)

Small quantities of Lactobacilli remains, however, and the oestrogen product at menarche will cause a thickening of the vaginal mucosa, prerequisite for the propagation of Lactobacilli, the dominant vaginal flora of adult female (Harstall & Corabian, 1998). The bacteria isolated from the vaginal secretion of women of child bearing age number around 10^7 to 10^8 CFU/ml fluid (Redondolopez *et al*., 1996).

This microflora composition continues until menopause, when it is replaced by a mixed flora unlike that of infant female, but with considerable portion of mycoplasma species and small quantities of anaerobic bacteria (including *Gardnerrella vaginalis*). When hormonal replacement therapy (HRT) is used, it will cause Lactobacilli to continue as the dominant flora (Forsum *et al.*, 2005 a).

1.2.3. Vaginitis

1.2.3.1. Definition and Symptoms of Vaginitis

Vaginitis is a name given to describe swelling, itching, burning or infection in the vagina that can be caused by several different organisms. Vaginal infection is the most common gynecological problem found in women of all ages, with most women having at least one form of vaginitis during their lives. Vaginal infections often occur when women's natural resistance is lowered by anxiety, tension, lack of sleep, poor diet and sexual activity with an infected partner (Quan, 2000).

In addition, the vaginal environment is influenced by a number of different factors including a woman's health, her personal hygiene, medications, hormones (particularly estrogen), and the health of her sexual partner. Any disturbances in these factors can trigger vaginitis. In general, the symptoms of vaginitis according to Egan & Lipsky (1999) are:

-Irritation and/ or itching of the genital area.

-Inflammation (irritation, redness and swelling) of the labia majora, labia minora and perineal area.

-Vaginal discharge.

-Foul vaginal odor.

-Discomfort or burning when urinating.

-Pain/irritation with sexual intercourse.

1.2.3.2. Types of Vaginitis

There are several types (or causes) of vaginitis:-

1.2.3.2.1. Infectious Vaginitis

Infectious vaginitis accounts for about 90% of all cases of vaginitis in reproductive age women (Brook, 2002), and is represented by bacterial infection, candidiasis and trichomoniasis. Bacterial infection includes both specific and non specific vaginitis caused by opportunistic pathogens and *Gardnerrela vaginalis* (the causative agent of bacterial vaginosis) (Joesoef *et al*., 1999). Other less common infection caused by gonorrhoeae, Chlamydia, herpes, campylobacter and some parasites (Egan & Lipsky, 1999).

1.2.3.2.2. Hormonal Vaginitis

Hormonal vaginitis includes atrophic vaginitis usually found in postmenapausal or post partum women. Sometimes, it can occur in young girls before puberty, in this situation, the estrogen support of the vagina is poor (Fang *et al*., 2001).

The relationship between vaginal microbiology, menses and oestrogen level is complex (Eschenbach, *et al*., 2000). Normally the vaginal epithelium is stimulated and builds up under the influence of oestrogen that affects the glycogen content which is broken down into sugars by the non bacterial enzymes which in turn are converted into lactic acid by Lactobacilli.

Wasiela *et al.,* (2001) found that interference with growth and glycogen content of the vaginal epithelium and change in the normal bacterial flora and pH of the vaginal secretion are predisposing factors toward vaginal disease and abnormal discharge.

Gloria *et al.,* (2000) described atrophic vaginitis as an inflammation or irritation of the vagina caused by thinning and shrinking of the vaginal tissue with decrease lubrication of the vaginal wall due to lack of oestrogen. The most common symptoms of atrophic vaginitis are thinning and redness of vaginal lining, vaginal skin inflammation ,sorness of the vaginal skin and painful intercourse.

Atrophic vaginitis can also occur in premenapausal women who take antiestroenic medcations or who have medical or surgical conditions that result in decreasing level of oestrogen, the thinned endometrium and increased vaginal pH level induced by oestrogen deficiency predispose to genitourinary tract infection (Kalogerki, *et al.*, 1996).

1.2.3.2.3. Irritation/Allergy Vaginitis

Irritant vaginitis can be caused by allergies to condomes, spermicides, soaps, perfumes, douches, lubricants and semen. It can also be caused by hot tubes, abrasion, tissue, tampons or topical medication (Hudson, 2007).

Since any member of synthetic chemicals and materials can induce an allergic reaction along the mucosal lining of the vagina, the best approach to treat all cases of irritant vaginitis is to avoid having synthetic chemicals and materials contact the vagina whenever possible. Scented soap should therefore never be used to clean the vaginal area, ferminine hygiene sprays should also be avoided as should contraceptive foams and suppositories in addition to tampons that could dry out and irritate the mucosal linning of the vagina just as soap can. (Stenchever, 2001).

1.2.3.2.4. Foreign Body Vaginitis

Foreign body vaginitis commonly caused by foreign bodies (retained tampons or condoms) cause extremely malodorous vaginal discharges. Treatment consists of removal, for which ring forceps may be useful. Further treatment is generally not necessary (Hudson, 2007).

1.2.3.2.5. Vaginitis due to Sexual Transmitted Diseases STDs

Sexually transmitted diseases can be a cause of vaginal discharge. Chlamydia and gonorrhea testing should be done whenever a sexually active individual complains of vaginal discharge even when the cervix appears normal (Rayan & Ray, 2004).

1.2.4. Microbiology of Vaginitis

1.2.4.1. Bacterial Infection

It had been focused on the common opportunistic pathogens that cause vaginitis in addition to bacterial vaginosis and Chlamydia.

1.2.4.1.1. *Staphylococcus aureus*

S. aureus is a gram positive, spherical in shape, it is single or in clusters and occasionally in short chains, it is non-motile, non spore forming, facultative anaerobe, it is catalase positive, oxidase negative, and coagulase positive, usually non capsular colonies appear opaque and are often pigmented golden-yellow. In common with other facultative anaerobes, *S. aureus* can grow in the absence of oxygen by has both fermentative and oxidative pathways (Masalha *et al*., 2001).

Several studies suggest that oxygen plays a role in the pathogenesis of *S. aureus* in both it's capacity to produce virulence factors and it's ability to persist and grow in different and often hospital environments (Yarwood & Schlievent, 2001).

S. aureus express many potential virulence factors such as surface proteins that promote colonization of host tissues, toxins that damage host tissues, in addition, to factors that inhibit phagocytosis and cause disease symptoms (Archer, 1998). So, *S. aureus* by these virulence factors can cause vaginitis, especially when there is adecrease in the number of Lactobacilli to maintain the normal vaginal ecosystem (Antonio *et al.,* 1999).

S. aureus is the major cause of hospital acquired (nosocomial) infection of surgical wound and epidermal skin disease in new born infant and food poisoning (Brook *et al*., 2004). Also, *S. aureus* can cause septicemia, folliculitis, boils, scalded skin syndrome, conjunctivitis and mastitis (Schneewind *et al*., 1995) and (Chibgu & Ezeronye, 2003).

1.2.4.1.2. *Group B Streptococcus-GBS*

Group B Streptococcus, taxonomically, known as *Streptococcus agalactiae,* is a gram positive cocci, non motile, non spore forming and spherical or ovoid, less than 2 µm in diameter, catalase negative, oxidase negative, facultatively anaerobic, fermentative metabolism with lactic acid being primary product. It grows poorly on nutrient media and prefers media enriched with blood or serum (Edward & Backer, 1995).

Vaginitis caused by *Streptococcus* presents acreamy, white discharge that is normally clear or white cloudy. There is usually no burning of the vulva as in case of yeast infection and the discharge is not very odorous like a bacterial vaginosis. There are several streptococcal species that can be present in the vagina: group A, B and D, about 70% of cases is due to group B and about 30% is due to group D, group A infection is rare. Streptococcal vaginitis can occur spontaneously but very often it is caused by the antibiotic treatment given for bacterial vaginosis (Frederick & Jelovesk, 2001).

The relative risk of vaginal infection with *Streptococcus agalactiae* in patient with purulent vaginal discharge is greater than that of candidiasis and lower than that of trichomoniasis (Brook *et al.*, 2007) but GBS infection is not classified within sexually transmitted diseases.

Streptococcus agalactia is now best known as a cause of postpartum infection as well as the most common cause of neonatal sepsis (Farley, 2001).

In pregnant women, GBS can cause chorioamnionitis, endometritis or genitourinary infection with bacteraemia, rarely can endocarditis or meningitis be observed (Berner, 2002).

These infections are related to its multifactorial virulence factors that include adherence, colonization, invation of epithelial cells, replication and the evation of host defense (Nizet *et al.,* 2000).

1.2.4.1.3. *Escherichia coli*

E.coli is one of the most important Enterobacteriaceae species, it is gram negative rods, usually motile,and produces polysaccharide capsule, positive test for indole, lysine decarboxylase and mannitol fermentation and produces gas from glucose. This bacteria is predominant among aerobic commensal bacteria in healthy human intestine (Smith & Scotland, 1993; Brook *et al*., 2004).

E.coli is one of the common organism in the microflora of pregnant as well as non pregnant women. Vaginal colonization with *E.coli* is associated with various genitourinary, obstetric and neonatal complication such as, severe form of pelvic inflammatory disease (Heinonen & Miettinen, 1994), urinary tract infection (Rajan *et al*., 1999), very low birth weight infants and early onset neonatal septicemia and meningitis. Vaginal *E.coli* has also been reported to be sexually transmissible to a male partner (Hebelka *et al*., 1993). The virulence factors of vaginal *E.coli* include adhesions, toxins, siderophores and invasion (Watt *et al*., 2003).

1.2.4.1.4. *Klebsiella pneumoniae*

K. pneumoniae is a gram negative, member of the family Enterobacteriaceae, that is characterized by polysaccharide capsule (which give their colonies their characteristic mucoid appearance), rod-shaped, facultative anaerobic, it is non motile, non-spore forming, flagellated, produces large sticky colonies when plated on nutrient media (Podschun & Ulmann, 1998).

Klebsiella pneumoniae is lactose fermenter, produce lysine decarboxylase but not ornithine decarboxylase and it is generally positive in the Vogas-Proskauer test and citrate, produces gas from glucose (Brook *et al*., 2004).

It can colonize the vagina specially, in women that have taken antibiotics due to high resistance rate and inhibition of other pathogens. The major virulence factors of *Klebsiella pneumoniae* is the thick capsule (its' antiphagocytic), and fimbriae (pili) (Podschun *et al*., 2001). This thick capsule is composed of complex acidic polysaccharides and form more than 70 serotypes (Greenwood *et al*., 2002).

The capsular material forms thick bundles of fibrillose structures covering the bacterial surface in massive layer (Amako *et al*., 1988)

1.2.4.1.5. *Listeria monocytogenes*

L. monocytogenes is gram positive rod-shaped bacterium, it is the agent of listeriosis, a serious infection that is caused by eating food contaminated with bacteria, the disease affect primarily pregnant women, new born infants and adults with weakened immune systems (Todar, 2008).

L. monocytogenes can also be isolated from vaginal secretion, specially in women with decrease number of

Lactobacilli since the later have anti-listeria activity and can protect against it especially the H_2O_2-producing one (Hawes *et al*., 1996).

Listeriosis is an uncommon infection, contaminated food is the usual source of infection and transmission appears to be blood-born following gastrointestinal infection (Danielian & Hall, 2005).

1.2.4.1.6. *Neisseria gonorrhoeae*

N. gonorrhoeae is gram negative intracellular, diplococcal organism that is sexually transmitted, this bacterium causes infection in the genital tract that may disseminate to organs. Infection of the genitals can result in a purulent (or pus like) discharge from the genitalia, which may be foul-smelling, inflammation, redness, swelling, dysuria and a burning-sensation during urination.

N. gonorrhoeae can also cause conjunctivitis, pharyngitis, in female with infection of the genitals can result also in pelvic inflammatory disease which if left untreated can result in infertility, pelvic inflammatory disease results in *N. gonorrhoeae* travels into the pelvic peritoneum (via cervix, endometrium and fallobian tubes). Patient should also be tested for other sexual transmitted infection specially Chlamydia infection since co-infection is frequent (Van Duynhoven, 1999).

1.2.4.1.7. *Staphylococcus epidermidis*

S.epiderimidis gram positive, catalase positive, oxidase negative, Coagulase negative. *S.epiderimidis* has a relatively small white colony, this type of staphylococci constitute a component of the normal flora of human. It is opportunistic pathogen that causes infection in debilitated or compromised patients (Kloss & Bannerman, 1994; Brook *et al*., 2004).

1.2.4.1.8. Bacterial Vaginosis

Bacterial vaginosis (BV) is an abnormality of the normal vaginal flora characterized by a reduced number of Lactobacilli, a higher pH and 100 folds increased numbers of potential pathogens including *Gardnerella vaginalis, Bacteroides, E.coli, GBS*, the anaerobes *Peptostreptococcus*, and *Mycoplasma hominis*,therefore,the presence of large number of *Lactobacilli* and low pH are important mechanism to protect against the growth of potential pathogenic organisms (Bennet *et al*., 2007).

Around 50% of women with B.V. are asymptomatic. If symptoms do occur, the most common is a thin, watery, malodorous, non itchy discharge. The criteria used to diagnose bacterial vaginosis: pH>4.5, the presence of thin watery discharge, fishy odour (with 10% KOH), clue cells on saline wet mount and/or gram stain (Goswami & Thomtou, 2006).

Spiegel *et al*.,(1983), developed a method which was first created to diagnose gram-stained vaginal smear. By this method, the bacteria were grouped into morphotypes, *Lactobacillus* morphotypes were called elongated bacteria and *Gardnerella* morphotypes were called short bacteria. Generally, no method for diagnosis of B.V. can at present be regarded as the best (Forsum *et al*., 2005 b).

In clinical practice, bacterial vaginosis, is diagnosed using the Amsel criteria (Amsel *et al*., 1983):-

1. Thin, white, yellow, homogeneous discharge.
2. Clue cells on microscopy.
3. pH of the vaginal fluid >4.5.
4. Release of fishy odour on adding 10% KOH solution.

At least three of the four criteria should be present for comfirmed diagnosis (National guidline for the management of bacterial vaginosis (2002). An alternative is to use a gram stained vaginal smear with the Hay/Ison criteria (Ison and Hay,2002) or the Nugent criteria (Nugent *et al*., 1991).

Untreated bacterial vaginosis may cause serious complication such as increase susceptibility to sexually transmitted infection including HIV, in addition to increase risk of (PID) pelvic inflammatory disease (Bailey *et al.*, 2004), it can be treated with metronidazole or clindamycine either orally or vaginally, however, there is usually a high rate of recurrence (Bradshow *et al*., 2006).

1.2.4.1.8.1 *Gardnerella vaginalis*

G.vaginalis is a facultatively anaerobic, non spore forming, non encapsulated, non motile, pleomorphic, gram variable rod. Growth is best at 35 ºC and is enhanced by carbon dioxide. It is indole, nitrate and urease negative, rare anaerobic strains exist, pili have been recognized on it's surface (Boustouller *et al*., 1987). It is oxidase negative, catalase negative, thin gram-variable rod shape.

The detection or quantitation of *G.vaginalis* in vaginal fluid can not be used as a diagnostic test for BV, but, increased prevelance and concentration of *G.vaginalis* in patients with this syndrome suggests that *G.vaginalis* plays a role in BV even through it is not the sole etiologic agent.

1.2.4.1.8.2. *Mycoplasma hominis*

M.hominis has been associated with BV in pregnant and non pregnant women (Martius *et al*., 1988). The persistence of *M.hominis* after therapy for BV was associated with the persistence of *Bacteroides* species (Koutsky *et al*., 1983). *M.hominis* was associated with an abnormal gas-liquid chromatograph and the presence of diamines wether or not *G.vaginalis* was also present.

1.2.4.1.8.3. *Mobiluncus* species

Mobiluncus spp. are the most recently recognized member of the vaginosis associated flora. *Mobiluncus* spp. are curved, gram-variable, motile organisms, they are slowly growing, fastidious organisms requiring an enriched medium for growth, they are indole, Catalase, oxidase and H_2S negative (Holst *et al*., 1990). They are generally stain gram variable or gram positive.

The prevelance of *Mobiluncus* spp. in the vagina has been determined in numerous studies and has been reviewed recently these species are also associated with BV (Holst, 1990).

1.2.4.1.9. *Chlamydia trachomatis*

Chlamydia trachomatis (an obligate intracellular bacteria) is considered to be the most commonly isolated sexually-transmitted organism. The patients infected with *Chlamydia trachomatis* are often a symptomatic but, they may be present with mucopurulent vaginal discharge or cervicitis (Bulletin, 1994).

C. trachomatis is naturally found living only inside human cells, Chlamydia can be transmitted during vaginal, anal or oral sex and can be passed from an infected mother to her baby during vaginal child's birth (Gerbase *et al.*,1998) Between half and three quarters of all women who have Chlamydia infection have no symptoms and do not know that they are infected.If untreated Chlamydia infection can cause serious reproductive and other health problems with both short term and long term consequences.

Approximately half of asymptomatic cases, will develop inflammatory disease.Symptoms that occur include unusual vaginal bleeding or discharge, pain in the abdomen, painful sexual intercourse (dyspareunia), fever, painful more frequently than usual urinary urgency (Gerbase *et al.*, 1998).

1.2.4.2. Fungal Infection (Candidiasis)

Candidiasis is a fungal infection (mycosis) of any of the candida species, of which *Candida albicans* is the most common (Walsh & Dixon, 1996). Yeast are commonly present in humans and their growth is normally limited by the human immune system and by other microorganisms such as bacteria occupying the same location (niches) in the human body (Mullery & Goroll, 2006). External use of detergent , douches or internal disturbances (hormonal or physiological) can perturb the normal vaginal flora, consisting of lactic acid bacteria such as Lactobacilli and result in over growth of Candida causing symptoms of infection such as local inflammation symptoms (Mardh *et al*., 2003).

Pregnancy and the use of oral contraceptives have been reported as risk factors (Shiefer, 1997). Diet has been found to affect rate of symptomatic candidiasis in some animal infection models (Yamaguchi *et al*., 2005). Hormonal replacement therapy and infertility treatment may also be predisposing factors (Nwokolo & Boag, 2000).

Infected women with *Candida albicans* may have severe itching, burning, soreness, irritation and a whitish-gray cottage cheese like discharge, often with a curd-like appearance.

1.2.4. 3 Protozoal Infection (*Trichomonas vaginalis*)

Trichomonas vaginalis is anaerobic parasitic flagellated protozoan, it is the causative agent of trichomoniasis, the most common pathogenic protozoan infection of humans in industrialized countries (Soper, 2004).

Trichomoniasis is a sexually transmitted infection which occurs if the normal acidity of the vagina is shifted from healthy semi-acidic pH (3.8-4.2) to much more basic one that is conductive to *T.vaginalis* growth (Sood, 2007). Some of symptoms of *T.vaginalis* include preterm delivery, low birth weight and increased mortality as well as predisposing to HIV infection, AIDs and cervical cancer (Schwebke & Burgess, 2004). *T.vaginalis* has also been reported in the urinary tract, fallobian tubes and pelvis and can cause pneumonia, bronchitis and oral lesions. Other symptoms include inflammation with increasing number of organisms, greenish-yellow frothy vaginal secretions and itching. Condoms are effective in preventing infection. Ten percent of the infected women will be presented with a strawberry cervix or vagina on examination. *T.vaginalis* was traditionally diagnosed via a wet mount. Currently the most common method of diagnosis is via over night culture (Ohlemeyer *et al*., 1998), the presence of trichomoniasis can also be diagnosed by PCR (Schirm *et al*., 2007). Treatment should be prescribed to any sexual partners as well because they may potentially be a symptomatic carriers (Cudmore *et al*., 2004).

1.2.4.4. Viral Infection

Viruses are a common cause of vaginitis one form caused by herpes simplex virus (HSV) which is often just called herpes infection. These infections are also spread by sexual contact. The primary symptoms of herpes vaginitis are pain associated with lesion or sores. These sores are usually visible on the vulva of the vagina but are occasionally inside the vagina and can only be seen during a gynecological exam (Merz, 1993). As with all sexually transmitted disease, women are more susceptible to acquiring genital herpes HSV-2 than men (Johnson, 2006). The risk of transmission from mother to her baby is higher, if the mother becomes infected at a round delivery (30-60%), but the risk falls to 3% if it is a recurrent infection and is less than 1 if there are no visible lesions. Generally the risk of transmission between men and women can be reduced by antiviral treatment (Corey *et al*., 2004).

1.2.5. The Probiotic Concept

Probiotics can be defined as: substances prepared with live microorganisms, which prevent infections through restoration of normal microbial flora when it has been altered or: live microorganisms either supplied exogenously or stimulated endogenously , which confer a health benefit or enhance host resistance against infection (Reid & Bruce ,2001).

The characteristics required for a lactobacillus strain to serve effectively as a probiotic include: human origin, avid and persistent adherence to vaginal epithelial cells, interference with the adhesion of other pathogens and production of H_2O_2 and other molecules capable of inhibiting the growth of pathogens (Cadieux *et al*.,2002).

It is believed that *Lactobacillus* protects the vagina from colonization by pathogens mainly by blocking the attachment of microorganisms to the vaginal epithelium and by excreting substances that inhibit their multiplication. There are three mechanisms by which *Lactobacillus* can prevent uropathogen adherence:1. blockage by exclusion in which Lactobacilli adhering to vaginal cell receptors exclude the attachment of recently arrived pathogens, 2.blockage by competition in which Lactobacilli and pathogens inoculated simultaneously compete for the receptors, 3. blockage by displacement, in which the previously attached pathogens are displaced by recently inoculated Lactobacilli (Andreu *et al*., 1995).

It was demonstrated that not all *Lactobacillus* strains express these properties with the same density (Osset *et al*., 2001).On the contrary,these are substantial differences among species and among strains from a single species, e.g. more than 75% of human vaginal *Lactobacillus* strains adhered poorly to vaginal epithelium, a quarter of the strains demonstrated greater adhesion,and 10% adhered in very large numbers (Sipsas *et al*.,2001)

Lactobacilli can inhibit the multiplication of pathogens by excreting certain substances, mainly H_2O_2, lactic acid and bacteriocin-like substances.

1.2.6. Lactobacilli

Lactobacilli are gram positive bacteria that occur singly in pairs or short chains, *Lactobacillus* species are oxidase negative, catalase negative, coagulase negative, faculatively anaerobes, non spore forming, no motile, can grow selectively on deMan-Rogosa-Sharpe broth or agar (MRS), there are many strain and species and each have an important role in maintaince the normal vaginal ecosystem(Sneath *et al* .,1994)

Lactobacilli are members of normal flora and are generally regarded as safe for use in humans specially in uropgenital tract infections, bacterial vaginosis, and recurrent vaginal candidiasis (Stapleton, 2003). The importance of Lactobacilli related to production of H_2O_2, bacteriocins and lactic acid.

1.2.6.1. Role of Hydrogen Peroxide in Vaginitis

Hydrogen peroxide (H_2O_2) has been considered a key factor in *Lactobacillus* antagonism against pathogens. This compound generates cytotoxic reactive oxygen species, superoxide anions and hydroxyl radicals in the vaginal fluid (Klebanoff *et al*., 1991). So that the presence of H_2O_2-producing Lactobacilli is very beneficial in preventing vaginitis since this compound is toxic to organisms that produce little or no H_2O_2-scavenging enzymes (e.g. catalase) and absence of H_2O_2 producing Lactobacilli will allow growth of catalase negative organisms such as those found among women with bacterial vaginosis (Eschenbach *et al*., 1989).

1.2.6.2. Role of Bacteriocin in Vaginitis

Bacteriocins are secreted oligopeptides, proteins or protein complexes, with antimicrobial activity against strains taxonomically related to the producer organisms (Jack *et al*., 1995).

Bacteriocins produced by lactic acid bacteria (Meyer & Nes, 1997), and especially Lactobacilli (Klaenhammer, 1993), may have a role not only in protecting vaginal environment, but also in gastrointestinal environment.

High level of bacteriocins-like compound was synthesized by some species of Lactobacilli with a bacteriocidal mode of action against opportunistic pathogens, *G.vaginalis, Candida albicans*.

In addition, H_2O_2 depenent activity alone was not sufficient to inhibit pathogens .So, simultaneous action of H_2O_2 and bacteriocin was highly effective against them. Thus, *lactobacillus* administered in the form of vaginal ovules, could be an attractive alternative approach for the prevention and treatment of vaginitis (Hawes *et al*., 1996).

1.2.6.3. Lactic Acid Production

Lactic acid bacteria are group of gram positive bacteria, non respiring, non spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates (Axelsson, 1998). Some of the family are homofermentive, that is,they only produce lactic acid, while others are hetrofermentive and produce lactic acid plus other volatile compounds and small amount of alcohol.

Despite their complexity, the whole basis of lactic acid fermentation centers on the ability of bacteria to produce acid, which then inhibit the growth of other non-desirable organisms. All lactic acid producers are microaerophillic, that is, they require small amounts of oxygen to function.

Homofermenters convert sugar primarily to lactic acid while hetrofermenters produce about 50% lactic acid plus 25% acetic acid and ethyl alcohol and 25% carbon dioxide. The hetrofermentative Lactobacilli produce mannitol and some species also produce dextran (Dirar, 1993).

1.2.7. Hormonal Factors That Affect Isolation of Lactobacilli

Hormones especially oestrogen have a role in isolation of Lactobacilli where oestrogen lower the vaginal pH and an acidic pH increase redox potential making the environment less favorable for anaerobic organisms. In addition, the increase in oestrogen level stimulate Lactobacilli adherence to epithelial cells and alter the vaginal cell charge (Nikolaitchouk, 2009). So in postmenapausal women with vaginitis and were treated with oestrogen, there was a decrease in pathogenic bacteria and increase in Lactobacilli species (Hyman *et al*.2005).

Cauci *et al*., (2002) found that women on hormonal replacement therapy showed a level of lactobacilli identical to that found in fertile women, so that, the positive influence of hormonal replacement therapy(HRT) on Lactobacilli is a known phenomenon.

Mardh *et al*., (1991) found that high level of oestrogen increases the glycogen concentration in vaginal epithelial cells, then the glycogen metabolism provides the glucose which constitute the main nutritional factor for Lactobacilli which convert it to lactic acid lowering the vaginal pH.

Other studies found that the availability of glucose that derived from glycogen metabolism potentially could also promote the growth of other microorganisms .

Donder *et al*., (2000) showed that post menopausal status involves major vaginal changes that are not confined to the glycogen content of epithelial cells, which can be modulated by hormonal replacement therapy. However, Millier *et al.,* (2000) found that these changes could be regarded as normal physiological condition rather than abnormal.

1.2.8. Treatment of Vagnitis

Vaginal infections are often a mixture of various etiological agents, which present challenging cases for treatment. So that the cause of infection determines the appropriate treatment. It may include oral or topical antibiotics and/or antifungal creams, antibacterial creams. A cream containing cortisome may also be used to relieve some of the irritation. If allergic reaction is involved an antihistamine may also be prescribed. For women who have irritation and inflammation caused by low level of estrogen (post menopausal), a topical estrogen cream may be prescribed (Pirotta, 2004).

Generally, empirical antimicrobial therapy must be comprehensive and should cover all likely pathogens. In clinical setting, the use of antibiotic combination is usually recommended for treatment of serious gram negative bacillary infection, this approach ensures coverage of broad range of organisms and polymicrobial infections, prevents emergence of bacterial subpopulations that may be resistant to one of antibiotic compound and provides additive or synergistic effects. Antibiotic monotherapy is recommended also (Angotti *et al*., 2007).

The most common drugs that have been used to treat vaginitis include cefotaxime, cephalexine,ciprofloxacine, erythromycin, azithromycin, clindamycin in addition to metranidazole and oestrogen to treat atrophic vaginitis (Anderson, 2004).

Regarding antifungal drugs, imidazole derivatives which have a fungicidal effect are effective in treatment by altering the permeability of the fungal cell membrane (Sobel *et al*., 2003).

Herbal suppositories for the treatment of bacterial vaginitis may include herbs such as Echinacea, althea and others which have antimicrobial activity and can kill off the overgrowth of bacteria, however, oral *Lactobacillus* can be supplemented in all conditions to normalize the vaginal flora (Zeger & Holt, 2003).

After treatment, the vaginal flora is often disturbed. The cause is reduced density of live flora (physiological barrier against pathogens) and the reduction of vaginal epithelium (physical barrier). As a result, post anti-infective treatment also requests the strengthening of the natural vaginal flora, which is done with local administration of *Lactobacillus* and potentially low dose of hormones (e.g. estriol) to increase the proliferation of epithelial cells (Ozkinay, 2005).

1.2.9. Prevention of Vaginitis

Women should prevent some habits like wearing hot, moist clothes that enhance growth of pathogens. Also they should avoid douches, sprays and other irritating compound that kill benificial bacteria or cause irritation to epithelial cells.

Practising safe sex to get rid of sexually transmitted infection. Treatment of both partners is very important since women are more liable than men to get sexually transmitted disease (Mashburn , 2000).

2.1 Patients :-

One hundred and ten (110) swabs were taken from women suffering from vaginitis, who were admitted to Babylon hospital for maternal and pediatrics, in addition to swabs taken from private clinics during the period from November 2008 to April 2009 in Babylon province . One hundred swabs were taken from married women, of these 100 swabs,20 swabs were from pregnant and 80 swabs were from non pregnant women. In addition, some of married women were with intrauterine device(70) while, women without intrauterine device constituted (30). Also, ten swabs from unmarried women.

2.2.Materials:-

2.2.1. Laboratory Instruments

No	Instruments	Company
1.	Autoclave	STERMITE, Japan
2.	Sensitive Electronic Balance	A and D, Japan
3.	Incubator	Memmert , Germany
4.	Distillator	GFL– Germany
5.	Oven	Memmert, Germany
6.	Gas pack Jar	Oxford, U.K.
7.	Cork borer	Memmert, Germany
8.	Centrifuge	Hermle, Japan
9.	Refrigerator	Concord, Italy
10.	Milipore filter.	Satorius membrane W .Germany
11.	Light microscope	Olympus, Japan
12.	Micropipette	Oxford, U.S.A.
13.	PH. meter	Hoeleze and Cheluis, KG, Germany
14.	Water Bath	Memmert ,Germany

2.2.2 Chemical and Biological Material

Name of material	Company/Countary
A. chemical materials	
K_2HPO_4, NaCl, $MgSO_4$, HCl, $CaCl_2$, KOH	Merk –Switzerland.
α-Naphtholamine,Tetramethyl-P-paraphylenediamine-dihydrochloride, methyl red, Amyl alcohol, Peptone .	B.D.H–England.
H_2O_2, glucose, 99% ethanol, TMB, CO_2 generating Kits	Fluka chemika Switzerland Oxford U.K
B- culture media	
Blood agar base, MacConkey agar, Muller Hinton agar, peptone water medium, nutrient agar media, Nutrient broth, Simmon citrate, Kligler-iron agar, MRS broth, MRS agar	Hi medium
C- stains	
Gram Stain Set	Crescent – Saudi

2.3 Methods

2.3.1 Collection of Specimens :-

The proper specimens collected for bacteriological analysis are described below. Those specimens were collected in a proper way to avoid any possible contamination. The samples were generally collected from women with vaginitis.

The swabs were inserted into the posterior fornix, upper part of the vagina and rotated there before withdrawing them.

A vaginal speculum was also used to provide a clear sight of the cervix and the swab was rubbed in and around the introitus of the cervix and withdrawn without contamination of the vaginal wall .

Swabs for culture were placed in tubes containing normal saline to maintain the swabs moist until being taken to the laboratory. Each specimen, except specimen for isolation of Lactobacilli (which was inoculated on selective media using deMan Rogosa sharpe (MRS) broth and agar and was incubated anaerobically using gas pack jar);was immediately inoculated on blood agar plates, nutrients agar, chocolate agar and MacConkey's, plates. All plates were incubated aerobically at 37 ºC for 24 – 48 hrs.

2.3.2 Preparation of the Reagents and Solutions:

2.3.2.1 Oxidase Reagent:

This reagent was prepared by dissolving 1gm (tetramethyl-paraphenylen–diamine-dihydrochloride) in 100 ml of D.W. and immediately was used for identification of bacteria (Baron *et al*., 1994).

2.3.2.2 Catalase Reagent :-

This reagent was prepared in (3%) solution using H_2O_2 diluted by D.W. and stored in a dark container and used for identification of the bacteria (Baron *et al*., 1994).

2.3.2.3 Phenol Red Reagent

It was prepared by dissolving 0.1 gm from phenol red dye in 300 ml of ethyl alcohol (95%) then the volume was completed to 500 ml by D.W. It was used to detect the acidity of the media (Baron *et al*.,1994).

2.3.2.4 Methyl Red Reagent

Methyl Red Reagent was prepared by dissolving 0.1 gm. of methyl red in 300 ml of 95% ethanol and then the volume was completed to 500 ml by D.W. (MacFaddin, 2000). It was used to detect complete glucose hydrolysis.

2.3.2.5 Vogus – Proskauer Reagent :-

It is composed of two solutions as below :-

a- α-Naphthol reagent was dissolved in 100 ml of 99% ethanol.

b- Potassium Hydroxide (KOH) solution:-

40 gm of KOH was dissolved in 100 ml of D.W, and it was used to detect the partial glucose hydrolysis (Collee *et al.*, 1996).

2.3.2.6 Kovac's Reagent :-

It was prepared by dissolving 5gm. of P-dimethylamine benzyladehyde in 75 of amyl–alcohol and was added to 35 ml of concentrate HCl acid. It was used to detect the indole production (Baron *et al.* , 1994).

2.3.2.7 McFarland Tube Standard (0.5) :-

A barium sulfate turbidity standard solution equivalent to a 0.5 McFarland standard was prepared as described by NCCLS (2003 a), as follows:-

A O.5– ml aliquot of 0.048 M $BaCl_2$ (1.175 % W/v $BaCl_2$. $2H_2O$) was assed to 99.5 ml of 0.18 H_2SO_4 (1% u/u) with constant stirring to maintain suspension.

Correct density of the turbidity standard was verified by using reading the absorbance at 625 nm. The absorbance should be 0.08 to 0.10 for the Mcfarland standard.

Barium sulfate suspension was distributed in 4 ml aliquots into screw–cap tubes, which were tightly sealed and stored in the dark at room temperature.

Barium sulfate turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and was inspected for uniformly turbid appearance.

Barium sulfate standard should be replayed or their densities verified monthly.

2-3-2-8.TrisBufferSolution:

This buffer was prepared by dissolving 12gm from the tris base (NH_2C (CH_3OH) $)_3$ TAAV company (Mwt.121.4) in small amount of D.W. and completed to 1 Litter. The PH was adjusted to 7 by adding HCl (0.1 N). This solution was used as a good buffer to maintain PH of the media for isolation of *Lactobacilli* (Mark *et al*., 2004).

2.3.3 Preparation of Culture Media: -

The general culture media described below were prepared according to the methods mentioned for each media and used in appropriate experiments .

2.3.3.1 Blood Agar Medium:

Blood agar medium were prepared according to MacFaddin, (2000) by dissolving 40 gm blood agar base in 1000 ml D.W. and autoclaved at 121 °C for 15 min, then cooled to 50 °C and 5% of fresh human blood was added. This medium was used to cultivate bacterial isolation and to determine their ability to lyse blood .

2.3.3.2 Chocolate Agar Medium :

Chocolate agar medium has been prepared by dissolving 40 gm. of blood agar base in 1000 ml D.W. and sterilized by autoclaving. Then 8% of human blood was added to the medium after cooling to 80 °C .

This medium was especially used for isolation and cultivation of bacteria that need 5-10 % CO_2 tension (Baron *et al* , 1994) .

2.3.3.3 MacConkey Agar Medium :

MacConkey agar medium has been prepared according to the method recommended by the manufacturing company and it was used for the primary isolation of most G-ve bacteria and differentiation of lactose fermentative from the non lactose fermentative (Collee *et al*. , 1996).

2. 3.3.4 Nutrient Agar Medium :

Nutrient agar medium has been prepared according to the manufacturing company.

It has been used for general experiment isolate culture, cultivation and activation of bacterial isolates when it is necessary (MacFaddin , 2000).

2.3.3.5 Mannitol Salt Agar Medium :

This media has been used as a selective media for isolation of *staphylococci* and differentiation of *S.aureus* (MacFaddin, 2000).

2.3.3.6 Eosine Methlene Blue (EMB) Medium :

Lactose fermenting colonies are either dark or possessed dark centers with transparent colorless peripheries, while organisms that do not ferment lactose remain uncolored (Murray *et al* ., 2003).

2.3.3.7 Muller- Hinton Agar:

Muller–Hinton agar has been prepared according to the method recommended by (Vall *et al*., 1999) and it has been used in antimicrobial susceptibility testing.

2.3.3.8 Kligler Iron Agar :

Kligler Iron agar has been used for determining glucose and lactose fermentation and possible hydrogen sulfide (H_2S) production as a first step in the identification of gram negative bacilli (MacFaddin, 2000).

2.3.3.9 Pepten Water Media

Pepten water media was prepared and used to detect the ability of bacteria to produce indole according to the method described by (Baron *et al*., 1994).

2.3.3.10 MR –VP Medium

MR–VP medium has been prepared and used to detect the partial and complete hydrolysis of glucose according to (MacFaddin,2000).

2.3.3.11 Simmon's Citrate Medium :

Simmon's citrate Medium has been used for determining the ability of bacteria to utilize citrate as the sole carbon source (MacFaddin, 2000).

2.3.3.12 De Man Rogosa Sharp Agar Medium:

This media has been used as a selective media for the isolation of Lactobacilli as it provide optimal conditions for their growth (De Man *et al*., 1980).

2.3.4 Laboratory Diagnosis:

According to the diagnosis procedures recommended by (MacFaddin, 2000; Collee *et al*., 1996; Baron *et al*., 1994) the isolation and identification of gram positive and gram negative bacteria in women with vaginitis were performed as follows:-

2.3.4.1. Microscopical Examination and Colonial Morphology:

A single colony was taken from each primary positive culture and it's identification depended on the morphology properties (colony size, shape, color and nature of pigments, translucency, edge, and elevation and texture). Then colonies were investigated by Gram stain to observe a specific shape, type of reaction, aggregation and specific intracellular compounds. After staining the bacteria by Gram stain, specific biochemical test were done to reach the final identification.

2.3.4.2. Physiological and Biochemical Tests

2.3.4.2.1. oxidase Test :

A filter paper circle was placed into a sterile plastic disposable petri dish and moistured with several drops of the freshly prepared oxidase reagent, then a small portion of the colony to be tested was removed and rubbed on the filter paper, changing the color to blue or purple within 10 seconds indicated for a positive result (Baron *et al.*,1994) .

2.3.4.2.2. Catalase Test: -

By streaking the nutrient agar medium with selected bacterial colonies and incubated at 37 C^o for 24 hrs. then transfer the growth by the steak and put it on the surface of a clean slide and add a drop of (3% H_2O_2); Positive results when the gas bubbles appear (Baron *et al*.,1994).

2.3.4.2.3. Coagulase Test

This enzyme was tested by two methods:-

A- Slide test for bound coagulase (clumping factor):

A drop of human plasma was placed on a clean, dry glass slide, a drop of D.W. was placed next to the drop of plasma as a control. By a sterile loop an amount of the isolated colony was emulsified with each drop. when clumping the plasma, bacteria was observed and a smooth homogenous in the control, the result was recorded positively (Baron *et al*.,1994).

B – Tube test for free coagulase**:**

Half ml of human plasma was placed in a glass tube and a visible portion of growth from isolated colonies was emulsified in the plasma by rubbing the material on the slide of the tube while holding the tube at an angle, then the suspension was incubated for 1-4 hours at 37 °C; the presence of clot that cannot be re-suspended by gentile shaking was recorded as a positive results; the organism that fails to colt the plasma within 24 hrs is considered as a coagulase negative (Baron *et al*.,1994).

2.3.4.2.4. Citrate Utilization Test:-

The surface of simmone's citrate slant medium was inoculated with colony of the tested bacteria and incubated at 37°C for 1-3 days. conversion of the indicators color from green to blue indicates that the organism was able to utilize citrate as a sole carbon source (Cruikshank *et al* ., 1975) .

2.3.4.2.5. Kliger's Iron Agar for H_2S Production :-

Only the colonies growing on MacConkey agar touched by a straight wire and inoculated on the media by stabbing the butt of the tube and streaking the slant. Fermentation was detected by a change in the indicator phenol red to yellow. The pH changes in the butt and the slant of medium were recorded after 18-48 hrs of incubation of gas formation which is usually visualized as bubbles in the medium, caused by the gas formed in the agar. Organisms can produce H_2S from black precipitate in the butt (Baron *et al*.,1994).

2.3.4.2.6. Indol Test :-

Tubes containing a peptone water medium were inoculated with the colony of the tested bacteria and incubated in 37°C for 28 hrs, then several drops of Kovac's reagent were added to the broth median, after shaking the appearance of red ring on the surface was regarded as a positive result (Cruikshankn *et al*., 1975).

2.3.4.2.7. Methyl Red Test :-

The tubes of the (MR-VP broth) were inoculated with the selected bacterial colonies and were incubated at 37° C for 24 hrs. Then (5 drops) of methyl red reagent were added to it. The appearance and observation of red color means a positive result and a complete analysis of glucose.(MacFaddin ,2000).

2.3.4.2.8. Vogus Proskauer Test :-

The tubes of (MR-VP broth) were seeded with specific bacterial culture and were incubated at 37 °C for 48 hrs, then the result was read by adding (0.6 ml of α-naphthol reagent) and (0.2 ml of 40 % KOH solution); appearance of red color after 15 min. means positive result due to partial analysis of glucose, which produce acetone or Acetyl Methyl–Carbinol (MacFaddin ,2000) .

2.3.4.2.9. Motility Test :-

This test was done by inoculating the tube that contained semisolid media with tested bacteria by stabbing method and was incubated at 37°C for 24-48 hrs. The disseminating of growth out the stability line was an indication for positive result (MacFaddin, 2000).

2.3.4.2.10. Mannitol Salt Agar Test :

The differentiation between *staphylococcus aureus* and other *staphylococci* e.g. *staphylococcus epidermidis* was done by sub-culturing selected colonies on mannitol salt agar for 24 hrs. at 37 ^{o}C colonies surrounded by a yellow halo indicating mannitol fermentation and isolates colony, is *S.aureus* (MacFaddin ,2000).

2.3.5.Detection of Defense Factors of Lactobacilli :-

2.3.5.1. Hydrogen Peroxide(H_2O_2) Production Test :

According to the qualitative method of (Eschenbach *et al*.,1989), Lactobacilli were streaked onto a 20 ml MRS agar plate containing 5 mg of 3,3$^{/}$; 5,5$^{/}$-tetrmethyl-benzidine (TMB) and (0.20) mg of horse radish peroxidase. Peroxidase generates O_2 from any H_2O_2 produced by the Lactobacilli and the TMB stains the colonies blue in the presence of O_2 after 48 hrs of anaerobic incubation, colonies that produce H_2O_2 on MRS agar thus appear dark blue while non-producer are colorless. The media were used within 48 hrs after preparation.

2.3.5.2. Bacteriocin Production Test :-

The method that used for bacteriocin production test by Lactobacilli was the cup assay method (Al-Qassab and Al-Khafaji, 1992)

- Lactobacilli isolates were grown in brain heart infusion broth + 5% glycerol then incubated anaerobically for 24-48 hrs.
- The growing bacterial isolates were heavily streaked on brain heart infusion agar 5% glycerol, plates then incubated anaerobically for 24-48 hrs.
- Agar discs were cut from the cultured agar layer using sterile 5 mm. cork borer.
- A volume of (0.1) ml of each clinical indicator isolates (we used here *S.aureus and E.coli*) grown for few hrs. in a shaking water both at 37 ºC to obtain (10^6-10^7 cell/ml) in each of nutrient broth was spreaded on nutrient agar plates.
- Agar discs were transferred carefully to the agar surface seeded with indicator isolates (*S.aureus, E.coli*) and incubated over night at 37° C.
- Sensitively patterns were recorded ,presence of inhibition zone around the agar disc indicate a positive result.

2.3.5.3. lactic Acid Production Test :

Tris-HCl (0.1 m) was used as a buffer pH 7 because we depended on pH measurement of the buffer before incubation and after 24-48 hrs. of anaerobic incubation and as follows:

- pH of the buffer was measured and it was 7.
- A loopfull of *Lactobacilli* isolates were inoculated and incubated for 24-48 hrs anaerobically.

- After 24 hrs, pH also was measured and a decrease in pH reading was attributed to production of lactic acid.
- After 48 hrs., the pH. Was also measured and finding of increase in pH. was attributed to entrance in another metabolic pathways. (Boskey *et al* .,2001).

2.3.5.4. Haemolysin Production:

Haemolysin production has been carried out by inoculating a blood agar medium with bacterial isolates at 37 ºC for 24–48 hrs. Appearance of clear zone around the colonies refers to complete haemolysis (β–haemolysis), greenish zone around the colonies refers to partial haemolysis (α-heamolysis), while, no change refers to non haemolysis (γ-haemolysis) (Doboy, *et al*., 1980).

2.3.5.5. Coagulate factor test :

As describied in 2.3.4.2.3

2.3.6. Effect of Antibiotics on Lactobacilli.

It was performed according to the comit'e Francais seL' Antibiogramme (1998), method in which fifty (50) micro liters of the pellet of an over night culture was diluted in MRS broth to about 10^7 CFU/ ml.

Muller-Hinton agar plates containing 5% sheep blood were flooded with this suspension in order to give confluent colonies and air dried for 15 min, and the discs impregnated with antibiotics were positioned on the plates. After 36 hrs. of anaerobic incubation at 37 ºC, the diameters of the bacteria–free zones were measured.

2.3.7. Antimicrobial Sensitivity Test:

It was performed according to Bauer – Kirby *et al*., (1966), method by using a pure culture of previously identified bacterial organism. The inoculum to be used in this test was prepared by adding growth from (5) isolated colonies grown on blood agar plate to 5 ml nutrient broth. This culture was then incubated at 37 °C for 18 hrs. to produce standardized bacterial suspension of moderate turbidity:

- A sterile swab was used to obtain an inoculum from this culture, that was streaked on a Muller – Hinton plate .
- The antibiotic discs were placed on the surface of the medium at evenly spaced intervals with flamed forceps or a disc applicator.
- Incubation was usually over night with an optimal time of 14 hrs. at 37 °C. Antibiotic inhibition zone were measured in(mm) using a caliper.

Inhibition zone diameter was interpreted according to that recommended by clinical laboratory standards institute documentations (CLSI, 2007). Antibiotic disc is prepared by oxide company with the following disc potency.

No	Type	Derivations	Disc Potency µg/ml
1.	Amoxicillin	Amx	10
2.	Amoxicillin +clavulanic acid (Amoxiclav)	Amc	20/10
3.	Cefotaxime	CTX	30
4.	Cephtazidime	CAZ	30
5.	Cefalexin	CL	30
6.	Amikacin	AK	30
7.	Gentamycin	GN	10
8.	Erythromycin	ER	15
9.	Azithromycin	AZM	15
10.	Ciprofloxacin	CIP	5

3.1: Role of Intrauterine Device as Predisposing Factor in Vaginitis

As shown in table (3-1),some women included in this study were with intrauterine device , it was found that the incidence of vaginitis was higher among married women (92.3%) than unmarried women (7.7%) and also it was showed that the incidence of vaginitis among married women that used intra uterine device (IUD)(71%) was higher than married women without IUD (29%), these findings were in agreement with that of Samar (2005), who found that about (87%) of women reported vaginitis or reproductive tract infection (RTI) due to IUD., Samar (2005) also found that there were significant differences in alteration of vaginal discharge and presence of RTI-related symptoms among women used IUD in comparison to women not used IUD.

A homogenous, malodorous thin, grey discharge, which resembled non specific vaginitis occurred in high percentages of women with intrauterine contraceptive device about four times more common than those women without it. Also the normal lactobacilli dominated microbial vaginal flora was replaced by many other opportunistic pathogens and certain anaerobic species among women used IUD with discharge (Kivigarvi *et al*., 2005).

Table (3-1): Distribution of women with vaginitis according to the use of intrauterine device (IUD).

<table>
<tr><th colspan="4">Women</th><th>No.of isolates</th><th>Percentage %</th></tr>
<tr><td rowspan="3">Married</td><td>Type</td><td>No. of isolates</td><td>%</td><td rowspan="3">120</td><td rowspan="3">92.3</td></tr>
<tr><td>With IUD</td><td>85</td><td>71</td></tr>
<tr><td>Without IUD</td><td>35</td><td>29</td></tr>
<tr><td colspan="4">Unmarried</td><td>10</td><td>7.7</td></tr>
<tr><td colspan="4">Total</td><td>130</td><td>100</td></tr>
</table>

IUD: Intrauterine device

3.2: Bacteriological Aspect of Vaginitis

3.2.1: Bacterial Isolation

In this study, (110) vaginal swabs were obtained from women suffering from vaginitis, all these swabs were subjected for culturing on different types of culture media and it was found that 105 samples gave positive bacterial culture where as five (5) samples showed no bacterial growth, as shown in table (3-2).

Out of 105 positive cultures, a total of 130 bacterial isolates were obtained,10 isolates from unmarried women and 120 bacterial isolates from married women. Of these 120 bacterial isolates, 15 isolates were of Lactobacilli and 105 isolates were of opportunistic pathogens,as shown in table (3-3).

Table (3-2): Frequency of positive and negative culture in women with vaginitis.

Women	**Positive culture**		**No growth**
	Opportunistic pathogens	**Lactobacilli**	
Pregnant	15	4	1
Non pregnant	65	11	4
Total	80	15	5

Table (3-3): Numbers of bacterial isolates among pregnant and non pregnant women with vaginitis.

Women	**Opportunistic bacterial isolates**	**Lactobacilli isolates**	**Total bacterial isolates**
Pregnant	31	4	35
Non pregnant	74	11	85
Total	105	15	120

The negative bacterial cultures may be attributed to consumption of antibiotics by the patients, or the presence of another type of causative agents that might need a special techniques for their detection such as viruses, Chlamydia and other agents.

From the results, it was shown that gram positive bacteria constituted (55.2%) from the total bacterial isolates compared to gram negative bacteria which constituted (44.8%) as shown in figure (3-1).

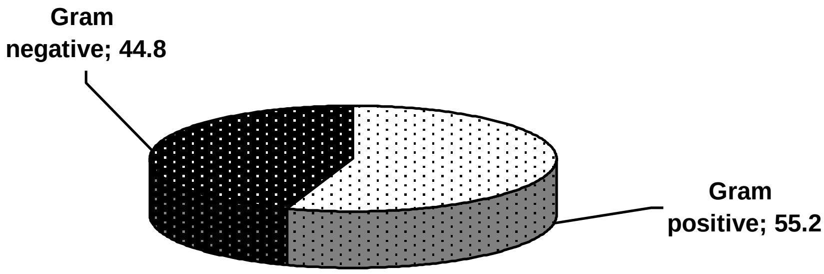

Figure (3-1): Percentage of gram positive and gram negative bacteria among women with vaginitis.

3.2.2: Opportunistic Bacterial Isolates in Women with Vaginitis

Thirty nine cases of vaginitis were caused by single bacterial type in non pregnant and sixteen in pregnant women, as shown in table (3-4), (3-5) respectively where *Staphylococcus aureus* constituted (8/39), *Escherichia coli* (5/39), followed by *Listeria monocytogenes* (7/39), *group B streptococci GBS* (5/39), *Pseudomonas aeruginosa* (4/39), *Klebsiella pneumoniae* (2/39) while other bacterial isolates constituted (5/39).

For pregnant women: there was different percentages of bacterial isolates and as shown in table (3-5).

Table (3-4): Types of opportunistic bacterial isolates from non pregnant women with vaginitis.

Bacteria	**Single isolate**	**Mixed isolates**	**Total isolates**	**Percentage (%)**
S.aureus	8	6	14	18.9
E.coli	5	7	12	16.2
L.monocytogenes	7	2	9	12.1
Group B streptococci GBS	5	3	8	10.8
P. aeruginosa	4	2	6	8.1
S. epidermidis	3	3	6	8.1
K. pneumoniae	2	4	6	8.1
Acinetobacter spp.	3	2	5	6.8
N.gonorrhoeae	1	2	3	4.1
Diphtheroid .	1	2	3	4.1
Corynebacterium spp.	0	2	2	2.7
Total	39	35	74	100

Table (3-5): Types of opportunistic bacterial isolates from pregnant women with vaginitis.

Bacteria	Single isolate	Mixed isolates	Total isolates	Percentage (%)
S.aureus	5	3	8	25.8
E.coli	3	5	8	25.8
Group B streptococci GBS	3	2	5	16.1
K. pneumoniae	2	2	4	12.9
S. epidermidis	2	1	3	9.7
P. aeruginosa	1	2	3	9.7
Total	16	15	31	100

These results were correlated with the results obtained by Donder *et al*., (2002), whereas, the results of bacteria isolated from pregnant women were similar to those obtained by Curzik *et al*.,(2001) and Rodringuez *et al*., (2001). Fourteen isolates (18.9%) of *Staphylococcus aureus* were isolated in this study in non pregnant while in pregnant women, eight (25.8%) isolates were isolated.These findings were in agreement with that of Mumtoz *et al*., (2008) who found that 24% of the patients with aerobic vaginitis were infected with *S.aureus* followed by enteric gram negative bacilli and other gram positive cocci.

Baron *et al*., (1994), showed that the percentage of *staphylococcus* species(*S.aureus*,*S.epidermidis*,*S.saprophyticus*) equal to 62% in vaginitis.

Hillier *et al*., (1993), studied the vaginal flora of some women and categorized them as normal in 50% of cases, intermediate in 27% and abnormal in 23%. They found that the organisms commonly found in women with normal smears were lactobacilli, coagulase negative staphylococci, *S.aureus*, *diphtheroids*, *candida* and *GBS*.

During pregnancy, the vaginal flora remains constant, there is no general increase in isolation of pathogenic bacteria as pregnancy progress, while *E.coli*, *S.aureus*, *Klebsiella* were acquired late in pregnancy (McDonald, 1997).

The results of this study also showed that most *S.aureus* isolates had the ability to produce β-haemolysis due to the presence of clear zone of haemolysis around the bacterial colonies formed on human blood agar, these results agreed with the results mentioned by Dinges *et al*., (2000).

12 isolates (16.2%) of *E.coli* were isolated in non pregnant and 8 isolates (25.8%) were isolated in pregnant women, these results agreed with the results mentioned by Michael, (1991), who isolated *E.coli* at percentage of (17.1%).

Vaginal *E.coli* had also been reported to be sexually transmissible to a male partner (Hebelka *et al*., 1993).

Regarding *group B Streptococci (GBS)* were also isolated (10.8%) in non pregnant and (16.1%) in pregnant women other studies have proved that the rate of isolation of *Streptococcus agalactiae* from vaginal swabs ranged from (5-40%) due to difference in the sample sites and cultural methods employed (Edward & Baker, 2000).

Maniatis *et al*., (1996), also reported that *Streptococcus agalactiae* isolated from cases of vaginitis were (10%) of all collected specimens. This bacteria regarded as species of female urogenital tract (Brook *et al*., 2007).

Zhu *et al*., (1996), reported that the rate of isolation of this bacteria from non pregnant women was (10.86%)

Klebsiella pneumoniae had also been isolated in this study (8.1%) in non pregnant, (12.9%) in pregnant woman. This bacteria is rarely present in healthy vagina, however, this study had confirmed presence of such bacteria in cases of vaginitis which may be attributed to: taking of antibioties by the patient women with vaginitis (especially β-lactam antibiotics) which inhibit the growth of other opportunistic pathogens, while. *Klebsiella* isolates are considered the most common resistant bacteria to most antibioties by producing extended spectrum Beta-lactamase (Bedenic *et al*., 2001; Levermore and Brown,2001). It also can be attributed to absence or decrease number of lactobacilli and their defence factors .

Pseudomonas aeruginosa had also been isolated (8.1%) from non pregnant women and (9.7%) from pregnant women. This bacteria had been isolated especially from women suffering from offensive odor, and from vaginal discharge, besides, the non pregnant women had an intrauterine device (Bonadio *et al.*, 2001; Takeyama *et al.*, 2002).

It is potentially opportunistic microorganism within the vagina, such microorganism may become increasing prevalent upon minor alterations of the vaginal environment.

Metcalf ,(2001) had isolated this bacteria from cases of vaginitis and showed that this bacteria was prevalent among non pregnant women using IUD.

Acinetobacter spp. were also isolated in this study (6.8%) from non pregnant women only.

In a local study in Hilla, Iraq, Al-Shukri (2003), pointed that *Acinetobacter* could be isolated from healthy vagina at a low rate. Other studies isolated this bacteria by a rate (3.1%) (Johnson ,1999).

Acinetobacter is diplococci, catalase positive, oxidase negative, and it recovered from the female genital tract (Geo *et al.*, 2001).

Only 3 isolates of *Neisseria gonorrhoeae* had been isolated in this study and mostly from women with infected husband, it is gram negative, intracellular diplococci organism that is sexually-transmitted, this organism cause infection in the genital tract that can disseminate to other organs (Romero *et al*., 1991).

3.2.3: Isolation of Lactobacilli

Eleven isolates of Lactobacilli had been isolated anaerobically from non pregnant women and four isolates from pregnant women, as shown in table (3-6).

Table (3-6): Distribution of Lactobacilli isolates among pregnant and non pregnant women.

Women	Number of lactobacilli isolates	Percentage (%)
Pregnant	4	26.7
Non pregnant	11	73.3
Total	15	100

Antonio *et al*., (1999) found that *Lactobacillus* colonized 71% of sexually active women

Although it is known that *Lactobacillus* is the predominant vaginal microorganism (10^7-10^8 CFU/ml fluid) in healthy premenapausal women, the composition and dynamics of the diverse Lactobacilli populations have been poorly characterized (Andreu ,2004).

The results correlated with Falagas *et al*., (2007) who reported that most female had only few species because even multiple colonization of lactobacilli species in the vagina but,it may produce some factors against other Lactobacilli to maintain dominance(Pavlov *et al*., 2000).

Andrew *et al*., (2003) found that although the presence of Lactobacilli may be used as a sign of healthy vaginal environment, the possibility that specific strain or combinations of strains of Lactobacilli being harmful should not be ignored, especially due to their ability to produce substance capable of inhibiting other normal vaginal microorganisms.

Other studies concentrated on importance of this bacteria as normal vaginal microflora that had the ability to regulate the growth of other vaginal flora, therefore any disturbances in Lactobacilli were highly correlated with the presence of these opportunistic microorganisms (Gilbert, 2003).

In Addition, results showed that the presence of Lactobacilli together with other opportunistic pathogens may be due to:

- Effects of antibiotics.
- Type of incubation as some Lactobacilli species are unable to produce some defense factors under anaerobic incubation.
- Antagonism among Lactobacilli species to maintain dominance.

3.2.3.1 Cultural Characteristic of Lactobacilli.

Lactobacilli considered as one of lactic acid bacteria which are gram positive, catalase negative, oxidase negative, coagulase negative, fermentive organism that produce lactic acid as a major end product of carbohydrate metabolism (Rouse *et al*., 2007).

Lactobacilli are indole negative, glucose positive, non spore forming, non motile and they are grown on selective media: On MRS agar anaerobically: The colonies were rounded, one mm in diameter, grey to green in color, smooth border, convex, as shown in figure (3-2) .

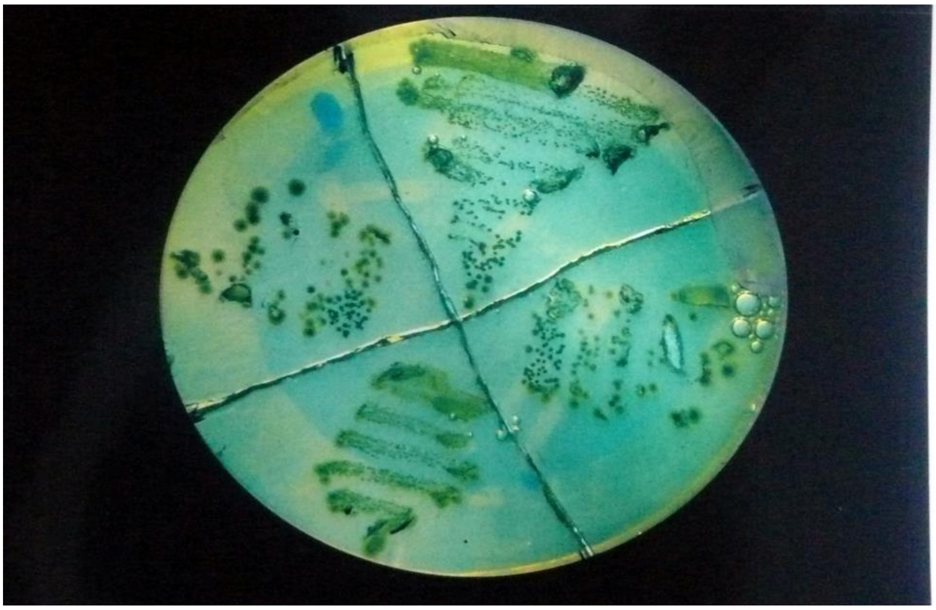

Figure (3-2): Colonies of lactobacilli on MRS (de-Man-Rogosa-Sharpe) agar.

3.2.3.2. Defense Factors of Lactobacilli as Probiotic Bacteria

Lactobacilli plays an important role in protection of the vagina from colonization by pathogens and this could be attributed to two main mechanisms: blocking the attachment of pathogens to the vaginal epithelium and by production of substances that inhibit their multiplication, Andreu, (2004). However, not all *Lactobacillus* strains express these properties with the same intensity, on the contrary, there are substantial differences among species and among strains from a single species.

Burtou *et al*, (2003), found that the instillation of Lactobacilli had the potential to make a significant impact on the health of women and therefore, it is important to understand how the vaginal microbes change and adapt to the presence of the Lactobacilli.

Reid *et al*., (2000) found that the potential importance of Lactobacilli is to protect the vagina from diseases, therefore, it has been used nowdays as probiotic to be determined.

The most important defence factors of Lactobacilli that were investigated in this study:

- Hydrogen peroxide (H_2O_2) production .
- Bacteriocin production .
- Lactic acid production .

3.2.3.2.1: Hydrogen Peroxide Production by Lactobacilli

The ability of Lactobacilli to produce hydrogen peroxide (H_2O_2) was investigated and the result showed that these isolates did not have the ability to produce H_2O_2, these results were in agreement with the results obtained by Dick *et al*., (2000), who found that only one isolate had the ability to generate H_2O_2 among the Lactobacilli isolated. Therefore, the presence of other opportunistic pathogens in the study may correlated with the absence of H_2O_2 production by Lactobacilli. Tomas *et al*., (2003) found that H_2O_2 production was higher under shaken aerobic cultures than anaerobic condition.

The results were in contrast with Donovan (2000) who found that some Lactobacilli strains had the ability to produce H_2O_2 at about 94-95%. Other studies found that although, all Lactobacilli produce lactic acid but, some were able to produce hydrogen peroxide and if it was produced, Lactobacilli will act as endogenous microbiocides in the vagina (Rabė and Hillier, 2003).

The results also disagreed with Shopova, (2003) who found that 93.3% of women colonized by Lactobacilli which were able to produce H_2O_2 and the remaining percentage were not able to produce it and represented as part of normal vaginal microflora.

Hawes *et al*., (1996) found that most isolates of lactobacilli produce some detectable H_2O_2. Other studies found that there was striking differences between the amount of H_2O_2 produced by different lactobacilli species and found that *L.cripatus* and *L.jensenii* did not produce H_2O_2 (Vallor *et al.*, 2001).

It had been demonstrated that although, H_2O_2 producing vaginal Lactobacilli have an inhibitory effect on colonization of the vagina by pathogenic bacteria, however, the extent of the contribution to the inhibition of the vaginal colonization due to H_2O_2 production by comparison with production of bacteriocin, organic acid and competition for adhesion site is not known (Aroutcheva *et al.*, 2001).

The lack of H_2O_2 production by Lactobacilli may be attributed also to the type of incubation and this agreed with Eschenbach *et al.*, (2000), who postulated that anaerobic Lactobacilli did not produce H_2O_2 while facultative one could produce H_2O_2 and may represented a non specific antimicrobial mechanism of the normal vaginal ecosystem. Also, the presence of genital infection lead to decrease the number of Lactobacilli that produce H_2O_2 and increase colonization of the vagina by Lactobacilli that do not produce it.

3.2.3.2.2: Bacteriocin Production by Lactobacilli

Bacteriocin production activity of Lactobacilli was studied and it was found that two strains (13.3%)of lactobacilli inhibited the growth of *E.coli* and other two strains (13.3%) inhibited the growth of *S.aureus*, and it was found that the largest zone of inhibition was (21 mm) on *E.coli* and (12mm) on *S.aureus*.

Table (3-7): Bacteriocin production by lactobacilli isolates.

***Lactobacilli* isolates**	**Zone of Inhibition growth /mm**	
	S.aureus	***E.coli***
1	-	-
2	-	-
3	-	+ (21 mm)*
4	+ (10 mm)*	-
5	-	-
6	+ (12 mm)*	-
7	-	-
8	-	+ (12 mm)*
9	-	-
10	-	-
11	-	-
12	-	-
13	-	-
14	-	-
15	-	-

* Zone of inhibition

The results agreed with the results mentioned by Aroutcheva *et al*., (2000) and Pavlov *et al*., (2000), who found that some vaginal Lactobacilli could inhibit other Lactobacilli by releasing bacteriocin.

Reily *et al*.,(2002) reported that some Lactobacilli exhibited potent antimicrobial activities in form of small, heat-stable, ribosomally-synthesized antimicrobial peptides called bacteriocin, these substances had bacteriocidal or bacteriostatic mode of action, generally, inhibiting microorganism that were closely related to the producing strains.

The result was compatible with the result obtained by Drider *et al*., (2006), who observed that some bacteriocin produced by Lactobacilli had narrow inhibitory spectrum, while Silva ,(2002), found that some Lactobacilli bacteriocin could inhibit *S.aureus*, *L.monocytogenes* by production of antilisterial bacteriocin.

The result also agreed with the result mentioned by Messens, *et al*., (2002) who found that Lactobacilli were able to produce bacteriocin-like inhibitory substances and could reduce the risk of vaginitis. Generally, bacteriocin is antimicrobial protein produced by bacteria that kill or inhibit growth of other bacteria,(Cleveland *et al*., 2001).

Bacteriocin produced by endogenous bacteria may be critical for maintain of normal microflora and host health by preventing invasion by exogenous pathogens, (Brook, 2007).

3.2.3.2.3: Lactic Acid Production

As shown in table (3-8): changes in pH measurement of the media that contain lactobacilli after 24 hr. of incubation were attributed to lactic acid production. The results showed pH of the broth decreased after 24 hr of anaerobic incubation of Lactobacilli isolates and increase or returned to previous value (7) after 48 hrs. of incubation.

The result agreed with the result mentioned by Aroutcheva *et al*., (2001), who found that the pH of the media of lactobacilli was caused by secretion of lactic acid and other organic acids, they also found that about 100% of lactic acid produced by lactobacilli but the percentage differ according to species.

The result also correlated with the result obtained by Simoes *et al*., (2000) who found that after 22 hrs. of incubation of Lactobacilli pH decreased by 2 or 0.8 points.

Boskey *et al*., (1999), demonstrated that the antimicrobial action of lactic acid is largely but not totally assigned to it's ability in the undissociated form to penetrate the cytoplasmic membrane resulting in reduced intracellular pH and disruption of transmembranous protein motive force.

This low pH is inhibitory for many potentially pathogenic organisms that colonized the vagina.

Ogawa, (2001), found that some species of Lactobacilli exert growth inhibitory and bacteriocidal activities on some strains of *E.coli* depend on it's lactic acid production and pH reduction effect.

Eschenbach *et al*., (2000) found that the low vaginal pH is usually accredited to lactic acid producing Lactobacilli, they also found that during times of high oestrogen, large amount of glycogen are deposited in the vaginal epithelium and that the glycogen is anaerobically metabolized to lactic acid, so that the vaginal bacteria mainly lactobacilli were the primary source of lactic acid production.

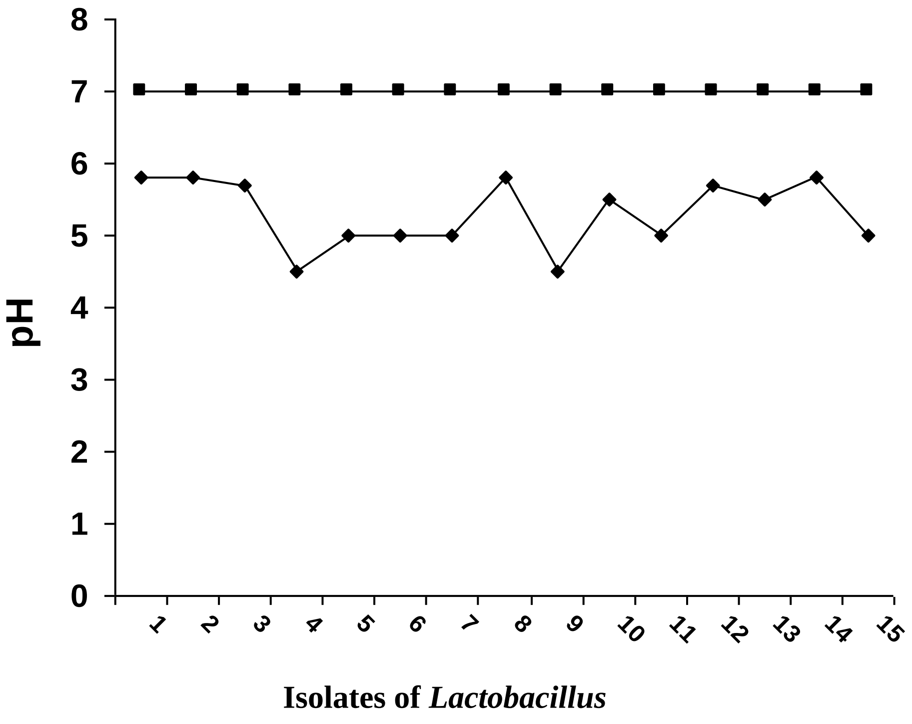

Figure (3-3): pH Changes in broth after anaerobic cultivation with Lactobacilli at 37 °C for 24 hrs.

3.3. Role of Lactobacilli as Probiotic Bacteria in Maintaing the Vaginal Health:

Lactobacilli play an importent role in maintaing the vaginal health by production of their defense factors and some of these defense factors have an inhibitory effect on some opportunistic pathogens (*S.aureus*, *E.coli*), this result agreed with the results mentioned by Ronnquist. *et al.,* (2006) who reported that the normal vagina of reproductive age women is predominately colonized with Lactobacilli which produce hydrogen peroxide, bacteriocins and lactic acid, these substances able to lower the vaginal pH, which create amore hostile environment for bacteria other than Lactobacilli if the number of Lactobacilli falls, the resulting increase in pH favors an overgrowth of anaerobic and facultative bacteria which can develop into vaginitis / vaginosis.

Hoyme and Saling, (2004) reported that the probiotic concept was also important in pregnant women by reducing the incidence of premature rupture of amniotic membranes which cause premature delivery and by the inverse association between Lactobacilli and group B *streptococci* and this very important for pregnant women since transfer of group B streptococci to neonates during delivery increases the risk of meningitis and other complications in newborn.

Anukam *et al*., (2006) mentioned that there was clearly potential for probiotics to help maintain good vaginal health and it was specially intriguing to note that even with the use of oral probiotic products, the vaginal milieu might be beneficially modified presumably, as a result of bacterial translocation from lower portion of the intestine to urogenital areas.

Sethi, *et al*., (2009) reported the role of Lactobacilli in preserving the vaginal health as being the predominant vaginal flora and mentioned that when Lactobacilli decreased due to many causes like douching, sexual practices and use of antibiotics; other pathogenic organisms would dominate. There is therefore, growing interest in the use of Lactobacilli of human origin as probiotics against genital infection (Garg *et al*., 2009).

Various clinical attempts had been carried out to assess the ability of Lactobacilli in prevention as well as treatment of genital infection (Dimitonova *et al*., 2007).

A recent study showed that the use of Lactobacilli containing-vaginal tablets helped in treatment of 61% of women suffering from vaginal infection in comparison to 19% of placebo treated patients (Mastromarino *et al*., 2009).

3.4. Effects of Antibiotics on Lactobacilli

Some antibiotics were used to show their effect on Lactobacilli isolates. It has been found that 93.3% of Lactobacilli isolates were resistant to vancomycin and ciprofloxacin, while the resistance for gentamycin were 53.3%, whereas some isolates were resistant to amoxicillin 40%, on the other hand, most of these isolates were susceptible with higher degree to erythromycin 93.4% (Figure 3-3).

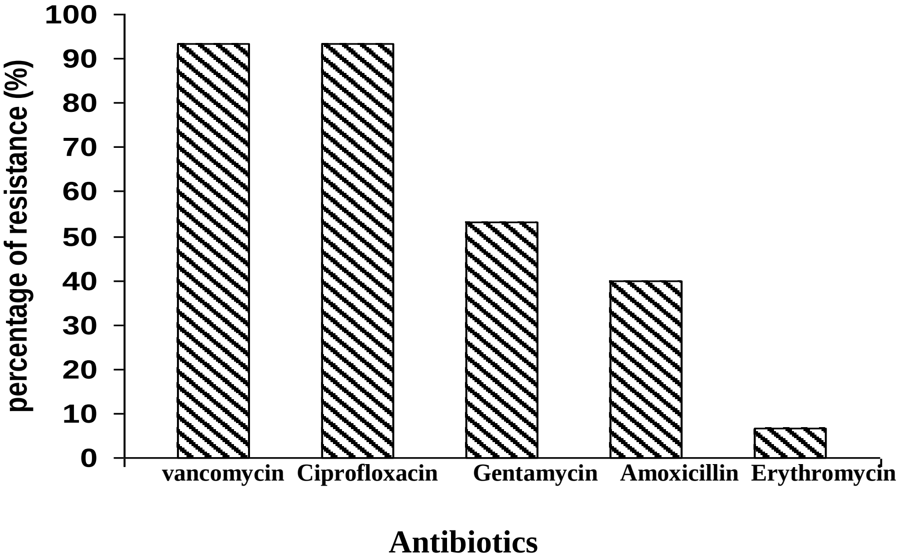

Figure (3-4): Antibiotics resistance of Lactobacilli.

The results showed that 93.3% of Lactobacilli isolates were resistant to vancomycin and ciprofloxacin, this result was in agreement with the result obtained by Coppola *et al*., (2005), who found that 100% of lactobacilli isolate were resistant to both ciprofloxacin and vancomycin and this resistance was important in selection of potentially probiotic Lactobacilli.

Robredo *et al*., (2000), found that several species of Lactobacilli including *L.rhamnosus* and *L.casei* were intrinsically resistant to vancomycin, these species had peptidoglycan precursors terminating with D-lactate instead of the target precursors for vancomycin activity terminating with D-alanine.

The result was in contrast with results of Mandar *et al*., (2001) who had indicated that there was mild to moderate resistance pattern of Lactobacilli against ciprofloxacin and vancomycin.

Alejdra *et al*., (2002), found that Lactobacilli were 50% resistant to vancomycin and most were sensitive to ciprofloxacin and this in contrast with our results. While the result also agreed with Wilks *et al*., (2004) who found that all isolates were ciprofloxacin resistant, while vancomycin resisted correlated with its species.

Regarding, gentamycin, the result showed that about 53.3% of Lactobacilli isolates were confer resistance to this antibiotic. This result was in agreement with Borriello *et al*., (2003) who found that probiotic Lactobacilli were 60% resistant to gentamycin. Other studies had recorded that there was an increase in the pattern of resistance of Lactobacilli species toward different inhibitors mainly protein synthesis (gentamycin, tobramicin) and nucleic acid inhibitors mainly (nalidexic acid and ciprofloxacin)(Mondar *et al*., 2001).

Elkins and Mullis, (2004) found that there was high percentage of Lactobacilli resistance to aminoglycosids (gentamycin, streptomycin, kanamycin).

Elmer, (2001) reported that although aminoglycosides have broad spectrum of activity, however, they are generally inactive against gram positive anaerobes and this is thought to occur because of membrane impermeability due to their characteristic multiple cationic charges, White, (1995) found that aminoglycoside have strong activity against aerobes because these bacteria generally have a high membrane potential which facilitate their entry by anelectrophoretic process.

As shown in figure (3-3), the result showed that about (40%) of isolate were resistant to amoxicillin, the result agreed with the result obtained by Herra *et al*., (1995), who found that Lactobacilli resistance to amoxicillin was at low percentage and the Lactobacilli were mildly resistant to amoxicillin.

Regarding erythromycin, the result showed that the resistance of Lactobacilli was (6.6%) and about (93.4%) were susceptible to erythromycin and this correlated with the result obtained by Wilks *et al*., (2004) who found that all isolates of Lactobacilli were erythromycin sensitive, In addition to Borriello, *et al*., (2003) who found that probiotic Lactobacilli were susceptible to erythromycin at high percentage, the result also agreed with the result obtained by Felten *et al*., (1999), who found that 90% of Lactobacilli were inhibited by erythromycin.

Charteris *et al*., (2001) reported that all Lactobacilli strains were resistant to vancomycin and sensitive to erythromycinn and ampicillin.

Danielson & Wind (2003) found that Lactobacilli resist to high level of antibiotics, these antibiotics have a broad spectrum of activity, however they are active also against gram positive anaerobes.

Mastromarino *et al*., (2002) found that few studies had been done on antimicrobial resistance of Lactobacilli for their non pathogenic nature as anaerobic commensals.

Zarazaga *et al*., (1999), observed that antibiotic resistance among Lactobacilli species and strains could be attributed to many factors including enzymatic inactivation, decrease intracellular drug accumulation or presence of gene that confer antibiotic resistance. Other studies carried out by Faro *et al*., (2001) showed that some Lactobacilli species were resistant to many tested antibiotics.

Charteris *et al*., (1998), found that Lactobacilli display a wide range of antibiotic resistance naturally.Plasmid-linked antibiotic resistance is not very common among Lactobacilli, since the transfer of antibiotic resistant genes may occur between phylogenically distant bacteria (Courvalin, 1994).

Ikaheimo, (1996) studied the effect of antimicrobial therapy on Lactobacilli and found that the antibiotic had an observable effect against the presence of Lactobacilli, these antibiotics can indiscriminately destroy both beneficial and pathogenic bacteria in the body, also the use of antibiotics could create an imbalance of the microflora with very negative effect, they cause the microflora to dislodge from epithelial cells of the vagina, thus allowing the attachment and proliferation of potentially putrefactive pathogenic organisms such as *Staphylococcus aureus, E.coli*, candida. So that the use of antibiotics by the patients to treat vaginitis should be very selective in order not to kill lactobacilli and this agreed with Reid, (2003) who reported that although antimicrobial agents are generally effective at eradicating the infection but, there was a high incidence of recurrence, the patient quality of life is affected and many women become frustrated by cycle of reported antimicrobial agent, whose effectiveness is diminished due to increasing development of microbial resistance, in addition, the use of antimicrobial agent is not only select resistance bacteria but, it can also disturb the balance of the body by killing friendly bacteria leading to urogenital tract infection.

3.5. Relationship Between Lactobacilli and Other Organisms Associated with Vaginitis

There was inverse relationship between Lactobacilli presence and the organism that caused vaginitis and the results showed that and attributed it to the production of defense factors of Lactobacilli, the results agreed with Sethi *et al*., (2009) who found that Lactobacilli constitute an important part of the urogenital tract microbiota and this indigenous bacteria considered as a natural resistant factor against potential pathogenic microorganisms and provide colonization resistant factor against opportunistic pathogens by producing autogenic regulation factors like lactic acid, hydrogen peroxide and bacteriocins.

Reid (2008) found that Lactobacilli produce a variety of substances such as bacteriocins which is toxic to other bacterial species, in addition acidification of the vagina due to lactic acid production is also protective while the production of hydrogen peroxide play the most important role against anaerobes and thus Lactobacilli producing H_2O_2 provide a protective role against vaginitis and acquisition of sexually transmitted infections.

3.6: Effect of Antibiotics on Bacterial Isolates

The effects of different antibiotics on bacterial isolates were investigated. The effect of amoxicillin and amoxiclav on bacterial isolates were studied as shown in figure (3-4), (3-5) respectively and it was shown that there was a relative increase in bacterial resistance (except for *L.monocytogenes*) to β-lactam antibiotic, this result agreed with the result obtained by Forbes, (2007), who had reported that *S.aureus* are the gram positive bacteria that most commonly produce β-lactamase enzymes which then breakdown the β-lactam ring and render it inactive, this is mediated by plasmid or by decreasing membrane permeability toward antimicrobial agents (Barid, 1996). In addition the low affinity of binding of antibiotics to PBP confer resistance to these antibiotics, this result was similar to those asserted by Anq *et al*., (2004), while in gram negative bacteria, resistance can be mediated by decrease uptake through the outer membrane porins.

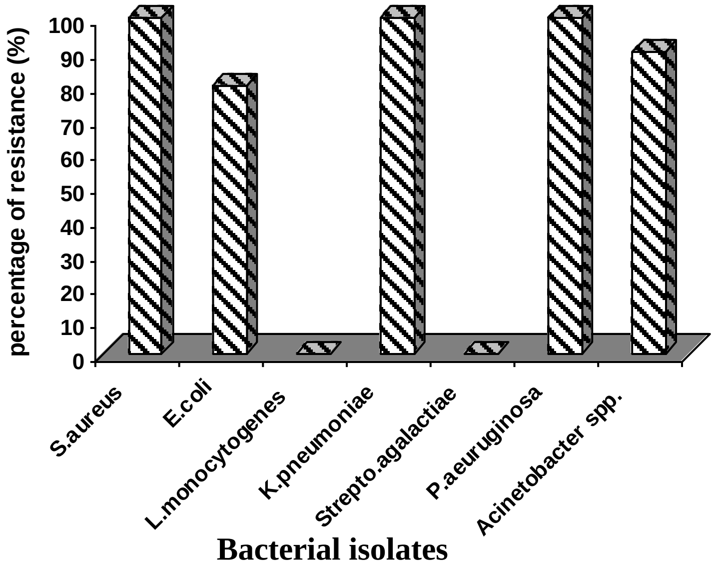

Figure (3-5): Percentage of bacterial resistance to amoxicillin.

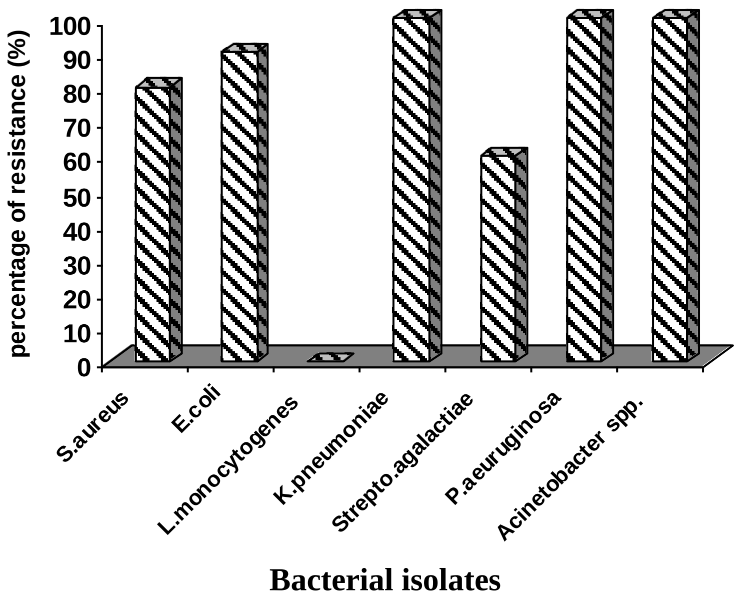

Figure (3-6): Percentage of bacterial resistance to amoxiclav.

The effects of some cephalosporin which include cephtazidime, cefalexine, cefotaxime on bacterial isolates had been also investigated as shown in figure (3-6), (3-7), (3-8), respectively. As shown most bacteria resist the action of ceftazidime and cephalexine (except *L.monocytogenes*).

Regarding cefotaxime: which is a third generation cephalosporin, the results showed that all isolates of *Klebsiella* spp, *S.aureus, P.aeruginosa* were (100%) resistant to cefotaxime while *E.coli* and *Streptococcus agalactiae* were both 50% resistant, and the resistance decreased gradually to (40%) for *Acinetobacter spp*. On the other hand, *L.monocytogenes* were completely (100%) sensitive to cefotaxime.

The resistance of *P.aeruginosa* isolates to cephalosporin may be due to synthesis of β-lactamse as well as loss of PBP by mutation, this bacteria exhibits intrinsic or acquired resistance to many antibiotics, *P.aeruginosa* are highly inherently resistant and this arises from combination of usually resisted outer membrane permeability and chromosomally-encoded β-lactamase, this agreed with the result mentioned by (Hankok & Speert, 2000) and(Bisklis *et al*., 2005).

S.aureus resistance result was agreed with Hanumanthappa *et al*., (2003) who pointed out that (82.4%) of *S.aureus* strains were resistant to cefotaxime.

Regarding *Klebsiella pneumoniae*, the resistance to cefotaxime was probably attributed to the fact that most clinical isolates of *K.pneumoniae* produce different plasmid and/or chromosomal-mediated β-lactamase enzymes, as plasmid mediated enzyme production is much more easily transferred to cells. There had been increased in β-lactamase resistance to third generation cephalosporin. These enzymes are called extended spectrum β-lactamase(Bedenic *et al*., 2001).

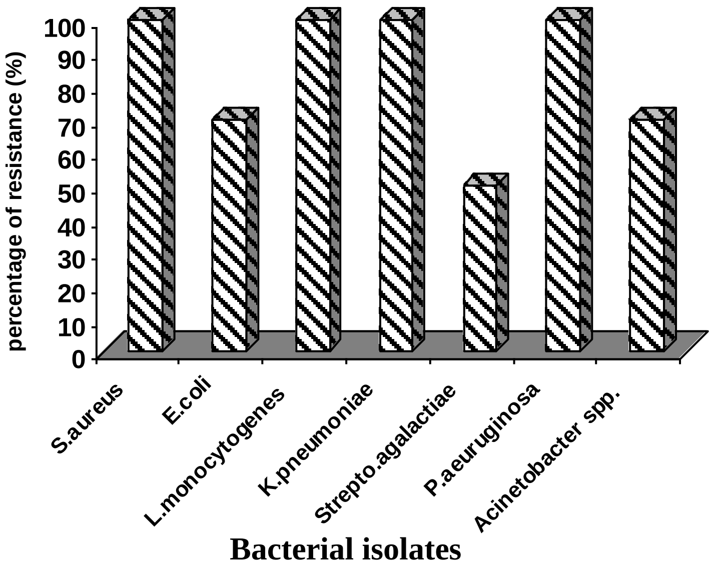

Figure (3-7): Percentage of bacterial resistance to cephtazidime.

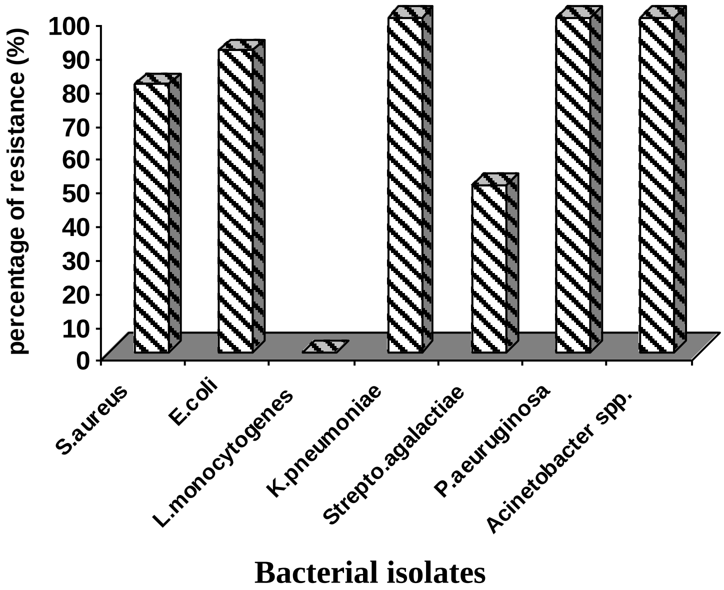

Figure (3-8): Percentage of bacterial resistance to cefalexine.

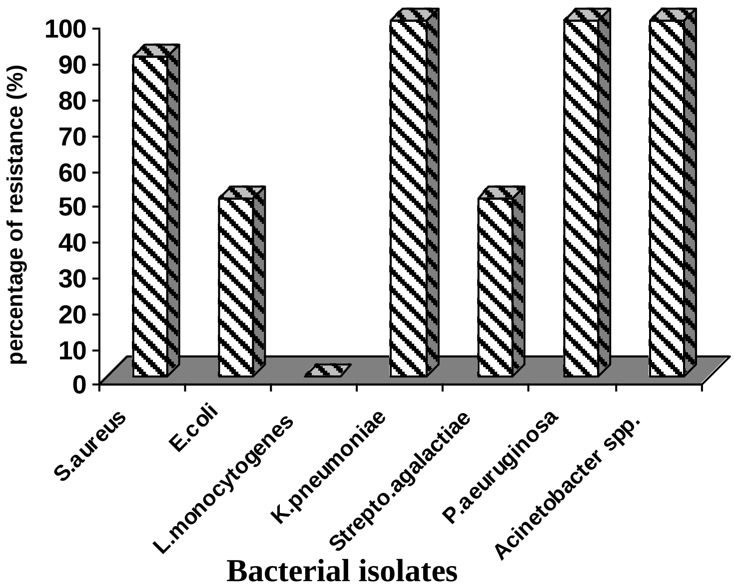

Figure (3-9): Percentage of bacterial resistance to cefotaxime.

Bacterial resistance to some of the macrolide group which includes azithromycin and erythromycin had also been investigated as shown in figure (3-9), (3-10), respectively.

The result showed that most bacteria were resistant to these antibiotics except L.monocytogenes and Streptococcus agalactiae which were completely susceptible to macrolid group followed by *S.aureus* . the results showed that the effect of this antibiotic was more on gram positive bacteria than on gram negative bacteria, this agreed with Forbes, (2007), who had reported that macrolides were not effective against most genera of gram negative bacteria.

Imamura *et al*., (2005), showed that azithromycin had bacteriocidal activity against *P.aeruginosa*, it directly disrupted outer membrane of *P.aeruginosa*, probably by acting to competitively displace of divalent cations from their binding sites on lipopolysaccharides (LPS) on the outer membrane, azithromycin was capable of permeabilizing the outer membrane of *P.aeruginosa* and that action was antagonized by mg^{+2} and this direct action of it may contribute to it's bacteriocdal activities against *P.aeruginosa*.

Regarding erythromycin, the result agreed with that mentioned by Chigbu & Ezerony, (2003) who found that (32%) of *S.aureus* were resistant to erythromycin.

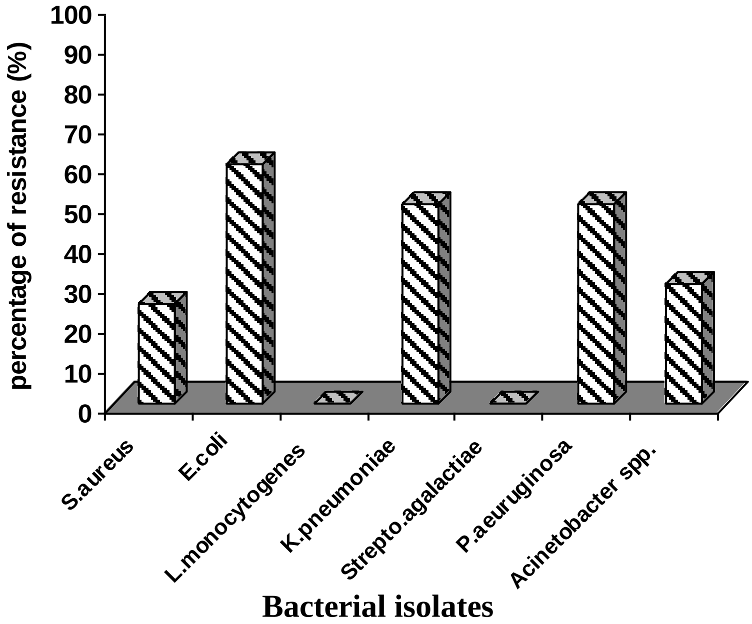

Figure (3-10): Percentage of bacterial resistance to azithromycin.

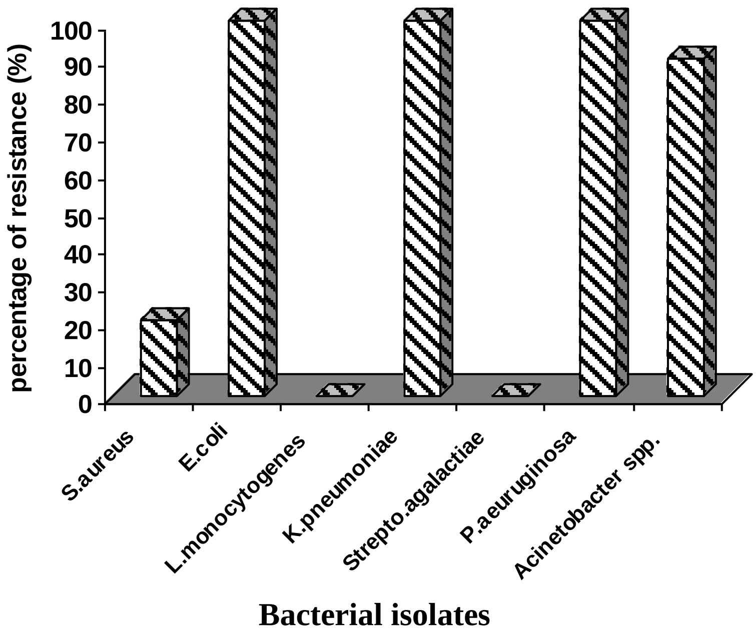

Figure (3-11): Percentage of bacterial resistance to erythromycin.

Bacterial resistance to aminoglycosides had also been studied which include gentamycin and amikacin as shown in figure (3-11), (3-12) respectively. The result showed that most bacteria were resistant to gentamycin except *L.monocytogenes* and *Streptococcus agalactiae*, which were completely 100% susceptible to gentamycin.

Amikacin can be considered the more effective antibiotics on gram positive and gram negative bacteria (Forbes, 2007).

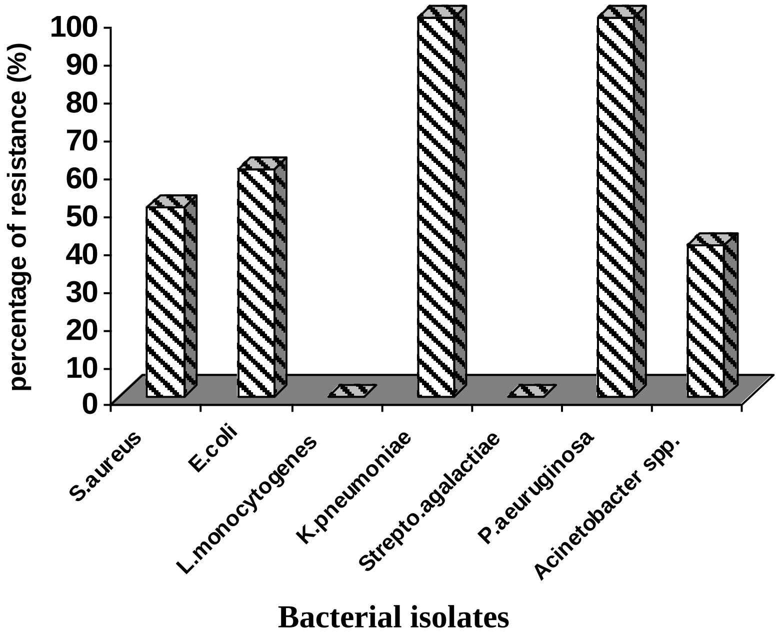

Figure (3-12): Percentage of bacterial resistance to gentamycin.

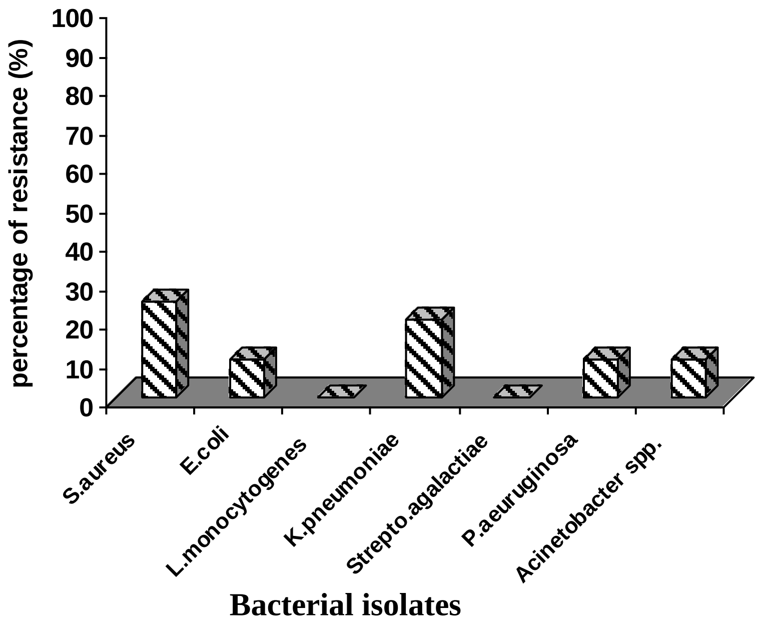

Figure (3-13): Percentage of bacterial resistance to amikacin.

The effect of ciprofloxacin was also studied as shown in figure (3-13), and it was found that most bacterial isolates were susceptible to ciprofloxacin and this agreed with Forbes, (2007), who had reported that fluoroquinolones are potent bacteriocidal agents and they have abroad spectrum activity that include gram positive and gram negative bacteria.

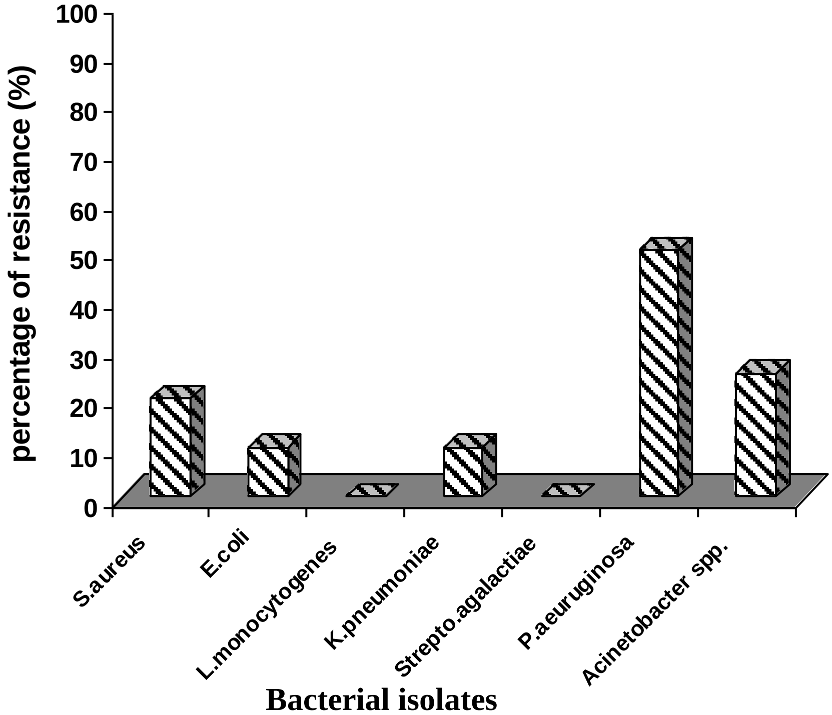

Figure (3-14): Percentage of bacterial resistance to ciprofloxacin.

3.7. Relationship Between Antibiotics That Affect Both Lactobacilli and Opportunistic Pathogens

It was shown in this study that some antibiotics were active against some opportunistic pathogens but inactive against Lactobacilli and unable to kill friendly bacteria like Lactobacilli and this observed by the effect of ciprofloxacin to which 93.3% of Lactobacilli isolates were resistant while it was considered the most effective against opportunistic pathogens. On the other hand, erythromycin to which 6.6% of Lactobacilli isolates were resistant while opportunistic pathogens showed higher degree of resistant to erythromycin except *L.monocytogenes* and *Streptococcus agalactiae*.

Regarding amoxicillin, the result showed that the opportunistic bacterial isolates had high resistance to β-lactam antibiotic while 40% of Lactobacilli isolates were resistant to amoxicillin.

Regarding other antibiotics there was different resistant pattern observed between both groups studied and from these results, the using of appropriate antibiotic which is highly selective is very important in order not to kill the friendly bacteria and to maintain the vaginal health like for e.g. prescribtion of ciprofloxacin to kill opportunistic pathogen and maintain Lactobacilli or avoiding erythromycin since 93.4% of Lactobacilli were sensitive to it.

Conclusions

According to this study, we can conclude the following:-

1. Lactobacilli have a protective role against vaginitis.
2. The production of H_2O_2 by *Lactobacillus* can be affected by the type of incubation *in vitro*.
3. The production of bacteriocin, lactic acid, H_2O_2 can be attributed to some species of Lactobacilli.
4. Antagonism between Lactobacilli species is present to maintain dominance.
5. Isolation of Lactobacilli are also affected by the type of antibiotic used to treat vaginitis where 93.3% of Lactobacilli isolates resist the effect of ciprofloxacin while 93.4 were sensitive to erythromycin
6. Vaginitis are more common in married than unmarried women especially those with intra uterine device.

Recommendations

According to this study, we can recommend the following points:

1.Using of proper antibiotics, which are highly selective against opportunistic pathogens and have no effect on Lactobacilli .

2.Studying of the probiotic role of Lactobacilli as a prophylactic and biotherapy means for disease other than vaginitis

3. Using of API system depending on enzymatic and biochemical tests for isolation and identification of lactic acid bacteria.

4. Introducting of new molecular techniques (PCR) for identification of Lactobacilli species and subspecies and correlate them with each virulence factors.

5. Studying other innate immune aspects of female urogenital tract.

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Contents

Dedication

To……

The memory of my mother,

My father,

My brothers and sister,

The Angel of mercy, my aunt,

The kind heart and my support in life

my husband Dr. Nawres

My flowers: Mustafa and Yousif

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List of Abbreviations

Symbol	Description
BV	Bacterial Vaginosis.
GBS	Group B *Streptococcus*
HRT	Hormonal Replacement Therapy.
lUD	Intrauterine Device.
MR	Methyl Red.
MRS	De Man-Rogosa-Sharpe.
PBPs	Pencillin Binding Proteins
PCR	Polymerase Chain Reaction
pH	Power of H^+ ion concentration
RTI	Reproductive Tract Infection
STD	Sexual Transmitted Disease
TMB	Tetra Methyl Benzidine
VP	Vogus Proskaur

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We, the examining committee, certify that we have read the thesis entitled (**A study of the Relationship Between Lactobacilli and Opportunistic Pathogens Associated with Vaginitis in Babylon Province**) and have examined the student (**Bara' Hamid Hadi AL-Greitty)** in its contents, and that in our opinion it is accepted as a thesis for the degree of Master of Science in Microbiology.

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A Study of the Relationship Between Lactobacilli and Opportunistic Bacterial Pathogens Associated with Vaginitis in Babylon Province

A Thesis

Submitted to the Council of the College of Medicine-University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology

By

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جامعة بابل
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دراسة العلاقة بين العُصيات اللبنية والمُمرِضات البكتيرية الانتهازية المتعلقة بالتهاب المهبل في محافظة بابل

رسالة
مقدمة الى مجلس كلية الطب/ جامعة بابل كجزء من متطلبات نيل درجة الماجستير في علم الاحياء المجهرية الطبية

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بسم الله الرحمن الرحيم

﴿ وَمَا أَنزَلْنَا عَلَيْكَ الْكِتَابَ إِلَّا لِتُبَيِّنَ
لَهُمُ الَّذِي اخْتَلَفُواْ فِيهِ وَهُدًى
وَرَحْمَةً لِّقَوْمٍ يُؤْمِنُونَ ﴾

صدق الله العلي العظيم

﴿ سورة النحل / الآية 64 ﴾

Abstract

In this study (110) vaginal swabs were obtained from women suffering from vaginitis and admitted to Babylon Hospital of Maternity and Paediatrics in Babylon province,swab samples from private clinics were also included.These swabs were distributed between married women(100) and unmarried women(10), pregnant(20) and non pregnant(80), with intrauterine device(70) and without intrauterine device(30). The period of sample collection was from November 2008 to April 2009.

The study involved the role of intrauterine device among married women with vaginitis and involved also the isolation of opportunistic bacterial isolates among pregnant and non pregnant women.

This study also involved studying the cultural characteristics of Lactobacilli, their probiotic role in maintaining vaginal health, in addition, the ability of Lactobacilli to produce some defense factors like hydrogen peroxide (H_2O_2), bacteriocin and lactic acid were also included.

The results showed that a total of 130 bacterial isolates were obtained,10 from unmarried women and 120 from married women.

The results revealed that intrauterine device (IUD) was a predisposing factor for vaginitis giving a percentage of (71%) among women used intrauterine device versus(29%) among women without it .

The most common species of opportunistic bacterial isolates were *Staphylococcus aureus* (44.7%) followed by *Escherichia coli(42%), Streptococcus agalactiae* (26.9%) , *Klebsiella pneumoniae (21%),*while other types of bacterial isolates differ between pregnant and non pregnant women.

The results showed that all Lactobacilli isolates were unable to produce hydrogen peroxide while, some isolates were able to produce bacteriocins that inhibit some isolates of opportunistic pathogens (*S.aureus, E.coli*).

This study also investigated the effects of antibiotics on Lactobacilli and on opportunistic bacterial isolates. The results revealed that 93.4% of Lactobacilli isolates were sensitive to erythromycin while 93.3% of Lactobacilli isolates were resistant to ciprofloxacin .The results also showed (40%, 53.3%) resistance of Lactobacilli toward amoxicillin and gentamycin respectively. These results were studied in relation to the effects of antibiotics on opportunistic bacterial isolates and it was found that most of these opportunistic isolates were sensitive to ciprofloxacin in contrast to erythromycin to which most of them were resistant except for *Listeria monocytogenes* and *Streptococcus agalactiae*. So, the study concluded that the types of antibiotics used to treat vaginitis must be very selective in order not to kill the beneficial bacteria(Lactobacilli) that help to preserve the vaginal health and ecosystem as being one of probiotic bacteria.

الخلاصة

تم في هذه الدراسة اخضاع 110 مسحة مهبلية للعزل وللتشخيص وتم استحصالها من النساء اللواتي يعانين من التهاب المهبل ويراجعن مستشفى بابل للولادة والاطفال في محافظة بابل بالاضافة الى المسحات التي اخذت من العيادات الخاصة .ووزعت هذه المسحات بين النساء المتزوجات(100) وغير المتزوجات(10) , الحوامل(20) وغير الحوامل(80) , اللواتي يستعملن موانع الحمل داخل الرحم (70) وغير المستعملات له (30). للفترة من تشرين الثاني 2008 الى نيسان 2009.

تضمنت هذه الدراسة دور مانع الحمل داخل الرحم لدى النساء المتزوجات المصابات بالتهاب المهبل كما شملت ايضاً عزل البكتريا الانتهازية من النساء الحوامل وغير الحوامل.

كما تضمنت الدراسة الكشف عن الخصائص الزرعية للعصيات اللبنية ودورها كمعزز حيوي في الحفاظ على صحة المهبل اضافة الى قدرة العصيات اللبنية على انتاج بعض العوامل الدفاعية مثل بيروكسيد الهيدروجين (H_2O_2)، والبكتروسين وحامض اللبنيك(lactic acid)

اظهرت نتائج الزرع 130عزلة بكتيرية حيث استحصلت120 عزلة بكتيرية من النساء المتزوجات و 10 عزلات من النساء الغير متزوجات

كما اشارت النتائج أن مانع الحمل داخل الرحم كان من العوامل المسببة لإلتهاب المهبل معطيا نسبة(71%) للنساء اللاتي يستعملن مانع الحمل داخل الرحم مقابل(29%) للنساء اللاتي لايستعملنه.

كانت اكثر انواع البكتيريا الانتهازية التي عزلت هي *S.aureus*(44.7%) جاءت بعدها *E.coli* (42%) ثم*Streptococcus agalactiae* (26.9%) ثم *K.pneumoniae*(21%) بينما اختلفت الانواع الاخرى من العزلات البكتيرية بين النساء الحوامل وغير الحوامل.

اظهرت النتائج إن جميع عزلات العصيات اللبنية *كانت غير قادرة* على انتاج بيروكسيد الهيدروجين بينما اظهرت بعض العزلات القابلية على انتاج البكتروسين الذي يمتلك القدرة على تثبيط بعض عزلات الممرضات الانتهازية مثل *(S.aureus* و*E.coli)*

شملت هذه الدراسة ايضاً تأثير المضادات الحيوية على عزل ونمو العصيات اللبنية وعلى البكتريا الانتهازية حيث كانت 93,4% من عزلات الـ Lactobacilli حساسة للأرثرومايسين بينما كانت 93,3% من هذه العزلات مقاومة للسبروفلوكساسين واظهرت النتائج بإن هناك(40%,53,3%) مقاومة بالنسبة للاموكسيلين والجنتامايسين من قبل Lactobacilli *على التوالي*.تم دراسة وربط هذه النتائج مع تأثير المضادات الحيوية على البكتريا الانتهازية وتبين أن معظم عزلات البكتريا الانتهازية كانت حساسة للسبروفلوكساسين عكس الأرثرومايسين الذي كانت مقاومة له بأستثناء بكتريا *Streptococcus agalactiae* و*L.monocytogenes*.*يستنتج من ذلك* أن نوع المضاد الحيوي الذي يستعمل لعلاج التهاب المهبل يجب ان يختار بشكل جيد كي لا يقتل البكتريا المفيدة(العصيات اللبنية) وللحفاظ على صحة المهبل وبيئته كون هذه البكتريا تمتلك دور المعززات الحيوية.

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Bara'

Appendix (1)

Morphological and Biochemical Features for Identification of Lactobacilli.

Test	Lactobacilli
-gram stain	Gram positive
-appearance	Single or pairs or short chains
Oxidase	-ve
Catalase	-ve
Coagulase	-ve
Indole	-ve
Glucose	+ve
Lactose	+ve
Spore	-ve
Mannitol	-ve
Incubation	Anaerobically
Motility	-ve
Selective media	mRS broth, mSR agar

Appendix (2)

Morphological and Biochemical Features for Identification of Gram Positive Isolates.

Test	GBS	*S.aureus*	*S.epidermidis*
Gram stain	Gram +ve cocci (pairs or chains)	Gram +ve cocci (clusters)	Gram +ve cocci (clusters)
Catalase	-ve	+ve	+ve
Oxidase	-ve	-ve	-ve
Hemolysin	Beta (narrow zone)	Beta	Gama
Coagulase	-ve	+ve	-ve
Growth on mannitol	-ve	Golden	White
Motility	-ve	-ve	-ve

Appendix (3)

Morphological and Biochemical Features for Identification of Gram Negative Isolates.

Test	*E.coli*	*K.pneumoniae*	*Actinobacter*	*P.aeruginosa*
Gram stain	Gram -ve short rods	Gram -ve short rods	Gram –ve coccobacilli (Dipplococci)	Gram -ve short rods
Catalase	+ve	+ve	+ve	+ve
Oxidase	-ve	-ve	+ve	+ve
Capsule	-ve	+ve	-ve	+ve
Hemolysin	-ve	-ve	-ve	+ve
Indole	+ve	-ve	-ve	-ve
M.R	+ve	-ve	-ve	-ve
Citrate	-ve	+ve	+ve	+ve
V.P	-ve	+ve	-ve	-ve
Urease	-ve	+ve	-ve	-ve
Growth on KIA	A/a	A/a	k/k	K/K
H_2S production	-ve	-ve	-ve	-ve
Motility	+ve	-ve	-ve	+ve
EMB	Metallic sheen	Centrally dark	Pale	Pale

Certification

We certify that this thesis was prepared under our supervision at the Department of Microbiology, College of Medicine, University of Babylon as partial fulfillment of the requirements for the degree of Master of Science in Medical Microbiology.

Supervisor	Supervisor
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In view of the available recommendation, I present this thesis for evaluation by the Examining Committee.

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1.1. Introduction

Vaginitis, is an infectious or non infectious inflammation of the vaginal mucosa, sometimes with inflammation of the vulva (external genitals). This inflammation often causes itching, burning, irritation, discharge and discomfort. It is one of the most common reasons for women to seek medical care (Egan & Lipsky, 1999).

Vaginitis falls into many forms: irritant, hormonal, foreign body, sexual transmitted diseases and infective. All types can cause great discomfort to a woman (Joesoef *et al*.,1999).

Irritant vaginitis can be due to allergic reactions to spermicides, condomes, soaps, douches, perfumes, medications and hot tubes in addition to abrasion, tampons and sanitary napkins (Hudson, 2007).

Hormonal vaginitis, is usually due to low levels of circulating estrogens in the body. This causes the lining of the vaginal canal to be thin and become atrophic. A thin vaginal lining may predispose a women to secondary infections. Typically a woman will complain of discharge, dryness, itching or burning. Estrogen is necessary to maintain the homeostasis of the vaginal flora and the proper pH of 4.5, The natural acidic environment of the vagina limits the growth of abnormal bacteria and maintains the presence of healthy bacteria (Fang *et al*., 2001).

Infective vaginitis may be caused by bacteria, fungi, parasites, viruses. The bacteriological agents associated with vaginitis include a wide variety of bacteria that are dominated by overgrowth and marked by deficiency of hydrogen peroxide-producing Lactobacilli. The most bacterial agents causing vaginitis include *S.aureus*, *E.coli*, *Group B streptococcus*, *L.monocytogenes*, *K.pneumoniae*, *Acinetobacter* spp., *N.gonorrhoea*, and the bacteria that cause bacterial vaginosis in addition to Chlamydia (Stenchever, 2001).

Other types of vaginitis due to sexual transmitted disease include *N.gonorrhoea*, Chlamydia, in addition to *Trichomonas vaginalis* which is one of the most common protozoan infection. Normally the acidic nature of the vagina renders the environment resistant to *Trichomonas* infection, symptoms present as profuse frothy discharge often bloody, green, yellow or grey, the discharge has unpleasant odor and vaginal itching, burning and pain may be present. The pH is between 5 and 7 (Soper, 2004).

The most important factor is to maintain the balance of the vaginal flora by the Lactobacilli and its protective and probiotic role in treating and preventing vaginal infection by producing antagonizing compounds which are hydrogen peroxide, lactic acid and bacteriocin (Hawes *et al*., 1996).

These properties of Lactobacilli lead to the use of this bacteria as probiotic in addition to being member of normal flora and are generally regarded as safe for use in humans. So that, in treatment of vaginitis, the normal flora disturbances must be restored by avoiding the use of wrong antibiotic that disturbs the normal vaginal flora (Andreu, 2004)

This study aims to:-

1. Isolate the Lactobacilli and evaluate their protective role against vaginitis.
2. Isolate and identify the common bacterial opportunistic pathogens in vaginitis.
3. Investigate some defense factors of Lactobacilli like hydrogen peroxide (H_2O_2), bacteriocins and lactic acid.
4. Study the effects of antibiotics on the bacterial isolates and compare their effect on the presence or absence of Lactobacilli.

1.2. Literatures Review

1.2.1. Female Genital Tract Anatomy

The mucosa of the vagina and outer portion of the cervix (ectocervix) are made up of a highly vascularized submucosa and a superficial non keratinized, stratified sequamous epithelium. This sequamous epithelium abruptly changes to simple stratified columnar epithelium at the transitional zone, which marks the beginning of the inner portion of the cervix (or endocervix), this is the site of hormonally regulated secretion of specialized mucous that facilitates sperm transport. The endocervix ends in the uterine cavity, the lining of which is referred to as either endometrium in the non pregnant state or decidua during pregnancy. Depending on hormonal stimulation, the endometrium varies from 1 to 6 mm in thickness and is made of several muscular layers. If pregnancy and implantation occurs, it hypertrophies further to become the nutrient-rich, intensely glandular decidua, if pregnancy does not occur, this layer is shed at the time of menses (Beckerman & Dudley, 2001).

The site of fertilization is the fallobian tube, a muscular membranes structure lined by a highly vascular mucosa (endosalpinx), which consists of ciliated and secretory cells (Beckerman & Dudley, 2001).

2.2.1. Development of the Normal Vaginal Flora

At birth, the vagina of the female infant is sterile, after only a few days, when the glycogen content of the vaginal epithelial cells has been elevated under the influence of the mother's oestrogen, the infant's vagina is colonized by the Lactobacilli migrating from the mother. This is in line with generally recognized fact that the normal bacterial flora in humans originates from the mother. In most instances, this flora continues to dominate throughout the individual's lifetime, and this is also the case with the vaginal flora though it is in the vagina's physiological environment during the life cycle (Cauci *et al*.,2002)

Small quantities of Lactobacilli remains, however, and the oestrogen product at menarche will cause a thickening of the vaginal mucosa, prerequisite for the propagation of Lactobacilli, the dominant vaginal flora of adult female (Harstall & Corabian, 1998). The bacteria isolated from the vaginal secretion of women of child bearing age number around 10^7 to 10^8 CFU/ml fluid (Redondolopez *et al*., 1996).

This microflora composition continues until menopause, when it is replaced by a mixed flora unlike that of infant female, but with considerable portion of mycoplasma species and small quantities of anaerobic bacteria (including *Gardnerrella vaginalis*). When hormonal replacement therapy (HRT) is used, it will cause Lactobacilli to continue as the dominant flora (Forsum *et al.*, 2005 a).

1.2.3. Vaginitis

1.2.3.1. Definition and Symptoms of Vaginitis

Vaginitis is a name given to describe swelling, itching, burning or infection in the vagina that can be caused by several different organisms. Vaginal infection is the most common gynecological problem found in women of all ages, with most women having at least one form of vaginitis during their lives. Vaginal infections often occur when women's natural resistance is lowered by anxiety, tension, lack of sleep, poor diet and sexual activity with an infected partner (Quan, 2000).

In addition, the vaginal environment is influenced by a number of different factors including a woman's health, her personal hygiene, medications, hormones (particularly estrogen), and the health of her sexual partner. Any disturbances in these factors can trigger vaginitis. In general, the symptoms of vaginitis according to Egan & Lipsky (1999) are:

-Irritation and/ or itching of the genital area.

-Inflammation (irritation, redness and swelling) of the labia majora, labia minora and perineal area.

-Vaginal discharge.

-Foul vaginal odor.

-Discomfort or burning when urinating.

-Pain/irritation with sexual intercourse.

1.2.3.2. Types of Vaginitis

There are several types (or causes) of vaginitis:-

1.2.3.2.1. Infectious Vaginitis

Infectious vaginitis accounts for about 90% of all cases of vaginitis in reproductive age women (Brook, 2002), and is represented by bacterial infection, candidiasis and trichomoniasis. Bacterial infection includes both specific and non specific vaginitis caused by opportunistic pathogens and *Gardnerrela vaginalis* (the causative agent of bacterial vaginosis) (Joesoef *et al*., 1999). Other less common infection caused by gonorrhoeae, Chlamydia, herpes, campylobacter and some parasites (Egan & Lipsky, 1999).

1.2.3.2.2. Hormonal Vaginitis

Hormonal vaginitis includes atrophic vaginitis usually found in postmenapausal or post partum women. Sometimes, it can occur in young girls before puberty, in this situation, the estrogen support of the vagina is poor (Fang *et al*., 2001).

The relationship between vaginal microbiology, menses and oestrogen level is complex (Eschenbach, *et al*., 2000). Normally the vaginal epithelium is stimulated and builds up under the influence of oestrogen that affects the glycogen content which is broken down into sugars by the non bacterial enzymes which in turn are converted into lactic acid by Lactobacilli.

Wasiela *et al*., (2001) found that interference with growth and glycogen content of the vaginal epithelium and change in the normal bacterial flora and pH of the vaginal secretion are predisposing factors toward vaginal disease and abnormal discharge.

Gloria *et al*., (2000) described atrophic vaginitis as an inflammation or irritation of the vagina caused by thinning and shrinking of the vaginal tissue with decrease lubrication of the vaginal wall due to lack of oestrogen. The most common symptoms of atrophic vaginitis are thinning and redness of vaginal lining, vaginal skin inflammation ,sorness of the vaginal skin and painful intercourse.

Atrophic vaginitis can also occur in premenapausal women who take antiestroenic medcations or who have medical or surgical conditions that result in decreasing level of oestrogen, the thinned endometrium and increased vaginal pH level induced by oestrogen deficiency predispose to genitourinary tract infection (Kalogerki, *et al*., 1996).

1.2.3.2.3. Irritation/Allergy Vaginitis

Irritant vaginitis can be caused by allergies to condomes, spermicides, soaps, perfumes, douches, lubricants and semen. It can also be caused by hot tubes, abrasion, tissue, tampons or topical medication (Hudson, 2007).

Since any member of synthetic chemicals and materials can induce an allergic reaction along the mucosal lining of the vagina, the best approach to treat all cases of irritant vaginitis is to avoid having synthetic chemicals and materials contact the vagina whenever possible. Scented soap should therefore never be used to clean the vaginal area, ferminine hygiene sprays should also be avoided as should contraceptive foams and suppositories in addition to tampons that could dry out and irritate the mucosal linning of the vagina just as soap can. (Stenchever, 2001).

1.2.3.2.4. Foreign Body Vaginitis

Foreign body vaginitis commonly caused by foreign bodies (retained tampons or condoms) cause extremely malodorous vaginal discharges. Treatment consists of removal, for which ring forceps may be useful. Further treatment is generally not necessary (Hudson, 2007).

1.2.3.2.5. Vaginitis due to Sexual Transmitted Diseases STDs

Sexually transmitted diseases can be a cause of vaginal discharge. Chlamydia and gonorrhea testing should be done whenever a sexually active individual complains of vaginal discharge even when the cervix appears normal (Rayan & Ray, 2004).

1.2.4. Microbiology of Vaginitis

1.2.4.1. Bacterial Infection

It had been focused on the common opportunistic pathogens that cause vaginitis in addition to bacterial vaginosis and Chlamydia.

1.2.4.1.1. *Staphylococcus aureus*

S. aureus is a gram positive, spherical in shape, it is single or in clusters and occasionally in short chains, it is non-motile, non spore forming, facultative anaerobe, it is catalase positive, oxidase negative, and coagulase positive, usually non capsular colonies appear opaque and are often pigmented golden-yellow. In common with other facultative anaerobes, *S. aureus* can grow in the absence of oxygen by has both fermentative and oxidative pathways (Masalha *et al*., 2001).

Several studies suggest that oxygen plays a role in the pathogenesis of *S. aureus* in both it's capacity to produce virulence factors and it's ability to persist and grow in different and often hospital environments (Yarwood & Schlievent, 2001).

S. aureus express many potential virulence factors such as surface proteins that promote colonization of host tissues, toxins that damage host tissues, in addition, to factors that inhibit phagocytosis and cause disease symptoms (Archer, 1998). So, *S. aureus* by these virulence factors can cause vaginitis, especially when there is adecrease in the number of Lactobacilli to maintain the normal vaginal ecosystem (Antonio *et al.,* 1999).

S. aureus is the major cause of hospital acquired (nosocomial) infection of surgical wound and epidermal skin disease in new born infant and food poisoning (Brook *et al*., 2004). Also, *S. aureus* can cause septicemia, folliculitis, boils, scalded skin syndrome, conjunctivitis and mastitis (Schneewind *et al*., 1995) and (Chibgu & Ezeronye, 2003).

1.2.4.1.2. *Group B Streptococcus-GBS*

Group B Streptococcus, taxonomically, known as *Streptococcus agalactiae,* is a gram positive cocci, non motile, non spore forming and spherical or ovoid, less than 2 µm in diameter, catalase negative, oxidase negative, facultatively anaerobic, fermentative metabolism with lactic acid being primary product. It grows poorly on nutrient media and prefers media enriched with blood or serum (Edward & Backer, 1995).

Vaginitis caused by *Streptococcus* presents acreamy, white discharge that is normally clear or white cloudy. There is usually no burning of the vulva as in case of yeast infection and the discharge is not very odorous like a bacterial vaginosis. There are several streptococcal species that can be present in the vagina: group A, B and D, about 70% of cases is due to group B and about 30% is due to group D, group A infection is rare. Streptococcal vaginitis can occur spontaneously but very often it is caused by the antibiotic treatment given for bacterial vaginosis (Frederick & Jelovesk, 2001).

The relative risk of vaginal infection with *Streptococcus agalactiae* in patient with purulent vaginal discharge is greater than that of candidiasis and lower than that of trichomoniasis (Brook *et al.*, 2007) but GBS infection is not classified within sexually transmitted diseases.

Streptococcus agalactia is now best known as a cause of postpartum infection as well as the most common cause of neonatal sepsis (Farley, 2001).

In pregnant women, GBS can cause chorioamnionitis, endometritis or genitourinary infection with bacteraemia, rarely can endocarditis or meningitis be observed (Berner, 2002).

These infections are related to its multifactorial virulence factors that include adherence, colonization, invation of epithelial cells, replication and the evation of host defense (Nizet *et al.,* 2000).

1.2.4.1.3. *Escherichia coli*

E.coli is one of the most important Enterobacteriaceae species, it is gram negative rods, usually motile,and produces polysaccharide capsule, positive test for indole, lysine decarboxylase and mannitol fermentation and produces gas from glucose. This bacteria is predominant among aerobic commensal bacteria in healthy human intestine (Smith & Scotland, 1993; Brook *et al*., 2004).

E.coli is one of the common organism in the microflora of pregnant as well as non pregnant women. Vaginal colonization with *E.coli* is associated with various genitourinary, obstetric and neonatal complication such as, severe form of pelvic inflammatory disease (Heinonen & Miettinen, 1994), urinary tract infection (Rajan *et al*., 1999), very low birth weight infants and early onset neonatal septicemia and meningitis. Vaginal *E.coli* has also been reported to be sexually transmissible to a male partner (Hebelka *et al*., 1993). The virulence factors of vaginal *E.coli* include adhesions, toxins, siderophores and invasion (Watt *et al*., 2003).

1.2.4.1.4. *Klebsiella pneumoniae*

K. pneumoniae is a gram negative, member of the family Enterobacteriaceae, that is characterized by polysaccharide capsule (which give their colonies their characteristic mucoid appearance), rod-shaped, facultative anaerobic, it is non motile, non-spore forming, flagellated, produces large sticky colonies when plated on nutrient media (Podschun & Ulmann, 1998).

Klebsiella pneumoniae is lactose fermenter, produce lysine decarboxylase but not ornithine decarboxylase and it is generally positive in the Vogas-Proskauer test and citrate, produces gas from glucose (Brook *et al*., 2004).

It can colonize the vagina specially, in women that have taken antibiotics due to high resistance rate and inhibition of other pathogens. The major virulence factors of *Klebsiella pneumoniae* is the thick capsule (its' antiphagocytic), and fimbriae (pili) (Podschun *et al*., 2001). This thick capsule is composed of complex acidic polysaccharides and form more than 70 serotypes (Greenwood *et al*., 2002).

The capsular material forms thick bundles of fibrillose structures covering the bacterial surface in massive layer (Amako *et al*., 1988)

1.2.4.1.5. *Listeria monocytogenes*

L. monocytogenes is gram positive rod-shaped bacterium, it is the agent of listeriosis, a serious infection that is caused by eating food contaminated with bacteria, the disease affect primarily pregnant women, new born infants and adults with weakened immune systems (Todar, 2008).

L. monocytogenes can also be isolated from vaginal secretion, specially in women with decrease number of

Lactobacilli since the later have anti-listeria activity and can protect against it especially the H_2O_2-producing one (Hawes *et al.*, 1996).

Listeriosis is an uncommon infection, contaminated food is the usual source of infection and transmission appears to be blood-born following gastrointestinal infection (Danielian & Hall, 2005).

1.2.4.1.6. *Neisseria gonorrhoeae*

N. gonorrhoeae is gram negative intracellular, diplococcal organism that is sexually transmitted, this bacterium causes infection in the genital tract that may disseminate to organs. Infection of the genitals can result in a purulent (or pus like) discharge from the genitalia, which may be foul-smelling, inflammation, redness, swelling, dysuria and a burning-sensation during urination.

N. gonorrhoeae can also cause conjunctivitis, pharyngitis, in female with infection of the genitals can result also in pelvic inflammatory disease which if left untreated can result in infertility, pelvic inflammatory disease results in *N. gonorrhoeae* travels into the pelvic peritoneum (via cervix, endometrium and fallobian tubes). Patient should also be tested for other sexual transmitted infection specially Chlamydia infection since co-infection is frequent (Van Duynhoven, 1999).

1.2.4.1.7. *Staphylococcus epidermidis*

S.epiderimidis gram positive, catalase positive, oxidase negative, Coagulase negative. *S.epiderimidis* has a relatively small white colony, this type of staphylococci constitute a component of the normal flora of human. It is opportunistic pathogen that causes infection in debilitated or compromised patients (Kloss & Bannerman, 1994; Brook *et al*., 2004).

1.2.4.1.8. Bacterial Vaginosis

Bacterial vaginosis (BV) is an abnormality of the normal vaginal flora characterized by a reduced number of Lactobacilli, a higher pH and 100 folds increased numbers of potential pathogens including *Gardnerella vaginalis, Bacteroides, E.coli, GBS*, the anaerobes *Peptostreptococcus*, and *Mycoplasma hominis*,therefore,the presence of large number of *Lactobacilli* and low pH are important mechanism to protect against the growth of potential pathogenic organisms (Bennet *et al*., 2007).

Around 50% of women with B.V. are asymptomatic. If symptoms do occur, the most common is a thin, watery, malodorous, non itchy discharge. The criteria used to diagnose bacterial vaginosis: pH>4.5, the presence of thin watery discharge, fishy odour (with 10% KOH), clue cells on saline wet mount and/or gram stain (Goswami & Thomtou, 2006).

Spiegel *et al*.,(1983), developed a method which was first created to diagnose gram-stained vaginal smear. By this method, the bacteria were grouped into morphotypes, *Lactobacillus* morphotypes were called elongated bacteria and *Gardnerella* morphotypes were called short bacteria. Generally, no method for diagnosis of B.V. can at present be regarded as the best (Forsum *et al*., 2005 b).

In clinical practice, bacterial vaginosis, is diagnosed using the Amsel criteria (Amsel *et al*., 1983):-

1. Thin, white, yellow, homogeneous discharge.
2. Clue cells on microscopy.
3. pH of the vaginal fluid >4.5.
4. Release of fishy odour on adding 10% KOH solution.

At least three of the four criteria should be present for comfirmed diagnosis (National guidline for the management of bacterial vaginosis (2002). An alternative is to use a gram stained vaginal smear with the Hay/Ison criteria (Ison and Hay,2002) or the Nugent criteria (Nugent *et al*., 1991).

Untreated bacterial vaginosis may cause serious complication such as increase susceptibility to sexually transmitted infection including HIV, in addition to increase risk of (PID) pelvic inflammatory disease (Bailey *et al.,* 2004), it can be treated with metronidazole or clindamycine either orally or vaginally, however, there is usually a high rate of recurrence (Bradshow *et al*., 2006).

1.2.4.1.8.1 *Gardnerella vaginalis*

G.vaginalis is a facultatively anaerobic, non spore forming, non encapsulated, non motile, pleomorphic, gram variable rod. Growth is best at 35 ºC and is enhanced by carbon dioxide. It is indole, nitrate and urease negative, rare anaerobic strains exist, pili have been recognized on it's surface (Boustouller *et al*., 1987). It is oxidase negative, catalase negative, thin gram-variable rod shape.

The detection or quantitation of *G.vaginalis* in vaginal fluid can not be used as a diagnostic test for BV, but, increased prevelance and concentration of *G.vaginalis* in patients with this syndrome suggests that *G.vaginalis* plays a role in BV even through it is not the sole etiologic agent.

1.2.4.1.8.2. *Mycoplasma hominis*

M.hominis has been associated with BV in pregnant and non pregnant women (Martius *et al*., 1988). The persistence of *M.hominis* after therapy for BV was associated with the persistence of *Bacteroides* species (Koutsky *et al*., 1983). *M.hominis* was associated with an abnormal gas-liquid chromatograph and the presence of diamines wether or not *G.vaginalis* was also present.

1.2.4.1.8.3. *Mobiluncus* species

Mobiluncus spp. are the most recently recognized member of the vaginosis associated flora. *Mobiluncus* spp. are curved, gram-variable, motile organisms, they are slowly growing, fastidious organisms requiring an enriched medium for growth, they are indole, Catalase, oxidase and H_2S negative (Holst *et al*., 1990). They are generally stain gram variable or gram positive.

The prevelance of *Mobiluncus* spp. in the vagina has been determined in numerous studies and has been reviewed recently these species are also associated with BV (Holst, 1990).

1.2.4.1.9. *Chlamydia trachomatis*

Chlamydia trachomatis (an obligate intracellular bacteria) is considered to be the most commonly isolated sexually-transmitted organism. The patients infected with *Chlamydia trachomatis* are often a symptomatic but, they may be present with mucopurulent vaginal discharge or cervicitis (Bulletin, 1994).

C. trachomatis is naturally found living only inside human cells, Chlamydia can be transmitted during vaginal, anal or oral sex and can be passed from an infected mother to her baby during vaginal child's birth (Gerbase *et al.*,1998) Between half and three quarters of all women who have Chlamydia infection have no symptoms and do not know that they are infected.If untreated Chlamydia infection can cause serious reproductive and other health problems with both short term and long term consequences.

Approximately half of asymptomatic cases, will develop inflammatory disease.Symptoms that occur include unusual vaginal bleeding or discharge, pain in the abdomen, painful sexual intercourse (dyspareunia), fever, painful more frequently than usual urinary urgency (Gerbase *et al.*, 1998).

1.2.4.2. Fungal Infection (Candidiasis)

Candidiasis is a fungal infection (mycosis) of any of the candida species, of which *Candida albicans* is the most common (Walsh & Dixon, 1996). Yeast are commonly present in humans and their growth is normally limited by the human immune system and by other microorganisms such as bacteria occupying the same location (niches) in the human body (Mullery & Goroll, 2006). External use of detergent , douches or internal disturbances (hormonal or physiological) can perturb the normal vaginal flora, consisting of lactic acid bacteria such as Lactobacilli and result in over growth of Candida causing symptoms of infection such as local inflammation symptoms (Mardh *et al*., 2003).

Pregnancy and the use of oral contraceptives have been reported as risk factors (Shiefer, 1997). Diet has been found to affect rate of symptomatic candidiasis in some animal infection models (Yamaguchi *et al*., 2005). Hormonal replacement therapy and infertility treatment may also be predisposing factors (Nwokolo & Boag, 2000).

Infected women with *Candida albicans* may have severe itching, burning, soreness, irritation and a whitish-gray cottage cheese like discharge, often with a curd-like appearance.

1.2.4. 3 Protozoal Infection (*Trichomonas vaginalis*)

Trichomonas vaginalis is anaerobic parasitic flagellated protozoan, it is the causative agent of trichomoniasis, the most common pathogenic protozoan infection of humans in industrialized countries (Soper, 2004).

Trichomoniasis is a sexually transmitted infection which occurs if the normal acidity of the vagina is shifted from healthy semi-acidic pH (3.8-4.2) to much more basic one that is conductive to *T.vaginalis* growth (Sood, 2007). Some of symptoms of *T.vaginalis* include preterm delivery, low birth weight and increased mortality as well as predisposing to HIV infection, AIDs and cervical cancer (Schwebke & Burgess, 2004). *T.vaginalis* has also been reported in the urinary tract, fallobian tubes and pelvis and can cause pneumonia, bronchitis and oral lesions. Other symptoms include inflammation with increasing number of organisms, greenish-yellow frothy vaginal secretions and itching. Condoms are effective in preventing infection. Ten percent of the infected women will be presented with a strawberry cervix or vagina on examination. *T.vaginalis* was traditionally diagnosed via a wet mount. Currently the most common method of diagnosis is via over night culture (Ohlemeyer *et al*., 1998), the presence of trichomoniasis can also be diagnosed by PCR (Schirm *et al*., 2007). Treatment should be prescribed to any sexual partners as well because they may potentially be a symptomatic carriers (Cudmore *et al*., 2004).

1.2.4.4. Viral Infection

Viruses are a common cause of vaginitis one form caused by herpes simplex virus (HSV) which is often just called herpes infection. These infections are also spread by sexual contact. The primary symptoms of herpes vaginitis are pain associated with lesion or sores. These sores are usually visible on the vulva of the vagina but are occasionally inside the vagina and can only be seen during a gynecological exam (Merz, 1993). As with all sexually transmitted disease, women are more susceptible to acquiring genital herpes HSV-2 than men (Johnson, 2006). The risk of transmission from mother to her baby is higher, if the mother becomes infected at a round delivery (30-60%), but the risk falls to 3% if it is a recurrent infection and is less than 1 if there are no visible lesions. Generally the risk of transmission between men and women can be reduced by antiviral treatment (Corey *et al*., 2004).

1.2.5. The Probiotic Concept

Probiotics can be defined as: substances prepared with live microorganisms, which prevent infections through restoration of normal microbial flora when it has been altered or: live microorganisms either supplied exogenously or stimulated endogenously , which confer a health benefit or enhance host resistance against infection (Reid & Bruce ,2001).

The characteristics required for a lactobacillus strain to serve effectively as a probiotic include: human origin, avid and persistent adherence to vaginal epithelial cells, interference with the adhesion of other pathogens and production of H_2O_2 and other molecules capable of inhibiting the growth of pathogens (Cadieux *et al*.,2002).

It is believed that *Lactobacillus* protects the vagina from colonization by pathogens mainly by blocking the attachment of microorganisms to the vaginal epithelium and by excreting substances that inhibit their multiplication. There are three mechanisms by which *Lactobacillus* can prevent uropathogen adherence:1. blockage by exclusion in which Lactobacilli adhering to vaginal cell receptors exclude the attachment of recently arrived pathogens, 2.blockage by competition in which Lactobacilli and pathogens inoculated simultaneously compete for the receptors, 3. blockage by displacement, in which the previously attached pathogens are displaced by recently inoculated Lactobacilli (Andreu *et al*., 1995).

It was demonstrated that not all *Lactobacillus* strains express these properties with the same density (Osset *et al*., 2001).On the contrary,these are substantial differences among species and among strains from a single species, e.g. more than 75% of human vaginal *Lactobacillus* strains adhered poorly to vaginal epithelium, a quarter of the strains demonstrated greater adhesion,and 10% adhered in very large numbers (Sipsas *et al*.,2001)

Lactobacilli can inhibit the multiplication of pathogens by excreting certain substances, mainly H_2O_2, lactic acid and bacteriocin-like substances.

1.2.6. Lactobacilli

Lactobacilli are gram positive bacteria that occur singly in pairs or short chains, *Lactobacillus* species are oxidase negative, catalase negative, coagulase negative, faculatively anaerobes, non spore forming, no motile, can grow selectively on deMan-Rogosa-Sharpe broth or agar (MRS), there are many strain and species and each have an important role in maintaince the normal vaginal ecosystem(Sneath *et al* .,1994)

Lactobacilli are members of normal flora and are generally regarded as safe for use in humans specially in uropgenital tract infections, bacterial vaginosis, and recurrent vaginal candidiasis (Stapleton, 2003). The importance of Lactobacilli related to production of H_2O_2, bacteriocins and lactic acid.

1.2.6.1. Role of Hydrogen Peroxide in Vaginitis

Hydrogen peroxide (H_2O_2) has been considered a key factor in *Lactobacillus* antagonism against pathogens. This compound generates cytotoxic reactive oxygen species, superoxide anions and hydroxyl radicals in the vaginal fluid (Klebanoff *et al*., 1991). So that the presence of H_2O_2-producing Lactobacilli is very beneficial in preventing vaginitis since this compound is toxic to organisms that produce little or no H_2O_2-scavenging enzymes (e.g. catalase) and absence of H_2O_2 producing Lactobacilli will allow growth of catalase negative organisms such as those found among women with bacterial vaginosis (Eschenbach *et al*., 1989).

1.2.6.2. Role of Bacteriocin in Vaginitis

Bacteriocins are secreted oligopeptides, proteins or protein complexes, with antimicrobial activity against strains taxonomically related to the producer organisms (Jack *et al*., 1995).

Bacteriocins produced by lactic acid bacteria (Meyer & Nes, 1997), and especially Lactobacilli (Klaenhammer, 1993), may have a role not only in protecting vaginal environment, but also in gastrointestinal environment.

High level of bacteriocins-like compound was synthesized by some species of Lactobacilli with a bacteriocidal mode of action against opportunistic pathogens, *G.vaginalis, Candida albicans*.

In addition, H_2O_2 depenent activity alone was not sufficient to inhibit pathogens .So, simultaneous action of H_2O_2 and bacteriocin was highly effective against them. Thus, *lactobacillus* administered in the form of vaginal ovules, could be an attractive alternative approach for the prevention and treatment of vaginitis (Hawes *et al*., 1996).

1.2.6.3. Lactic Acid Production

Lactic acid bacteria are group of gram positive bacteria, non respiring, non spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates (Axelsson, 1998). Some of the family are homofermentive, that is,they only produce lactic acid, while others are hetrofermentive and produce lactic acid plus other volatile compounds and small amount of alcohol.

Despite their complexity, the whole basis of lactic acid fermentation centers on the ability of bacteria to produce acid, which then inhibit the growth of other non-desirable organisms. All lactic acid producers are microaerophillic, that is, they require small amounts of oxygen to function.

Homofermenters convert sugar primarily to lactic acid while hetrofermenters produce about 50% lactic acid plus 25% acetic acid and ethyl alcohol and 25% carbon dioxide. The hetrofermentative Lactobacilli produce mannitol and some species also produce dextran (Dirar, 1993).

1.2.7. Hormonal Factors That Affect Isolation of Lactobacilli

Hormones especially oestrogen have a role in isolation of Lactobacilli where oestrogen lower the vaginal pH and an acidic pH increase redox potential making the environment less favorable for anaerobic organisms. In addition, the increase in oestrogen level stimulate Lactobacilli adherence to epithelial cells and alter the vaginal cell charge (Nikolaitchouk, 2009). So in postmenapausal women with vaginitis and were treated with oestrogen, there was a decrease in pathogenic bacteria and increase in Lactobacilli species (Hyman *et al*.2005).

Cauci *et al*., (2002) found that women on hormonal replacement therapy showed a level of lactobacilli identical to that found in fertile women, so that, the positive influence of hormonal replacement therapy(HRT) on Lactobacilli is a known phenomenon.

Mardh *et al*., (1991) found that high level of oestrogen increases the glycogen concentration in vaginal epithelial cells, then the glycogen metabolism provides the glucose which constitute the main nutritional factor for Lactobacilli which convert it to lactic acid lowering the vaginal pH.

Other studies found that the availability of glucose that derived from glycogen metabolism potentially could also promote the growth of other microorganisms .

Donder *et al*., (2000) showed that post menopausal status involves major vaginal changes that are not confined to the glycogen content of epithelial cells, which can be modulated by hormonal replacement therapy. However, Millier *et al.,* (2000) found that these changes could be regarded as normal physiological condition rather than abnormal.

1.2.8. Treatment of Vagnitis

Vaginal infections are often a mixture of various etiological agents, which present challenging cases for treatment. So that the cause of infection determines the appropriate treatment. It may include oral or topical antibiotics and/or antifungal creams, antibacterial creams. A cream containing cortisome may also be used to relieve some of the irritation. If allergic reaction is involved an antihistamine may also be prescribed. For women who have irritation and inflammation caused by low level of estrogen (post menopausal), a topical estrogen cream may be prescribed (Pirotta, 2004).

Generally, empirical antimicrobial therapy must be comprehensive and should cover all likely pathogens. In clinical setting, the use of antibiotic combination is usually recommended for treatment of serious gram negative bacillary infection, this approach ensures coverage of broad range of organisms and polymicrobial infections, prevents emergence of bacterial subpopulations that may be resistant to one of antibiotic compound and provides additive or synergistic effects. Antibiotic monotherapy is recommended also (Angotti *et al*., 2007).

The most common drugs that have been used to treat vaginitis include cefotaxime, cephalexine,ciprofloxacine, erythromycin, azithromycin, clindamycin in addition to metranidazole and oestrogen to treat atrophic vaginitis (Anderson, 2004).

Regarding antifungal drugs, imidazole derivatives which have a fungicidal effect are effective in treatment by altering the permeability of the fungal cell membrane (Sobel *et al*., 2003).

Herbal suppositories for the treatment of bacterial vaginitis may include herbs such as Echinacea, althea and others which have antimicrobial activity and can kill off the overgrowth of bacteria, however, oral *Lactobacillus* can be supplemented in all conditions to normalize the vaginal flora (Zeger & Holt, 2003).

After treatment, the vaginal flora is often disturbed. The cause is reduced density of live flora (physiological barrier against pathogens) and the reduction of vaginal epithelium (physical barrier). As a result, post anti-infective treatment also requests the strengthening of the natural vaginal flora, which is done with local administration of *Lactobacillus* and potentially low dose of hormones (e.g. estriol) to increase the proliferation of epithelial cells (Ozkinay, 2005).

1.2.9. Prevention of Vaginitis

Women should prevent some habits like wearing hot, moist clothes that enhance growth of pathogens. Also they should avoid douches, sprays and other irritating compound that kill benificial bacteria or cause irritation to epithelial cells.

Practising safe sex to get rid of sexually transmitted infection. Treatment of both partners is very important since women are more liable than men to get sexually transmitted disease (Mashburn , 2000).

2.1 Patients :-

One hundred and ten (110) swabs were taken from women suffering from vaginitis, who were admitted to Babylon hospital for maternal and pediatrics, in addition to swabs taken from private clinics during the period from November 2008 to April 2009 in Babylon province . One hundred swabs were taken from married women, of these 100 swabs,20 swabs were from pregnant and 80 swabs were from non pregnant women. In addition, some of married women were with intrauterine device(70) while, women without intrauterine device constituted (30). Also, ten swabs from unmarried women.

2.2.Materials:-

2.2.1. Laboratory Instruments

No	Instruments	Company
1.	Autoclave	STERMITE, Japan
2.	Sensitive Electronic Balance	A and D, Japan
3.	Incubator	Memmert , Germany
4.	Distillator	GFL– Germany
5.	Oven	Memmert, Germany
6.	Gas pack Jar	Oxford, U.K.
7.	Cork borer	Memmert, Germany
8.	Centrifuge	Hermle, Japan
9.	Refrigerator	Concord, Italy
10.	Milipore filter.	Satorius membrane W .Germany
11.	Light microscope	Olympus, Japan
12.	Micropipette	Oxford, U.S.A.
13.	PH. meter	Hoeleze and Cheluis, KG, Germany
14.	Water Bath	Memmert ,Germany

2.2.2 Chemical and Biological Material

Name of material	Company/Countary
A. chemical materials	
K_2HPO_4, NaCl, $MgSO_4$, HCl, $CaCl_2$, KOH	Merk –Switzerland.
α-Naphtholamine,Tetramethyl-P-paraphylenediamine-dihydrochloride, methyl red, Amyl alcohol, Peptone .	B.D.H–England.
H_2O_2, glucose, 99% ethanol, TMB, CO_2 generating Kits	Fluka chemika Switzerland Oxford U.K
B- culture media	
Blood agar base, MacConkey agar, Muller Hinton agar, peptone water medium, nutrient agar media, Nutrient broth, Simmon citrate, Kligler-iron agar, MRS broth, MRS agar	Hi medium
C- stains	
Gram Stain Set	Crescent – Saudi

2.3 Methods

2.3.1 Collection of Specimens :-

The proper specimens collected for bacteriological analysis are described below. Those specimens were collected in a proper way to avoid any possible contamination. The samples were generally collected from women with vaginitis.

The swabs were inserted into the posterior fornix, upper part of the vagina and rotated there before withdrawing them.

A vaginal speculum was also used to provide a clear sight of the cervix and the swab was rubbed in and around the introitus of the cervix and withdrawn without contamination of the vaginal wall .

Swabs for culture were placed in tubes containing normal saline to maintain the swabs moist until being taken to the laboratory. Each specimen, except specimen for isolation of Lactobacilli (which was inoculated on selective media using deMan Rogosa sharpe (MRS) broth and agar and was incubated anaerobically using gas pack jar);was immediately inoculated on blood agar plates, nutrients agar, chocolate agar and MacConkey's, plates. All plates were incubated aerobically at 37 ºC for 24 – 48 hrs.

2.3.2 Preparation of the Reagents and Solutions:

2.3.2.1 Oxidase Reagent:

This reagent was prepared by dissolving 1gm (tetramethyl-paraphenylen–diamine-dihydrochloride) in 100 ml of D.W. and immediately was used for identification of bacteria (Baron *et al.*, 1994).

2.3.2.2 Catalase Reagent :-

This reagent was prepared in (3%) solution using H_2O_2 diluted by D.W. and stored in a dark container and used for identification of the bacteria (Baron *et al.*, 1994).

2.3.2.3 Phenol Red Reagent

It was prepared by dissolving 0.1 gm from phenol red dye in 300 ml of ethyl alcohol (95%) then the volume was completed to 500 ml by D.W. It was used to detect the acidity of the media (Baron *et al.*,1994).

2.3.2.4 Methyl Red Reagent

Methyl Red Reagent was prepared by dissolving 0.1 gm. of methyl red in 300 ml of 95% ethanol and then the volume was completed to 500 ml by D.W. (MacFaddin, 2000). It was used to detect complete glucose hydrolysis.

2.3.2.5 Vogus – Proskauer Reagent :-

It is composed of two solutions as below :-

a- α -Naphthol reagent was dissolved in 100 ml of 99% ethanol.

b- Potassium Hydroxide (KOH) solution:-

40 gm of KOH was dissolved in 100 ml of D.W, and it was used to detect the partial glucose hydrolysis (Collee *et al.,* 1996).

2.3.2.6 Kovac's Reagent :-

It was prepared by dissolving 5gm. of P-dimethylamine benzyladehyde in 75 of amyl–alcohol and was added to 35 ml of concentrate HCl acid. It was used to detect the indole production (Baron *et al.* , 1994).

2.3.2.7 McFarland Tube Standard (0.5) :-

A barium sulfate turbidity standard solution equivalent to a 0.5 McFarland standard was prepared as described by NCCLS (2003 a), as follows:-

A O.5– ml aliquot of 0.048 M $BaCl_2$ (1.175 % W/v $BaCl_2$. $2H_2O$) was assed to 99.5 ml of 0.18 H_2SO_4 (1% u/u) with constant stirring to maintain suspension.

Correct density of the turbidity standard was verified by using reading the absorbance at 625 nm. The absorbance should be 0.08 to 0.10 for the Mcfarland standard.

Barium sulfate suspension was distributed in 4 ml aliquots into screw–cap tubes, which were tightly sealed and stored in the dark at room temperature.

Barium sulfate turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and was inspected for uniformly turbid appearance.

Barium sulfate standard should be replayed or their densities verified monthly.

2-3-2-8.TrisBufferSolution:

This buffer was prepared by dissolving 12gm from the tris base (NH_2C (CH_3OH) $)_3$ TAAV company (Mwt.121.4) in small amount of D.W. and completed to 1 Litter. The PH was adjusted to 7 by adding HCl (0.1 N). This solution was used as a good buffer to maintain PH of the media for isolation of *Lactobacilli* (Mark *et al*., 2004).

2.3.3 Preparation of Culture Media: -

The general culture media described below were prepared according to the methods mentioned for each media and used in appropriate experiments .

2.3.3.1 Blood Agar Medium:

Blood agar medium were prepared according to MacFaddin, (2000) by dissolving 40 gm blood agar base in 1000 ml D.W. and autoclaved at 121 °C for 15 min, then cooled to 50 °C and 5% of fresh human blood was added. This medium was used to cultivate bacterial isolation and to determine their ability to lyse blood .

2.3.3.2 Chocolate Agar Medium :

Chocolate agar medium has been prepared by dissolving 40 gm. of blood agar base in 1000 ml D.W. and sterilized by autoclaving. Then 8% of human blood was added to the medium after cooling to 80 °C .

This medium was especially used for isolation and cultivation of bacteria that need 5-10 % CO_2 tension (Baron *et al* , 1994) .

2.3.3.3 MacConkey Agar Medium :

MacConkey agar medium has been prepared according to the method recommended by the manufacturing company and it was used for the primary isolation of most G-ve bacteria and differentiation of lactose fermentative from the non lactose fermentative (Collee *et al*. , 1996).

2. 3.3.4 Nutrient Agar Medium :

Nutrient agar medium has been prepared according to the manufacturing company.

It has been used for general experiment isolate culture, cultivation and activation of bacterial isolates when it is necessary (MacFaddin , 2000).

2.3.3.5 Mannitol Salt Agar Medium :

This media has been used as a selective media for isolation of *staphylococci* and differentiation of *S.aureus* (MacFaddin, 2000).

2.3.3.6 Eosine Methlene Blue (EMB) Medium :

Lactose fermenting colonies are either dark or possessed dark centers with transparent colorless peripheries, while organisms that do not ferment lactose remain uncolored (Murray *et al* ., 2003).

2.3.3.7 Muller- Hinton Agar:

Muller–Hinton agar has been prepared according to the method recommended by (Vall *et al*., 1999) and it has been used in antimicrobial susceptibility testing.

2.3.3.8 Kligler Iron Agar :

Kligler Iron agar has been used for determining glucose and lactose fermentation and possible hydrogen sulfide (H_2S) production as a first step in the identification of gram negative bacilli (MacFaddin, 2000).

2.3.3.9 Pepten Water Media

Pepten water media was prepared and used to detect the ability of bacteria to produce indole according to the method described by (Baron *et al*., 1994).

2.3.3.10 MR –VP Medium

MR–VP medium has been prepared and used to detect the partial and complete hydrolysis of glucose according to (MacFaddin,2000).

2.3.3.11 Simmon's Citrate Medium :

Simmon's citrate Medium has been used for determining the ability of bacteria to utilize citrate as the sole carbon source (MacFaddin, 2000).

2.3.3.12 De Man Rogosa Sharp Agar Medium:

This media has been used as a selective media for the isolation of Lactobacilli as it provide optimal conditions for their growth (De Man *et al*., 1980).

2.3.4 Laboratory Diagnosis:

According to the diagnosis procedures recommended by (MacFaddin, 2000; Collee *et al*., 1996; Baron *et al*., 1994) the isolation and identification of gram positive and gram negative bacteria in women with vaginitis were performed as follows:-

2.3.4.1. Microscopical Examination and Colonial Morphology:

A single colony was taken from each primary positive culture and it's identification depended on the morphology properties (colony size, shape, color and nature of pigments, translucency, edge, and elevation and texture). Then colonies were investigated by Gram stain to observe a specific shape, type of reaction, aggregation and specific intracellular compounds. After staining the bacteria by Gram stain, specific biochemical test were done to reach the final identification.

2.3.4.2. Physiological and Biochemical Tests

2.3.4.2.1. oxidase Test :

A filter paper circle was placed into a sterile plastic disposable petri dish and moistured with several drops of the freshly prepared oxidase reagent, then a small portion of the colony to be tested was removed and rubbed on the filter paper, changing the color to blue or purple within 10 seconds indicated for a positive result (Baron *et al.*,1994) .

2.3.4.2.2. Catalase Test: -

By streaking the nutrient agar medium with selected bacterial colonies and incubated at 37 C^{o} for 24 hrs. then transfer the growth by the steak and put it on the surface of a clean slide and add a drop of (3% H_2O_2); Positive results when the gas bubbles appear (Baron *et al*.,1994).

2.3.4.2.3. Coagulase Test

This enzyme was tested by two methods:-

A- Slide test for bound coagulase (clumping factor):

A drop of human plasma was placed on a clean, dry glass slide, a drop of D.W. was placed next to the drop of plasma as a control. By a sterile loop an amount of the isolated colony was emulsified with each drop. when clumping the plasma, bacteria was observed and a smooth homogenous in the control, the result was recorded positively (Baron *et al*.,1994).

B – Tube test for free coagulase**:**

Half ml of human plasma was placed in a glass tube and a visible portion of growth from isolated colonies was emulsified in the plasma by rubbing the material on the slide of the tube while holding the tube at an angle, then the suspension was incubated for 1-4 hours at 37 °C; the presence of clot that cannot be re-suspended by gentile shaking was recorded as a positive results; the organism that fails to colt the plasma within 24 hrs is considered as a coagulase negative (Baron *et al*.,1994).

2.3.4.2.4. Citrate Utilization Test:-

The surface of simmone's citrate slant medium was inoculated with colony of the tested bacteria and incubated at 37°C for 1-3 days. conversion of the indicators color from green to blue indicates that the organism was able to utilize citrate as a sole carbon source (Cruikshank *et al* ., 1975) .

2.3.4.2.5. Kliger's Iron Agar for H_2S Production :-

Only the colonies growing on MacConkey agar touched by a straight wire and inoculated on the media by stabbing the butt of the tube and streaking the slant. Fermentation was detected by a change in the indicator phenol red to yellow. The pH changes in the butt and the slant of medium were recorded after 18-48 hrs of incubation of gas formation which is usually visualized as bubbles in the medium, caused by the gas formed in the agar. Organisms can produce H_2S from black precipitate in the butt (Baron *et al*.,1994).

2.3.4.2.6. Indol Test :-

Tubes containing a peptone water medium were inoculated with the colony of the tested bacteria and incubated in 37°C for 28 hrs, then several drops of Kovac's reagent were added to the broth median, after shaking the appearance of red ring on the surface was regarded as a positive result (Cruikshankn *et al*., 1975).

2.3.4.2.7. Methyl Red Test :-

The tubes of the (MR-VP broth) were inoculated with the selected bacterial colonies and were incubated at 37° C for 24 hrs. Then (5 drops) of methyl red reagent were added to it. The appearance and observation of red color means a positive result and a complete analysis of glucose.(MacFaddin ,2000).

2.3.4.2.8. Vogus Proskauer Test :-

The tubes of (MR-VP broth) were seeded with specific bacterial culture and were incubated at 37 °C for 48 hrs, then the result was read by adding (0.6 ml of α-naphthol reagent) and (0.2 ml of 40 % KOH solution); appearance of red color after 15 min. means positive result due to partial analysis of glucose, which produce acetone or Acetyl Methyl–Carbinol (MacFaddin ,2000) .

2.3.4.2.9. Motility Test :-

This test was done by inoculating the tube that contained semisolid media with tested bacteria by stabbing method and was incubated at 37°C for 24-48 hrs. The disseminating of growth out the stability line was an indication for positive result (MacFaddin, 2000).

2.3.4.2.10. Mannitol Salt Agar Test :

The differentiation between *staphylococcus aureus* and other *staphylococci* e.g. *staphylococcus epidermidis* was done by sub-culturing selected colonies on mannitol salt agar for 24 hrs. at 37 ^{o}C colonies surrounded by a yellow halo indicating mannitol fermentation and isolates colony, is *S.aureus* (MacFaddin ,2000).

2.3.5.Detection of Defense Factors of Lactobacilli :-

2.3.5.1. Hydrogen Peroxide(H_2O_2) Production Test :

According to the qualitative method of (Eschenbach *et al*.,1989), Lactobacilli were streaked onto a 20 ml MRS agar plate containing 5 mg of 3,3$^{/}$; 5,5$^{/}$-tetrmethyl-benzidine (TMB) and (0.20) mg of horse radish peroxidase. Peroxidase generates O_2 from any H_2O_2 produced by the Lactobacilli and the TMB stains the colonies blue in the presence of O_2 after 48 hrs of anaerobic incubation, colonies that produce H_2O_2 on MRS agar thus appear dark blue while non-producer are colorless. The media were used within 48 hrs after preparation.

2.3.5.2. Bacteriocin Production Test :-

The method that used for bacteriocin production test by Lactobacilli was the cup assay method (Al-Qassab and Al-Khafaji, 1992)

- Lactobacilli isolates were grown in brain heart infusion broth + 5% glycerol then incubated anaerobically for 24-48 hrs.
- The growing bacterial isolates were heavily streaked on brain heart infusion agar 5% glycerol, plates then incubated anaerobically for 24-48 hrs.
- Agar discs were cut from the cultured agar layer using sterile 5 mm. cork borer.
- A volume of (0.1) ml of each clinical indicator isolates (we used here *S.aureus and E.coli*) grown for few hrs. in a shaking water both at 37 ºC to obtain (10^6-10^7 cell/ml) in each of nutrient broth was spreaded on nutrient agar plates.
- Agar discs were transferred carefully to the agar surface seeded with indicator isolates (*S.aureus*, *E.coli*) and incubated over night at 37° C.
- Sensitively patterns were recorded ,presence of inhibition zone around the agar disc indicate a positive result.

2.3.5.3. lactic Acid Production Test :

Tris-HCl (0.1 m) was used as a buffer pH 7 because we depended on pH measurement of the buffer before incubation and after 24-48 hrs. of anaerobic incubation and as follows:

- pH of the buffer was measured and it was 7.
- A loopfull of *Lactobacilli* isolates were inoculated and incubated for 24-48 hrs anaerobically.

- After 24 hrs, pH also was measured and a decrease in pH reading was attributed to production of lactic acid.
- After 48 hrs., the pH. Was also measured and finding of increase in pH. was attributed to entrance in another metabolic pathways. (Boskey *et al* .,2001).

2.3.5.4. Haemolysin Production:

Haemolysin production has been carried out by inoculating a blood agar medium with bacterial isolates at 37 ºC for 24–48 hrs. Appearance of clear zone around the colonies refers to complete haemolysis (β–haemolysis), greenish zone around the colonies refers to partial haemolysis (α-heamolysis), while, no change refers to non haemolysis (γ-haemolysis) (Doboy, *et al*., 1980).

2.3.5.5. Coagulate factor test :

As describied in 2.3.4.2.3

2.3.6. Effect of Antibiotics on Lactobacilli.

It was performed according to the comit'e Francais seL' Antibiogramme (1998), method in which fifty (50) micro liters of the pellet of an over night culture was diluted in MRS broth to about 10^7 CFU/ ml.

Muller-Hinton agar plates containing 5% sheep blood were flooded with this suspension in order to give confluent colonies and air dried for 15 min, and the discs impregnated with antibiotics were positioned on the plates. After 36 hrs. of anaerobic incubation at 37 ºC, the diameters of the bacteria–free zones were measured.

2.3.7. Antimicrobial Sensitivity Test:

It was performed according to Bauer – Kirby *et al*., (1966), method by using a pure culture of previously identified bacterial organism. The inoculum to be used in this test was prepared by adding growth from (5) isolated colonies grown on blood agar plate to 5 ml nutrient broth. This culture was then incubated at 37 °C for 18 hrs. to produce standardized bacterial suspension of moderate turbidity:

- A sterile swab was used to obtain an inoculum from this culture, that was streaked on a Muller – Hinton plate .
- The antibiotic discs were placed on the surface of the medium at evenly spaced intervals with flamed forceps or a disc applicator.
- Incubation was usually over night with an optimal time of 14 hrs. at 37 °C. Antibiotic inhibition zone were measured in(mm) using a caliper.

Inhibition zone diameter was interpreted according to that recommended by clinical laboratory standards institute documentations (CLSI, 2007). Antibiotic disc is prepared by oxide company with the following disc potency.

No	Type	Derivations	Disc Potency µg/ml
1.	Amoxicillin	Amx	10
2.	Amoxicillin +clavulanic acid (Amoxiclav)	Amc	20/10
3.	Cefotaxime	CTX	30
4.	Cephtazidime	CAZ	30
5.	Cefalexin	CL	30
6.	Amikacin	AK	30
7.	Gentamycin	GN	10
8.	Erythromycin	ER	15
9.	Azithromycin	AZM	15
10.	Ciprofloxacin	CIP	5

3.1: Role of Intrauterine Device as Predisposing Factor in Vaginitis

As shown in table (3-1),some women included in this study were with intrauterine device , it was found that the incidence of vaginitis was higher among married women (92.3%) than unmarried women (7.7%) and also it was showed that the incidence of vaginitis among married women that used intra uterine device (IUD)(71%) was higher than married women without IUD (29%), these findings were in agreement with that of Samar (2005), who found that about (87%) of women reported vaginitis or reproductive tract infection (RTI) due to IUD., Samar (2005) also found that there were significant differences in alteration of vaginal discharge and presence of RTI-related symptoms among women used IUD in comparison to women not used IUD.

A homogenous, malodorous thin, grey discharge, which resembled non specific vaginitis occurred in high percentages of women with intrauterine contraceptive device about four times more common than those women without it. Also the normal lactobacilli dominated microbial vaginal flora was replaced by many other opportunistic pathogens and certain anaerobic species among women used IUD with discharge (Kivigarvi *et al*., 2005).

Table (3-1): Distribution of women with vaginitis according to the use of intrauterine device (IUD).

<table>
<tr><th colspan="4">Women</th><th>No.of isolates</th><th>Percentage %</th></tr>
<tr><td rowspan="3">Married</td><td>Type</td><td>No. of isolates</td><td>%</td><td rowspan="3">120</td><td rowspan="3">92.3</td></tr>
<tr><td>With IUD</td><td>85</td><td>71</td></tr>
<tr><td>Without IUD</td><td>35</td><td>29</td></tr>
<tr><td colspan="4">Unmarried</td><td>10</td><td>7.7</td></tr>
<tr><td colspan="4">Total</td><td>130</td><td>100</td></tr>
</table>

IUD: Intrauterine device

3.2: Bacteriological Aspect of Vaginitis

3.2.1: Bacterial Isolation

In this study, (110) vaginal swabs were obtained from women suffering from vaginitis, all these swabs were subjected for culturing on different types of culture media and it was found that 105 samples gave positive bacterial culture where as five (5) samples showed no bacterial growth, as shown in table (3-2).

Out of 105 positive cultures, a total of 130 bacterial isolates were obtained,10 isolates from unmarried women and 120 bacterial isolates from married women. Of these 120 bacterial isolates, 15 isolates were of Lactobacilli and 105 isolates were of opportunistic pathogens,as shown in table (3-3).

Table (3-2): Frequency of positive and negative culture in women with vaginitis.

Women	**Positive culture**		**No growth**
	Opportunistic pathogens	**Lactobacilli**	
Pregnant	15	4	1
Non pregnant	65	11	4
Total	80	15	5

Table (3-3): Numbers of bacterial isolates among pregnant and non pregnant women with vaginitis.

Women	**Opportunistic bacterial isolates**	**Lactobacilli isolates**	**Total bacterial isolates**
Pregnant	31	4	35
Non pregnant	74	11	85
Total	105	15	120

The negative bacterial cultures may be attributed to consumption of antibiotics by the patients, or the presence of another type of causative agents that might need a special techniques for their detection such as viruses, Chlamydia and other agents.

From the results, it was shown that gram positive bacteria constituted (55.2%) from the total bacterial isolates compared to gram negative bacteria which constituted (44.8%) as shown in figure (3-1).

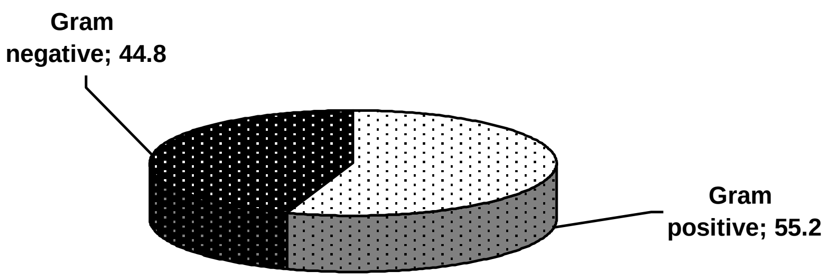

Figure (3-1): Percentage of gram positive and gram negative bacteria among women with vaginitis.

3.2.2: Opportunistic Bacterial Isolates in Women with Vaginitis

Thirty nine cases of vaginitis were caused by single bacterial type in non pregnant and sixteen in pregnant women, as shown in table (3-4), (3-5) respectively where *Staphylococcus aureus* constituted (8/39), *Escherichia coli* (5/39), followed by *Listeria monocytogenes* (7/39), *group B streptococci GBS* (5/39), *Pseudomonas aeruginosa* (4/39), *Klebsiella pneumoniae* (2/39) while other bacterial isolates constituted (5/39).

For pregnant women: there was different percentages of bacterial isolates and as shown in table (3-5).

Table (3-4): Types of opportunistic bacterial isolates from non pregnant women with vaginitis.

Bacteria	Single isolate	Mixed isolates	Total isolates	Percentage (%)
S.aureus	8	6	14	18.9
E.coli	5	7	12	16.2
L.monocytogenes	7	2	9	12.1
Group B streptococci GBS	5	3	8	10.8
P. aeruginosa	4	2	6	8.1
S. epidermidis	3	3	6	8.1
K. pneumoniae	2	4	6	8.1
Acinetobacter spp.	3	2	5	6.8
N.gonorrhoeae	1	2	3	4.1
Diphtheroid .	1	2	3	4.1
Corynebacterium spp.	0	2	2	2.7
Total	39	35	74	100

Table (3-5): Types of opportunistic bacterial isolates from pregnant women with vaginitis.

Bacteria	**Single isolate**	**Mixed isolates**	**Total isolates**	**Percentage (%)**
S.aureus	5	3	8	25.8
E.coli	3	5	8	25.8
Group B streptococci GBS	3	2	5	16.1
K. pneumoniae	2	2	4	12.9
S. epidermidis	2	1	3	9.7
P. aeruginosa	1	2	3	9.7
Total	16	15	31	100

These results were correlated with the results obtained by Donder *et al*., (2002), whereas, the results of bacteria isolated from pregnant women were similar to those obtained by Curzik *et al*.,(2001) and Rodringuez *et al*., (2001). Fourteen isolates (18.9%) of *Staphylococcus aureus* were isolated in this study in non pregnant while in pregnant women, eight (25.8%) isolates were isolated.These findings were in agreement with that of Mumtoz *et al*., (2008) who found that 24% of the patients with aerobic vaginitis were infected with *S.aureus* followed by enteric gram negative bacilli and other gram positive cocci.

Baron *et al.*, (1994), showed that the percentage of *staphylococcus* species(*S.aureus,S.epidermidis,S.saprophyticus*) equal to 62% in vaginitis.

Hillier *et al.*, (1993), studied the vaginal flora of some women and categorized them as normal in 50% of cases, intermediate in 27% and abnormal in 23%. They found that the organisms commonly found in women with normal smears were lactobacilli, coagulase negative staphylococci, *S.aureus*, *diphtheroids*, *candida* and *GBS*.

During pregnancy, the vaginal flora remains constant, there is no general increase in isolation of pathogenic bacteria as pregnancy progress, while *E.coli*, *S.aureus*, *Klebsiella* were acquired late in pregnancy (McDonald, 1997).

The results of this study also showed that most *S.aureus* isolates had the ability to produce β-haemolysis due to the presence of clear zone of haemolysis around the bacterial colonies formed on human blood agar, these results agreed with the results mentioned by Dinges *et al.*, (2000).

12 isolates (16.2%) of *E.coli* were isolated in non pregnant and 8 isolates (25.8%) were isolated in pregnant women, these results agreed with the results mentioned by Michael, (1991), who isolated *E.coli* at percentage of (17.1%).

Vaginal *E.coli* had also been reported to be sexually transmissible to a male partner (Hebelka *et al.*, 1993).

Regarding *group B Streptococci (GBS)* were also isolated (10.8%) in non pregnant and (16.1%) in pregnant women other studies have proved that the rate of isolation of *Streptococcus agalactiae* from vaginal swabs ranged from (5-40%) due to difference in the sample sites and cultural methods employed (Edward & Baker, 2000).

Maniatis *et al*., (1996), also reported that *Streptococcus agalactiae* isolated from cases of vaginitis were (10%) of all collected specimens. This bacteria regarded as species of female urogenital tract (Brook *et al*., 2007).

Zhu *et al*., (1996), reported that the rate of isolation of this bacteria from non pregnant women was (10.86%)

Klebsiella pneumoniae had also been isolated in this study (8.1%) in non pregnant, (12.9%) in pregnant woman. This bacteria is rarely present in healthy vagina, however, this study had confirmed presence of such bacteria in cases of vaginitis which may be attributed to: taking of antibioties by the patient women with vaginitis (especially β-lactam antibiotics) which inhibit the growth of other opportunistic pathogens, while. *Klebsiella* isolates are considered the most common resistant bacteria to most antibioties by producing extended spectrum Beta-lactamase (Bedenic *et al*., 2001; Levermore and Brown,2001). It also can be attributed to absence or decrease number of lactobacilli and their defence factors .

Pseudomonas aeruginosa had also been isolated (8.1%) from non pregnant women and (9.7%) from pregnant women. This bacteria had been isolated especially from women suffering from offensive odor, and from vaginal discharge, besides, the non pregnant women had an intrauterine device (Bonadio *et al.*, 2001; Takeyama *et al.*, 2002).

It is potentially opportunistic microorganism within the vagina, such microorganism may become increasing prevalent upon minor alterations of the vaginal environment.

Metcalf ,(2001) had isolated this bacteria from cases of vaginitis and showed that this bacteria was prevalent among non pregnant women using IUD.

Acinetobacter spp. were also isolated in this study (6.8%) from non pregnant women only.

In a local study in Hilla, Iraq, Al-Shukri (2003), pointed that *Acinetobacter* could be isolated from healthy vagina at a low rate. Other studies isolated this bacteria by a rate (3.1%) (Johnson ,1999).

Acinetobacter is diplococci, catalase positive, oxidase negative, and it recovered from the female genital tract (Geo *et al.*, 2001).

Only 3 isolates of *Neisseria gonorrhoeae* had been isolated in this study and mostly from women with infected husband, it is gram negative, intracellular diplococci organism that is sexually-transmitted, this organism cause infection in the genital tract that can disseminate to other organs (Romero *et al*., 1991).

3.2.3: Isolation of Lactobacilli

Eleven isolates of Lactobacilli had been isolated anaerobically from non pregnant women and four isolates from pregnant women, as shown in table (3-6).

Table (3-6): Distribution of Lactobacilli isolates among pregnant and non pregnant women.

Women	Number of lactobacilli isolates	Percentage (%)
Pregnant	4	26.7
Non pregnant	11	73.3
Total	15	100

Antonio *et al*., (1999) found that *Lactobacillus* colonized 71% of sexually active women

Although it is known that *Lactobacillus* is the predominant vaginal microorganism (10^7-10^8 CFU/ml fluid) in healthy premenapausal women, the composition and dynamics of the diverse Lactobacilli populations have been poorly characterized (Andreu ,2004).

The results correlated with Falagas *et al*., (2007) who reported that most female had only few species because even multiple colonization of lactobacilli species in the vagina but,it may produce some factors against other Lactobacilli to maintain dominance(Pavlov *et al*., 2000).

Andrew *et al*., (2003) found that although the presence of Lactobacilli may be used as a sign of healthy vaginal environment, the possibility that specific strain or combinations of strains of Lactobacilli being harmful should not be ignored, especially due to their ability to produce substance capable of inhibiting other normal vaginal microorganisms.

Other studies concentrated on importance of this bacteria as normal vaginal microflora that had the ability to regulate the growth of other vaginal flora, therefore any disturbances in Lactobacilli were highly correlated with the presence of these opportunistic microorganisms (Gilbert, 2003).

In Addition, results showed that the presence of Lactobacilli together with other opportunistic pathogens may be due to:

- Effects of antibiotics.
- Type of incubation as some Lactobacilli species are unable to produce some defense factors under anaerobic incubation.
- Antagonism among Lactobacilli species to maintain dominance.

3.2.3.1 Cultural Characteristic of Lactobacilli.

Lactobacilli considered as one of lactic acid bacteria which are gram positive, catalase negative, oxidase negative, coagulase negative, fermentive organism that produce lactic acid as a major end product of carbohydrate metabolism (Rouse *et al*., 2007).

Lactobacilli are indole negative, glucose positive, non spore forming, non motile and they are grown on selective media: On MRS agar anaerobically: The colonies were rounded, one mm in diameter, grey to green in color, smooth border, convex, as shown in figure (3-2) .

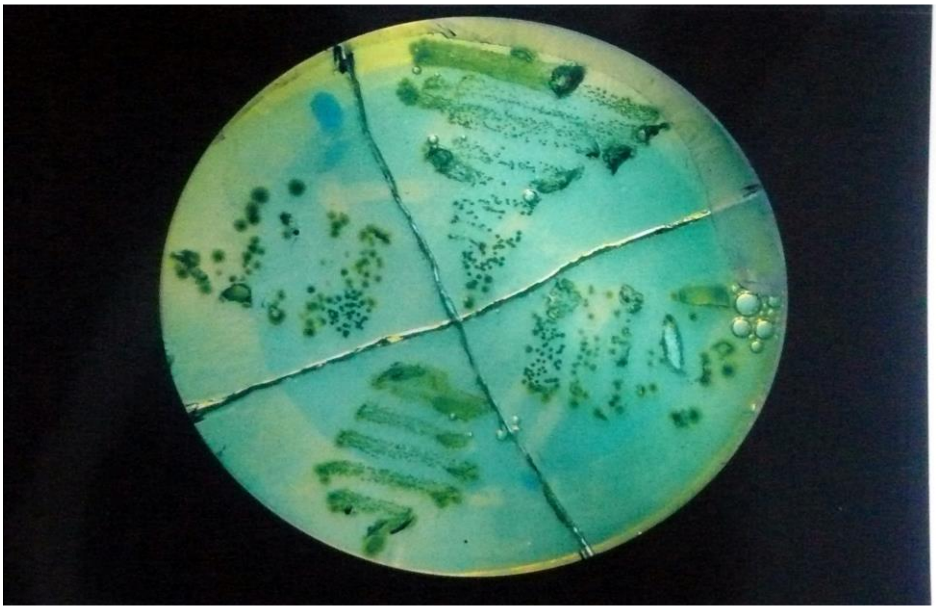

Figure (3-2): Colonies of lactobacilli on MRS (de-Man-Rogosa-Sharpe) agar.

3.2.3.2. Defense Factors of Lactobacilli as Probiotic Bacteria

Lactobacilli plays an important role in protection of the vagina from colonization by pathogens and this could be attributed to two main mechanisms: blocking the attachment of pathogens to the vaginal epithelium and by production of substances that inhibit their multiplication, Andreu, (2004). However, not all *Lactobacillus* strains express these properties with the same intensity, on the contrary, there are substantial differences among species and among strains from a single species.

Burtou *et al*, (2003), found that the instillation of Lactobacilli had the potential to make a significant impact on the health of women and therefore, it is important to understand how the vaginal microbes change and adapt to the presence of the Lactobacilli.

Reid *et al*., (2000) found that the potential importance of Lactobacilli is to protect the vagina from diseases, therefore, it has been used nowdays as probiotic to be determined.

The most important defence factors of Lactobacilli that were investigated in this study:

- Hydrogen peroxide (H_2O_2) production .
- Bacteriocin production .
- Lactic acid production .

3.2.3.2.1: Hydrogen Peroxide Production by Lactobacilli

The ability of Lactobacilli to produce hydrogen peroxide (H_2O_2) was investigated and the result showed that these isolates did not have the ability to produce H_2O_2, these results were in agreement with the results obtained by Dick *et al*., (2000), who found that only one isolate had the ability to generate H_2O_2 among the Lactobacilli isolated. Therefore, the presence of other opportunistic pathogens in the study may correlated with the absence of H_2O_2 production by Lactobacilli. Tomas *et al*., (2003) found that H_2O_2 production was higher under shaken aerobic cultures than anaerobic condition.

The results were in contrast with Donovan (2000) who found that some Lactobacilli strains had the ability to produce H_2O_2 at about 94-95%. Other studies found that although, all Lactobacilli produce lactic acid but, some were able to produce hydrogen peroxide and if it was produced, Lactobacilli will act as endogenous microbiocides in the vagina (Rabė and Hillier, 2003).

The results also disagreed with Shopova, (2003) who found that 93.3% of women colonized by Lactobacilli which were able to produce H_2O_2 and the remaining percentage were not able to produce it and represented as part of normal vaginal microflora.

Hawes *et al*., (1996) found that most isolates of lactobacilli produce some detectable H_2O_2. Other studies found that there was striking differences between the amount of H_2O_2 produced by different lactobacilli species and found that *L.cripatus* and *L.jensenii* did not produce H_2O_2 (Vallor *et al*., 2001).

It had been demonstrated that although, H_2O_2 producing vaginal Lactobacilli have an inhibitory effect on colonization of the vagina by pathogenic bacteria, however, the extent of the contribution to the inhibition of the vaginal colonization due to H_2O_2 production by comparison with production of bacteriocin, organic acid and competition for adhesion site is not known (Aroutcheva *et al*., 2001).

The lack of H_2O_2 production by Lactobacilli may be attributed also to the type of incubation and this agreed with Eschenbach *et al*., (2000), who postulated that anaerobic Lactobacilli did not produce H_2O_2 while facultative one could produce H_2O_2 and may represented a non specific antimicrobial mechanism of the normal vaginal ecosystem. Also, the presence of genital infection lead to decrease the number of Lactobacilli that produce H_2O_2 and increase colonization of the vagina by Lactobacilli that do not produce it.

3.2.3.2.2: Bacteriocin Production by Lactobacilli

Bacteriocin production activity of Lactobacilli was studied and it was found that two strains (13.3%)of lactobacilli inhibited the growth of *E.coli* and other two strains (13.3%) inhibited the growth of *S.aureus*, and it was found that the largest zone of inhibition was (21 mm) on *E.coli* and (12mm) on *S.aureus*.

Table (3-7): Bacteriocin production by lactobacilli isolates.

***Lactobacilli* isolates**	**Zone of Inhibition growth /mm**	
	S.aureus	***E.coli***
1	-	-
2	-	-
3	-	+ (21 mm)*
4	+ (10 mm)*	-
5	-	-
6	+ (12 mm)*	-
7	-	-
8	-	+ (12 mm)*
9	-	-
10	-	-
11	-	-
12	-	-
13	-	-
14	-	-
15	-	-

* Zone of inhibition

The results agreed with the results mentioned by Aroutcheva *et al*., (2000) and Pavlov *et al*., (2000), who found that some vaginal Lactobacilli could inhibit other Lactobacilli by releasing bacteriocin.

Reily *et al*.,(2002) reported that some Lactobacilli exhibited potent antimicrobial activities in form of small, heat-stable, ribosomally-synthesized antimicrobial peptides called bacteriocin, these substances had bacteriocidal or bacteriostatic mode of action, generally, inhibiting microorganism that were closely related to the producing strains.

The result was compatible with the result obtained by Drider *et al*., (2006), who observed that some bacteriocin produced by Lactobacilli had narrow inhibitory spectrum, while Silva ,(2002), found that some Lactobacilli bacteriocin could inhibit *S.aureus*, *L.monocytogenes* by production of antilisterial bacteriocin.

The result also agreed with the result mentioned by Messens, *et al*., (2002) who found that Lactobacilli were able to produce bacteriocin-like inhibitory substances and could reduce the risk of vaginitis. Generally, bacteriocin is antimicrobial protein produced by bacteria that kill or inhibit growth of other bacteria,(Cleveland *et al*., 2001).

Bacteriocin produced by endogenous bacteria may be critical for maintain of normal microflora and host health by preventing invasion by exogenous pathogens, (Brook, 2007).

3.2.3.2.3: Lactic Acid Production

As shown in table (3-8): changes in pH measurement of the media that contain lactobacilli after 24 hr. of incubation were attributed to lactic acid production. The results showed pH of the broth decreased after 24 hr of anaerobic incubation of Lactobacilli isolates and increase or returned to previous value (7) after 48 hrs. of incubation.

The result agreed with the result mentioned by Aroutcheva *et al*., (2001), who found that the pH of the media of lactobacilli was caused by secretion of lactic acid and other organic acids, they also found that about 100% of lactic acid produced by lactobacilli but the percentage differ according to species.

The result also correlated with the result obtained by Simoes *et al*., (2000) who found that after 22 hrs. of incubation of Lactobacilli pH decreased by 2 or 0.8 points.

Boskey *et al*., (1999), demonstrated that the antimicrobial action of lactic acid is largely but not totally assigned to it's ability in the undissociated form to penetrate the cytoplasmic membrane resulting in reduced intracellular pH and disruption of transmembranous protein motive force.

This low pH is inhibitory for many potentially pathogenic organisms that colonized the vagina.

Ogawa, (2001), found that some species of Lactobacilli exert growth inhibitory and bacteriocidal activities on some strains of *E.coli* depend on it's lactic acid production and pH reduction effect.

Eschenbach *et al*., (2000) found that the low vaginal pH is usually accredited to lactic acid producing Lactobacilli, they also found that during times of high oestrogen, large amount of glycogen are deposited in the vaginal epithelium and that the glycogen is anaerobically metabolized to lactic acid, so that the vaginal bacteria mainly lactobacilli were the primary source of lactic acid production.

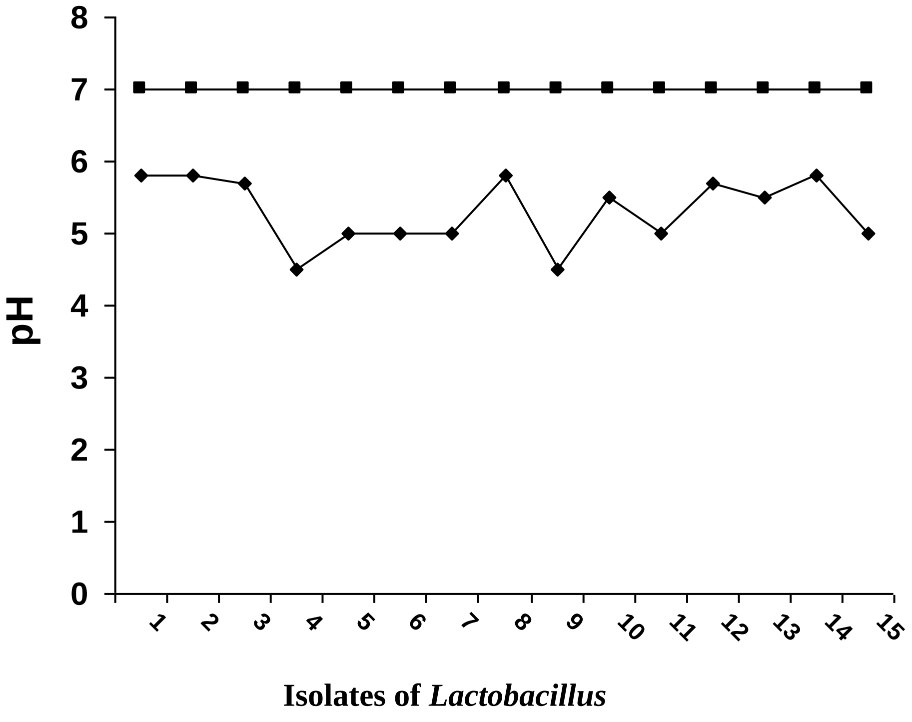

Figure (3-3): pH Changes in broth after anaerobic cultivation with Lactobacilli at 37 °C for 24 hrs.

3.3. Role of Lactobacilli as Probiotic Bacteria in Maintaing the Vaginal Health:

Lactobacilli play an importent role in maintaing the vaginal health by production of their defense factors and some of these defense factors have an inhibitory effect on some opportunistic pathogens (*S.aureus*, *E.coli*), this result agreed with the results mentioned by Ronnquist. *et al.,* (2006) who reported that the normal vagina of reproductive age women is predominately colonized with Lactobacilli which produce hydrogen peroxide, bacteriocins and lactic acid, these substances able to lower the vaginal pH, which create amore hostile environment for bacteria other than Lactobacilli if the number of Lactobacilli falls, the resulting increase in pH favors an overgrowth of anaerobic and facultative bacteria which can develop into vaginitis / vaginosis.

Hoyme and Saling, (2004) reported that the probiotic concept was also important in pregnant women by reducing the incidence of premature rupture of amniotic membranes which cause premature delivery and by the inverse association between Lactobacilli and group B *streptococci* and this very important for pregnant women since transfer of group B streptococci to neonates during delivery increases the risk of meningitis and other complications in newborn.

Anukam *et al*., (2006) mentioned that there was clearly potential for probiotics to help maintain good vaginal health and it was specially intriguing to note that even with the use of oral probiotic products, the vaginal milieu might be beneficially modified presumably, as a result of bacterial translocation from lower portion of the intestine to urogenital areas.

Sethi, *et al*., (2009) reported the role of Lactobacilli in preserving the vaginal health as being the predominant vaginal flora and mentioned that when Lactobacilli decreased due to many causes like douching, sexual practices and use of antibiotics; other pathogenic organisms would dominate. There is therefore, growing interest in the use of Lactobacilli of human origin as probiotics against genital infection (Garg *et al*., 2009).

Various clinical attempts had been carried out to assess the ability of Lactobacilli in prevention as well as treatment of genital infection (Dimitonova *et al*., 2007).

A recent study showed that the use of Lactobacilli containing-vaginal tablets helped in treatment of 61% of women suffering from vaginal infection in comparison to 19% of placebo treated patients (Mastromarino *et al*., 2009).

3.4. Effects of Antibiotics on Lactobacilli

Some antibiotics were used to show their effect on Lactobacilli isolates. It has been found that 93.3% of Lactobacilli isolates were resistant to vancomycin and ciprofloxacin, while the resistance for gentamycin were 53.3%, whereas some isolates were resistant to amoxicillin 40%, on the other hand, most of these isolates were susceptible with higher degree to erythromycin 93.4% (Figure 3-3).

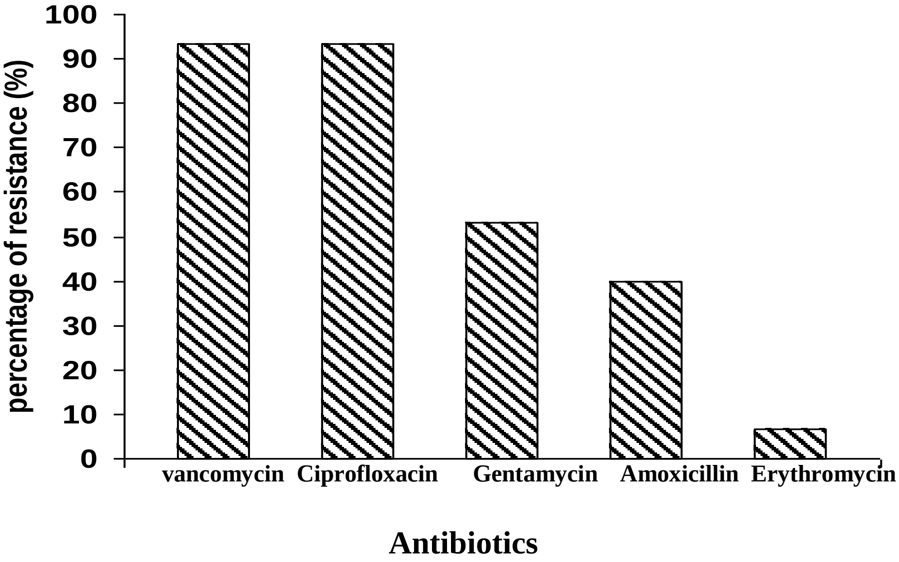

Figure (3-4): Antibiotics resistance of Lactobacilli.

The results showed that 93.3% of Lactobacilli isolates were resistant to vancomycin and ciprofloxacin, this result was in agreement with the result obtained by Coppola *et al*., (2005), who found that 100% of lactobacilli isolate were resistant to both ciprofloxacin and vancomycin and this resistance was important in selection of potentially probiotic Lactobacilli.

Robredo *et al*., (2000), found that several species of Lactobacilli including *L.rhamnosus* and *L.casei* were intrinsically resistant to vancomycin, these species had peptidoglycan precursors terminating with D-lactate instead of the target precursors for vancomycin activity terminating with D-alanine.

The result was in contrast with results of Mandar *et al*., (2001) who had indicated that there was mild to moderate resistance pattern of Lactobacilli against ciprofloxacin and vancomycin.

Alejdra *et al*., (2002), found that Lactobacilli were 50% resistant to vancomycin and most were sensitive to ciprofloxacin and this in contrast with our results. While the result also agreed with Wilks *et al*., (2004) who found that all isolates were ciprofloxacin resistant, while vancomycin resisted correlated with its species.

Regarding, gentamycin, the result showed that about 53.3% of Lactobacilli isolates were confer resistance to this antibiotic. This result was in agreement with Borriello *et al*., (2003) who found that probiotic Lactobacilli were 60% resistant to gentamycin. Other studies had recorded that there was an increase in the pattern of resistance of Lactobacilli species toward different inhibitors mainly protein synthesis (gentamycin, tobramicin) and nucleic acid inhibitors mainly (nalidexic acid and ciprofloxacin)(Mondar *et al*., 2001).

Elkins and Mullis, (2004) found that there was high percentage of Lactobacilli resistance to aminoglycosids (gentamycin, streptomycin, kanamycin).

Elmer, (2001) reported that although aminoglycosides have broad spectrum of activity, however, they are generally inactive against gram positive anaerobes and this is thought to occur because of membrane impermeability due to their characteristic multiple cationic charges, White, (1995) found that aminoglycoside have strong activity against aerobes because these bacteria generally have a high membrane potential which facilitate their entry by anelectrophoretic process.

As shown in figure (3-3), the result showed that about (40%) of isolate were resistant to amoxicillin, the result agreed with the result obtained by Herra *et al.*, (1995), who found that Lactobacilli resistance to amoxicillin was at low percentage and the Lactobacilli were mildly resistant to amoxicillin.

Regarding erythromycin, the result showed that the resistance of Lactobacilli was (6.6%) and about (93.4%) were susceptible to erythromycin and this correlated with the result obtained by Wilks *et al.*, (2004) who found that all isolates of Lactobacilli were erythromycin sensitive, In addition to Borriello, *et al.*, (2003) who found that probiotic Lactobacilli were susceptible to erythromycin at high percentage, the result also agreed with the result obtained by Felten *et al.*, (1999), who found that 90% of Lactobacilli were inhibited by erythromycin.

Charteris *et al*., (2001) reported that all Lactobacilli strains were resistant to vancomycin and sensitive to erythromycinn and ampicillin.

Danielson & Wind (2003) found that Lactobacilli resist to high level of antibiotics, these antibiotics have a broad spectrum of activity, however they are active also against gram positive anaerobes.

Mastromarino *et al*., (2002) found that few studies had been done on antimicrobial resistance of Lactobacilli for their non pathogenic nature as anaerobic commensals.

Zarazaga *et al*., (1999), observed that antibiotic resistance among Lactobacilli species and strains could be attributed to many factors including enzymatic inactivation, decrease intracellular drug accumulation or presence of gene that confer antibiotic resistance. Other studies carried out by Faro *et al*., (2001) showed that some Lactobacilli species were resistant to many tested antibiotics.

Charteris *et al*., (1998), found that Lactobacilli display a wide range of antibiotic resistance naturally.Plasmid-linked antibiotic resistance is not very common among Lactobacilli, since the transfer of antibiotic resistant genes may occur between phylogenically distant bacteria (Courvalin, 1994).

Ikaheimo, (1996) studied the effect of antimicrobial therapy on Lactobacilli and found that the antibiotic had an observable effect against the presence of Lactobacilli, these antibiotics can indiscriminately destroy both beneficial and pathogenic bacteria in the body, also the use of antibiotics could create an imbalance of the microflora with very negative effect, they cause the microflora to dislodge from epithelial cells of the vagina, thus allowing the attachment and proliferation of potentially putrefactive pathogenic organisms such as *Staphylococcus aureus, E.coli*, candida. So that the use of antibiotics by the patients to treat vaginitis should be very selective in order not to kill lactobacilli and this agreed with Reid, (2003) who reported that although antimicrobial agents are generally effective at eradicating the infection but, there was a high incidence of recurrence, the patient quality of life is affected and many women become frustrated by cycle of reported antimicrobial agent, whose effectiveness is diminished due to increasing development of microbial resistance, in addition, the use of antimicrobial agent is not only select resistance bacteria but, it can also disturb the balance of the body by killing friendly bacteria leading to urogenital tract infection.

3.5. Relationship Between Lactobacilli and Other Organisms Associated with Vaginitis

There was inverse relationship between Lactobacilli presence and the organism that caused vaginitis and the results showed that and attributed it to the production of defense factors of Lactobacilli, the results agreed with Sethi *et al*., (2009) who found that Lactobacilli constitute an important part of the urogenital tract microbiota and this indigenous bacteria considered as a natural resistant factor against potential pathogenic microorganisms and provide colonization resistant factor against opportunistic pathogens by producing autogenic regulation factors like lactic acid, hydrogen peroxide and bacteriocins.

Reid (2008) found that Lactobacilli produce a variety of substances such as bacteriocins which is toxic to other bacterial species, in addition acidification of the vagina due to lactic acid production is also protective while the production of hydrogen peroxide play the most important role against anaerobes and thus Lactobacilli producing H_2O_2 provide a protective role against vaginitis and acquisition of sexually transmitted infections.

3.6: Effect of Antibiotics on Bacterial Isolates

The effects of different antibiotics on bacterial isolates were investigated. The effect of amoxicillin and amoxiclav on bacterial isolates were studied as shown in figure (3-4), (3-5) respectively and it was shown that there was a relative increase in bacterial resistance (except for *L.monocytogenes*) to β-lactam antibiotic, this result agreed with the result obtained by Forbes, (2007), who had reported that *S.aureus* are the gram positive bacteria that most commonly produce β-lactamase enzymes which then breakdown the β-lactam ring and render it inactive, this is mediated by plasmid or by decreasing membrane permeability toward antimicrobial agents (Barid, 1996). In addition the low affinity of binding of antibiotics to PBP confer resistance to these antibiotics, this result was similar to those asserted by Anq *et al*., (2004), while in gram negative bacteria, resistance can be mediated by decrease uptake through the outer membrane porins.

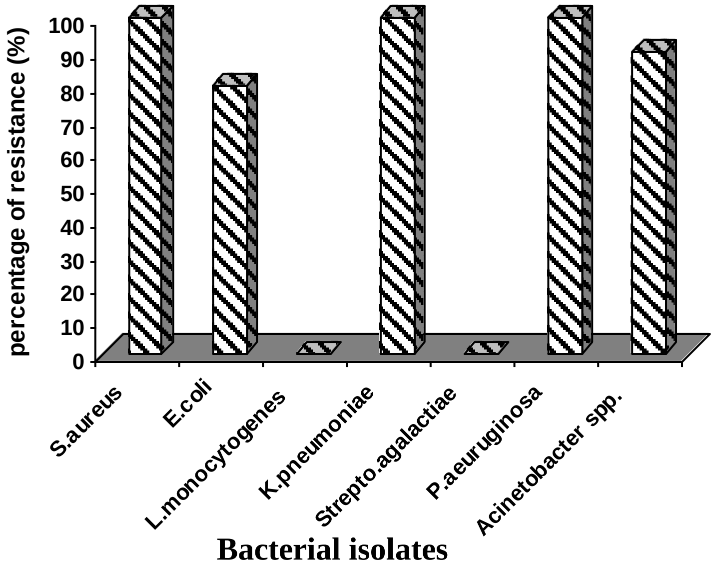

Figure (3-5): Percentage of bacterial resistance to amoxicillin.

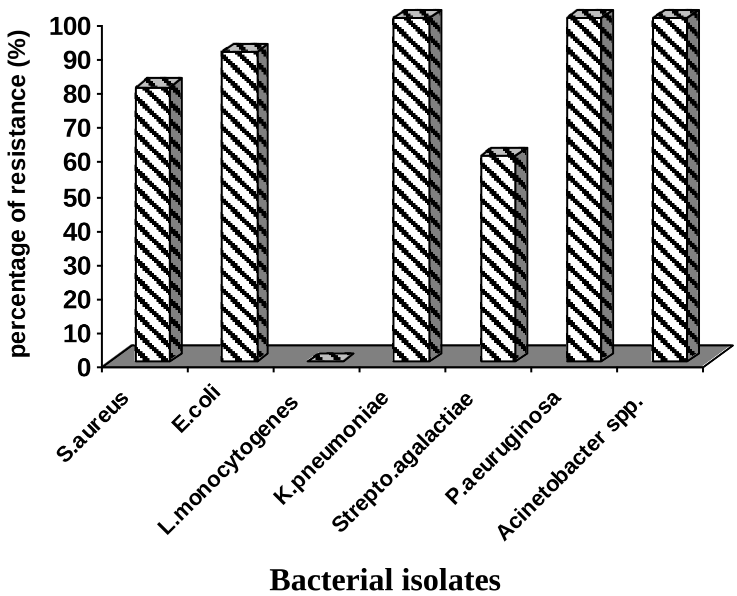

Figure (3-6): Percentage of bacterial resistance to amoxiclav.

The effects of some cephalosporin which include cephtazidime, cefalexine, cefotaxime on bacterial isolates had been also investigated as shown in figure (3-6), (3-7), (3-8), respectively. As shown most bacteria resist the action of ceftazidime and cephalexine (except *L.monocytogenes*).

Regarding cefotaxime: which is a third generation cephalosporin, the results showed that all isolates of *Klebsiella* spp, *S.aureus, P.aeruginosa* were (100%) resistant to cefotaxime while *E.coli* and *Streptococcus agalactiae* were both 50% resistant, and the resistance decreased gradually to (40%) for *Acinetobacter spp*. On the other hand, *L.monocytogenes* were completely (100%) sensitive to cefotaxime.

The resistance of *P.aeruginosa* isolates to cephalosporin may be due to synthesis of β-lactamse as well as loss of PBP by mutation, this bacteria exhibits intrinsic or acquired resistance to many antibiotics, *P.aeruginosa* are highly inherently resistant and this arises from combination of usually resisted outer membrane permeability and chromosomally-encoded β-lactamase, this agreed with the result mentioned by (Hankok & Speert, 2000) and(Bisklis *et al*., 2005).

S.aureus resistance result was agreed with Hanumanthappa *et al*., (2003) who pointed out that (82.4%) of *S.aureus* strains were resistant to cefotaxime.

Regarding *Klebsiella pneumoniae*, the resistance to cefotaxime was probably attributed to the fact that most clinical isolates of *K.pneumoniae* produce different plasmid and/or chromosomal-mediated β-lactamase enzymes, as plasmid mediated enzyme production is much more easily transferred to cells. There had been increased in β-lactamase resistance to third generation cephalosporin. These enzymes are called extended spectrum β-lactamase(Bedenic *et al*., 2001).

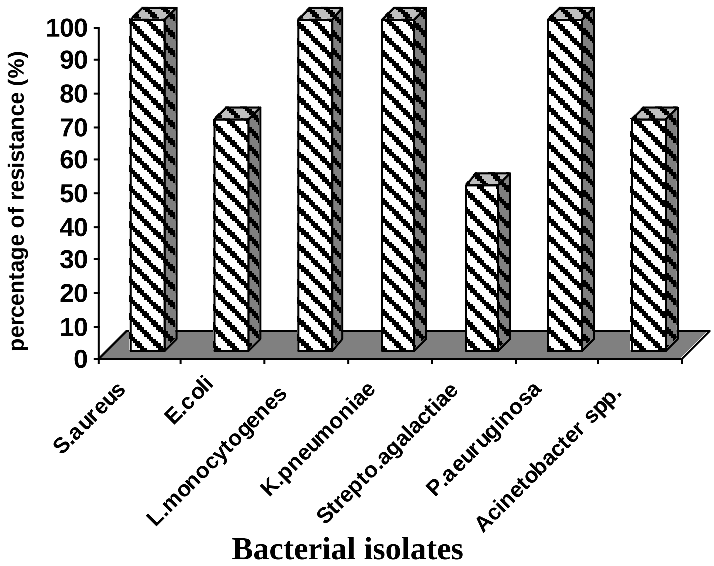

Figure (3-7): Percentage of bacterial resistance to cephtazidime.

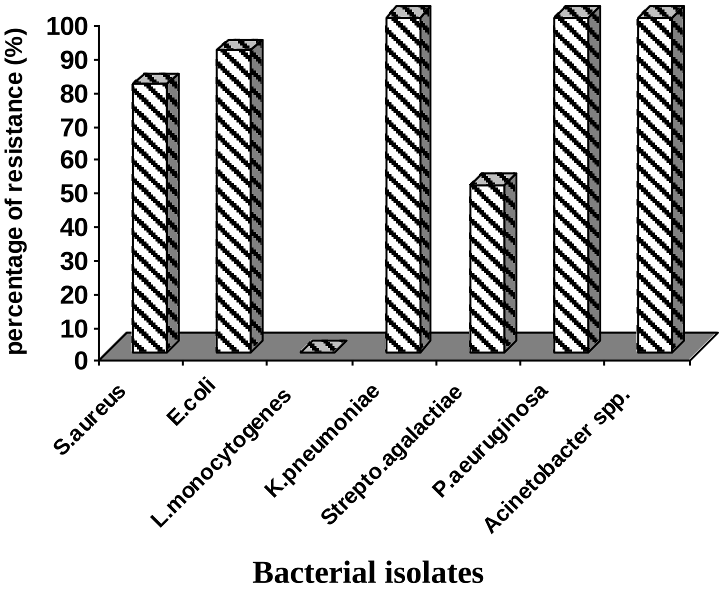

Figure (3-8): Percentage of bacterial resistance to cefalexine.

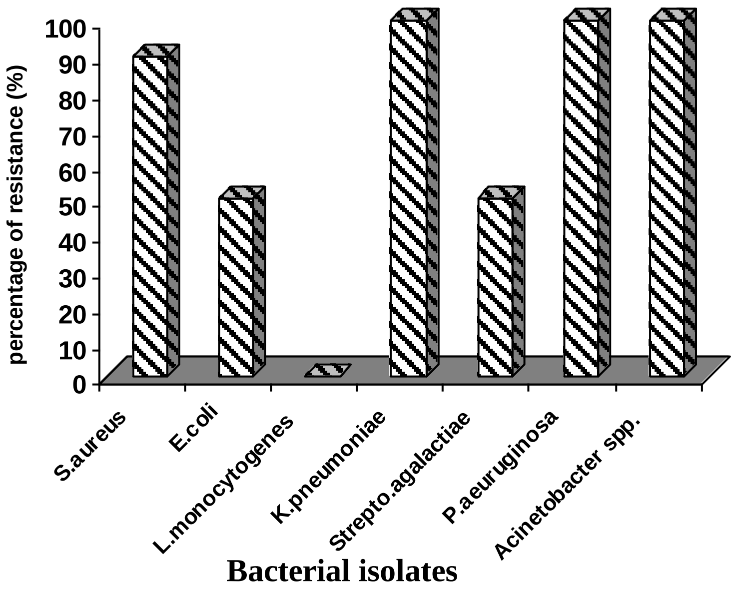

Figure (3-9): Percentage of bacterial resistance to cefotaxime.

Bacterial resistance to some of the macrolide group which includes azithromycin and erythromycin had also been investigated as shown in figure (3-9), (3-10), respectively.

The result showed that most bacteria were resistant to these antibiotics except L.monocytogenes and Streptococcus agalactiae which were completely susceptible to macrolid group followed by *S.aureus* . the results showed that the effect of this antibiotic was more on gram positive bacteria than on gram negative bacteria, this agreed with Forbes, (2007), who had reported that macrolides were not effective against most genera of gram negative bacteria.

Imamura *et al*., (2005), showed that azithromycin had bacteriocidal activity against *P.aeruginosa*, it directly disrupted outer membrane of *P.aeruginosa*, probably by acting to competitively displace of divalent cations from their binding sites on lipopolysaccharides (LPS) on the outer membrane, azithromycin was capable of permeabilizing the outer membrane of *P.aeruginosa* and that action was antagonized by mg^{+2} and this direct action of it may contribute to it's bacteriocdal activities against *P.aeruginosa*.

Regarding erythromycin, the result agreed with that mentioned by Chigbu & Ezerony, (2003) who found that (32%) of *S.aureus* were resistant to erythromycin.

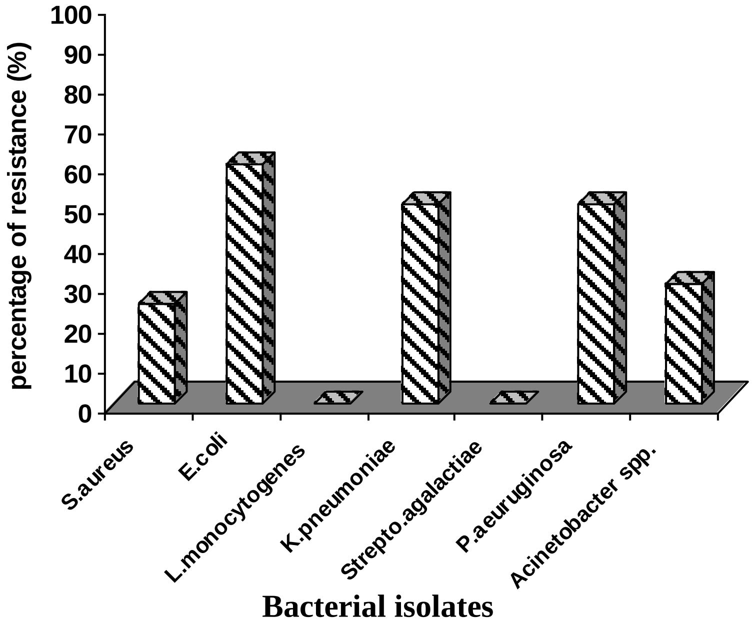

Figure (3-10): Percentage of bacterial resistance to azithromycin.

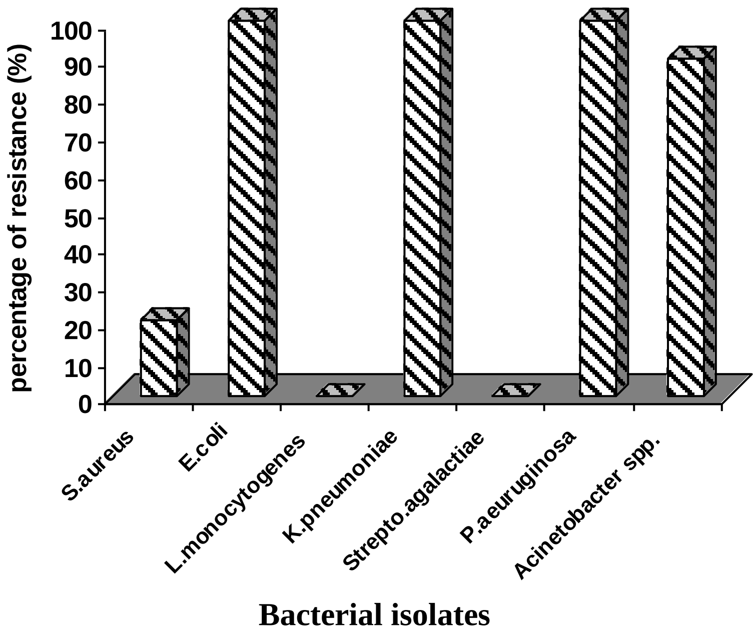

Figure (3-11): Percentage of bacterial resistance to erythromycin.

Bacterial resistance to aminoglycosides had also been studied which include gentamycin and amikacin as shown in figure (3-11), (3-12) respectively. The result showed that most bacteria were resistant to gentamycin except *L.monocytogenes* and *Streptococcus agalactiae*, which were completely 100% susceptible to gentamycin.

Amikacin can be considered the more effective antibiotics on gram positive and gram negative bacteria (Forbes, 2007).

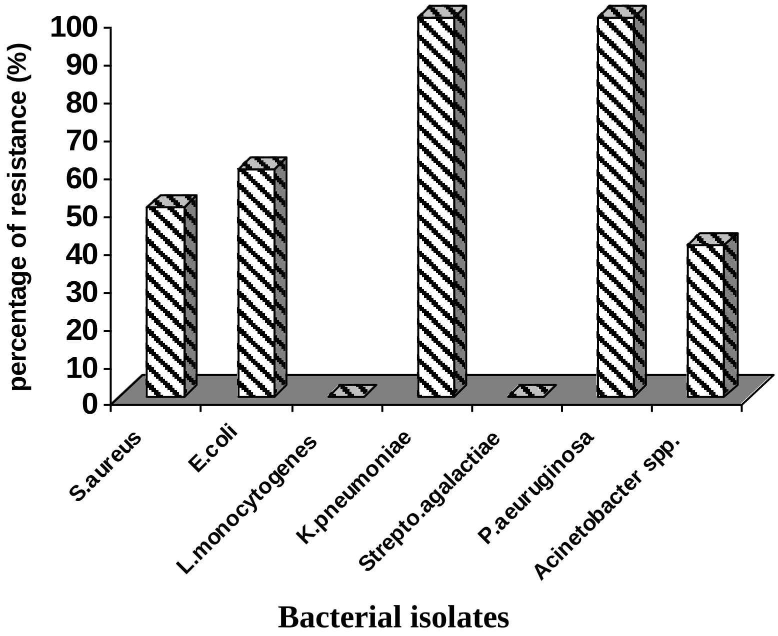

Figure (3-12): Percentage of bacterial resistance to gentamycin.

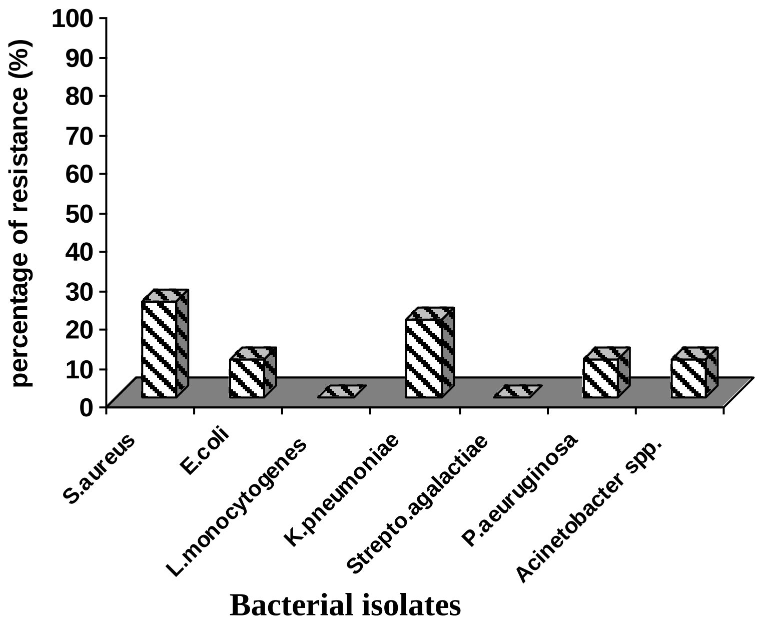

Figure (3-13): Percentage of bacterial resistance to amikacin.

The effect of ciprofloxacin was also studied as shown in figure (3-13), and it was found that most bacterial isolates were susceptible to ciprofloxacin and this agreed with Forbes, (2007), who had reported that fluoroquinolones are potent bacteriocidal agents and they have abroad spectrum activity that include gram positive and gram negative bacteria.

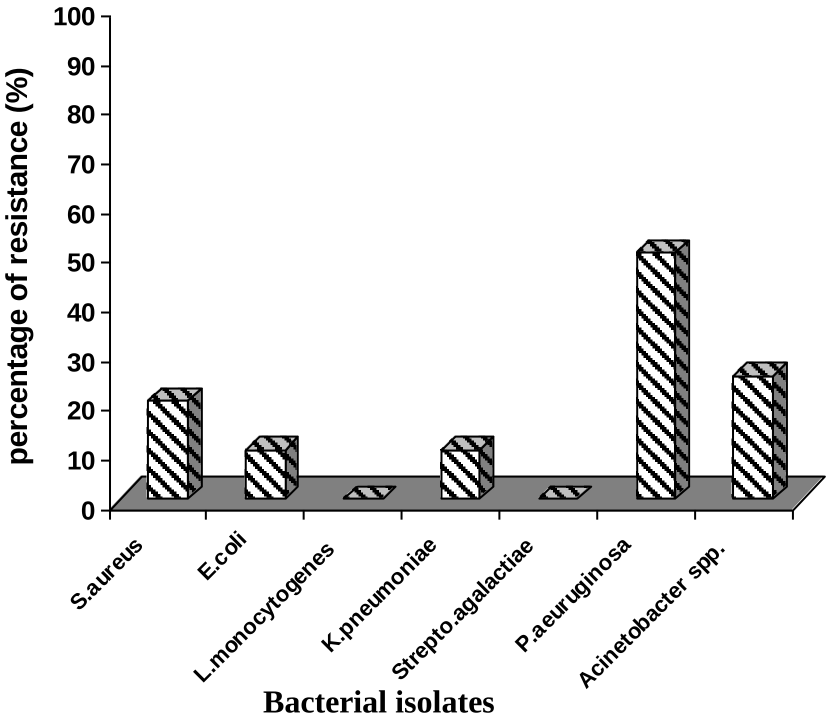

Figure (3-14): Percentage of bacterial resistance to ciprofloxacin.

3.7. Relationship Between Antibiotics That Affect Both Lactobacilli and Opportunistic Pathogens

It was shown in this study that some antibiotics were active against some opportunistic pathogens but inactive against Lactobacilli and unable to kill friendly bacteria like Lactobacilli and this observed by the effect of ciprofloxacin to which 93.3% of Lactobacilli isolates were resistant while it was considered the most effective against opportunistic pathogens. On the other hand, erythromycin to which 6.6% of Lactobacilli isolates were resistant while opportunistic pathogens showed higher degree of resistant to erythromycin except *L.monocytogenes* and *Streptococcus agalactiae*.

Regarding amoxicillin, the result showed that the opportunistic bacterial isolates had high resistance to β-lactam antibiotic while 40% of Lactobacilli isolates were resistant to amoxicillin.

Regarding other antibiotics there was different resistant pattern observed between both groups studied and from these results, the using of appropriate antibiotic which is highly selective is very important in order not to kill the friendly bacteria and to maintain the vaginal health like for e.g. prescribtion of ciprofloxacin to kill opportunistic pathogen and maintain Lactobacilli or avoiding erythromycin since 93.4% of Lactobacilli were sensitive to it.

Conclusions

According to this study, we can conclude the following:-

1. Lactobacilli have a protective role against vaginitis.
2. The production of H_2O_2 by *Lactobacillus* can be affected by the type of incubation *in vitro*.
3. The production of bacteriocin, lactic acid, H_2O_2 can be attributed to some species of Lactobacilli.
4. Antagonism between Lactobacilli species is present to maintain dominance.
5. Isolation of Lactobacilli are also affected by the type of antibiotic used to treat vaginitis where 93.3% of Lactobacilli isolates resist the effect of ciprofloxacin while 93.4 were sensitive to erythromycin
6. Vaginitis are more common in married than unmarried women especially those with intra uterine device.

Recommendations

According to this study, we can recommend the following points:

1.Using of proper antibiotics, which are highly selective against opportunistic pathogens and have no effect on Lactobacilli .

2.Studying of the probiotic role of Lactobacilli as a prophylactic and biotherapy means for disease other than vaginitis

3. Using of API system depending on enzymatic and biochemical tests for isolation and identification of lactic acid bacteria.

4. Introducting of new molecular techniques (PCR) for identification of Lactobacilli species and subspecies and correlate them with each virulence factors.

5. Studying other innate immune aspects of female urogenital tract.

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Contents

Dedication

To……

The memory of my mother,

My father,

My brothers and sister,

The Angel of mercy, my aunt,

The kind heart and my support in life

my husband Dr. Nawres

My flowers: Mustafa and Yousif

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List of Abbreviations

Symbol	Description
BV	Bacterial Vaginosis.
GBS	Group B *Streptococcus*
HRT	Hormonal Replacement Therapy.
lUD	Intrauterine Device.
MR	Methyl Red.
MRS	De Man-Rogosa-Sharpe.
PBPs	Pencillin Binding Proteins
PCR	Polymerase Chain Reaction
pH	Power of H^+ ion concentration
RTI	Reproductive Tract Infection
STD	Sexual Transmitted Disease
TMB	Tetra Methyl Benzidine
VP	Vogus Proskaur

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We, the examining committee, certify that we have read the thesis entitled (**A study of the Relationship Between Lactobacilli and Opportunistic Pathogens Associated with Vaginitis in Babylon Province**) and have examined the student (**Bara' Hamid Hadi AL-Greitty)** in its contents, and that in our opinion it is accepted as a thesis for the degree of Master of Science in Microbiology.

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