Republic of Iraq
Ministry of Higher Education
And Scientific Research
Al-Mustansiriyah University College of Science

Effects of some Environmental Growth Conditions on Autoaggregation of *Lactobacilli spp.* Isolated from Bacterial Vaginitis Infected Females

A Thesis

Submitted to the Council of College of Science

The University of Mustansiriyah

In Partial Fulfillment of the Requirements for

The Degree of Master of Science in Biology/Microbiology

By

Susan Abdul Raheem Hasan

B. Sc. Microbiology
2002

Supervised By

Dr. Mohammed F. Ahmed — **Assistant. Prof**

Dr. Safa'a A. Abd Al Razzaq — **Consultant Physician**

2013/ July **1434/ Ramadan**

Certification

We certify that this thesis ***“*Effects of some Environmental Growth Conditions on Autoaggregation of *Lactobacilli spp.* Isolated from Bacterial Vaginitis Infected Females*"*** by **Susan Abdul Raheem Hasan** was prepared under our supervision at the Biology Department in the College of Science, University of Al-Mustansiriyah as a partial requirement for the achievement of the degree of Master of Science/ Microbiology.

Signature:	**Signature:**
Supervisor: Dr. Mohammed F. Ahmed	**Co-Supervisor: Dr. Safa’a A. Abd Al Razzaq**
Assist Professor	**Consultant Physician**
Date: / / 2013	**Date: / / 2013**
Biology department/ College of Science/ Al-Mustansiriyah University	**Medicine City**

In view of the available recommendations, I forward this thesis for the debate by an Examination committee.

Signature:

Prof. Dr. Abd AL-Amer N. AL-Rikabi

Date: / / 2013

Head of Biology Department

College of Science- Al-Mustansiriyah University

بسم الله الرحمن الرحيم

يُؤْتِي الْحِكْمَةَ مَن يَشَاءُ وَمَن يُؤْتَ الْحِكْمَةَ فَقَدْ أُوتِيَ خَيْرًا كَثِيرًا

وَمَا يَذَّكَّرُ إِلَّا أُولُو الْأَلْبَابِ

صدق الله العظيم

سورة البقرة / الآية (269)

Dedication

TO the spirit of my mother who gave me everything, but I can't gave her anything.

The one supported me from my birthday until her death.

For her memory, this is the only thing that I can ever gave.

Acknowledgements

First and foremost, I owe my deepest gratitude to the Almighty God, for His blessings that make me enable to complete my research.

I am heartily thankful to my supervisor, Dr. Mohammed F. Ahmed, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding the project. His wide knowledge and his logical way of thinking have been of great value for me. His understanding, encouraging and personal guidance have provided a good basis for the present research. I will never forget that he has been my inspiration as I hurdle all the obstacles in the completion this research.

I also would like to acknowledge the contributions to my co-supervisor, Dr. Safa'a A. Abd Al Razzaq whom gave attention to keep the concepts and information clear in developing of my research.

Spesial thank to Dr. Jamila Al Saadi for her help as the first one who gave me a hand in my work.

I wish to express my appreciation to my colleagues in the laboratory, especially Miss Israa Al-Kadmy for her technical help, and friendly support. The timely assistance provided by the staff members of the department is gratefully acknowledged.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of this research.

SUSAN

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviations	Key
bp	base pair
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
PCR	Polymerase chain reaction
rpm	Revolution per minute
TBE	Tris Borate EDTA
UTI	Urinary tract infection
UV	Ultra violate radiation
w/v	Weight per volume
kb	kilo base
μg	Microgram
mg	Milligram
min	Minute
HIV	Human immunodeficiency virus
μL	Microliter
pH	Potency of hydrogen ions
BV	Bacterial vaginosis

Abbreviations	Key
rRNA	ribosomal Ribonucleic acid
spp.	species
L.	*Lactobacillus*
MRS	Man Rogosa Sharpe
PBS	phosphate buffere saline
Hrs.	hours
LAB	Lactic Acid Bacteria
RFLP	Restriction Fragment Length Polymorphism
PFGE	Pulse-Field Gel Electrophoresis
DGGE	Denaturing Gradient Gel Electrophoresis
TGGE	Temperature Gradient Gel Electrophoresis
Temp.	Temperature
No.	Number
OD	optical density
t	time

Summary

Of forty two adult females suffering from bacterial vaginitis, only ten isolates of Lactobacilli spp. were isolated. In addition, two isolates from healthy females were used as a control in this study**.**

To prevent contamination with other vaginal pathogenic bacteria, isolation procedure of Lactobacilli spp. from bacterial vaginitis infected females required a shifting in growth medium conditions. This shifting included an alternative change in pH from 6.2 to 4.0 and again to 6.2.

The results of molecular diagnosis using 16S rRNA of these ten bacterial vaginitis Lactobacilli isolates indicated that six isolates were diagnosed as *Lactobacillus crispatus*, while the other four isolates were diagnosed as *Lactobacillus gasseri*. In addition, two healthy vaginal Lactobacilli isolates were diagnosed as *Lactobacillus crispatus* and *Lactobacillus gasseri*. The diagnosis protocol included application of a specific forward and reverse 16S rRNA primers and amplicon size of 154bp (for *Lactobacillus crispatus*) and 322bp (for *Lactobacillus gasseri*). For both primers, the optimum annealing temperature was found to be 52°C**.**

The results of the visual analysis of autoaggregation showed that *L. gasseri* demonstrated a huge masses of autoaggregation, compared to *L. crispatus* that revealed a smaller masses of autoaggregation.

The visual analysis of autoaggregation showed that *L. gasseri* demonstrated a huge masses of autoaggregation, compared to *L. crispatus* that revealed a smaller masses of autoaggregation.

The results of autoaggregation growth factors by using spectrophotometric method indicates that, regardless to aeration status, the highest percentage of autoaggregation occurred when growth pH was 5 and at a temperature of 37°C. It gave a values of 70% (for *L. gasseri)* and 61% (for *L. crispatus*). While the lowest percentage of autoaggregation taken place when the growth pH was 8 and at growth temperature of 44°C. It gives a values of 27% (for *L. gasseri*) and 8% (for *L. crispatus*).

Anaerobic conditions showed a highest autoaggregation percentage compared to aerobic conditions. It gave a values of 50% (for *L. gasseri*) at anaerobic conditions, (compared to 35% at aerobic conditions), while *L. crispatus* gave a value of autoaggregation of 30% at anaerobic conditions, (compared to 26% at aerobic conditions).

With respect to growth conditions, no obvious differences were noticed upon growing of the Lactibacilli at hyperthermic (38-39°C) and hypothermic (35-36°C) temperatures for *L. gasseri* and *L. crispatus*.

Introduction

Lactobacilli indigenous to the human vagina are beneficial to women's health (Redondo-Lopez *et al*., 1990). These bacteria can inhibit other potentially harmful microorganisms by producing lactic acid, hydrogen peroxide (H2O2), and antimicrobial substances (Hallen *et al*., 1992; Klebanoff *et al.*, 1991; Zheng *et al*., 1994). In most healthy women, Lactobacilli are the dominant species in the vagina. Theoretically, the anaerobic bacteria are suppressed by Lactobacilli (Hallen *et al*., 1992; Klebanoff *et al.*, 1991) and cannot replace Lactobacilli unless the latter is first diminished. However, the group of anaerobic bacteria commonly outnumber Lactobacilli, causing a microbial imbalance called bacterial vaginosis (BV) (Amsel *et al*., 1983; Eschenbach *et al*., 1989; Eschenbach *et al*., 1988; Hill, 1993; Sobel, 1997; Spiegel, 1991). BV is a clinical condition that is characterized by decreased Lactobacilli and an increased number of anaerobic gram-negative rods, *Gardnerella* species, and genital mycoplasmas (Eschenbach *et al*., 1988; Sobel, 1997; Spiegel, 1991). Women who suffer from BV may have an increased discharge that often has an unpleasant fishy odor. BV It has been associated with many health risks, including preterm birth of low-birth-weight infants, midtrimester pregnancy loss, amniotic fluid infection, postpartum endometritis, pelvic inflammatory disease, and gynecologic postoperative infections (Hay *et al*., 1994; Hillier *et al*., 1995; Hillier *et al*., 1995;

Martius *et al*., 1988; McGregor *et al*., 1995). Recently, a lack of vaginal Lactobacilli or the presence of BV was found to promote human immunodeficiency virus transmission (Cohen *et al*., 1995; Martin *et al*., 1999; Sewankambo *et al*., 1997). The cause of BV is currently unknown, and it is unclear what causes the decrease of vaginal Lactobacilli. Several possible mechanisms by which vaginal Lactobacilli decrease have been proposed. These include douching (Harwood *et al*., 1996); the use of spermicide, such as nonoxynol-9 (Hooton *et al*., 1991); and treatment with antibiotics for other infections (Kilic *et al*., 2001; Andreu, 2004). The aggregation ability comprises autoaggregation, (The bacteria ability to form multicellular aggregates) has been shown to play an important role in colonization of the urogenital tract (Mastromarino *et al*., 2002; Reid *et al*., 1990), characterized by clumping of cells of the same strain, and coaggregation,in which genetically distinct cells are involved (Kolenbrander, 1988).

The autoaggregation ability is dependent on environmental factors (such as pH and heat conditions) (Ekmekci *et al.*, 2009). Moreover, the cell surface properties of bacteria are thought to play an important role in autoaggregation. It has been suggested that lipoteichoic acids, proteins, and carbohydrates on the bacterial surface, soluble proteins, or pheromones are involved in the aggregation ability of bacteria (Ocaňa and Nader-Macias, 2002).

However, the identification of *Lactobacillus* isolates by phenotypic methods is difficult because it requires, in several cases, determination of bacterial properties beyond those of the common fermentation tests (for example, cell wall analysis and electrophoretic mobility of lactate dehydrogenase) (Kandler and Weiss, 1986). Moreover, the derivation of simple yet rapid identification methods is therefore required in order to deal with the large numbers of *Lactobacillus* isolates obtained during microbial ecological studies of ecosystems. However, the use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons (Patel, 2001). According to this type of identification, the taxonomy of *Lactobacillus* has expanded. For example, *L. acidophilus,* which previously cannot be distinguished biochemically has been subdivided into six distinct species; *L. acidophilus, L. crispatus, L. gasseri, L. gallinarum, L. amylivorus* and *L. johnsonii* (Du Plessis and Dicks, 1995).

Therefore, the objective of this study are:

1- To isolate and identify Lactobacilli spp. isolated from females, who are suffering from urogenital tract infections, besides normal females, as a control, by using classical and molecular diagnosis of 16S rRNA.

2- To investigate the effects of pH, temperature, aerobic and anaerobic condition on the autoaggregation ability of these BV Lactobacilli isolates by using visual and spectrophotometric methods for autoaggregation analysis.

Literature review

1.1 *Lactobacillus*:

Lactobacillus called Döderlein's bacillus, is a genus of Gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria (Makarova *et al.*, 2006). They are a major part of the lactic acid bacteria group, named as such because most of its members convert lactose and other sugars to lactic acid. Orla-Jensen laid the foundations for a classification based on four genera of lactic acid bacteria: *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* (Orla-Jensen, 1919). In 1928, Thomas first described Döderlein's bacilli as *Lactobacillus acidophilus* (Thomas, 1928). The traditional phenotypic methods that were available, and which are still very important in current classifications, are: morphology, mode of glucose fermentation, growth at certain "cardinal" temperatures (e.g. 10°C and 45°C), and range of sugar utilization (Axelsson, 1998). These and other characteristics have not been useful for discriminating the closely related bacteria in the ecological niche of the normal human vagina, which mainly belong to the *Lactobacillus* (Carlsson *et al*., 1975). Other earlier studies using the classic phenotypic identification methods demonstrated heterogeneity of the flora, for reviews see Redondo-Lopez and Zhong (Redondo-Lopez, 1990; Zhong *et al*., 1998). Modern phylogeny of Lactobacilli presents six or seven different groups based on

16S rDNA sequences: *L. buchneri* group, *L. casei* and *L. sakei* group, *L. delbrüeckii* or *acidophilus* group, *L. plantarum* group, *L. reuteri* group and finally *L. salivarius* group (Hammes *et al*., 2006). DNA-DNA hybridization as well as phenotypic characters was used by Giorgi (Giorgi *et al*., 1987) for the study of vaginal Lactobacilli isolated from asymptomatic women, and these were identified as *L. gasseri*, *L. jensenii* and *L. crispatus*.

Lactobacilli have usually been considered to be non-pathogenic to man and have in recent years been actively investigated for their potentially beneficial effects (Harty *et al*., 1994). There is much interest in the use of Lactobacilli as probiotics against human gastrointestinal disorders (Song *et al*., 2000). The healthy human vagina is dominated by Lactobacilli, which play an important role in protecting the host from urogenital infections (Boris *et al*., 1998; Martin *et al*., 1999; McLean *et al*., 2000). Furthermore, it is widely recognized that the microbial balance between Lactobacilli as the dominating flora and other, mainly gram-negative anaerobes can be upset and frequently result in the syndrome of bacterial vaginosis (Spiegel, 1991).

1.2. Classification:

According to Winn *et al*., (2006) *Lactobacillus* is classified as follows:

Kingdom: Eubacteria

Phylum: Firmicutes

Class: Bacilli

Order: Lactobacillales

Family: Lactobacillaceae

Genus: *Lactobacillus*

1.3. Morphology:

Lactobacillus is Gram-positive, non-spore forming, the species are varying from long, straight rods, ranging from (0.5-1.2) µm by (1-10) µm in size, seen as single or in short or long chains (Kandler and Weiss, 1986; karthikeyan and Santosh, 2009). Colonies on agar media are usually small (2-5) mm with entire margin, convex, smooth and opaque without pigment (Kandler and Weiss, 1986).

1.4. Diagnosis:

1.4.1. Classical Diagnosis:

The identification of *Lactobacillus* isolates by phenotypic methods is difficult because it requires, in several cases, determination of bacterial properties beyond those of the common fermentation tests (for example, cell wall analysis and electrophoretic mobility of lactate dehydrogenase) (Kandler and Weiss, 1986). In general, about 17 phenotypic tests are required to identify a *Lactobacillus* isolate accurately to the species level (Hammes and Vogel, 1995). For thirty-two years between 1928 and 1960, the dominant *Lactobacillus* species in the vagina was believed to be *Lactobacillus acidophilus* (Thomas, 1928; Rogosa and Sharpe, 1960). Moreover, the derivation of simple yet rapid identification methods is therefore required in order to deal with the large numbers of *Lactobacillus* isolates obtained during microbial ecological studies of ecosystems.

1.4.2. Molecular diagnosis:

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001).

According to this type of identification, the taxonomy of Lactobacilli has expanded. For example, *L. acidophilus,* which previously cannot be distinguished biochemically has been subdivided into six distinct species; *L. acidophilus, L. crispatus, L. gasseri, L. gallinarum, L. amylivorus* and *L. johnsonii* (Du Plessis and Dicks, 1995). Due to such complexity, the aim of the present work was designed to isolate, identify and diagnose of the vaginal Lactobacilli from females, who are suffering from urogenital tract infections, in addition to the normal females, in Baghdad city using molecular technique.

1.4.2.1. Molecular diagnosis of *lactobacillus*:

The taxonomy of LAB based on comparative 16S ribosomal RNA (rRNA) sequencing analysis has revealed that some taxa generated on the basis on phenotypic features do not correspond with the phylogenetic relations. Molecular techniques, especially polymerase chain reaction (PCR) based methods, such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse-field gel electrophoresis (PFGE), are regarded important for specific characterization and detection of LAB strains (Gevers *et al*., 2001; Holzapfel *et al*., 2001). Recently, culture-independent approaches have been applied for the detection of intestinal microbiota (Zoetendal *et al*., 2002). Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) analysis of faecal 16S rDNA gene and its rRNA amplicons have shown to be powerful approaches in determining and monitoring the bacterial community in feces (Zoetendal *et al*., 1998).

1.4.2.2. Application of molecular diagnosis for bacterial diagnosis:

Marked changes have occurred in bacterial classification since the application of molecular technologies to this task. The impetus for major change has resulted from the observation that 16S ribosomal RNA (rRNA) sequences can be used as evolutionary chronometers (Woese, 1987). Some regions of the 16S rRNA molecule are conserved throughout all bacterial species and can be used to align sequences obtained from different isolates. From a practical point of view, the 16S rRNA gene sequences (rDNA) can be used in the reliable identification of many bacterial species through the derivation of specific oligonucleotide probes or polymerase chain reaction (PCR) based techniques (Langendijk *et al*., 1995; Vandamme *et al*., 1996; Wang *et al*., 1996; Welling *et al*., 1997).

1.4.2.3. Application of molecular diagnosis for *Lactobacillus* diagnosis:

Traditionally, *Lactobacillus* and species have been identified on the basis of cell morphology, analysis of fermentation products and associated enzyme activities, and the ability to utilize various carbohydrate substrates. The application of these approaches in the classification and identification of Lactic Acid Bacteria has been the subject of several reviews and will not be covered in this article (Hammes and R. F., 1995; Pot *et al*., 1994; Sgorbati, 1995; Tannock, 1999). Suffice it to say that, in general, phenotypic methods suffer from a lack of reproducibility generated by conditions of culture related to different laboratories, and to the diversity of strains (biotypes) that comprise the recognised species (Ballongue, 1993; Hammes and R. F., 1995; Sgorbati, 1995). Nucleic acids are universal in cellular biology, and the nucleotide base sequence of these molecules is not influenced by culture conditions. Analysis of nucleic acids thus provides a basis for identification methods that are reproducible from one laboratory to another. Genotypic approaches hold the most promise for the rapid and accurate identification of Lactobacilli.

1.5. Existence:

Bacteria of the genus *Lactobacillus* have been proposed as probiotic microorganisms to restore the ecological equilibrium of the intestinal, respiratory, and urogenital tracts (Hammes *et al*., 1995). *Lactobacillus* species are phylogenetically diverse and are found naturally in milk, plants, meats, and the mucosal surfaces (oral, intestinal, and reproductive tracts) of humans and animals.

1.6. Importance of *Lactobacillus*:

Lactobacilli are believed to interfere with pathogens by different mechanisms. The first is competitive exclusion of genitourinary pathogens from receptors present on the surface of the genitourinary epithelium (Chan *et al*., 1985; Velraeds *et al*., 1996). Second, lactobacilli coaggregate with some uropathogenic bacteria (Redondo-Lo´pez, 1990), a process that, when linked to the production of antimicrobial compounds, such as lactic acid, hydrogen peroxide, bacteriocin-like substances (McGroarty and G. Reid, 1988; Reid *et al*., 1988), and possibly biosurfactants (Velraeds *et al*., 1996), would result in inhibition of the growth of the pathogen. Adherence of bacteria to epithelial cells has been shown to be an important factor in the colonization of mucous membranes.

1.7. Effect of Lactobacilli:

1.7.1. Effects on dairy product:

Lactic acid bacteria (LAB) are known to produce a variety of antimicrobial compounds, of which bacteriocins are the most promising as they can be used as natural and safe food preservatives. Bacteriocins are ribosomally synthesized peptides that not only inhibit bacteria closely related to the producer strain but also food-borne pathogens and spoilage bacteria (Klaenhammer, 1988). Currently, due to consumer demand for reduction of chemical additives, bacteriocins have attracted increasing interest. Although several LAB strains have been reported as bacteriocin producers, their affectivity ranges from narrow to broad spectrum types. Also, some bacteriocins could exhibit their antibacterial activity not only under acidic conditions, but also under neutral and/or weak alkaline conditions (Hata *et al.,* 2009). Therefore, Biochemical characterization of bacteriocins in terms of their stability, host range and mode of antimicrobial action is essential so as to evaluate their possible potential as food preservatives. They also represent one of the most prominent groups of microorganisms, having been used for centuries in the bioprocessing of foods, notably in fermented dairy products, vegetables and meats and in sourdough (Goh and Klaenhammer, 2010).

1.7.2. Effects on intestine:

LAB have been cited to be part of human (Holzapfel *et al*., 2001; Reid, 2001; Schrezenmeir, 2001; Sghir *et al*., 2000). LAB constitute an integral part of the healthy gastrointestinal (GI) microecology and are involved in the host metabolism (Fernandes *et al*., 1987). LAB along with other gut microbiota ferment various substrates like lactose, biogenic amines and allergenic compounds into short-chain fatty acids and other organic acids and gases (Gibson and Fuller., 2000; Gorbach, 1990; Jay, 2000). LAB synthesize enzymes, vitamins, antioxidants and bacteriocins (Fernandes *et al*., 1987; Knorr, 1998). With these properties, intestinal LAB constitute an important mechanism for the metabolism and detoxification of foreign substances entering the body (Salminen, 1990).

1.7.3. Effects on mouth:

It is estimated that more than 1010 bacteria per gram of dental plaque colonize the human oral cavity. More than half of them still remain uncultivable. Their existence is only known because a fingerprint in form of a sequence from a gene fragment, most often from 16S rDNA, could be traced in a clinical sample (Aas *et al*., 2005). All oral microorganisms form biofilms on surfaces such as the oral mucosa, the tongue, or the surface of the teeth. Many supragingivally predominant bacteria belong to the Firmicutes phylum (Gram-positive rods and cocci of low G+C content) with the lactic acid producing bacteria (LAB) as the largest and clinically important subgroup (Kilian, 2005; Marsh and Martin, 2009). LAB are main constituents of the commensal microbiota of the human oral cavity, but form also part of the biofilms colonizing the upper respiratory, intestinal and urinary tracts. In the oral cavity, they are thought to play major roles in dental plaque formation and oral biofilm homeostasis. However, under conditions of prolonged shifts of biofilm composition, LAB may induce dental caries through excessive lactic acid formation (Marsh and Nyvad, 2008), and upon penetration into the blood stream LAB may cause in susceptible individuals a variety of life-threatening conditions such as endocarditis, septicemia, or meningitis (Baddour, 1994; Husni, 1997).

1.7.4. Effects on female genitourinary tract system:

It was 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 H2O2 provide a protective role against virginities and acquisition of sexually transmitted infections (Reid, 2008). The colonization of vaginal mucosa by engineered microbicide-secreting *Lactobacillus* strains is therefore seen as an economical and long-lasting means of enhancing this natural mucosal barrier for the prevention of human immunodeficiency virus (HIV) infection via vaginal intercourse (Liu *et al*., 2006; 2008). Vaginal mucosal microfloras are typically dominated by Gram-positive *Lactobacillus* species, usually *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. iners*, which serve as an important natural barrier to HIV infection (Antonio *et al*., 1999; Va´squez *et al*., 2002; Pavlova *et al*., 2002; Iqbal *et al*., 2008).

Biosurfactants are compounds produced and released by some *Lactobacillus* strains which accumulate at interfaces and help the microorganisms to bind to collagen on epithelial cells. They are found to inhibit adhesion of pathogens involved in urogenital tract infections (Velraeds *et al*., 1996; Reid *et al*., 1999).

Vaginal Lactobacilli have also demonstrated the capability to adhere to vaginal epithelia and competitively exclude pathogens to enable barrier protection of the vaginal epithelium (Rodendo-Lopez *et al*., 1990). A study by Boris and colleagues found that some *Lactobacillus* strains elicit adherence to vaginal epithelia by bacterial cell’s glycoprotein and carbohydrate moieties. Lactobacilli have a higher affinity for vaginal cell receptors and compete against *G. vaginalis* and *Candida albicans* for attachment sites. Therefore, the pathogens were displaced through the receptor binding interference mechanism (Boris *et al*., 1998).

Besides that, Lactobacilli keep a high oxireduction potential in the vaginal environment, which inhibits multiplication of strictly anaerobic bacteria (Aroutcheva *et al*., 2001). The absence hydrogen peroxide-producing Lactobacilli have been related to a higher risk of BV, recurrent urinary tract infection by *E. coli* and increased susceptibility to the infection by Human Immunodeficiency Virus (HIV-1) (Reid *et al*., 1990; Tomas *et al*., 2003).

1.8. *Lactobacillus* as probiotic:

Bacteria of the genus *Lactobacillus* have been proposed as probiotic microorganisms to restore the ecological equilibrium of the intestinal, respiratory, and urogenital tracts (Hammes *et al*., 1995). This type of bacterial replacement therapy has been widely used as fermented milks to prevent diarrhea in humans and animals (Fuller, 1992; Hudault *et al*., 1997). They have also been increasingly considered for their use in women to prevent genital and urinary tract infections (Redondo-Lopez *et al*., 1990; McGroarty, 1993; Boris and Barb´es, 2000; Reid, 1999; Foxman *et al*., 2000). Urinary tract infections (UTIs) affect millions of women each year, with an annual societal cost of billions of dollars (Foxman, 1990). Importantly, more than one quarter of women with a UTI will have a recurrent infection within six months (Stamm and Hooton, 1993). There are few established options for prevention of UTI other than the use of prophylactic antibiotics (Gupta *et al*., 1999).

However, resistance to commonly used antibiotics is increasing among bacterial cystitis isolates (Eschenbach *et al*., 1989). Therefore, effective nonantibiotic methods of prevention are needed. One potential alternative may be a *Lactobacillus* probiotic. A growing body of evidence suggests that vaginal H202- producing Lactobacilli may have a protective effect against urogenital infections, including UTI (Martin *et al*., 1999; Antonio *et al*., 1999). It is hypothesized that Lactobacilli prevent uropathogen colonization of the vagina, a necessary step in ascending infection of the bladder.

1.9. Aggregation of *Lactobacilli*:

The aggregation ability comprises autoaggregation, characterized by clumping of cells of the same strain, and coaggregation, in which genetically distinct cells are involved (Kolenbrander, 1988). Autoaggregation and coaggregation are involved in the microbial colonization of the gastrointestinal and urogenital tracts, but it is not known if these phenomena and the persistence of Lactobacilli in the intestinal or vaginal tract are related (Ocaňa and Nader-Macias, 2002).

As demonstrated in studies by numerous groups (Drago *et al*., 1997; Mastromarino *et al*., 2002; Kos *et al*., 2003; Castagliuolo *et al*., 2005), microbial aggregation is a desirable property of probiotic bacteria. In those studies, autoaggregation ability (Kos *et al*., 2003) and coaggregation ability with pathogenic Escherichia coli (Drago *et al*., 1997) of potentially probiotic gastrointestinal Lactobacilli were determined. Mastromarino *et al*., (2002) studied the coaggregation of vaginal Lactobacilli with *Candida albicans* and *Gardnerella vaginalis*.

The experimental results reported by Castagliuolo *et al*., (2005) indicated for the first time that the aggregation property (both auto-aggregation and coaggregation) of *Lactobacillus crispatus M247* is a relevant probiotic characteristic to exert protective effects on colitis in mice.

1.9.1. What is aggregation?

Cell aggregation seems to involve the interaction of cell surface components such as lipoteichoic acid, proteins, and carbohydrates, as well as soluble proteins (Clewell and Weaver, 1989; Reniero *et al*., 1991). Studies on the mechanism of autoaggregation in Lactobacilli showed that proteins present in the culture supernatant and proteins or lipoproteins located on the cell surface are involved in cell aggregation. Furthermore, it was observed that spent culture supernatants of autoaggregating Lactobacilli mediate not only the aggregation of cells of the producer strain, but also aggregation of other lactic acid bacteria and even *Escherichia coli* (Schachtsiek *et al*., 2004).

1.9.2. Importance of aggregation:

Lactic acid bacteria (LAB) grow in a variety of habitats, such as the mucosa and intestines of humans and animals, as well as fermenting foods and feed (Hammes and Hertel, 2003). Their ability to form multicellular aggregates has been shown to play an important role in colonization of the oral cavity (Kolenbrander**,** 2000) and the urogenital tract (Mastromarino *et al*., 2002; Reid *et al*., 1990), as well as in genetic exchange via conjugation (e.g., in *Enterococcus faecalis* (Bensing and G. M. Dunny, 1993) and *Lactococcus lactis*) (Gasson, 1992). The aggregation ability comprises autoaggregation, characterized by clumping of cells of the same strain, and coaggregation, in which genetically distinct cells are involved (Kolenbrander, 1988; Roos *et al*., 1999). Both types of aggregation have been described previously for Lactobacilli, including *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus reuteri* (Boris *et al*., 1997; Cesena *et al*., 2001; Kmet, 1995; Reniero, 1992; Vandevoorde *et al*., 1992).

1.9. 3. Types of aggregations:

A- Coaggregation:

However, the coaggregation (aggregation between genetically different strains) between Lactobacilli and pathogens could prevent the access of the latter to the tissues and their adhesion to epithelia, avoiding the establishment of a vaginal infection (Boris *et al*., 1997).

The co-aggregation can create a microenvironment around the pathogen with a higher concentration of inhibitory substances and it can also block the dissemination of pathogens to tissue receptors (Mastromarino *et al*., 2002; Reid 2001).

B-Autoaggregation:

The auto-aggregation ability, or formation of multicellular clumps between micro-organisms of the same strain, is one of the proposed mechanisms to explain the protective role of Lactobacilli in the human vagina (Boris *et al*., 1997). This property, related to the adhesion ability to epithelial vaginal cells, could cause the Lactobacilli to produce a biofilm on the vaginal epithelia, which prevents the entry of pathogens (Lepargneur and Rousseau, 2002).

The ability of auto-aggregation of vaginal Lactobacilli is an intrinsic characteristic and may substantially increase the colonization of environments with short residence times (Ocaña and Nader-Macías, 2002). According to Juarez-Tomás *et al*., 2005 the ability of autoaggregation is higher in acid environments where probiotic Lactobacilli are more adapted to survive and represents the first step towards the formation of biofilms by Lactobacilli strains, which helps to inhibit the overgrowth and proliferation of pathogenic microorganisms (Kos *et al*., 2003; Strus *et al*., 2005).

However, the effect of several culture conditions on the auto-aggregation phenomenon was not deeply evaluated until now (Boris *et al*., 1997; Ventura *et al*., 2002).

1.9.4. Auto-aggregation under different growth conditions:

According to the auto-aggregation results obtained at different growth times, the auto-aggregation ability of a vaginal *Lactobacillus* strain grown under several culture conditions was systematically and statistically evaluated for the first time (Tomas *et al*., 2005). Their results indicate that the physico-chemical factors tested (initial pH and temperature) affected in a different way the growth and the auto-aggregation ability of *L. johnsonii CRL 1294*. The growth of this microorganism was significantly higher at 37°C, initial pH 6.5, in MRS broth. At a lower temperature (30°C), the lag phase was longer and the growth rate was lower in growth media. At 44°C the growth was evidenced in MRS. These results suggest that in extreme conditions, such as unfavorable high temperature, *L. johnsonii* could utilize the nutrients of MRS.

The effect of pH on the autoaggregation percentages was more significant than those of temperature, obtaining the higher values at pH 5 or 6.5. A higher temperature of incubation (44°C) did not inhibit the auto-aggregation ability of *L. johnsonii CRL 1294*.

1.9.5. Factors affecting aggregation:

In order to know the factors which affect the auto-aggregating ability of, *Lactobacillus* the extent of auto-aggregation under different culture conditions was assessed. The environmental conditions, the cellular functions and activities influenced by regulator systems operating under high-cell density conditions, as the quorum sensing signals, are included between those factors (Kjelleberg and Molin, 2002; McNeill and Hamilton, 2003).

1.9.5.1. pH:

The potentially probiotic Lactobacilli would be exposed to vaginal environment with fluctuating conditions, such as different pH values (pH 4.0– 4.5 in normal women; pH 5.0– 6.0 in women with bacterial vaginosis, pH close to 7.0 around the menstruation) (Larsen, 1993).

Therefore, the aggregating micro-organisms will survive and proliferate under conditions that promote the approach of partner cells (Rickard *et al*., 2003).

The optimal conditions for a decrease of pH were coincident with the optimum growth conditions. The higher aggregation obtained at low pH could be explained by modifications of the bacterial surface charge, such as a decreasing of Coulomb repulsive forces, which could promote the approach of the cells (Vandevoorde *et al*., 1992). This fact could be relevant in the vaginal ecosystem, where a normal pH < 4.5 could favour the cellular interaction between Lactobacilli to form a protective biofilm on the vaginal mucosae.

1.9.5.2. Temperature:

The autoaggregation ability is dependent on environmental factors (such as pH and heat conditions). Optimum autoaggregation occurred at room temperature, and heat treatment of Lactobacilli reduced autoaggregation scores (Ekmekci *et al*., 2009). There is some evidence to suggest that heat-sensitive surface components on Lactobacilli and uropathogens are also involved in certain aggregation reactions (Jabra-Rizk *et al*., 1999).

A higher temperature of incubation (44°C) did not inhibit the auto-aggregation ability of *L. johnsonii CRL 1294* and the growth of this microorganism was significantly higher at 37°C (Toma´s *et al*., 2009).

Materials and methods

2.1. Equipment:

Equipment	Manufacturing company and origin
Autoclave	Sakura (Japan)
Anaerobic jar	China
Vortex	Lab coo (Germany)
Electrophoresis system , power supply and Transilliuminator	LKB –Sweden
Compound Microscope	Olympus (Japan)
Electric balance	Precisa (Swaziland)
Water bath	Tafesa Hannover (Germany)
Polymerase Chain Reaction thermal cycler	TECHNE, TC-3OOO, Bibby scientific Ltd, (USA)
Hot plate	Takashima (Japan)
Incubator	Yamato (Japan)

Oven	Marubeni (Japan)
Optical density reader	India
Refrigerator	Korea
Swabs	China
Micropipette	Brand (Germany)
Digital camera	Sony(China)
Wooden sticks	China
Sterile swab for streaking	Lab. Service(S.P.A.)
Plastic Test tubes 10 ml	AFCO(Jordan)& S A R
Microwave oven	China
Pasture pipette	China
Parfilm	Chicago
Pettri dish	S A R
pH meter	Radiometer (England)

2.2. Media:

Medium	**Company**
Agar-agar	India
MRS agar	India
MRS broth	India

2.3. Chemicals:

Chemicals	**Company and origin**
EDTA (ethylene diamine tetra acetic acid), HCL (hydrochloric acid), H_2O_2 (hydrogen peroxide 3%), NaOH (sodium hydroxide), NaCL (sodium chloride) and Seder oil.	BHD (England)
Agarose, 10x TBE buffer, Ethidium bromide	Promega (USA)
Gram‘s stain including crystal violet, iodine ,acetone and safranin solutions	Sera and Vaccines Institute (Iraq)

Bromothymel blue	India
Safranin	India
pH paper	China
KH2PO4	BHD (England)
K2HPO4	BHD (England)
Glycerol	BHD (England)
Acetone	BHD (England)

2.4. Glassware:

Glassware	**Company**
Slides& cover slide	Jordan
Universal tube	Jordan
Vacuum tube	China
Flask (100ml,250ml,500ml,1000ml)	Germany and China
Screw caps	China

2.5. Sterilization method:

Moist heat sterilization was used to sterilize media and some solutions that are not affected by heating, using autoclave under 15 bar/in² pressures at 121 °C for 15 minutes, while dry sterilization was used to sterilize glassware at 160-180 °C for 2-3 hrs. For solutions which may be denaturized by heat (Atlas *et al.*, 1995).

2.6. Culture media preparation and sterilization:

MRS medium was previously used by Pendharkar *et al*, 2013. The growth medium used to cultivate the bacterial strains used in this study were prepared according to the manufacturer's instructions as follow:

2.6.1. Agar agar:

Addition of HCL caused liguification of the MRS agar. To overcome this problem, agar-agar was added before sterilization at a quantity of 1gm for each 100ml of MRS agar to prevent liguification.

2.6.2. MRS broth:

MRS broth was used for anaerobic and aerobic cultivation of Lactobacilli (Kos *et al.*, 2003). This medium was prepared according to the instructions of the manufacturing companies by dissolving 55.15gm of MRS broth in 1L distilled water. They were sterilized by autoclave at

121 °C for 15 minute under pressure 15 bar/in^2, and then incubated at 37°C for 24 hour to ensure their sterility.

2.6.3. MRS agar:

The media were prepared according to the instructions of the manufacturing companies, which are usually fixed on the container of the media. It has been prepared by adding 67.15 gm. of this medium to 1000 ml of distilled water then heated until complete dissolve and sterilized by autoclave at 121 ^oC, 15 pound/inch^2 for 15 minutes, After cooling poured in Petri dish, then incubated at 37°C for 24 hour to ensure their sterility.

2.6.4. Phosphate buffer solution:

Eighty gm of NaCl, 0.34gm of KH_2PO_4, and 1.12gm of K_2HPO_4 were all dissolved in 1000 ml of D.W. The pH was adjusted to 7.3, then the solution was sterilized in autoclave (Forbes *et al*., 2007).

2.7. Gram stain preparation:

Gram Stain Kit consists of:

- Violet stain solution.
- Lugo Iodine solution.
- Alcohol Acetone solution.

- Basic Fuchsine solution.

1. Flood the slide with Crystal Violet (the primary stain).
2. After 1 minute, rinse the slide with water.
3. Flood the slide with Iodine (Iodine is a mordant that binds with Crystal violet and is then unable to exit the Gram+ peptidoglycan cell wall.)
4. After 1 minute, rinse the slide with water.
5. Flood the slide with Acetone Alcohol. (Alcohol is a decolorizer that will remove the stain from the Gram-negative cells.)
6. After 10 or 15 seconds, rinse the slide with water. (Do not leave the decolorizer on too long or it may remove stain from the Gram-positive cells as well.)
7. Flood slide with Safrinin (the counterstain).
8. After 1 minute, rinse the slide with water.
9. Gently blot the slide dry. It is now ready to be viewed under oil immersion (1000x TM) with a bright-field compound microscope.

After this staining procedure, the Gram + cells will appear purple, having retained the primary stain. The Gram – cells will appear pink, having retained the counterstain after the primary stain was removed by the decolorizer (Baron *et al*., 1999).

2.8. Catalase test:

A. Catalase test reagent has been prepared by dissolving 3 ml of H2O2 in 100 ml of distilled water, used to detect the production of catalase (Andrew *et al*., 1996).

B. The slide method was used for the detection of catalase enzyme activity. This test was used to detect the ability of bacteria to produce the enzyme catalase. It was carried out by mixing a single isolated colony transferred by woody stick with a drop of Hydrogen peroxide (3%), the production of gas bubbles indicates the positive result and the few or non-production of gas bubbles indicates the negative result Forbes *et al*; (2007). Negative results indicated that the result is positive.

2.9. Endospore stain:

The protocol for differentially staining endospores and vegetative cells is as follows:

1. Place the heat-fixed bacterial slide over screened water bath and then apply the primary stain malachite green.

2. Allow the slide to sit over the steaming water bath for 5 minutes, reapplying stain if it begins to dry out.

3. Remove the slide from the water bath and rinse the slide with water until water runs clear.

4. Flood slide with the counterstain safrinin for 20 seconds and then rinse.

5. View specimen under oil immersion (magnification of 1000xTM) with a light microscope.

After this staining procedure, the endospores will appear green, having retained the primary stain, malachite green. The vegetative cells (bacteria are in the active, metabolizing state) will appear pink, having retained the counterstain, safrinin (Andrew *et al.*, 1996). Negative results indicated that the result is positive.

2.10. Motility test:

A sterile, cool inoculating wire is used to obtain inoculum from a pure culture of the test organism. The needle is stabbed into motility stab media approximately two-thirds of the depth of the media. The wire is then pulled out of the media as close as possible to the location where it entered. The tube is incubated for approximately 1-2 labs and observed for evidence of motility. A non-motile organism will have a clearly defined edge as it grows on the stab line. Motile organisms will be turbid throughout the tube or have fuzzy, diffuse growth at the edges. Some organisms are so motile that the entire tube becomes very turbid (cloudy) (Cowan, 1975). Negative results indicated that the result is positive.

2.11. The volunteers and sample number:

Forty-two Lactobacilli isolates were obtained from females volunteers, their ages ranged between 14 to 50 years old. All samples were collected during period between November; 2011 to March; 2012.

2.12. Specimens collection and treatment:

The specimens were taken from the vagina of females. Vaginal swab technique was used for sample collection. The swabbing was supervised by gynecologist consult then the swab were rolled onto a slide for Gram stain of vaginal smear and the swab was then streaked on MRS agar plates. However, three subcultures on MRS agar at different pH were done. The first and the third subcultures were applied on MRS agar that had an original medium pH (i.e. without pH change, which is 6.2), while the pH of the second subculture was adjusted at 4. All MRS agar plates incubated anaerobically at 37ºC for 48 hours using anaerobic jar (De Man *et al.*, 1960).

Result of catalase , endospore stain , and motility of the isolated colonies, that taken from the final subculture, were all negative, while morphological characteristics and Gram stains of these isolates were positive (Kandler and Weiss.1986).

2.13. Bacteriological analysis:

2.13.1. Samples storage:

A. Short time preservation:

Single pure colony of bacterial isolate was streaked on the MRS agar culture plates and on the MRS agar slants. Incubated at 37ºC for 48 hours, sealed well and stored at 4 ºC in the refrigerator one month for the plates and three months for the slants (Harely and Prescott, 2002).

B. Long time preservation:

The bacterial isolate was inoculated into the MRS broth and incubated at 37ºC for 48 hours then the broth culture was preserved by adding glycerol to a final concentration of 20% and stored at -20ºC for 12-18 months (Karch *et al.*, 1995).

2.13.2. Morphological and biochemical identification:

2.13.2.1. Morphological identification:

A- Microscopical characteristics:

All the *Lactobacillus* bacteria were Gram stained and examined under high magnification (100X lens) by light microscope.

B- Colonial characteristics:

The colonies of *Lactobacillus* are small (2-5) mm with entire margin, smooth, convex and opaque without pigments; on MRS agar media (Kandler and Weiss, 1986).

2.13.2.2. Biochemical identification:

The biochemical characteristics depended on (catalase test, endospore stain, motility test) and the results were considered according to the response of the bacteria in accord with Harrigan and MaCance, 1976 studies.

2.14. Molecular identification:

2.14.1. Primers preparation (BioCorp):

The primers that are used in PCR amplification were diluted by adding nuclease free water according to the manufacture companies information. 754 µl of free nuclease water, added to F. primer to get 100 pmol/µl. Then, 10 µl of previous solution diluted by adding 90 µl of free nuclease water to get final volume of 10 pmol/µl.

For Reverse primer, 588 µl of free nuclease water added to the R. primer to get 100 pmol/µl. Then, 10 µl of previous solution diluted by adding 90 µl of free nuclease water to get final volume of 10 pmol/µl. This primer were prepared for *L. gasseri*.

For *L. crispatus*, 750 µl of free nuclease water added to Forward primer to get 100 pmol/µl. Then, 10 µl of previous solution diluted by adding 90µl of free nuclease water to get final volume of 10 pmol/µl.

For Reverse primer, 800µl of free nuclease water added to the R. primer to get 100 pmol/µl. Then, 10 µl of previous solution diluted by adding 90 µl of free nuclease water to get final volume of 10 pmol/µl. After primer preparation, it is ready for preparation of PCR mixture.

2.14.2. DNA extraction:

Boiling method that described by (Ruppé *et al*., 2009) was applied for DNA extraction. Briefly, this method included transferring of a pure isolates to Eppendrof's tubes that were previously containing 500µL of free nuclease water. These tubes were then boiled in a water bath for 10 min. After centrifugation (13000 rpm) for (5 min), supernatant was used as template DNA for the PCR.

2.14.3. Preparation of PCR mixture:

The primers that were used in PCR amplification were diluted by adding nuclease free water according to the manufacture companies' information as described in (3.14.1). The reaction mixture was prepared according to the procedure that suggested by the manufacture company. We used 7 microliters of the DNA template were mixed with PCR mixture that composed from 12.5 µl of Green Master Mix, 2.5 µl from each primers forward and reverse, and 3 µl of nuclease free water to get final volume of 25 µl.

2.14.4. PCR amplification procedure:

PCR programs applied in this study were based on method described by Yan *et al*., (2009). The PCR reaction conditions were as follow: predenatured at 95°c for 10 minutes, melt at 95°c for 30 seconds; optimization annealing at 51,53,50,52°c for 30 seconds; extension at 72°c for 30 seconds; 40 cycles; a final extension at 72°c for 8 minutes. PCR primers were shown in Table (2.1); the PCR products were visualized after electrophoresis in 0.8% agarose gels and staining with ethidium bromide as seen in Figure 3.3, 3.4.

Table 2.1: PCR primers and running programs

Species	primers	Sequences	annealing Temp. (ºc)	cycle NO.
L. crispatus	452F	5′-GATAGAGGTAGTAACTGGCCTTTA-3′	52	40
	1023 R	5′-CTTTGTATCTCTACAAATGGCACTA-3′		
L. gasseri	L.gassF	5′-AGCGAGCTTGCCTAGATGAATTTG-3′	52	40
	L.gassR	5′-TCTTTTAAACTCTAGACATGCGTC-3′		

2.14.5. Agarose Gel Electrophoresis:

The amplified PCR product were analyzed by agarose gel electrophoresis according to Sambrook and Russell, (2001) method and were performed as follows:

a. Agarose was prepared at 1% concentration for verifying PCR products. Agarose (1 gram) was dissolved in 100 ml of 1X TBE, then it was melted by heating with stirring. The agarose was left to cool at 60°C, ethidium bromide was added then it was poured into the tapped tray.

b. A comb was placed near one edge of the gel.

c. The gel was left to harden until it became opaque, then the comb and the tape were gently removed.

d. TBE (1X) buffer was poured into gel tank and the tray was placed horizontally in electrophoresis tank.

e. The amplified PCR products were directly applied since the PCR master mix already containing loading buffer.

f. Five microliters of the DNA ladder (10kbp) was loaded in single lane which served as marker during the electrophoresis process.

g. The power supply was set at 75 V for 45min to 1 hour for PCR products.

h. After electrophoresis the gel was exposed to UV using UV transilliuminator and then photographed using digital camera (Sony-Japan).

2.15. Effects of some environmental factors on auto-aggregation:

Two methods were used for the studying and analysis of the effects of some environmental factors on auto-aggregation, these included visual method and spectrophotometric method.

2.15.1. Studying of autoaggregation visually:

The following steps were followed for studying the analysis of autoaggregation visually which is based on the method described by (Tomás *et al*., 2005) with some modification.

A. The bacteria including *L. gasseri*, *L. crispatus* and non-autoaggregation bacteria (*streptocoocus ssp.*) were grown on MRS broth (in universal tube, size 40 ml) for 24hrs. at 37°C.

B. After incubation period the tubes were visually noticed and the aggregation was scored as positive when visible particles, formed by the aggregated cells, gravitated to the bottom of the tube and/or adhered cells to its wall, leaving a clear supernatant fluid.

C. The tubes were photographed using digital camera model (DSC-W530, Sony corp.).

2.15.2. Studying of autoaggregation spectrophotometrically:

The following procedure were followed for the Studying of autoaggregation spectrophotometrically:

Lactobacillus spp. was grown overnight at 37ºC in MRS broth, centrifuged at 6,000g for 15min and cell pellets were resuspended in phosphate buffered saline (PBS) to obtain an optical density (O.D.) of 0.6 at 600nm Auto-aggregation inversely correlated with O.D. and it was monitored every 1hrs. for up to 4hrs. (Tomás *et al*., 2005).

The following steps were followed for the detection of autoaggregation percentage values. According to the method described by Tomás *et al*., 2005).

A. Lactobacilli species was grown overnight at 37°C in MRS broth that prepared as described in section (2.6.2).

B. After incubation period, the tubes were centrifuged at 6000 rpm for 15min.

C. The cell pellets were resuspend in PBS (prepared as described in section 2.6.4).

D. The pellets were washed twice in PBS.

E. The pellets were resuspended again in PBS to give a final optical density of 0.6 at 600nm as measured by a spectrophotometer.

F. Samples consisted of 5ml of each species with OD measured at 600nm. The samples were measured for 4hrs. (Including 30min intervals).

G. The percentage of autoaggregation was expressed according to the following equation [mentioned by Vandervoorde, (1992)]:

$$\text{Auto-aggregation \%} = \frac{\text{OD initial} - \text{OD final}}{\text{OD initial}} \times 100$$

Where OD initial is the OD at initial time (t = 0) of autoaggregation assay, and OD final is the OD at each time after beginning this assay (t = 1, 2, 3 and 4 hrs.).

2.15.3. Some Environmental Factors affecting autoaggregation:

Three environmental factors were considered in the present study included temperature, pH, and aeration situations. Three different temperatures were applied i.e. 30, 37, 44 °C. For pH, three different values were used i.e. 5, 6.2, 8, and aerobic and anaerobic conditions. Implication of the first two variables (i.e. temperature and pH) were performed according to the following illustration (table 2.2):

Temperature

pH

T / pH	30	37	44
5	30 , 5	37 , 5	44 , 5
6.2	30 , 6.2	37 , 6.2	44 , 6.2
8	30 , 8	37 , 8	44 , 8

Table 2.2: In each square, first value indicates temperature, and second value indicates pH.

Each square considered as a unique experiment, and then the squares were assayed according to the following steps:

1. Before sterilization of the media, the pH were adjusted to it's specific degree according to the table 3.1.

2. After sterilization each tube was inoculated with a loop full inoculum of original sample.

3. All samples were kept in anaerobic jar and transferred to incubator previously adjusted at a recommended temperature described in table 3.1.

4. After incubation time, i.e. 24 hours. The tubes were shaked by vortex for 2 seconds. The activated cultures were harvested by centrifugation at 6,000 x *g* for 15 min, washed twice in phosphate- buffered saline (PBS) and resuspended in PBS to give a final optical density of 0.6 at 600 nm as measured by a spectrophotometer.

5. The same steps (from 1 to 4) were applied on the isolates but under aerobic conditions.

6. All obtained OD were expressed in figures 3.7 to 3.19 in the next chapter.

2.16. Number of replicates:

Each experiment in this work were done in three replicates and the average values were considered.

2.17. Exhibition of the results:

The figure of the results were plotted as a histographs by using Microsoft Office Excel Worksheet. Mean and standard deviation of the numerical values were also obtained this program. Tables were drawn by the application of Microsoft Office Word Document. The percentage values of autoaggregation were computed according to the equation mentioned in section (2.15.2.g.).

Results and discussion

3.1. Isolation of a pure culture of Lactobacilli *spp.*:

According to the morphological and biochemical characteristics, forty-two isolates were obtained and described as Lactobacilli *spp*. These isolates were collected from females, aged between 14 and 50 years. For isolation procedure of Lactobacilli *spp.*, and due to diversity of vaginal microflora (particularly in BV infected females) (Hillier, 1998) (first), and to get rid of contamination with non-target bacteria (second), some modifications were done (in the present work) for this purpose. Such a modification included three steps of subculturing of the bacteria that are grown at 37 ^{0}C and anaerobically on MRS agar, but at different pH. In the first step of subculturing, the bacteria were allowed to grow on MRS agar having a normal media pH (which is 6.2). In the second step, the colonies were transferred to MRS agar having a pH of 4, and in the third step of subculturing, the colonies were again shifted to MRS agar having normal MRS medium pH (i.e. 6.2). This final (third step), allowed the development of pure colonies of Lactobacilli on MRS agar. We think, such a procedure guaranteed the isolation of this bacterium from BV suffering females. However, such a modifications are in consistence with Pulugurth (2010) report, in spite of it did not consider the pH aspects, and it ensured the repeated isolation of Lactobacilli as an essential step in diagnosis confirmation.

The current work also considered additional two isolates, which obtained from healthy females, and applied them as a control (for comparison) with the BV infected females.

3.2. Morphological and Biochemical identification of Lactobacilli:

All forty-two isolates (including the two controls) have demonstrated the same morphological and biochemical characteristics. However, figures 3.1 shows the colonial characteristics of Lactobacilli *spp*. on MRS agar, at ideal conditions (i.e. pH 6.2, 37ºC, and anaerobically). The colonies showed a typical morphological characteristics, which are: small colonies without pigment, white or cream color, and round in shape. This description is in agree with that reported in (Bergey's Manual of Determinative Bacteriology).

Figure (3.1): Colonial characteristics of *Lactobacillus spp.* on MRS agar at growth conditions of pH 6.2, 37ºC, and anaerobically.

The microscopical characteristics of the Lactobacilli *spp*. isolates are illustrated in figure 3.2. This figure demonstrates that these bacteria are Gram positive, nonsporing bacilli which constitute the usual microscopical description of this bacterium as described by many authors (Cannon *et al.,* 2005; Madigan and Oren, 1999; Antonio *et al.,* and 1999; Redondo-Lopez *et al.*, 1990).

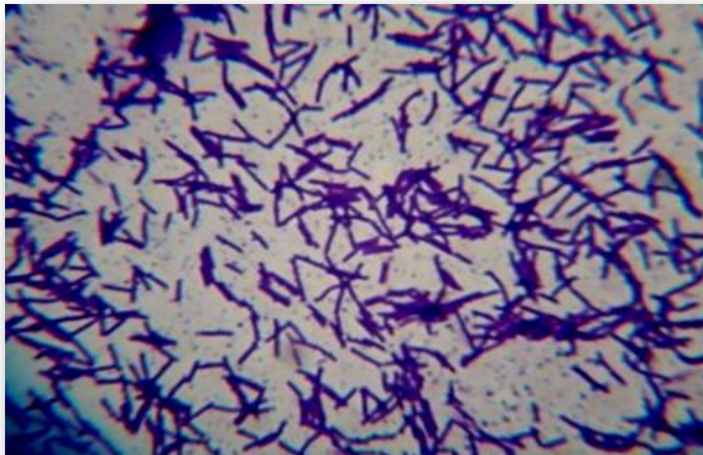


Figure (3.2): Microscopical characteristics of the *Lactobacilli spp.* isolates (100x).

The biochemical characteristics including (catalase test, endospore stain method, motility test) results were negative for all tests and all the isolates stained positively with Gram stain. These characteristics classify the isolates of the present research as genus of Lactobacilli *spp*. as reported by (Harrigan and MaCance, 1976; and Atlas *et al.*, 1995).

3.3. Molecular identification:

Twelve randomly chosen isolates (from original forty two isolates) of Lactobacilli were considered for molecular diagnosis. Bacterial DNA extraction, PCR amplification of 16S rRNA gene were performed as described previously by (YAN *et al.*, 2009). The primers 452F, 1023R, *L.gassF*, *L.gassR*, were used as the identifications primers. Some optimization on the thermal cycling program was done, this optimization included the application of 52ºC as annealing temperature. Table (2.1) (in materials and methods chapter) illustrates the program used in this study for 16S rRNA analysis. The optimization in the annealing temperature allowed a better resolution for molecular diagnosis compared to the other temperatures i.e. (50 ºC, 51 ºC, 53 ºC), which gave, somewhat, a less obvious resolution. This fact is shown in figure (3.3) and (3.4) that demonstrates a clear profile of DNA sequencing.

However, the literature has reported the application of a wide range of annealing temperatures, for example, Frank *et al.*, (2008) have demonstrated the application of annealing temperatures of 48°C, 54°C, and 60°C. Moreover, Flint and Angert (2005**)** have used an annealing temperature of 56ºC when they showed the development of a strain-specific assay for the detection of viable *Lactobacillus* on cattle feed. These variations in the annealing temperatures of the present work with that of the literature could be explained to the variation of the sources of the isolates and the technique used for the detection.

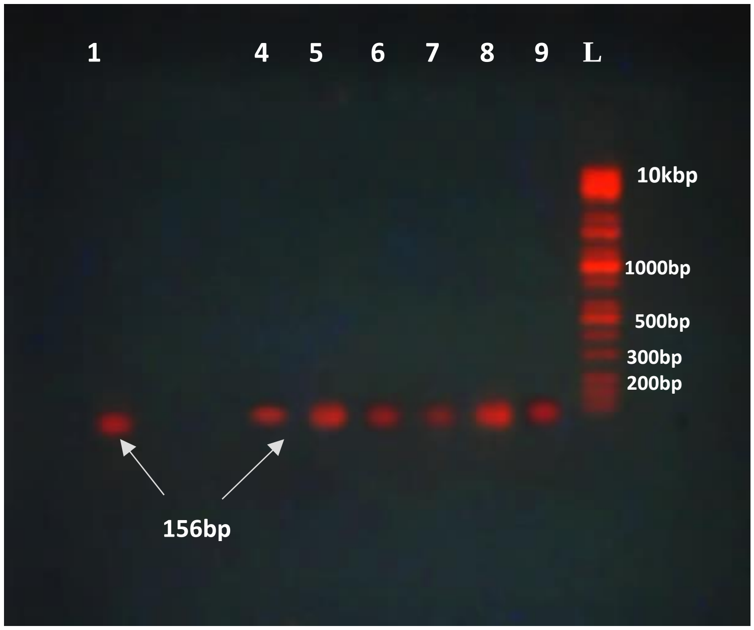


Figure (3.3): Agarose gel electrophoresis (1 % agarose, supplied with ethidium bromide at 75v) for 16S rRNA gene for the detection of *Lactobacillus crispatus* (amplified size 154bp as compared with 10kbp DNA ladder (L)) using template DNA prepared by boiling method. Lines 1, 4, 5, 6, 7, 8, 9 represents positive results.

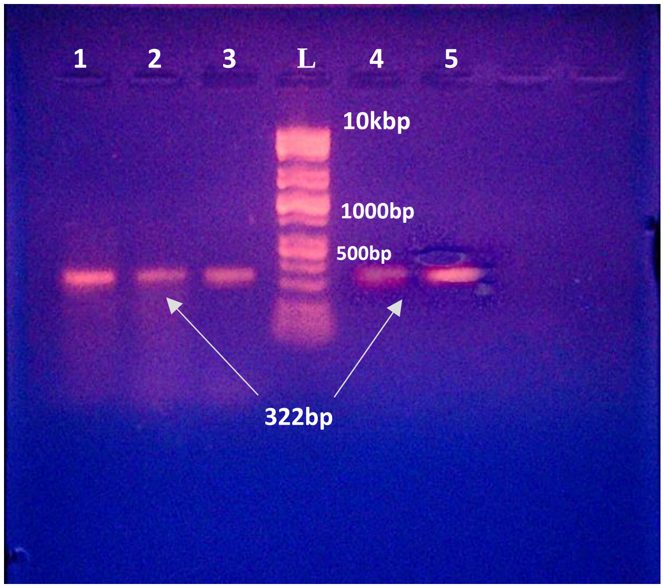


Figure (3.4): Agarose gel electrophoresis (1 % agarose, supplied with ethidium bromide at 75 v) for 16S rRNA gene for detection of *Lactobacillus gasseri* (amplified size 322bp as compared with 10kbp DNA ladder (L)) using template DNA prepared by boiling method Lines 1, 2, 3, 4, 5 represents positive results.

Table (3.1) shows the result of Lactobacilli diagnosis, it illustrates that the species obtained from chosen vaginal Lactobacilli spp. isolates are include *Lactobacillus crispatus* and *Lactobacillus gasseri.* Yan *et al.*, (2009) found that *L. crispatus* constitutes (67%), and Burton *et al*., 2003 reported that, of the 14 subjects harboring Lactobacilli, *L. crispatus* constitutes only 7 (50%) in Canada. Verhelst *et al*., (2004) have reported that *L. crispatus* percentage is (66.6%), also *L. crispatus* was 51.9% by

(Ravel *et al*., 2011). Moreover, Tamrakar *et al*., (2007) have studied 98 healthy, pregnant Japanese women and found that *L. crispatus* was (61.2%), and *L. gasseri* (33.7%).Vasquez *et al*., (2002) have mentioned the presence of *L. crispatus* in 47.8% and *L. gasseri* in 30.4% of 23 Swedish women. The present study dealt with the 16S rDNA sequence analysis confirms the DNA homology studies of Pavlova *et al*., (2002); Giorgi *et al*., (1987), Antonio *et al*., (1999) and Song *et al*., (1999), who have found that the most prevalent species of vaginal Lactobacilli in women from Italy, the United States and Japan, respectively, were homologous to the type strains of *L. crispatus*, and *L. gasseri*, that support the results of the present study.

Table (3.1): The species of bacteria and their percentages (for 12 samples chosen randomly from forty-two isolates).

Lactobacillus spp.	No. of identified species	Percentages of *Lactobacillus* species
L. crispatus	7	58.3
L. gasseri	5	41.6
Total	12	100

On the other hand, Damlen *et al*., (2011) study has reported that the usual vaginal Lactobacilli percentages are *L. crispatus* and constitute percentage of (22%), and *L. gasseri* (10%), and (Balkus *et al.*, 2012) showed that the only species that found in human vagina was *L. crispatus* (34%). These differences in the present and other works may be attributed to geographical distribution (Shi *et al*., 2009), technique used (Shi *et al*., 2009), and/or socioeconomic situation of the studied case.

According to our knowledge, the results of the present work considered as a first report in Baghdad city concern with the molecular diagnosis, besides the optimization of certain elements in molecular diagnosis of vaginal Lactobacilli spp. isolated from BV infected females. However, such a precise diagnosis is considered very critical in the development of suitable bacterial replacement therapy for the treatment of vaginosis.

Moreover, additional work is required in this field in order to plot a complete phylogenetic map for the distribution of these bacteria in Baghdad and other cities in Iraq.

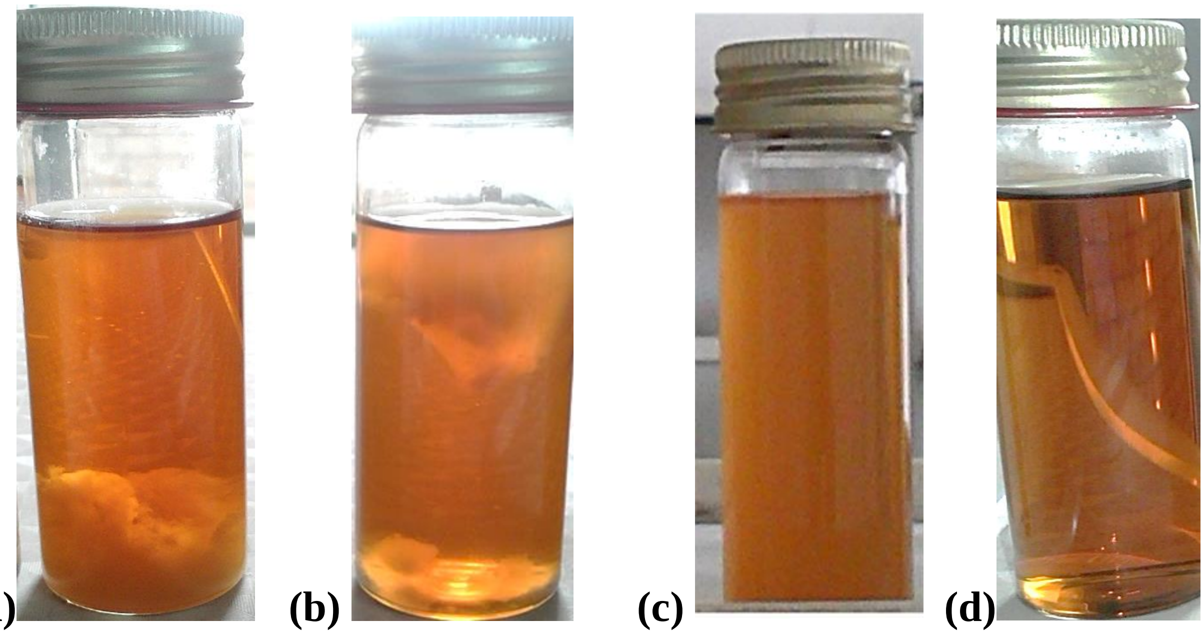
3.4. Effects of some environmental factors on auto-aggregation:

Two methods were used for the studying and analysis of the effects of some environmental factors on auto-aggregation, these included visual method and spectrophotometric method.

3.4.1. Analysis of autoaggregation using visual method:

Visual analysis of autoaggregation are illustrated in figure (3.5) and figure (3.6). Figure 3.5, shows the analysis at growth conditions at pH 5, 37^{o}C, anaerobically. From this figure it is obvious that *L. gasseri* (figure 3.5, a) is forming a clear huge masses of autoaggregation, compared to *L. crispatus* (figure 3.5, b), that revealed a smaller masses of autoaggregation. However, both of these two bacteria, when compared with non-aggregate forming bacteria (figure 3.5, c), they have both formed a considerable visual autoaggregation. These results are in agree with Reid *et al*., (1988), Boris *et al*., (1997), and Del Re *et al*., (2000) who have mentioned that aggregation abilities that may form a barrier that prevents colonization by pathogenic microorganisms. This is on one hand, on the other hand, Ekmekci *et al*; (2009) have mentioned that *L. gasseri* develops a better biomass of autoaggregation. However, the above mentioned studies are supporting the results of the present study. Moreover, these results also confirm a previous report of Kos *et al*., (2003) who mention a better growth of the Lactobacilli *spp*. on MRS broth than on MRS agar it could be the reason for slightly better autoaggregation of cells grown on MRS broth. However, Antikainen, (2007) has stated that the observed autoaggregation could be related to cell surface component, because it was not lost after washing and suspending of the cells in PBS, and this could be explained the relationship between autoaggregation and adhesiveness of *Lactobacillus* that are mediated by proteinaceous components on the cell surface.

Figure 3.6, represents visual analysis of autoaggregation at growth conditions of pH 8, 37^0C, anaerobically. When this figure is compared with figure 3.5 (i.e. growth at the same conditions, but at pH 8), it seems that the ability of autoaggregation for both bacteria (i.e. *L. gasseri* and *L. crispatus*) have reduced. It is obvious that this reduction in autoaggregation ability was due to the elevation of growth pH (i.e. from pH 5 to pH 8), since many previous reports support this fact (Tomas *et al*., 2005).



(a) (b) (c) (d)

Figure (3.5): Visual analysis of autoaggregation at growth conditions of 37°C, pH 5, and anaerobically (Mag.1X).

(a) = *Lactobacillus gasseri*; (b) = *Lactobacillus crsipatus*; and (c) non-aggregative bacteria; (d) = control (blank).

Figure (3.6): Visual analysis of autoaggregation at growth conditions of 37ºC, pH 8, and anaerobically (Mag.1X).

(a) = *Lactobacillus gasseri*; (b) = *Lactobacillus crsipatus*; and (c) non-aggregative bacteria; (d) = control (blank).

3.4.2. Analysis of autoaggregation using spectrophotometric method:

3.4.2.1. Effect of pH at growth temperature of 37°C and at anaerobic conditions:

Figure (3.7) shows the percentage of autoaggregation at pH 6.2, temperature 37°C and at anaerobic conditions for *Lactobacillus crispatus* (figure 3.7, a) and for *Lactobacillus gasseri* (figure 3.7, b). It illustrates that the highest and lowest autoaggregation values for *L. crispatus* is 33% (for isolate No. 3) and 27% (for isolate No. 4, 6) respectively. While for normal isolate (i.e. non-infected female), the percentage values of autoaggregation is 33% (figure 3.7, a). For *Lactobacillus gasseri*, the highest and lowest percentage values of autoaggregation are 52% (for isolate No. 4) and 47% (for isolate No. 2) respectively. While for normal isolate, the percentage value of autoaggregation value was 53% (figure 3.7, b). The mean values of the percentage of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 30% (SD ± 2.71) (for *Lactobacillus crispatus*) and 50% (SD ± 2.55 respectively (figure 3.7, c). However Ekmekci *et al*., (2009) have reported that the percentage values of autoaggregation are 30% for *L. crispatus s*and 51% for *L. gasseri*. These results are in accord with the results of the present study. Moreover, Gil *et al*., (2010) have demonstrated that the values of autoaggregation were approximately 32%, for *L. crispatus*, and 25%, for *L. gasseri*. These results are in contradict with the results of the present study. Anyway, these variations could be attributed to the differences in the socioeconomic situation and (or) bacteriological analysis technique used.

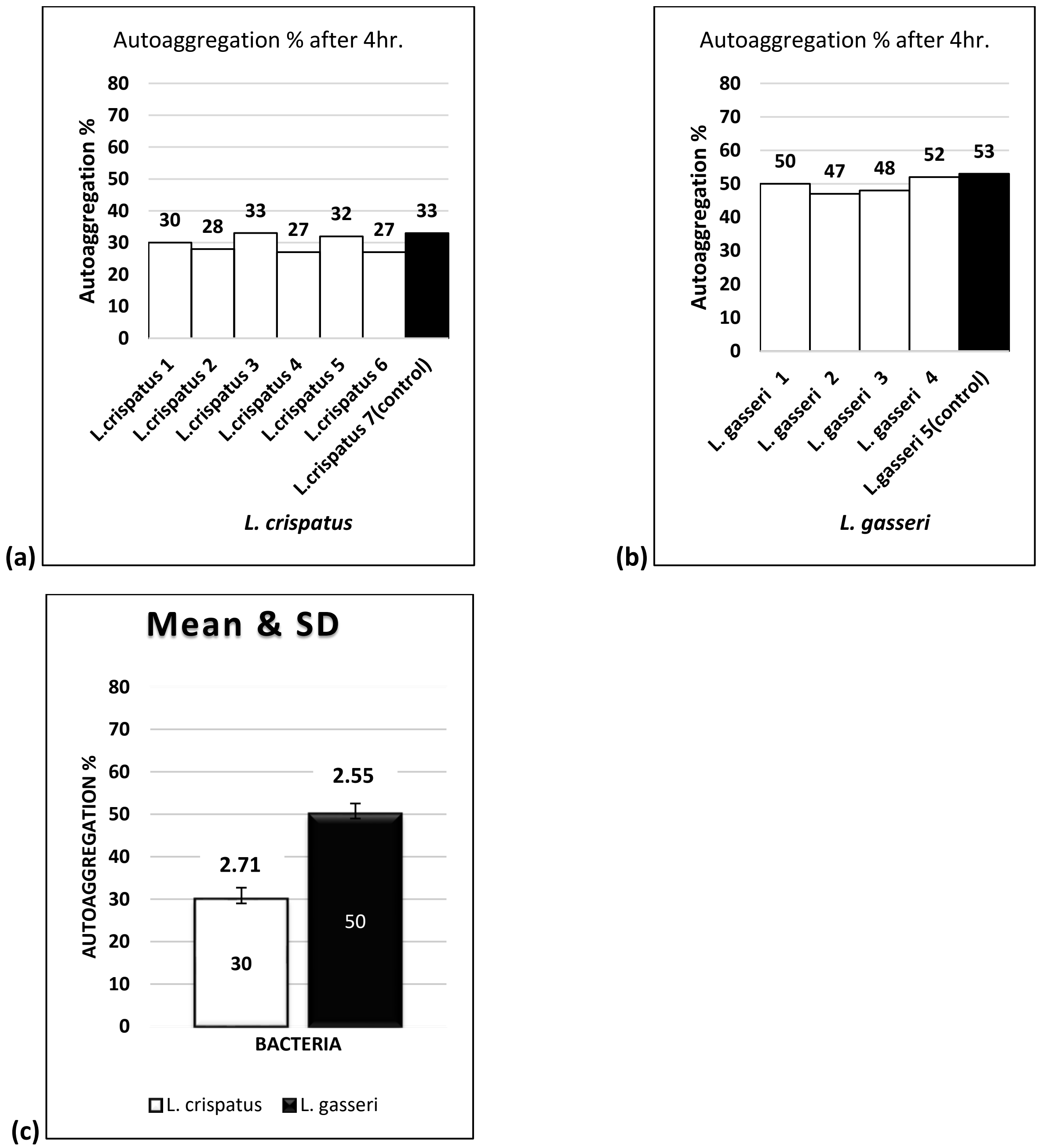


Figure (3.7): Autoaggregation values at pH=6.2, temperature 37°C, under anaerobic conditions: (a) for *L. crispatus*, (b) for *L. gasseri*, and (c) mean and SD values for (a) and (b).

Figure 3.8 reveals the percentage of autoaggregation at pH 5, temperature 37°C and at anaerobic conditions for *Lactobacillus crispatus* [figure 3.8 (a)] and (b) for *Lactobacillus gasseri*. It illustrates that the highest and lowest percentages of autoaggregation values for *L. crispatus* is 63% (for isolates No. 4, 6) and 58% (for isolates No. 1, 3, 5) respectively. While for normal isolate (i.e. non-infected females) the percentage of autoaggregation value was 65% (figure 3.8, a). For *Lactobacillus gasseri*, the highest percentages values of autoaggregation were 72% (for isolate No. 1) and 65% (for isolate No. 4) respectively. While for normal female isolate, the autoaggregation value was 75% (figure 3.8,b).The mean values of the percentage of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 61% (SD ± 2.94), (for *Lactobacillus crispatus*) and 70% (SD ± 3.81), (for *Lactobacillus gasseri*) respectively (figure 3.8, c). Moreover, It have been reported that the percentage values of autoaggregation were 65% (for *L. crispatus*) and 75% (for *L. gasseri*) [Ekmekci *et al*., (2009)]. These results are in accord with the present study.

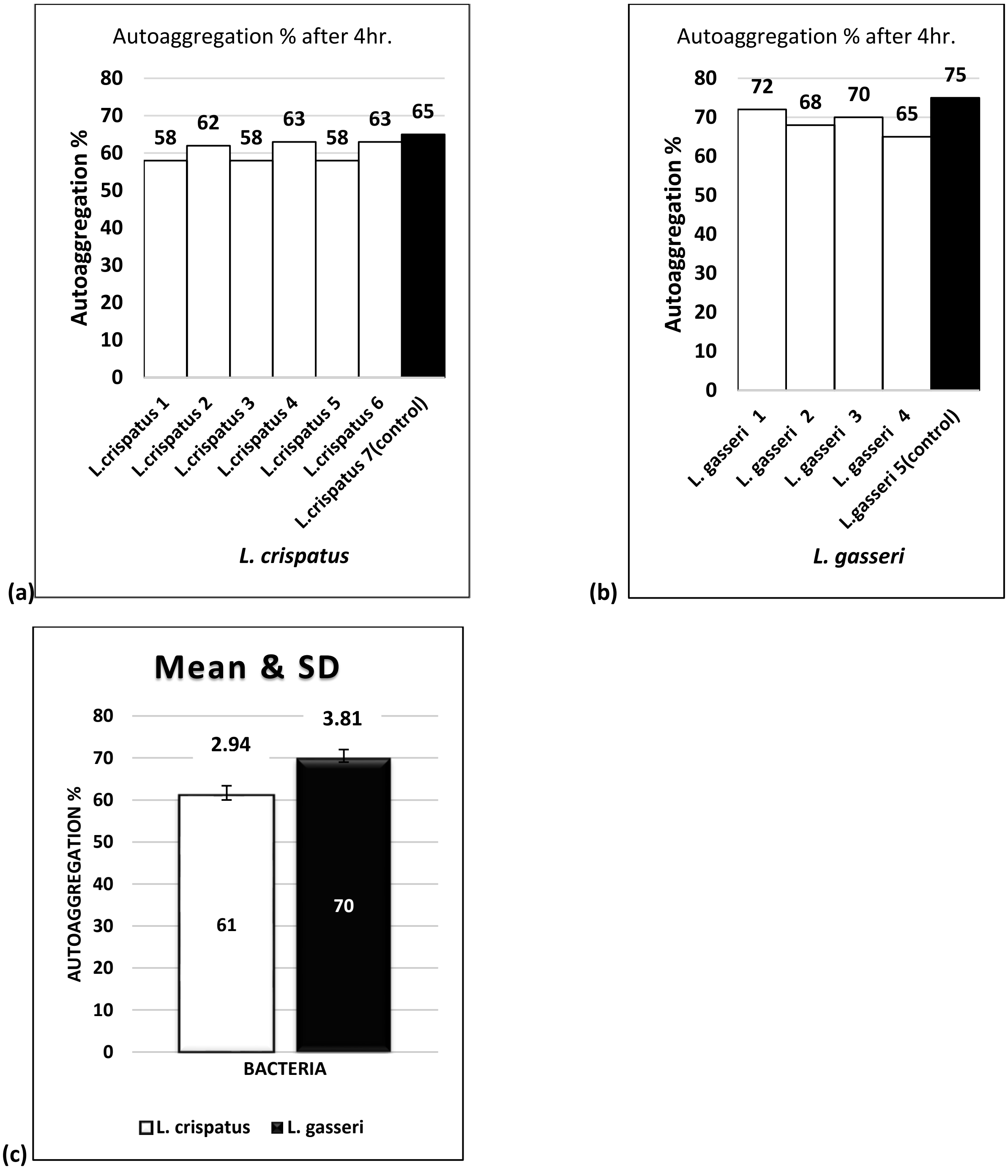


Figure (3.8): Autoaggregation values at pH= 5, temperature 37°C, under anaerobic conditions. (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

Figure 3.9 shows the percentage value of autoaggregation at pH 8, temperature 37°C and at anaerobic conditions for *Lactobacillus crispatus* [figure 3.9 (a) and (b)] for *Lactobacillus gasseri*. It illustrates that the highest and lowest percentages of autoaggregation values for *L. crispatus* were 28% (for isolates No. 2, 4, 5) and 23% (for isolate No. 3) respectively. While the percentage of autoaggregation value of normal isolate (i.e. non-infected female) was 30% (figure 3.9, a). For *Lactobacillus gasseri*, the highest percentage value of autoaggregation was 47% (for isolates No. 1, 3) and 43% (for isolate No. 2) respectively. While in normal isolate, the percentage of autoaggregation value was 48% (figure 3.9, b). The mean values of the percentages of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 27% (SD ± 2.31), (for *Lactobacillus crispatus*) and 46% (SD ± 2), (for *Lactobacillus gasseri*) respectively (figure 3.9, c). Ekmekci *et al*., (2009) have showed that the percentages values of autoaggregation are 25% for *L. crispatus* and 45% (for *L. gasseri*), at pH 9. However, these results are in accord with the results of the present study.

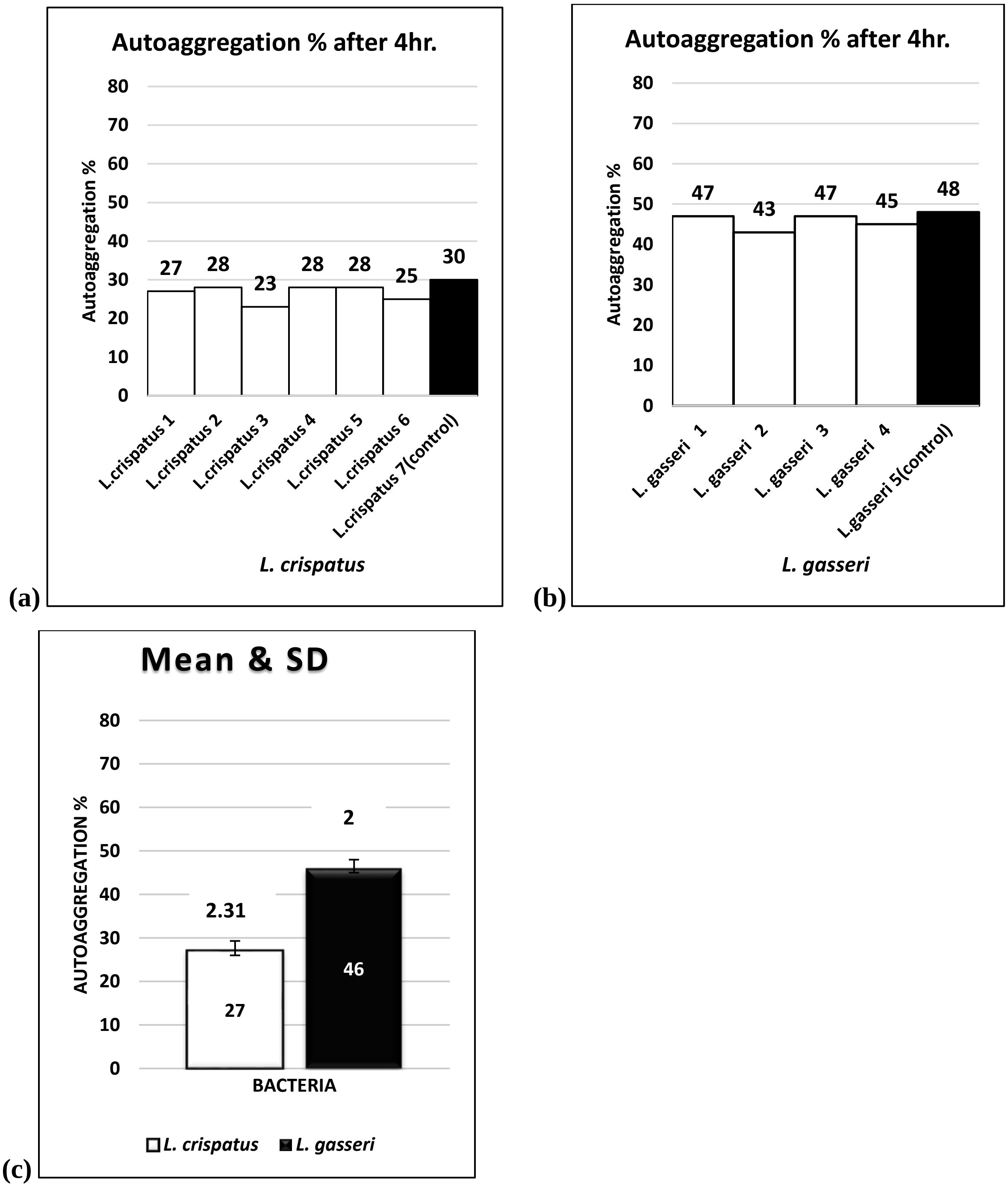


Figure (3.9): Autoaggregation values at pH= 8, temperature 37°C, under anaerobic conditions. (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

3.4.2.2. Effect of pH at growth temperature of 30 °C and at anaerobic conditions:

The percentage of autoaggregation values at pH 6.2, temperature 30°Cand at anaerobic conditions are demonstrated in figure, 3.10 (for *Lactobacillus crispatus*, figure 3.10, a, and for *Lactobacillus gasseri* figure 3.10, b. It illustrates that the highest and lowest percentages of autoaggregation values for *L. crispatus* is 27% (for isolate No. 2) and 22% (for isolate No. 4) respectively. While for normal isolate (i.e. non-infected female), the percentage autoaggregation value was 28% (figure 3.10, a). For *Lactobacillus gasseri*, the highest and lowest percentage value of autoaggregation was 47% (for isolate No. 1) and 42% (for isolate No. 3) respectively. While for normal isolate, the percentage of autoaggregation value was 48% (figure 3.10, b). The mean values of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 25% (SD ± 2.08), (for *Lactobacillus crispatus*) and 45% (SD ± 2.55), respectively (figure 3.10, c). Unfortunately, no reports were found in the literature concerning study of the percentage(s) of autoaggregation values at pH 6.2, temperature 30°C, and at anaerobic conditions.

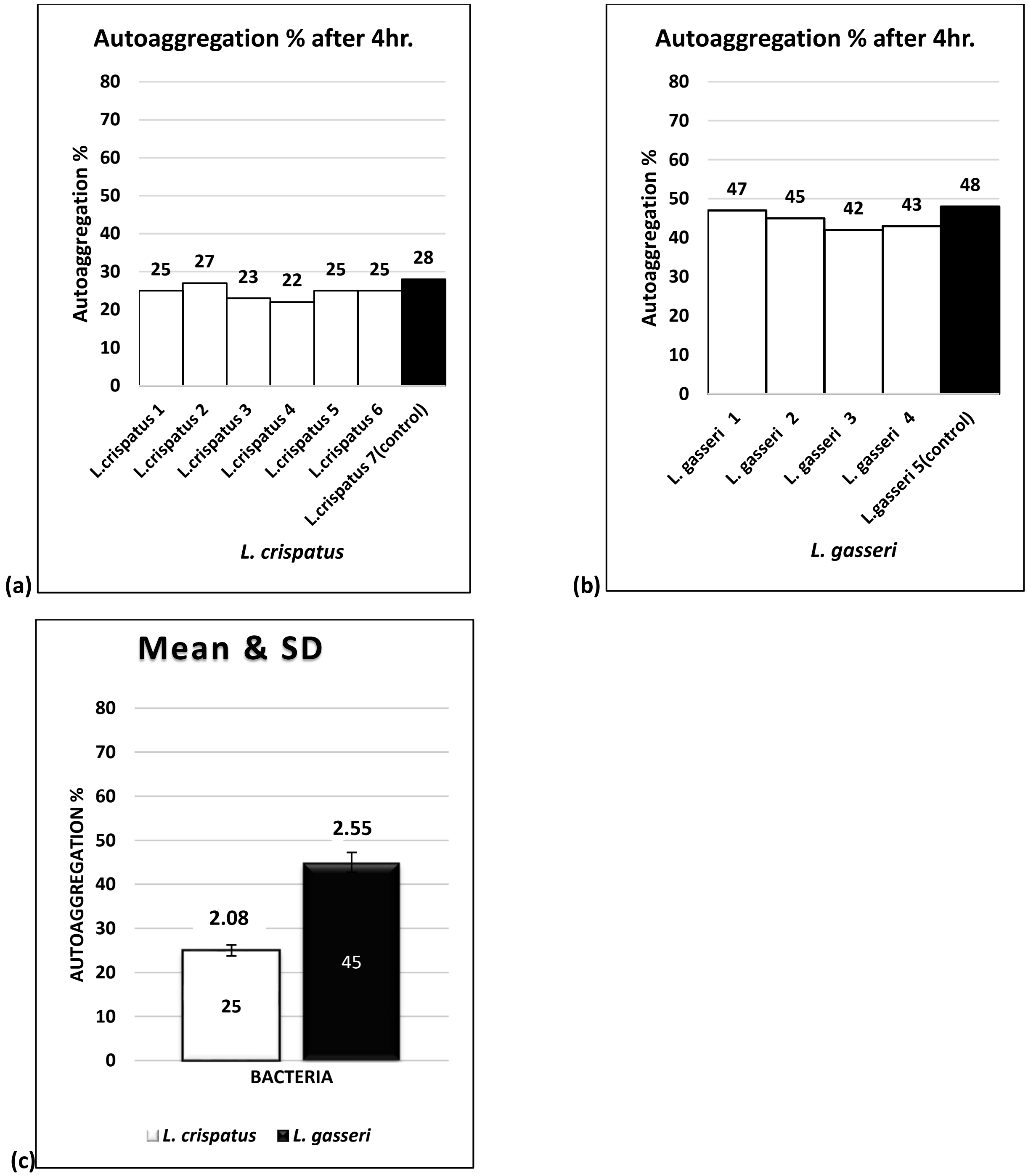


Figure 3.10: Autoaggregation values at pH= 6.2, temperature 30°C, under anaerobic conditions. (a) For *L. crispatus*, (b) for *L. gasseri*, and (c) mean and SD values for (a) and (b).

Figure 3.11 shows the percentage of autoaggregation values at pH 5, temperature 30°C and in anaerobic conditions for *Lactobacillus crispatus* (figure 3.11, a), and for *Lactobacillus gasseri* (figure 3.11, b). It illustrates that the highest and lowest percentage of autoaggregation values for *L. crispatus* are 30% (for isolate No. 2) and 25% (for isolates No. 3, 4) respectively. While in normal isolate (i.e. non-infected female) the percentage of autoaggregation value was 32% (figure 3.11, a). For *Lactobacillus gasseri*, the highest and lowest percentage values of autoaggregation were 50% (for isolate No. 4) and 43% (for isolate No. 2) respectively. While for normal isolate, the autoaggregation value was 50% (figure 3.11, b). The mean values of the percentage of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 28% (SD ± 2.71), (for *Lactobacillus crispatus*) and 47% (SD ± 3.08), respectively (figure 3.11, c). Toma´s *et al*., (2005) have reported that the value of the percentage of autoaggregation for *L. jensenii*, at pH 5, 30°C was 76.73%. The results of the present study is lower than that mentioned by Toma's *et al* report. Again these variations could be explained various reasons including the species used and the technique applied for bacterial analysis.

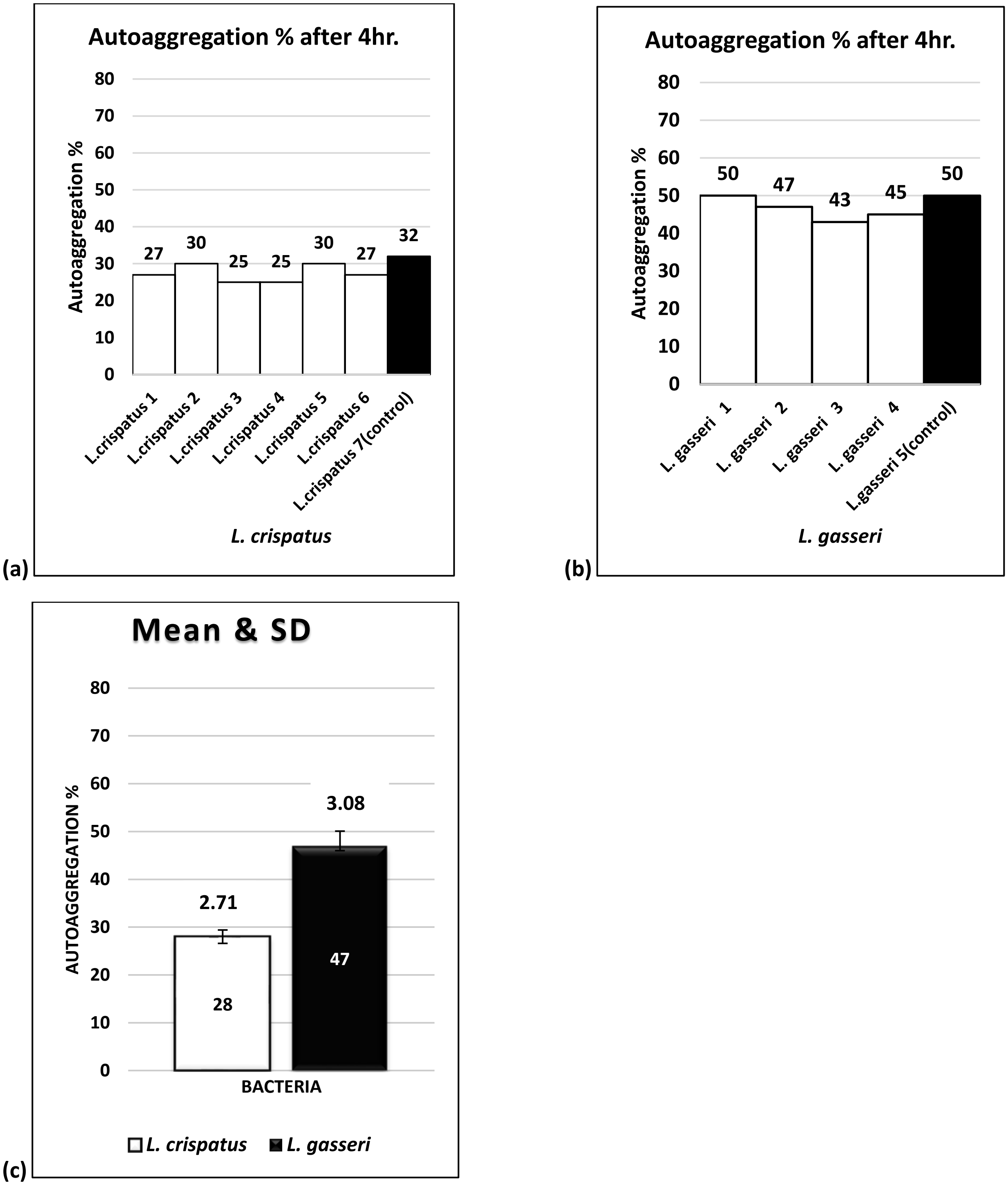


Figure (3.11): Autoaggregation values at pH= 5, temperature 30°C, under anaerobic conditions. (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

Figure 3.12 shows autoaggregation at pH 8, temperature 30°C and at anaerobic conditions for *Lactobacillus crispatus* [figure 3.12 (a)] and (b) for *Lactobacillus gasseri*. It illustrates that the highest and lowest autoaggregation values for *L. crispatus* was 25% (for isolate No.4) and 20% (for isolate No. 3) respectively. While for normal isolate (i.e. non-infected female) autoaggregation value was 25 (figure 3.12, a). For *Lactobacillus gasseri*, the highest values of autoaggregation were 43% (for isolate No. 3) and 40% (for isolates No. 2, 4) respectively. While for normal isolate, the autoaggregation value was 45% (figure 3.12, b). The mean percentage values of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 23% (SD ± 1.73), (for *Lactobacillus crispatus*) and 42% (SD ± 2.12), respectively (figure 3.12, c). These results indicate that at a high values of pH, e.g. pH8, the percentage of the value of autoaggregation decreases. This was clear when the value of autoaggregation at pH 8 (figure 3.12) is compared with the value of autoaggregation at lower pH, e.g. pH 6.2 (figure 3.7) and pH 5 (figure 3.8). However, these results seem to be in agree with Giraud *et al.*, (1991) and Toma's *et al* (2005) studies.

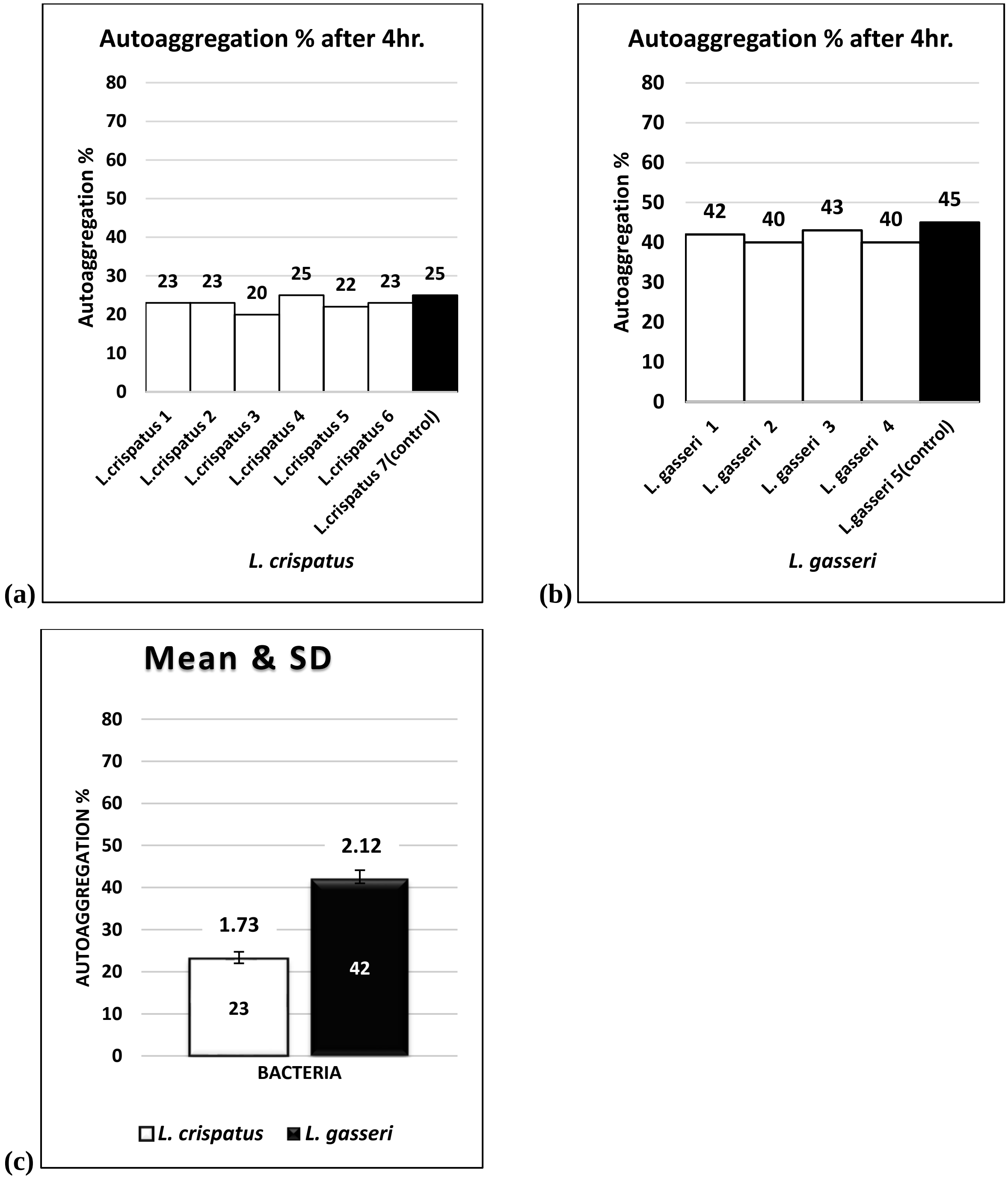
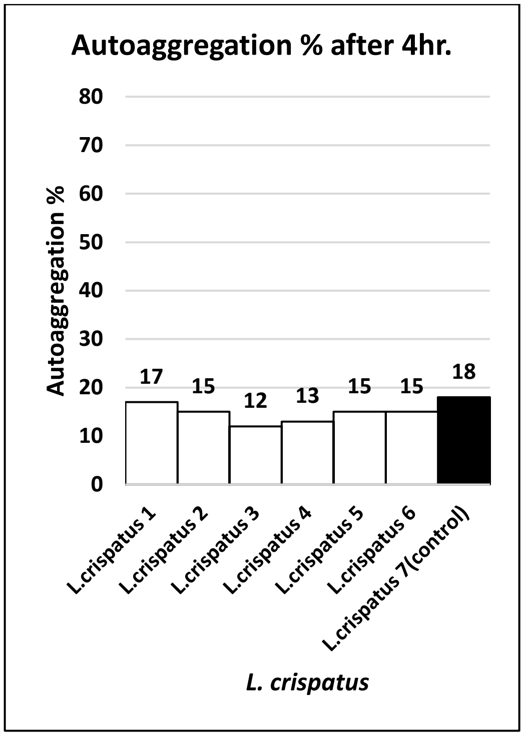


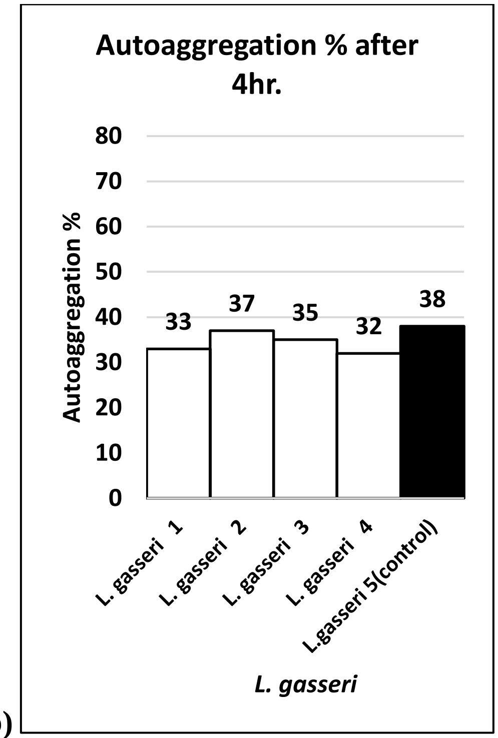
Figure (3.12): Autoaggregation values at pH= 8, temperature 30°C, under anaerobic conditions. (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

3.4.2.3. Effect of pH at growth temperature of 44°C and at anaerobic conditions:

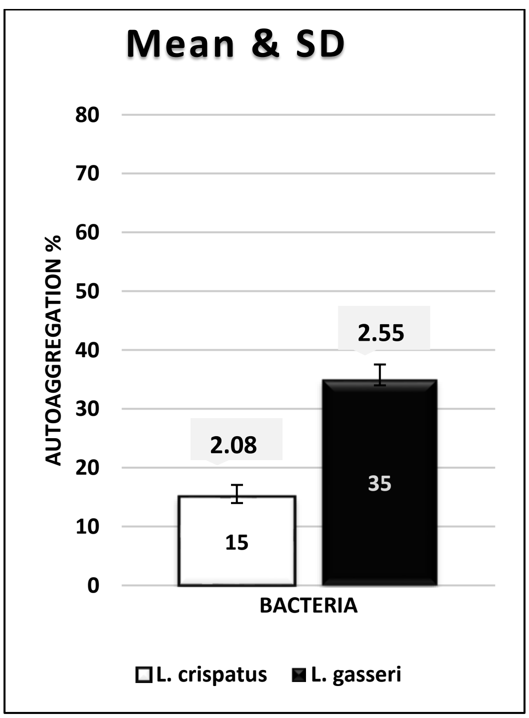
The percentage of autoaggregation at pH 6.2, temperature 44°C and in anaerobic conditions for *Lactobacillus crispatus* (figure 3.13, a) for *Lactobacillus gasseri* (figure 3.13, b). It reveals that the highest and lowest autoaggregation percentage values for *L. crispatus* are17% (for isolate No.1) and 12% (for isolate No. 3) respectively. While for normal isolate (i.e. non-infected female) autoaggregation value percentage was 18% (figure 3.13, a). For *Lactobacillus gasseri*, the highest and lowest percentage values of autoaggregation were 37% (for isolate No. 4) and 32% (for isolate No. 2) respectively. While for normal isolate, the percentage of autoaggregation value was 38% (figure 3.13, b). The mean percentage values of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 15% (SD ± 2.08), (for *Lactobacillus crispatus*), and 35% (SD ± 2.55) respectively (figure 3.13, c). From these results it seem that high temperatures (e.g. 44°C, figure 3.13) have a drastic effect on autoaggregation, this was obvious, when comparing the percentages of the values of autoaggregation (at 44°C) with that of temperature at 37°C (figure 3.7). However, Toma's *et al.,* (2005) have stated that *lactobacilli spp*. grow better 37°C than at 44°C. So, if this fact reflect also autoaggregation, then Toma's *et al* report support our finding. However, additional work is required to support the present finding.



(a)



(b)



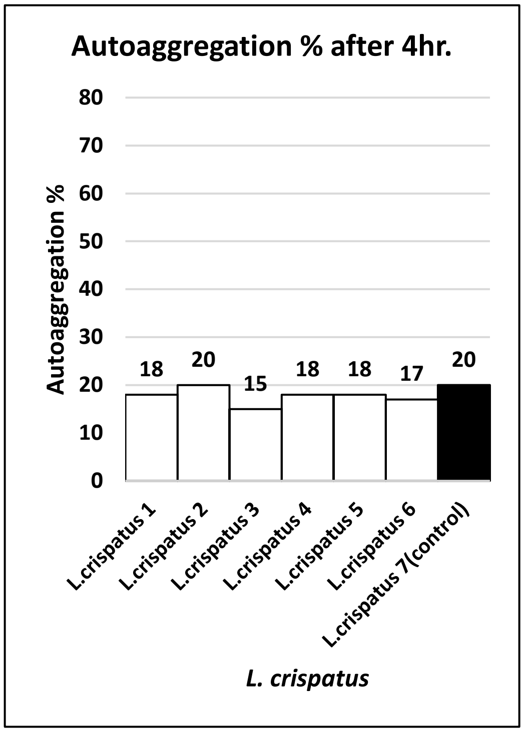
(c)

Figure (3.13): Autoaggregation values at pH= 6.2, temperature 44°C, under anaerobic conditions. (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

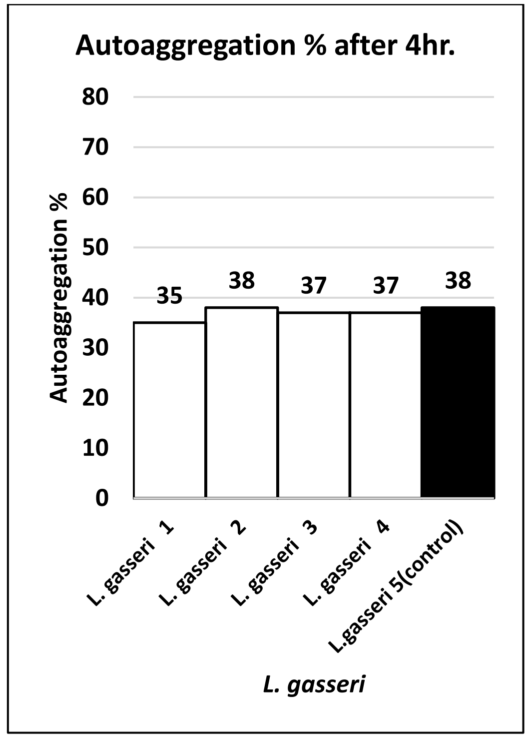
Figure 3.14 shows the percentage values of autoaggregation at pH 5, temperature 44°C and in anaerobic conditions for *Lactobacillus crispatus* (figure 3.14, a) and for *Lactobacillus gasseri* (figure 3.14, b). It illustrates that the highest and lowest percentage of autoaggregation values for *L. crispatus* are 20% (for isolate No.2) and 15% (for isolate No. 3) respectively. While for normal isolate (i.e. non-infected female) the percentage value of autoaggregation is 20% (figure 3.14, a). For *Lactobacillus gasseri*, the highest and lowest of the percentage values of autoaggregation were 38% (for isolate No. 2) and 35% (for isolate No. 1) respectively. While for normal isolate, the autoaggregation value was 53% (figure 3.14, b). The mean values of the percentages autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 18% (SD ± 1.73) (for *Lactobacillus crispatus*) and 37% (SD ± 1.23) respectively, (figure 3.14, c). It has been reported that Toma´s *et al*., (2005) have showed that the percentage value of autoaggregation were 67.76% for *L. johnsonii* pH 5, 44°C for exponentially growing bacteria. This result contradict with the results of the present study. Again, these variations may be attributed to the differences in the socioeconomic situation and (or) bacteriological analysis technique that applied.

The percentage of autoaggregation values at pH 8, temperature 44°C and in anaerobic conditions for *Lactobacillus crispatus* are illustrated in figure 3.15, a, and for *Lactobacillus gasseri* in figure 3.15,b. This figure shows that the highest and lowest autoaggregation value percentages for

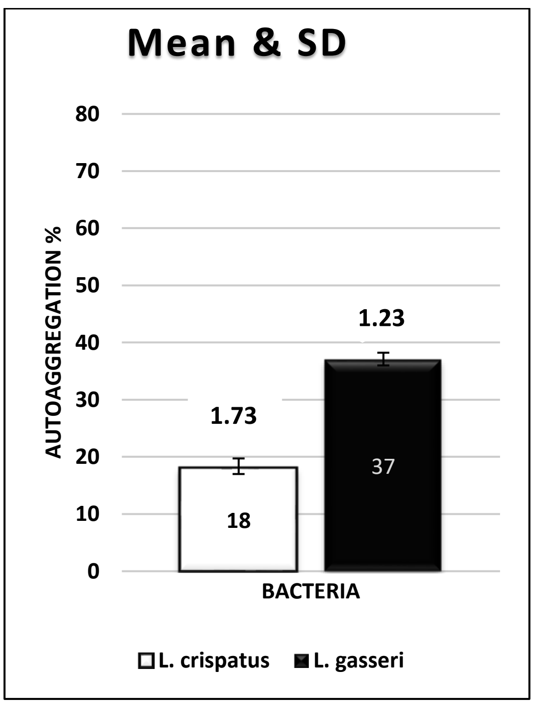
L. crispatus is 10% (for isolate No.2) and 5% (for isolate No. 3) respectively. While for normal isolate (i.e. non-infected female) autoaggregation value percentage 10% (figure 3.15, a). For *Lactobacillus gasseri*, the highest and lowest percentage values of autoaggregation were 28% (for isolate No. 2) and 25% (for isolate No. 4) respectively. While for normal isolate, the percentage value of autoaggregation was 28% (figure 3.15, b). The mean percentage value of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 8% (SD ± 1.73) and 27% (SD ± 1.23) respectively (figure 3.15, c). It have been reported that the percentage value of autoaggregation were 54.23% for *L. johnsonii* pH 8, 44°C (Toma´s *et al*., (2005). Since no previous report was seen concerning the application the same species and at the same environmental conditions (that applied in the present study) for studying autoaggregation, we have urged to compare the mean values of autoaggregation of the present study, which are 8% (for *Lactobacillus crispatus*) and 27% (for *Lactobacillus gasseri*) with that of Toma's *et al*, study (which is 54.23%). However, there appears a clear difference between the data of the two studies in spite of the application the same method and equation for finding the percentages values of autoaggregation. This difference may be explained to the different species that applied in the two studies (hence Toma's *et al* report used *Lactobacillus johnsonii*).



(a)



(b)



(c)

Figure (3.14): Autoaggregation values at pH= 5, temperature 44°C, under anaerobic conditions. (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

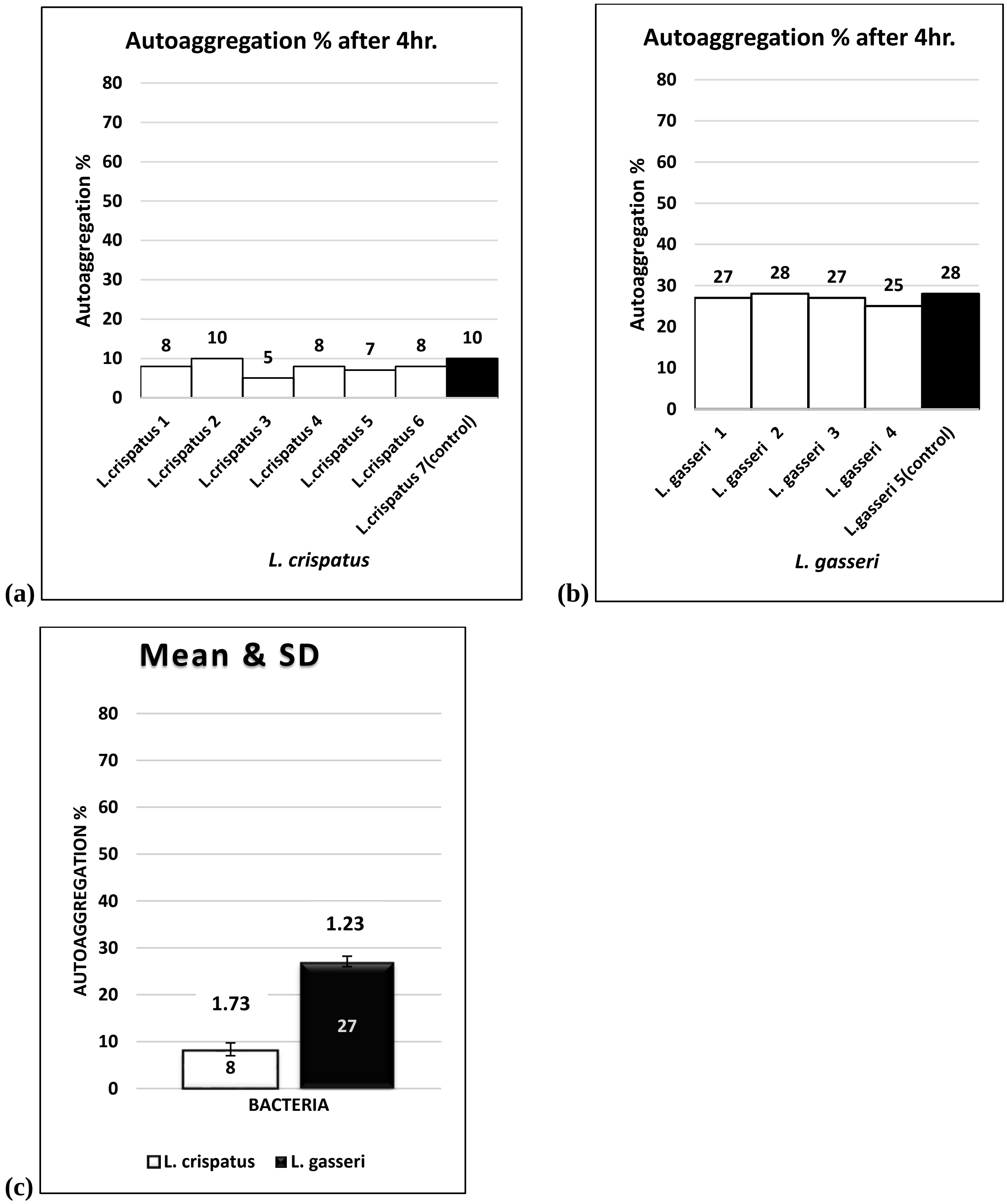


Figure (3.15): Autoaggregation values at pH= 8, temperature 44°C, under anaerobic conditions. (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

3.4.2.4. Effect of aerobic conditions:

Figure 3.16 demonstrates the percentage values of autoaggregation at pH 6.2, temperature 37°C and in aerobic conditions for *Lactobacillus crispatus* (figure 3.16, a) and for *Lactobacillus gasseri* (figure 3.16, b). It illustrates that the highest and lowest autoaggregation values for *L. crispatus* was 28% (for isolates No.3, 5) and 23% (for isolates No. 2, 4) respectively. While for normal isolate (i.e. non-infected female), the percentage value of autoaggregation was 30% (figure 3.16, a). For *Lactobacillus gasseri*, the highest percentage value of autoaggregation was 37% (for isolate No. 3) and 30% (for isolate No. 2) respectively. While for normal isolate, the percentage value of autoaggregation was 40% (figure 3.16, b). The mean percentage values of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 26% (SD ± 2.71) and 35% (SD ± 3.81) respectively (figure 3.16, c). When the results of this section (i.e. growth under aerobic conditions) is compared with that of anaerobic conditions (figure, 3.7), it is clear to observe that autoaggregation is preferred under anaerobic condition (compared to that of aerobic condition). This fact was obvious when a comparison between the data of the two data is done, hence percentage values of autoaggregation under anaerobic conditions for *Lactobacillus crispatus* and *Lactobacillus gasseri* were 30% and 50% respectively (figure, 3.7), while the percentage values of autoaggregation under anaerobic conditions for *Lactobacillus crispatus* and *Lactobacillus gasseri* under aerobic conditions were 26% and 35% (figure, 3.16). Moreover, Gupta *et al*., (2011) have stated that anaerobic condition is more preferred, physiologically, than aerobic condition. In addition, Ekmekci *et al*., (2009) have showed that the percentage values of autoaggregation, under aerobic conditions, were 21% for *L. crispatus* and 47% for *L. gasseri* 12. These results are in accord with the results of the present study.

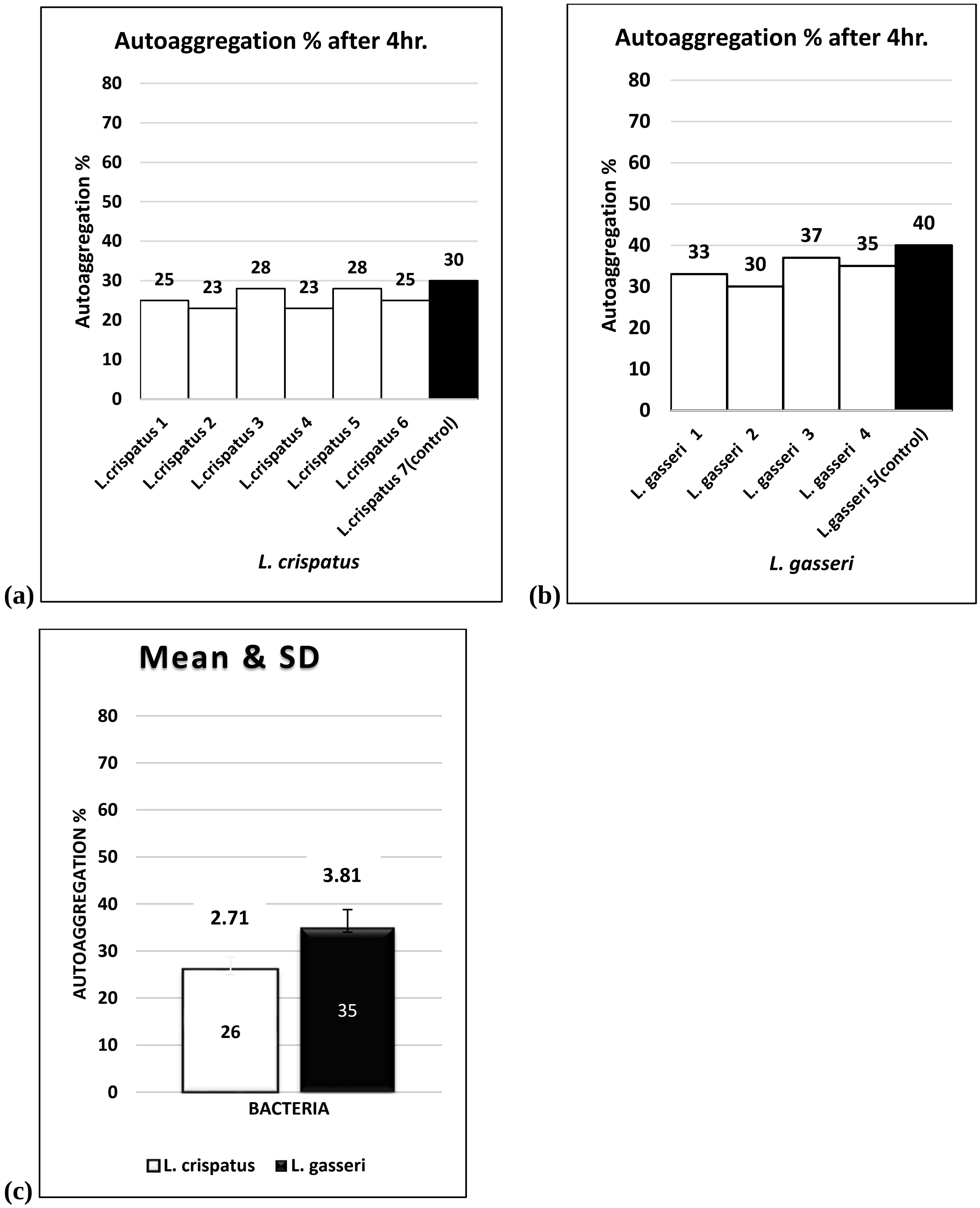
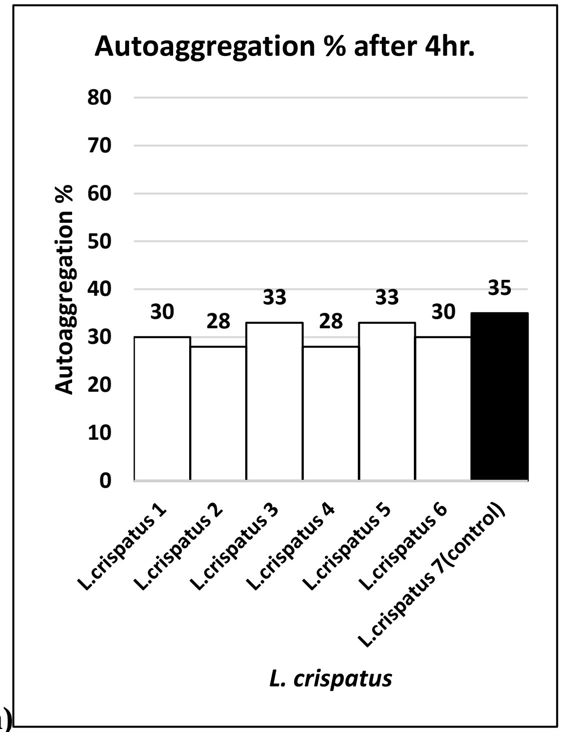


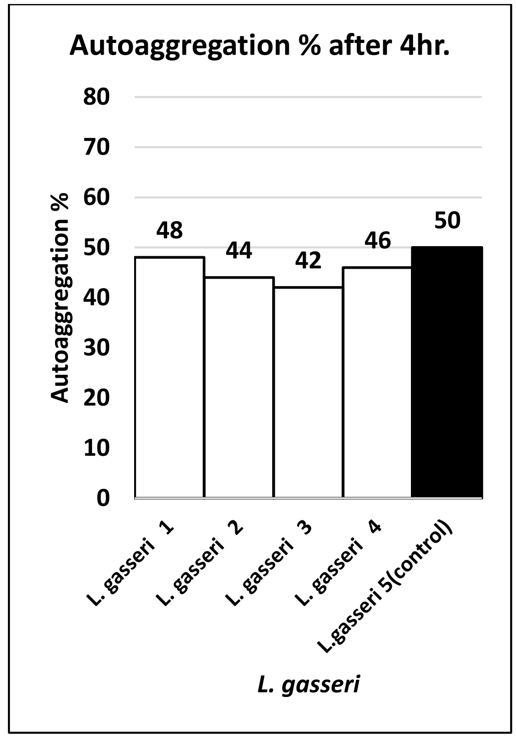
Figure (3.16): Autoaggregation values at pH= 6.2, temperature 37°C, under aerobic conditions. (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

3.4.2.5 Effect of hyper- and hypothermic temperature:

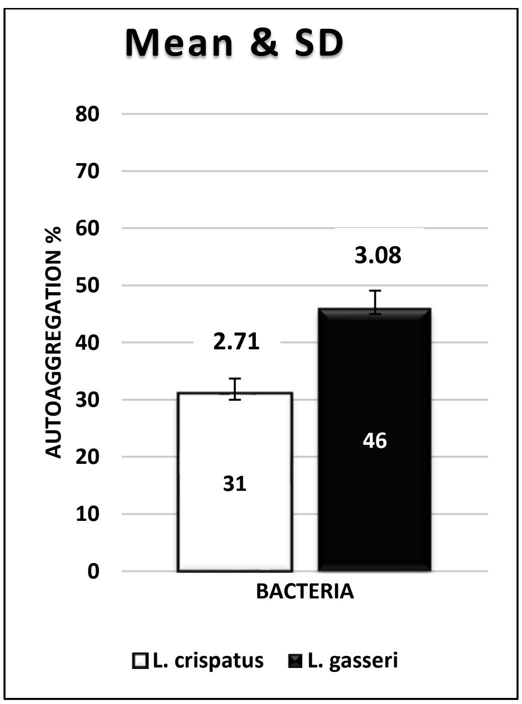
The percentage values of autoaggregation at pH 6.2, temperature 39°C and in anaerobic conditions for *Lactobacillus crispatus* are illustrated in figure 3.17, a, and for *Lactobacillus gasseri* in figure 3.17, b. It illustrates that the highest and lowest percentage values of autoaggregation for *L. crispatus* is 30% (for isolates No.1, 6) and 28% (for isolates No. 2, 4) respectively. While for normal isolate (i.e. non-infected female) the percentage value of autoaggregation was 35% (figure 3.17, a). For *Lactobacillus gasseri*, the highest and lowest percentage values of autoaggregation were 48% (for isolate No. 4) and 42% (for isolate No.1) respectively. While for the normal isolate, the percentage value of autoaggregation value was 50% (figure 3.17, b). The mean percentage values of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 31% (SD ± 2.71) and 46% (SD ± 3.08) respectively (figure 3.17, c).



(a)



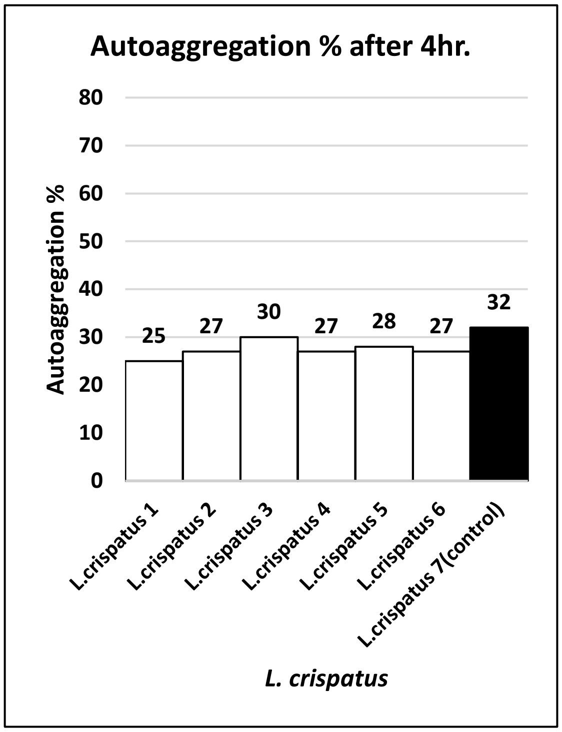
(b)



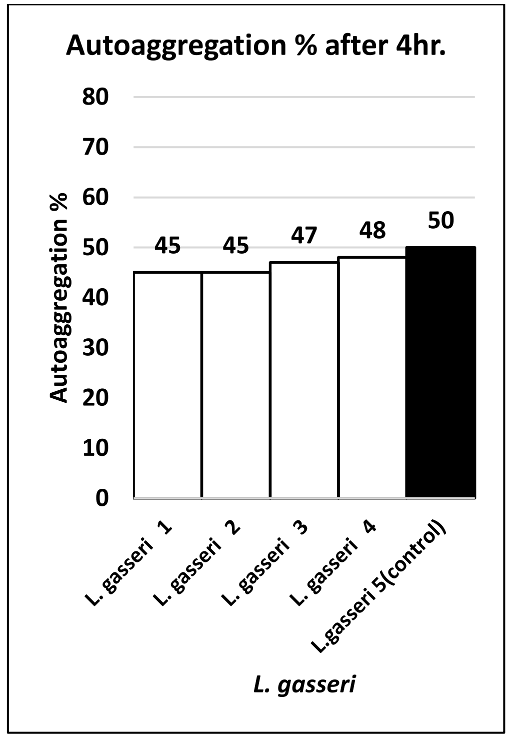
(c)

Figure (3.17): Autoaggregation values at pH= 6.2, temperature 39°C, under anaerobic conditions (Hyperthermia). (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

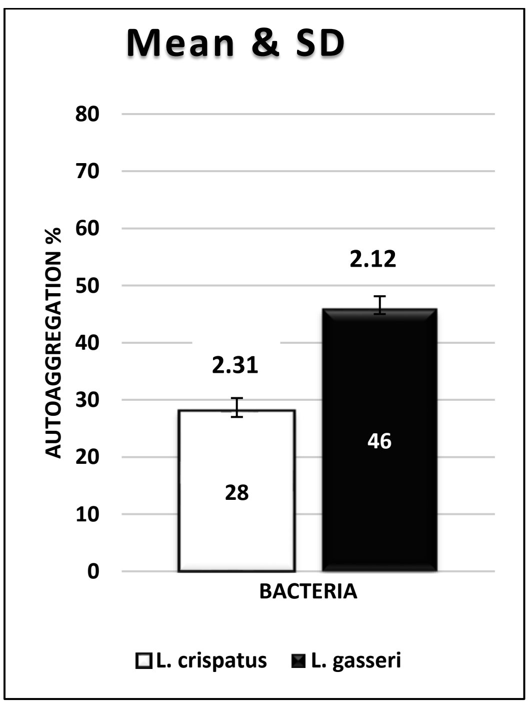
Figure 3.18 shows autoaggregation at pH 6.2, temperature 35°C and at anaerobic conditions for *Lactobacillus crispatus* (figure 3.18, a) and for *Lactobacillus gasseri* (figure 3.18, b). It illustrates that the highest and lowest percentage values of autoaggregation for *L. crispatus* was 30% (isolate No.3) and 25% (isolate No. 1) respectively. While for normal isolate (i.e. non-infected female) the percentage value of autoaggregation was 32% (figure 3.18, a). For *Lactobacillus gasseri*, the highest the percentage values of autoaggregation were 48% (for isolate No. 4) and 45 (for isolates No. 1, and 2) respectively. While for normal isolate, the autoaggregation percentage value was 50% (figure 3.18, b). The mean percentage values of autoaggregation of *Lactobacillus crispatus* and *Lactobacillus gasseri* were 28% (SD ± 2.31) and 47% (SD ± 2.121) respectively (figure 3.18, c).



(a)



(b)



(c)

Figure (3.18): Autoaggregation values at pH= 6.2, temperature 35°C, under anaerobic conditions (Hypothermia). (a) For *L. crispatus*, (b) for *L. gasseri* and (c) mean and SD values for (a) and (b).

The results of figures 3.7, 3.17 and 3.18 are summarized in figure 3.19. This figure shows that the mean percentage value of autoaggregation of *Lactobacillus gasseri* (which is 47.6% in figure 3.19, b) is higher than the mean percentage value of *Lactobacillus crispatus* (which is 26.6% in figure 3.19, a). However, these results are in agree with Ecmekci *et al*., (2009) who studied some factors affecting the autoaggregation ability on vaginal *Lactobacilli* isolated from Turkish women and certified that the percentages values of autoaggregation of vaginal *Lactobacillus gasseri* is higher than *Lactobacillus crispatus*. On the other hand, it is well known that the hyperthermic temperature between 38-39^{o}C (KB, 2009), while the hypothermic temperature between 35-36^{o}C (DF *et al*., 1987). Figure 3.19 demonstrate that percentage values of autoaggregation for both bacterial species (*Lactobacillus crispatus* and *Lactobacillus gasseri*) does not affected upon application of hyperthermic (39^{o}C) and hypothermic (35^{o}C) temperatures. This was obvious from observing the standard deviation values (of the mean of the percentage values of autoaggregation) of both species which are ±1.53 (of mean 29.6%) (For *Lactobacillus crispatus*) (Figure 3.19, a), and ±2.08 (of mean 47.6%) (For *Lactobacillus gasseri*)(Figure 3.19, b). Moreover, it has been reported that during hyperthermic and hypothermic diseases, the microbial physiology and ecology of human body is changed (Melis *et al*., 2000), but it seems that this is not the case in the present study. This fact could be supported by Eschenbach *et al*., (1989) report that suggests the Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis, and due to their inhibitory activity against pathogenic bacteria (Ravaei *et al*., 2013), this may lead to the restoration of the activity of autoaggregation even when they grow at hyper- or hypothermic temperatures.

However, no previous report was noticed in the literature concerning applications of hypothermic (35-36) °C or hyperthermic (38-39) °C temperatures with respect to autoaggregation for vaginal *Lactobacilli*.

The results of the present study indicated that autoaggregation ability is dependent on environmental factors (such as pH, temperature, and aeration conditions).

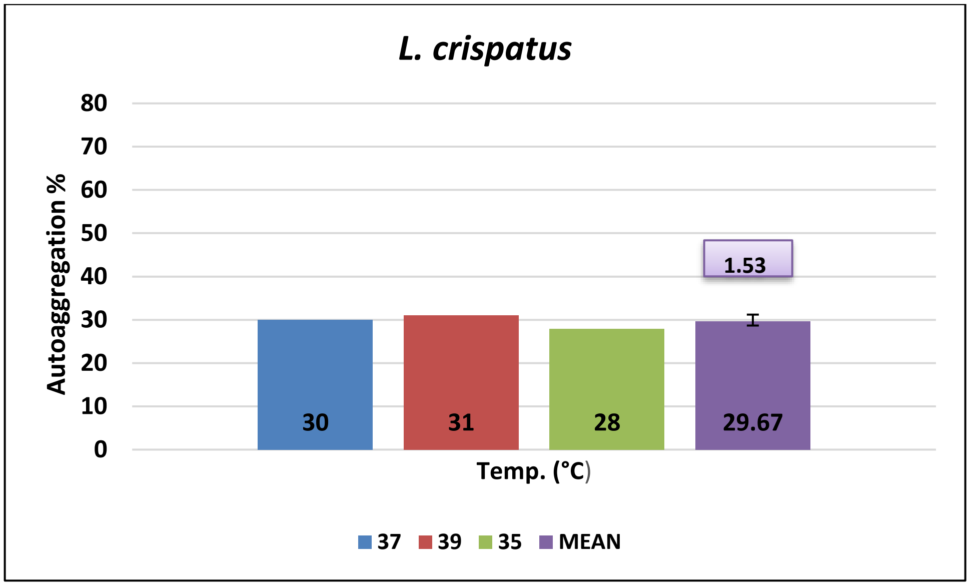
However, the percentage of autoaggregation is increase with the decreasing of the pH of the growth medium; many authors (Kos *et al*., 2003 and Strus *et al*., 2005) support this finding. Moreover, Kos *et al*., (2003) and Strus *et al*., (2005) reported that the ability of autoaggregation is higher in acid environments where probiotic *Lactobacilli* are more adapted to survive and represents the first step towards the formation of biofilms by *Lactobacilli* strains, which helps to inhibit the overgrowth and proliferation of pathogenic microorganisms.

On the other hand, this work showed that high temperature of the growth of *Lactobacilli* reduces autoaggregation scores. There are some evidences to suggest that heat-sensitive surface components on *Lactobacilli* and uropathogens are also involved in certain aggregation reactions (Jabra-Rizk *et al*., 1999).

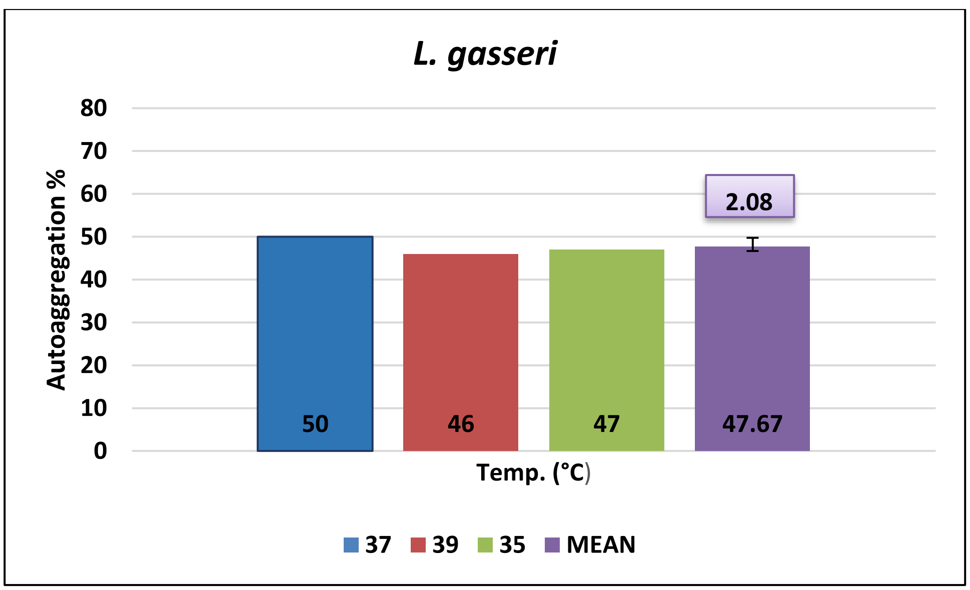
The effect of pH on the autoaggregation percentages was more significant than those of temperature, obtaining the higher values at pH 5 or 6.2. A higher aggregation obtained at low pH could be explained by modifications of the bacterial surface charge, such as a decreasing of Coulomb repulsive forces, which could promote the approach of the cells (Vandevoorde *et al*., 1992). This fact could be relevant in the vaginal ecosystem, where a normal pH < 4.5 could favor the cellular interaction between *Lactobacilli* to form a protective biofilm on the vaginal mucosae.

It have been shown that the enzyme which produced lactic acid from pyruvic acid was lactic acid dehydrogenase. So that assumed a higher lactic acid produced by higher enzyme activity of cells. Increasing temperature and pH caused decreasing enzyme activity and producing lactic acid. The pH of fermentation was shown the number of producing lactic acid of bacteria, the lower pH caused by higher producing lactic acid (Luwihana *et al*., 2011).

Additional studies are required to elucidate this hypothesis, as the biofilm establishment and development is a complex process affected by multiple factors (Kjelleberg and Molin, 2002; Rickard *et al*., 2003). The environmental conditions, the cellular functions and activities influenced by regulator systems operating under high-cell density conditions, as the quorum sensing signals, are included between those factors (Kjelleberg and Molin 2002; McNeill and Hamilton 2003).



(a)



(b)

Figure (3.19): The percentage and mean values of autoaggregation at 37, 39, and 35 °C, for *L. crispatus* (a) and *L. gasseri* (b).

Conclusions

1- Isolation procedure of *Lactobacilli spp.* from bacterial vaginitis infected females requires a shifting in growth medium conditions. This shifting included an alternative change in pH from 6.2 to 4.0 and again to 6.2.

2- The optimum annealing temperature for the primers was found to be 52°C for 16S rRNA *Lactobacillus crispatus* and *Lactobacillus gasseri*.

3- Visual analysis of autoaggregation showed that *L. gasseri* demonstrated huge masses of autoaggregation, compared to *L. crispatus* that revealed smaller masses of autoaggregation.

4- Spectrophotometric method showed that the optimum conditions for autoaggregation are at pH 5, temperature 37^0C, and at anaerobic condition.

5- Anaerobic conditions showed a highest autoaggregation percentage compared to aerobic conditions.

6- Compared to the normal temperature, no differences in autoaggregation were noticed upon growing of the *Lactobacilli* at hyperthermic (38-39°C) and hypothermic (35-36°C).

Recommendations

1. Work is required in this field in order to plot a complete phylogenetic map for the distribution of these bacteria in Baghdad and other cities in Iraq.

2. A better understanding of the species composition and ecology of bacterial ecosystems may help to develop better prophylaxis against BV and HIV.

3. Vaginal colonization of women with these species may be advantageous in the maintenance of a normal microflora and the prevention of sexually transmitted diseases. Randomized, controlled trials will be needed to test this hypothesis.

5. Future studies are encouraged to assess technological properties of those microorganisms for clinical use, including determination of their viability and stability in pharmaceutical preparations such as capsules resistant to gastrointestinal tract for oral intake and ovules/capsules for intravaginal administration.

6. The *Lactobacilli* used in this study may protect the vaginal epithelium through a barrier created by autoaggregation. Consequently, they may be excellent candidates for eventual use as a probiotic. Studies to further evaluate their feasibility as such are needed.

7. An acidifying agent, such as vitamin C, being a particularly safe product with very low risk of systemic adverse effects, could play an important role in prophylaxis for those women with high vaginal pH suggestive of disordered vaginal flora, including those conditions (such as pregnancy, recurrent BV episodes, diabetes and risky sexual habit), where long-lasting treatments and repeated cycles after each menses are required.

8. Other environmental conditions, that affect autoaggregation, for example the cellular functions and activities influenced by regulator systems operating under high-cell density conditions, as the quorum sensing signals, should be imlicated with autoaggregation.

References

A

- **Antonio** M.A., Hawes S.E., and Hillier S.L. (1999). The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *Journal of Infectious Diseases*. 180, 1950-1956.
- **Aas** JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. 2005. Defining the normal bacterial flora of the oral cavity. *J ClinMicrobiol*, 43:5721-5732.
- **Amsel**, R., P. A. Totten, C. A. Spiegel, K. S. C. Chen, D. A. Eschenbach, and K. K. Holmes. 1983. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *Am. J. Med*. 74:14–22.
- **Andreu** A. 2004. *Lactobacillus* as probiotic for preventing urogenital infection. *Rev Med Microbial*; 15: 1-6.

- **Andrew** G. F., Gerald J. C., Barrie P. M., and Anthony S. (1996). *Practical medical microbiology* 4thed. New York- London.
- **Antikainen**Jenni. 2007. Surface proteins of *Lactobacillus crispatus*: Adhesive properties and cell wall anchoring. *Department of Biological and Environmental Sciences*, Faculty of Biosciences, University of Helsinki.
- **Antonio** M. A. D., S. E. Hawes, and Hillier S. L. 1999. The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J. Infect. Dis.* 180:1950–1956.
- **Aroutcheva** A., Gariti D., Simon M., Shott S., Faro J., Simoes JA, Gurguis A, Faro S. (2001). Defense factors of vaginal *Lactobacilli*. *Am.J. Obstet. Gynecol.*, 185, 375-379.
- **Atlas** R.M., Brown A.E. and Parks L.C. (1995). Laboratory manual of experimental microbiology. 1st ed. Mosby, St. Louis, U.S.A.
- **Axelsson** L. 1998. Lactic Acid Bacteria: Classification and Physiology. p. 1-72. *In* S.

B

- **Baddour** LM. 1994. Virulence factors among gram-positive bacteria in experimental endocarditis. *Infect Immun*. 62:2143-2148.
- **Balkus** J.E., Mitchell C., Agnew K., Liu C., Fiedler T., Cohn S.E., Lugue A., Coombs R., Fredricks D.N., and Hitti J. 2012. Detection of hydrogen peroxide-producing *Lactobacillus* species in the vagina: a comparison of culture and quantitative PCR among HIV-1 seropositive women. *BMC Infectious Diseases*. 12:188.
- **Ballongue** J. 1993. *Bifidobacteria* and probiotic action. *In* S. Salminen and A. von Wright (ed.), Lactic acid bacteria. p. 357-428. Marcel Dekker Inc., New York.
- **Baron** E.J., Peterson L.R., and Finegold S. (1999). Diagnostic Microbiology. 9thed. Baily and Scott's. The C.V. Mosby Company.
- **B. A.**Bensing, and G. M. Dunny. 1993. Cloning and molecular analysis of genes affecting expression of binding substances, the recipient-encoded receptor(s) mediating mating aggregate formation in *Enterococcus faecalis. J. Bacteriol*. 175:7421–7429.
- **Boris** S, Barb´es C. 2000. Role played by *Lactobacilli* in controlling the population of vaginal pathogens. *Microbes and Infection.* 2(5):543–546.

- **Boris** S., J. E. Sua´rez, F. Va´zquez, and C. Barbe´s. 1998. Adherence of human vaginal *Lactobacilli* to vaginal epithelial cells and interaction with uropathogens. *Infect. Immun*. 66:1985–1989.
- **Boris** S., J. E. Suarez, and C. Barbes. 1997. Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. *J. Appl. Microbiol*. 83:413–420.
- **Burton** JP, Cadieux PA, Reid G. 2003. Improved understanding ofthe bacterial vaginal microbiota of women before and afterprobiotic instillation. *Appl Environ Microbiol*; 69: 97-101.

C

- **Cannon** JP, Lee TA, Bolanos JT, Danziger LH.2005. Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. *Eur J ClinMicrobiol Infect Dis*. 24(1):31-40.
- **Caries**: The Disease and Its Clinical Management. 2 edition. Edited by: Fejerskov O, Kidd E. Chichester. UK: Wiley-Blackwell; 2008:163-187.
- **Carlsson** J. and L. Gothefors. 1975. Transmission of *Lactobacillus jensenii* and *Lactobacillus acidophilus* from mother to child at time of delivery. *J ClinMicrobiol*. 1:124-8.

- **Castagliuolo** I., Galeazzi F., Ferrari S., Elli M., Brun P., Cavaggioni A., Tormen D., Sturniolo G., et al. (2005). Beneficial effect of auto-aggregating *Lactobacillus crispatus* on experimental induced colitis in mice. *FEMS Immunol Med Microbiol*. 43, 197–204.
- **Cesena** C., L. Morelli, M. Alander, T. Siljander, E. Tuomola, S.Salminen, T. Mattila-Sandholm, T. Vilpponen-Salmela, and A. vonWright. 2001. *Lactobacillus crispatus*and its nonaggregating mutant in human colonization trails. *J. Dairy Sci*. 84:1001–1010.
- **Chan** R. C. Y., G. Reid, R. T. Irvin, A. W. Bruce, and J. W. Costerton. 1985. Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. *Infect. Immun*. 47:84–89.
- **Clewell** D. B. and K. E. Weaver. (1989). Sex pheromones and plasmid transfer in *Enterococcus faecalis*. *Plasmid* 21,175-184.
- **Cohen**, C. R., A. Duerr, N. Pruithithada, S. Rugpao, S. L. Hillier, P. Garcia, and K. Nelson. 1995. Bacterial vaginosis and HIV seroprevalence among female commercial sex workers in Chiang Mai, Thailand. *AIDS*. 9:1093–1097.
- **Cowan** S.T., and Steel K.J. (1975). Manual for the identification of medical bacteria, 2nd ed. Cambridge university press, London.

D

- **Damelin** L.H., Paximadis M., Mavri D., Birkhead M., Lewis D. A., and Tiemessen C. T. 2011. Identification of predominant culturable vaginal *Lactobacillus* species and associated bacteriophages from women with and without vaginal discharge syndrome in South Africa. *Journal of Medical Microbiology*. 60, 180–183.
- **DeMan** J.C., Rogosa M., and Sharpe M.E. (1960). A medium for the cultivation of *Lactobacilli. Journal of Applied Bacteriology* 23, 130-135.
- **DelRe** B., Sgorbati B., Miglioli M., and Palenzona D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacteriumlongum*. *Letters in Applied Microbiology*. 31, 438–442.
- **DF**Danzl, RS Pozos, PS Auerbach, S Glazer, W Goetz, E Johnson, J Jui, P Lilja, JA Marx, J Miller, *etal*., (1987). Multicenter hypothermia survey. *Ann Emerg Med*. 16(9):1042-55.

- **Drago** L., Gismondo M.R., Lombardi A., de Haen C. and Gozzini, L. (1997). Inhibition of in vitro growth of enteropathogens by new *Lactobacillus* isolates of human intestinal origin. *FEMS Microbiol Lett.* 153, 455–463.
- **DuPlessis** EM and Dicks LM. (1995). Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus amylovorus, Lactobacillus gallinarum, Lactobacillus gasseri* and *Lactobacillus johnsonii*. *Curr. Microbiol*. 31: 114-118.

E

- **Ekmekci** Havva, Aslim Belma, and Darilmaz Derya Onal. 2009. Some factors affecting the autoaggregation ability of vaginal *Lactobacilli* isolated from Turkish women. *Arch. Biol. Sci*., Belgrade, 61 (3), 407-412.
- **Eschenbach** D A, Davick P R, Williams B L, Klebanoff S J, Young-Smith K, Critchlow C M, and Holmes K K. (1989). Prevalence of hydrogen peroxide-producing *Lactobacillus species* in normal women and women with bacterial vaginosis. *J Clin Microbiol*. 27(2): 251–256.

- **Eschenbach**, D. A., S. L. Hillier, C. Critchlow, C. Stevens, T. De Rousen, and K. K. Holmes. 1988. Diagnosis and clinical manifestations of bacterial vaginosis. *Am. J. Obstet. Gynecol.* 158:819–828.

F

- **Fernandes** C.F., K.M. Shahani, M.A. Amer. 1987. Therapeutic role of dietary *Lactobacilli* and lactobacillic fermentated dairy products. *FEMS Microbiol.* Rev. 46, 343-356.
- **Flint** Joseph F., Angert Esther R. 2005. Development of a strain-specific assay for detection of viable *Lactobacillus spp*. HOFG1 after application to cattle feed.*J Microbiol Methods*. 61 (2):235-43.
- **Forbes** B.A., Daniel F.S., and Alice S.W. (2007). Bailey and Scott's diagnostic microbiology. 12th. ed. *Mosby Elsevier company*, USA.
- **Foxman** B., Barlow R., Arcy H. D., Gillespie B., and Sobel J. D. 2000. Urinary tract infection: self-reported incidence and associated costs. *Annals of Epidemiology*, vol. 10, no. 8, pp. 509–515.

- **Foxman** B.1990. Recurring urinary tract infection: incidence and risk factors. *American Journal of Public Health*. vol. 80, no. 3, pp. 331–333.
- **Frank** Jeremy A., Reich Claudia I., Sharma Shobha, Weisbaum Jon S., Wilson Brenda A., and Olsen Gary J. 2008. Critical evaluation of Two Primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and environmental microbiology*. Vol. 74, No. 8, p. 2461–2470.
- **Fuller** R. Probiotics: their development and use. In: Fuller R, Heidt PJ, Rusch V, Van der Waaij D, eds. *Probiotics: Prospectsof Use in Opportunistic Infections.* Herborn Dill, Germany: Institute for Microbiology and Biochemistry; 1992:1–7.

G

- **Gasson** M. J., S. Swindell, S. Maeda, and H. M. Dodd. 1992. Molecular rearrangement of lactose plasmid DNA associated with high-frequency transfer and cell aggregation in *Lactococcuslactis*712. *Mol. Microbiol*. 6:3213–3223.

- **GB** Melis, MT Ibba, B Steri, P Kotsonis, V Matta, AM Paoletti. 2000. Role of pH as a regulator of vaginal physiological environment. *Minerva Ginecol.* 52(4):111-21.
- **Gevers** D., G. Huys, J. Swings. 2001. Applicability of rep-PCR finger printing for identification of *Lactobacillus* species. *FEMS Microbiol. Lett*. 205, 31-36.
- **Gibson** G.R., R. Fuller. 2000. Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and probiotics for human use. *J. Nutr*. 130, 391S- 395S.
- **Gil** Natalia F., Martinez Rafael C.R., Gomes Bruna C., Nomizo Auro, De Martinis Elaine C. P. (2010).Vaginal *Lactobacilli* as potential probiotics against *Candida spp*. *Brazilian Journal of Microbiology*. 41: 6-14.
- **Giorgi** A., S. Torriani, F. Dellaglio, G. Bo, E. Stola, and L. Bernuzzi. 1987. Identification of vaginal *Lactobacilli* from asymptomatic women. *Microbiologica*. 10:377-84.
- **Giraud** Eric, Lelong Bertrand, and Raimbault Maurice. 1991. Influence and of pH and initial lactate concentration on the growth of *Lactobacillus plantarum*. *Appl Microbio1 Biotechnol*. 36:96-99.

- **Goh** Y.J. and Klaenhammer T. R. 2010. Functional Roles of Aggregation-Promoting-Like Factor in Stress Tolerance and Adherence of *Lactobacillus acidophilus* NCFM. *Applied and Environmental Microbiology*. p. 5005–5012.
- **Gorbach** S. L. 1990. Lactic acid bacteria and human health. Ann. Med. 22, 37-41.
- **Gupta** K., Scholes D., and Stamm W. E. 1999. Increasing prevalence of antimicrobial resistance among uropathogens causing acute uncomplicated cystitis in women. *Journal of the American Medical Association*, vol. 281, no. 8, pp. 736–738.
- **Gupta** S, Abu-Ghannam N, Scannell AGM. (2011).Growth and kinetics of *Lactobacillus plantarum* in the fermentation of edible Irish brown seaweeds. *Food and Bioproducts Processing*. Vol. 89, no. 4, pp. 346-355.

H

- **Hallen** A., C. Jarstrand, and C. Påhlson. 1992. Treatment of bacterial vaginosis with *Lactobacilli*. *Sex. Transm. Dis.* 19:146–148.

- **Hammes** W, Weiss N, Holzapfel W. 1995.The genera *Lactobacillus* and *Carnobacterium*. In: Balows A, Tr¨uper H, Dworkin M,Harder W, Schleifer KH, eds. *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Applications. Vol II.* 2nd ed. New York, NY: Springer:1536–1594.
- **Hammes** W. P. and R. F. Vogel. 1995. The genus *Lactobacillus*, *In*: B. J. B. Wood and W. H. Holzapfel (ed.).The lactic acid bacteria. Volume 2. The genera of lactic acid bacteria. Blackie Academic and Professional, London. p. 19-54.
- **Hammes** W. P. and Vogel R. F. The genus *Lactobacillus*. In: Wood B J B, Holzapfel W H, editors; Wood B J B, Holzapfel W H, editors. The lactic acid bacteria. 2. The genera of lactic acid bacteria. London, United Kingdom: Blackie Academic and Professional; 1995. pp. 19-54.
- **Hammes**W. P. and C. Hertel.2003. The genera *Lactobacillus* and *Carnobacterium*. *In* M. Dworkin *et al*. (ed.).The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed., release 3.15.[Online.] Springer-Verlag, NewYork, N.Y. http://link.springer-ny.com/link/ service/books/10125/.
- **Hammes** W. P. and C. Hertel. 2006. The Genera *Lactobacillus* and *Carnobacterium*. p. 320-403. *In* M. Dwarkin (ed.), The Prokaryotes, 3rd ed, vol. 4. Springer, New York.

- **Hammes** W., Weiss N., Holzapfel W. The genera *Lactobacillus* and *Carnobacterium*. In: Balows A, Tr¨uper H, Dworkin M, Harder W, Schleifer KH, eds. *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Applications. Vol II.* 2nd ed. New York, NY: Springer; 1995:1536–1594.
- **Harley** J.P., and Prescott L.M. 2002. Laboratory Exercises in Microbiology. 5th.ed. The McGraw-Hill Companies, Inc., New York.
- **Harrigan** W.F. and McCance M.E. 1976. *Laboratory Methods in Food and Dairy Microbiology*. Academic Press Inc., London.
- **Harty** D. W. S., Oakley H. J., Patrikakis M., Hume E. B. H., Knox K. W. 1994. Pathogenic potential of *Lactobacilli*. *International Journal of Food Microbiology*. Vol. 24 pp. 179-189.
- **Harwood** B., R. Mittendorf, D. Judge, S. Dayal, and C. Walker. 1996. Patterns of vaginal douching and their association with vaginal bacteriosis. *Infect. Dis. Obstet. Gynecol.* 4:51.
- **Hata** T, Alemu M, Kobayashi M, Suzuki C, Nitisinprasert S, Ohmomo S. 2009. Characterization of a bacteriocin produced by *Enterococcus faecalis* N1-33 and its application as a food preservative. *Journal of Food Protection*; 72: 524–530.

- **Hay** P. E., R. F. Lamont, D. Taylor-Robinson, D. J. Morgan, C. A. Ison, and J. Pearson. 1994. Abnormal bacterial colonization of the genital tract and subsequent preterm delivery and late miscarriage. *Br. Med. J.* 308:295–298.
- **Hill** G. B. 1993. The microbiology of bacterial vaginosis. *Am. J. Obstet. Gynecol.* 169:450–454.
- **Hillier** SL. 1998. The vaginal microbial ecosystem and resistance to HIV. *AIDS Res Hum Retroviruses.* 14 (Suppl 1): S17–21.
- **Hillier**, S. L., M. A. Krohn, E. Cassen, T. R. Easterling, L. K. Rabe, and D. A. Eschenbach. 1995. The role of bacterial vaginosis and vaginal bacteria in amniotic fluid infection in women in preterm labor with intact fetal membranes. *Clin. Infect. Dis.* 20(Suppl. 2):S276–278.
- **Hillier**, S. L., R. P. Nugent, D. A. Eschenbach, M. A. Krohn, R. S. Gibbs, D. H. Martin, M. F. Cotch, R. Edelman, J. G. Pastorek II, A. V. Rao, D. McNellis, J. A. Regan, J. C. Carey, M. A. Klebanoff, and the Vaginal Infections and Prematurity Study Group. 1995. Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. *N. Engl. J. Med.* 333:1737–1742.

- **Holzapfel** W.H., P. Haberer, R. Geisen, J. Björkroth, U. Schillinger. 2001. Taxonomy and important features of probiotic microorganisms in food nutrition. *Am. J. Clin. Nutr.* 73, 365S-373S.
- **Hooton**, T. M., C. I. Fennell, A. M. Clark, and W. E. Stamm. 1991. Nonoxynol- 9: differential antibacterial activity and enhancement of bacterial adherence to vaginal epithelial cells. *J. Infect. Dis.* 164:1216–1219.
- **Hudault** S, Li´evin V, Bernet-Camard M-F, Servin AL. 1997. Antagonistic activity exerted in vitro and in vivo by *lactobacillus casei* (strain GG) against *Salmonella typhimurium*C5 infection. *Applied and Environmental Microbiology*. 63(2):513–518.
- **Husni** RN, Gordon SM, Washington JA, Longworth DL. 1997. *Lactobacillus* bacterimia and endocarditis: Review of 45 cases. *Clin Infect Dis*, 25:1048-1055.

I

- **Iqbal** S. M. and R. Kaul. 2008. Mucosal innate immunity as a determinant of HIV susceptibility. *Am. J. Reprod. Immunol*. 59**:**44–54.

J

- **Jabra-Rizk** M. A., Falker W. A., Merz W. G., Kelley J. I., Baqui A. A. M. A., and T. F. Meiller. (1999). Coaggregation of *Candida dubliniensis* with *Fusobacterium nucleatum*. *J. Clin. Microbiol.* 37,1463-1468.
- **Jay** J.M. 2000. Fermentation and fermented dairy products, In *Modern Food Microbiology*, 6th edition. pp. 113-130. An Aspen Publication, Aspen Publishers, Inc. Gaithersburg, USA.

K

- **Kandler** O, Weiss N. Regular, nonsporing gram-positive rods. In: Sneath P H A, editor; Sneath P H A, editor. Bergey's manual of systematic bacteriology. Vol. 2. Baltimore, Md: Williams and Wilkins; 1986. pp. 1208-1234.

- **Kandler** O. and Weiss N. (1986). Genus *Lactobacillus.* In: Bergey´s Manual of Systematic Bacteriology, Vol. 2.(Edited by Sneath, P. H. A.; Mair N. S. and Hold J. G.), William and Wilkins Co., Baltimore, USA.
- **Karch** H., Russman H., Schmidt H., Schwarzkoph A., and Heesemann J. 1995. Long shedding and clonal turnover of EHEC0157 in diarrhea disease. *J. Clin. Microbiol*.33:1602.
- **karthikeyan** V. and Santosh S. W. (2009). Isolation and partial characterization of bacteriocin produced from *Lactobacillus plantarum. Afr. J. Microbial. Res.*, 3(5):233-239.
- **KB** Laupland. (2009). Fever in the critically ill medical patient. *Crit Care Med.* 37 (7 Suppl): S273-8.
- **Kilian** M. 2005.*Streptococcus* and *Lactobacillus*. In Topley and Wilson’s Microbiology and Microbial Infections. Edited by: Borriello P, Murray PR, Funke G. London: Hodder Arnold; 833-881.
- **Kilic** Ali O., Pavlova Sylvia I., Alpay Sengul, Sirri Kilic S., and Tao Lin. 2001. Comparative study of vaginal *Lactobacillus* phages isolated from women in the United States and turkey: prevalence, morphology, host range, and DNA homology. *Clinical and Diagnostic Laboratory Immunology*. p. 31–39.

- **Kjelleberg** S. and Molin S. (2002). Is there a role for quorum sensing signals in bacterial biofilms? *Curr Opin Microbiol*.5, 254–258.
- **Klaenhammer** T.R. 1988. Bacteriocins of lactic acid bacteria. *Biochimie* 70(3): 337 – 349.
- **Klebanoff** S. J., S. L. Hillier, D. A. Eschenbach, and A. M. Waltersdorph. 1991. Control of the microbial flora of the vagina by H2O2-generating *Lactobacilli*. *J. Infect. Dis.* 164:94–100.
- **Kmet** V., M. L. Callegari, V. Bottazzi, and L. Morelli. 1995. Aggregation promoting factor in pig intestinal *Lactobacillus* strains. *Lett. Appl. Microbiol.* 21:351–353.
- **Knorr** D. 1998. Technology aspects related to microorganisms in functional foods. *Trends Food Sci*. 9, 295-306.
- **Kolenbrander** P. E. 2000. Oral microbial communities: biofilms, interactions, and genetic systems. *Annu. Rev. Microbiol*. 54:413–439.
- **Kolenbrander** P. E. (1988). Intergeneric coaggregation among human oral bacteria and ecology of dental plaque. *Annu. Rev. Microbiol.* 42, 627-656.

- **Kos** B., Suskovic J., Vukovic S., Simpraga M., Frece J., and Matosic S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus M92*. *J ApplMicrobiol*. 94, 981–987.

L

- **Langendijk** P. S., F Schuts, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. F. Wilkinson, and G. J. Welling. 1995. Quantitative fluorescence *in situ* hybridization of *Bifidobacterium spp*. with genus-specific 16S rRNA-targetedprobes and its application in fecal samples. *Appl. Environ. Microbiol*. 61:3069-3075.
- **Larsen** B. (1993). Vaginal flora in health and disease. *Clin Obstet Gynecol*.36, 107–121.
- **Lepargneur** J.P. and Rousseau V. (2002). Protective role of the Do¨derlein flora. *J Gynecol Obstet Biol Reprod* 31, 485–494.
- **Liu** X., Lagenaur L. A., Lee P. P. and Xu Q. (2008). Engineering human vaginal *Lactobacillus* for surface expression of two-domain CD4. *Appl Environ Microbiol* 74, 4626–4635.

- **Liu** X., Lagenaur L. A., Simpson D. A., Essenmacher K. P., Frazier- Parker C. L., Liu Y., Tsai D., Rao S. S., Hamer D. H. and other authors. (2006). Engineered vaginal *Lactobacillus* strain for mucosal delivery of the human immunodeficiency virus inhibitor cyanovirin-N. *Antimicrob Agents Chemother* 50, 3250–3259.
- **Luwihana** Sri, Yulianto Wisnu Adi, and Nugraheni Esti. (2011). Effect of temperature and ph on growth of *Lactobacillus acidophilus fncc 0015* and acceptability of noni (*morindacitrifolia*) probiotic drink .The 3rd International Conference of Indonesian Society for Lactic Acid Bacteria (3rd IC-ISLAB) : Better Life with Lactic Acid Bacteria: Exploring Novel Functions of Lactic Acid Bacteria.

M

- **Madigan** MT, Oren A. 1999. Thermophilic and halophilic extremophiles. *Curr Opin Microbiol*, 2(3):265-269.
- **Makarova** K., Slesarev A., Wolf Y., Sorokin A., Mirkin B., Koonin E., Pavlov A., Pavlova N. *etal*. (2006). Comparative genomics of the lactic acid bacteria. *ProcNatlAcadSci U S A.* 103 (42): 15611–6. doi:10.1073/pnas.0607117103. PMC 1622870. PMID 17030793.

- **Marsh** PD, Martin MV. 2009. Oral Microbiology. 5 edition. Edinburgh: Churchill Livingstone Elsevier.
- **Marsh** PD, Nyvad B. 2008. The oral microflora and biofilms on teeth. In Dental Caries: The Disease and Its Clinical Management. 2 edition. Edited by: Fejerskov O, Kidd E. Chichester. UK: Wiley-Blackwell; 163-187.
- **Martin** H. L., B. A. Richardson, P. M. Nyange, L. Lavreys, S. L. Hillier, B. Chohan, K. Mandaliya, J. O. Ndinya-Achola, J. Bwayo, and J. Kreiss. 1999. Vaginal *Lactobacilli*, microbial flora, and risk of human immunodeficiency virus type one and sexually transmitted disease acquisition. *J. Infect. Dis*. 180:1863–1868.
- **Martius** J., M. A. Krohn, S. L. Hillier, W. E. Stamm, K. K. Holmes, and D. A. Eschenbach. 1988. Relationship of vaginal *Lactobacillus* species, cervical Chlamydia trachomatis, and vaginosis to preterm birth. *Obstet. Gynecol*. 71:89–95.
- **Mastromarino** P., Brigidi P., Macchia S., Maggi L., Pirovano F., Trinchieri V., Conte U., and Mateuzzi D. (2002). Characterization and selection of vaginal *Lactobacillus* for the preparation of vaginal tablets. *J ApplMicrobiol*. 93, 884–893.

- **McGregor** J. A., J. I. French, R. Parker, D. Draper, E. Patterson, W. Jones, K. Thorsgard, and J. McFee. 1995. Prevention of premature birth by screening and treatment for common genital tract infections. Results of a prospective controlled evaluation. *Am. J. Obstet. Gynecol.* 173:157–167.
- **McGroarty** J. A. and G. Reid. 1988. Detection of a *Lactobacillus* substance that inhibits *Escherichia coli*. *Can. J. Microbiol*. 34:974–978.
- **McGroarty** JA. 1993. Probiotic use of *Lactobacilli* in the human female urogenital tract. *FEMS Immunology and Medical Microbiology*. 6(4):251–264.
- **McLean** N. W., and I. J. Rosenstein. 2000. Characterization and selection of a *Lactobacillus* species to re-colonise the vagina of women with recurrent bacterial vaginosis. *J. Med. Microbiol*. 49:543–552.
- **McNeill** K., and Hamilton I.R. (2003). Acid response of biofilm cells of *Streptococcus mutans*. *FEMS MicrobiolLett*. 221, 25–30.

O

- **Ocaňa** V. S. and M. E. Nader-Macías. (2002). Vaginal *Lactobacilli*: self- and coaggregation ability. *Brit. J. Biomed. Sci.* 59, 183-190.
- **Orla-Jensen** S.1919. The Lactic Acid Bacteria. Host and Son, Copenhagen.

P

- **Patel** J. B. 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Mol. Diagn.* 6:313–321.
- **Pavlova** S. I., A. O. Kilic, S. S. Kilic, J.-S. So, M. E. Nader-Macias, J. A. Simoes, and L. Tao. 2002. Genetic diversity of vaginal *Lactobacilli* from women in different countries based on 16S rRNA gene sequences. *J ApplMicrobiol*2002; 92: 451– 459.

- **Pendharkar** Sonal, Magopane Tebogo, Larsson Per-Göran, Bruyn Guy de, Gray Glenda E, Hammarström Lennart, and Marcotte Harold. 2013. Identification and characterization of vaginal *Lactobacilli* from South African women. *BMC Infectious Diseases*. 13:43.
- **Pot** B., W. Ludwig, K. Kersters, and K-H Schleifer. 1994. Taxonomy of lactic acid bacteria.*In*: L. de Vuyst and E. J. Vandamme (ed.), Bacteriocins of lactic acid bacteria. Microbiology, genetics and applications. Blackie Academic and Professional, London. p. 13-90
- **Pulugurtha** Shamala. 2010. A diagnosis of an infection of the urinary tract and *Lactobacillus*. Internet site: http:// www.Livestrong.com/article/251934.

R

- **Ravaei** Amin, poor Zoheir Heshmati, Salehi Taghi Zahraei, Tamai Iradj Ashrafi, Ghane Masood, and pour Jalal Derakhshan. (2013). Evaluation of Antimicrobial Activity of Three *Lactobacillus spp*. against Antibiotic Resistance *Salmonella typhimurium. Advanced Studies in Biology*, Vol. 5, no. 2, 61 – 70.

- **Ravel** Jacques, Gajer Pawel, Abdo Zaid, Schneider G. Maria, Koeniga Sara S. K., Mc Culle Stacey L.,Karlebach Shara, Gorle Reshma, Russell Jennifer, Tacket Carol O., Brotman Rebecca M., Davis Catherine C., Ault Kevin, Peralta Ligia, and Forney Larry J. (2011). Vaginal microbiome of reproductive-age women. *PNAS*,vol. 108, suppl. 1,4680–4687.
- **Redondo-Lopez** V., R. L. Cook, and J. D. Sobel. 1990. Emerging role of *Lactobacilli* in the control and maintenance of the vaginal bacterial microflora. *Rev. Infect. Dis.* 12:856–872.
- **Reid** G. 1999. The scientific basis for probiotic strains of *Lactobacillus*. *Applied and Environmental Microbiology*. 65(9): 3763–3766.
- **Reid** G. 2001. Probiotic agents to protect the urogenital tract against infection. *Am. J. Clin. Nutr*. 73, 437S-443S.
- **Reid** G. 2008. Probiotic *Lactobacilli* for urogenital health in women. *J ClinGastroenterol*; 42 (3): 234-236.
- **Reid** G., Heinemann C., Velraeds M., van der Mei H., and Busscher H. (1999). Biosurfactants produced by *Lactobacillus*. *Methods Enzymol.310*, 426- 433.

- **Reid** G., MacGroarty J.A., Domingue P.A.G., Chow A.W., Bruce A.W., Eisen A., Costerton J.W. (1990). Coaggregation of urogenital bacteria *in vitro* and *in vivo*. *Current Microbiol.* 20, 47-52.
- **Reid** G., McGroarty J.A., Angotti R., and Cook R.L. (1988). *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. *Canadian Journal of Microbiology.* 34, 344–351.
- **Reniero** R., Cocconcelli P. S., Bottazzi V., and L. Morelli. (1991). High-frequency conjugation in *Lactobacillus* mediated by an aggregation-promoting factor. *J. Gen. Microbiol.* 138, 763-768.
- **Rickard** A.H., Gilbert P., High N.J., Kolenbrander P.E., and Handley P.S. (2003). Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol*. 11, 94–100.
- **Rogosa** M. and Sharpe ME. (1960). Species differentiation of human vaginal *Lactobacilli*. *J. General Microbiol*. 20: 382-386.
- **Roos** S., S. Lindgren, and H. Jonsson. 1999. Autoaggregation of *Lactobacillus reuteri*is mediated by a putative DEAD-box helicase. *Mol. Microbiol.* 32:427–436.

- **Ruppé** E., Hem S., Lath S., Gautier V., Ariey F., Sarthou J.L., Monchy D., and Arlet G. (2009). CTX-M β-Lactamases in *Escherichia coli* from Community-acquired Urinary Tract Infections. *Cambodia.Emerg InfectDis*. 15(5):741-748.

S

- **Salminen** S. 1990. The role of intestinal microbiota in preserving intestinal integrity and health with special reference to lactic acid bacteria. *Ann. Med*. 22, 42.
- **Sambrook** J., Russell D.W., (2001). Molecular Cloning: A Laboratory Manual. 3rd edition. Cold Spring Harbor, NewYork, USA. pp: 5.2-5.14, 8.2.
- **Schachtsiek** M., Hammes P. W., and C. Hertel. (2004). Characterization of *Lactobacillus coryniformis DSM 2001T* surface protein Cpf mediating coaggregation with and aggregation among pathogens. *Appl. Environ. Microbiol*. 70**,** 7078-7085.
- **Schrezenmeir** J., M. de Vrese. 2001. Probiotics, prebiotics, and synbiotics – approaching a definition. *Am. J. Clin. Nutr*. 73, 361S-364S.

- **Sewankambo** N., R. H. Gray, M. J. Wawer, L. Paxton, D. Mc Nairn, F. Wabwire-Mangen, D. Serwadda, C. Li, N. Kiwanuka, S. L. Hillier, L. Rabe, 38 Kilic¸ *et al*.,*Clin. Diagn. LAB. IMMUNOL*. C. A. Gaydos, T. C. Quinn, and J. Konde-Lule. 1997. HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. *Lancet* 350:546–550.
- **Sghir** A., G. Gramet, A. Suau, V. Rochet, P. Pochart, J. Dore. 2000. Quantification of bacterial groups within human fecal microbiota by oligonucleotide probe hybridization. *Appl. Environ. Microbiol*. 66, 2263-2266.
- **Sgorbati** B., B. Biavati, and D. Palenzona. 1995. The genus *Bifidobacterium*. *In*: B. J. B. Wood and W. H. Holzapfel (ed.).The lactic acid bacteria. Volume 2. The genera of lactic acid bacteria. Blackie Academic and Professional, London. p. 279-306.
- **Shi** Y., Chen L., Tong J., and Xu C. 2009. Preliminary characterization of vaginal microbiota in healthy Chinese women using cultivation-independent methods. *Japan Society of Obstetrics and Gynecology*. *Res.* Vol. 35, No. 3: 525–532.
- **Sobel** J. D. 1997. Vaginitis. *N. Engl. J. Med.* 337:1896–1903.

- **Song** Y.L., Kato N., Matsumiya Y., Liu C.X., Kato H., and Watanabe K. (1999). Identification of and hydrogen peroxide production by fecal and vaginal *lactobacilli* isolated from Japanese women and newborn infants. *Journal of Clinical Microbiology*. 37, 3062-3064.
- **Song** Y-L., Kato N., Liu C-X., Matsumiya Y., Kato H., Watanabe, K. 2000. Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. *FEMS Microbiology Letters*. Vol. 187 pp. 167-173.
- **Spiegel** C. A. 1991. Bacterial vaginosis. *Clin. Microbiol*. Rev. 4:485–502.
- **Stamm** W. E., and Hooton T. M. 1993. Management of urinary tract infections in adults. *New England Journal of Medicine*, vol. 329, no. 18, pp. 1328–1334.
- **Strus** M., Kucharska A., Kukla G., Brzychczy-Włoch M., Maresz K., Heczko P.B. (2005). The *in vitro* activity of vaginal *Lactobacillus* with probiotic properties against *Candida*. *Infect. Dis. Obstet. Gynecol*., 13, 69-75.

7

- **Tamrakar** R., Yamada T., Furuta I. *et al*., 2007. Association between *Lactobacillus* species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. *BMC Infectious Diseases*, vol. 7, and article 128.
- **Tannock** G.W. 1999. Probiotics: A Critical Review. Horizon Scientific Press, Wymondham, U.K.
- **Thomas** S. 1928. Döderlein's bacillus: *Lactobacillus acidophilus*. *J Infect Dis*. 43:218-227.
- **Toma´s** M.S. Jua´rez, Wiese B., and Nader-Macı´as M.E. 2005. Effects of culture conditions on the growth and auto-aggregation ability of vaginal *Lactobacillus johnsonii CRL 1294*. *Journal of Applied Microbiology*. 99, 1383–1391.
- **Toma´s** M.S. Juarez, Bru E., Wiese B., Holgado A.A.P. de Ruiz, Nader-Macı´as M.E. (2005). Influence of pH, temperature and culture media on the growth and bacteriocin production by vaginal *Lactobacillus salivarius CRL 1328*. *Journal of Applied Microbiology*, 93, 714–724.

- **Tomas** M.S., Bru, E., Nader-Macías M.E. (2003). Comparison of the growth and hydrogen peroxide production by vaginal *Lactobacilli* under different culture conditions. *Am. J. Obstet. Gynecol.*, 188, 35-44.

V

- **Va´squez** A., T. Jakobsson, S. Ahrne´, U. Forsum, and G. Molin. 2002. Vaginal *Lactobacillus* flora of healthy Swedish women. *J. Clin. Microbiol.* 40: 2746–2749.
- **Vandamme** P., B. Pot, M. Gillis, P. de Vos, K. Kersters, and J. Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microb. Rev*. 60: 407-438.
- **Vandevoorde** L., Christiaens H., and Verstraete W. (1992). Prevalence of coaggregation reactions among chicken *Lactobacilli*. *J. Appl. Bacteriol*. 72,214-219.
- **Velraeds** M.M., Van Der Mei H., Reid G., and Busscher H. (1996). Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. *Appl. Environ. Microbiol62*, 1958–1963.

- **Ventura** M., Jankovic I., Walker D.C., Pridmore R.D., and Zink R. (2002). Identification and characterization of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*. *Appl Environ Microbiol*. 68, 6172–6181.

- **Verhelst** Rita, Verstraelen Hans, Claeys Geert, Verschraegen Gerda, Delanghe Joris, Simaey Leen Van, Ganck Catharine De, Temmerman Marleen and Vaneechoutte Mario. (2004). Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between *Atopobium vaginae*, *Gardnerella vaginalis* and bacterial vaginosis. *BMC Microbiology*. 4:16.

W

- **Wang** R-F, W-W. Cao and C. E. Cerniglia. 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. *Appl. Environ. Microbiol*. 62:1242-1247.

- **Welling** G. W., P. Elfferich, G. C. Raangs, A. C. M. Wildeboer-Veloo, G. J. Jansen, and J. E. Degener. 1997. 16S ribosomal RNA-targeted oligonucleotide probes for monitoring of intestinal tract bacteria. *Scand. J. Gastroenterol*. 32 Supplement 222: 17-19.
- **Winn** W.C, Allen S. D., Janda W. M., Koneman E. W., Procop W., Schreckenberger P.C. and Woods G. L. (2006). Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed, Lippincott Williams and Wilkins, Philadelphia, USA.
- **Woese** C. R. 1987. Bacterial evolution. *Microbiol. Rev.* 51: 221-271.

Y

- **YAN** Dong-hui, LÜ Zhi and SU Jian-rong. (2009). Comparison of main *Lactobacillus* species between healthy women and women with bacterial vaginosis. *Chin Med J.* 122(22):2748-2751.

Z

- **Zheng**, H., T. M. Alcorn, and M. S. Cohen. 1994. Effects of H2O2-producing *Lactobacilli* on *Neisseria gonorrhoeae* growth and catalase activity. *J. Infect. Dis.* 170:1209–1215.
- **Zhong** W., K. Millsap, H. Bialkowska-Hobrzanska, and G. Reid. 1998. Differentiation of *Lactobacillus* Species by Molecular Typing. *Appl Environ Microbiol*. 64:2418-23.
- **Zoetendal** E.G., A. Von Wright, T. Vilpponen-Salmela, K. Ben-Amor, A.D.L. Akkermans, W.M. de Vos. 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Appl. Environ. Microbiol*. 68, 3401-3407.
- **Zoetendal** E.G., A.D.L. Akkermans, W.M. de Vos. 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host specific communities of active bacteria. *Appl. Environ. Microbiol*. 64, 3854-3859.

Appending 1: The OD values of Spectrophotometer for bacterial isolates under different growth conditions (the values measured every 30min. for 4hrs., which presence each figure in chapter 3).

TABLE (1)

T =37, pH =6.2, Anaerobic conditions

No. of sample	Zero min.	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.	210 min.	240 min.
L.crispatus 1	0.6	0.55	0.51	0.47	0.45	0.43	0.43	0.42	0.42
L.crispatus 2	0.6	0.55	0.53	0.49	0.47	0.45	0.44	0.44	0.43
L.crispatus 3	0.6	0.54	0.50	0.48	0.45	0.43	0.42	0.40	0.40
L.crispatus 4	0.6	0.57	0.56	0.54	0.53	0.50	0.46	0.45	0.44
L.crispatus 5	0.6	0.56	0.55	0.53	0.49	0.45	0.42	0.42	0.41
L.crispatus 6	0.6	0.58	0.55	0.53	0.50	0.48	0.45	0.45	0.44
L.crispatus 7 (control)	0.6	0.54	0.50	0.48	0.45	0.43	0.42	0.40	0.40
L. gasseri 1	0.6	0.54	0.48	0.43	0.39	0.35	0.33	0.31	0.30
L. gasseri 2	0.6	0.56	0.51	0.47	0.44	0.40	0.36	0.33	0.32
L. gasseri 3	0.6	0.55	0.50	0.47	0.43	0.39	0.35	0.33	0.31
L. gasseri 4	0.6	0.53	0.46	0.41	0.37	0.35	0.32	0.31	0.29
L. gasseri 5 (control)	0.6	0.51	0.45	0.40	0.37	0.35	0.35	0.30	0.28

TABLE (2)

T= 37, pH = 5, Anaerobic conditions

No. of sample	**Zero min.**	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**150 min.**	**180 min.**	**210 min.**	**240 min.**
L.crispatus 1	0.6	0.54	0.48	0.42	0.37	0.33	0.29	0.24	0.25
L.crispatus 2	0.6	0.53	0.46	0.40	0.35	0.30	0.25	0.25	0.23
L.crispatus 3	0.6	0.53	0.47	0.43	0.38	0.33	0.27	0.23	0.25
L.crispatus 4	0.6	0.52	0.45	0.38	0.34	0.30	0.27	0.24	0.22
L.crispatus 5	0.6	0.54	0.47	0.42	0.38	0.33	0.29	0.27	0.25
L.crispatus 6	0.6	0.51	0.44	0.37	0.33	0.29	0.26	0.23	0.22
L.crispatus 7 (control)	0.6	0.51	0.45	0.36	0.31	0.28	0.24	0.22	0.21
L. gasseri 1	0.6	0.50	0.42	0.34	0.28	0.23	0.20	0.18	0.17
L. gasseri 2	0.6	0.53	0.44	0.36	0.30	0.26	0.22	0.20	0.19
L. gasseri 3	0.6	0.52	0.43	0.35	0.29	0.24	0.22	0.20	0.18
L. gasseri 4	0.6	0.51	0.44	0.35	0.30	0.27	0.23	0.22	0.21
L. gasseri 5 (control)	0.6	0.49	0.41	0.33	0.27	0.22	0.19	0.17	0.15

TABLE (3)

T = 37, pH = 8, Anaerobic conditions

No. of sample	**Zero min.**	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**150 min.**	**180 min.**	**210 min.**	**240 min.**
L.crispatus 1	0.6	0.57	0.54	0.52	0.50	0.48	0.46	0.45	0.44
L.crispatus 2	0.6	0.57	0.54	0.51	0.49	0.48	0.46	0.44	0.43
L.crispatus 3	0.6	0.57	0.54	0.52	0.50	0.49	0.47	0.47	0.46
L.crispatus 4	0.6	0.57	0.55	0.52	0.50	0.48	0.46	0.44	0.43
L.crispatus 5	0.6	0.58	0.55	0.52	0.49	0.47	0.45	0.43	0.43
L.crispatus 6	0.6	0.58	0.55	0.52	0.49	0.46	0.44	0.45	0.45
L.crispatus 7 (control)	0.6	0.56	0.54	0.51	0.48	0.46	0.43	0.43	0.42
L. gasseri 1	0.6	0.56	0.51	0.47	0.44	0.40	0.36	0.33	0.32
L. gasseri 2	0.6	0.56	0.51	0.47	0.45	0.41	0.36	0.33	0.34
L. gasseri 3	0.6	0.56	0.51	0.47	0.44	0.41	0.34	0.33	0.32
L. gasseri 4	0.6	0.56	0.51	0.47	0.44	0.40	0.36	0.33	0.33
L. gasseri 5 (control)	0.6	0.55	0.50	0.46	0.44	0.40	0.33	0.32	0.31

TABLE (4)

T = 30, pH = 6.2, Anaerobic conditions

No. of sample	**Zero min.**	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**150 min.**	**180 min.**	**210 min.**	**240 min.**
L.crispatus 1	0.6	0.58	0.55	0.52	0.50	0.48	0.48	0.46	0.45
L.crispatus 2	0.6	0.56	0.54	0.50	0.48	0.46	0.45	0.45	0.44
L.crispatus 3	0.6	0.57	0.54	0.52	0.50	0.50	0.47	0.46	0.46
L.crispatus 4	0.6	0.57	0.55	0.53	0.51	0.49	0.49	0.48	0.47
L.crispatus 5	0.6	0.58	0.55	0.52	0.49	0.48	0.46	0.45	0.45
L.crispatus 6	0.6	0.58	0.55	0.53	0.50	0.48	0.46	0.46	0.45
L.crispatus 7 (control)	0.6	0.58	0.55	0.52	0.49	0.47	0.45	0.44	0.43
L. gasseri 1	0.6	0.56	0.51	0.47	0.44	0.40	0.36	0.33	0.32
L. gasseri 2	0.6	0.56	0.50	0.47	0.45	0.41	0.37	0.34	0.33
L. gasseri 3	0.6	0.55	0.50	0.46	0.42	0.38	0.36	0.36	0.35
L. gasseri 4	0.6	0.56	0.50	0.47	0.45	0.41	0.37	0.34	0.34
L. gasseri 5 (control)	0.6	0.56	0.51	0.47	0.44	0.40	0.36	0.32	0.31

TABLE (5)

T= 30, pH= 5, Anaerobic conditions

No. of sample	Zero min.	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.	210 min.	240 min.
L.crispatus 1	0.6	0.58	0.54	0.51	0.49	0.47	0.45	0.45	0.44
L.crispatus 2	0.6	0.57	0.53	0.49	0.47	0.45	0.45	0.43	0.42
L.crispatus 3	0.6	0.58	0.55	0.52	0.49	0.48	0.46	0.46	0.45
L.crispatus 4	0.6	0.58	0.55	0.53	0.51	0.49	0.48	0.46	0.45
L.crispatus 5	0.6	0.57	0.53	0.49	0.47	0.45	0.44	0.42	0.42
L.crispatus 6	0.6	0.57	0.55	0.52	0.50	0.48	0.47	0.45	0.44
L.crispatus 7 (control)	0.6	0.54	0.50	0.48	0.45	0.43	0.42	0.41	0.41
L. gasseri 1	0.6	0.56	0.50	0.46	0.43	0.39	0.35	0.31	0.30
L. gasseri 2	0.6	0.56	0.50	0.47	0.44	0.40	0.36	0.33	0.32
L. gasseri 3	0.6	0.55	0.50	0.45	0.40	0.37	0.35	0.35	0.34
L. gasseri 4	0.6	0.56	0.50	0.46	0.44	0.40	0.36	0.33	0.33
L. gasseri 5 (control)	0.6	0.55	0.50	0.46	0.43	0.39	0.35	0.31	0.30

TABLE (6)

T = 30, pH = 8, Anaerobic conditions

No. of sample	**Zero min.**	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**150 min.**	**180 min.**	**210 min.**	**240 min.**
L.crispatus 1	0.6	0.58	0.56	0.54	0.51	0.49	0.47	0.46	0.46
L.crispatus 2	0.6	0.57	0.55	0.53	0.51	0.49	0.49	0.47	0.46
L.crispatus 3	0.6	0.58	0.56	0.54	0.53	0.53	0.51	0.49	0.48
L.crispatus 4	0.6	0.58	0.55	0.53	.51	0.49	0.48	0.46	0.45
L.crispatus 5	0.6	0.58	0.56	0.54	0.52	0.50	0.50	0.48	0.47
L.crispatus 6	0.6	0.57	0.54	0.52	0.50	0.49	0.47	0.47	0.46
L.crispatus 7 (control)	0.6	0.58	0.55	0.52	0.50	0.48	0.48	0.46	0.45
L. gasseri 1	0.6	0.56	0.51	0.46	0.41	0.38	0.36	0.36	0.35
L. gasseri 2	0.6	0.56	0.50	0.47	0.44	0.41	0.38	0.36	0.36
L. gasseri 3	0.6	0.55	0.50	0.45	0.40	0.37	0.35	0.34	0.34
L. gasseri 4	0.6	0.56	0.50	0.47	0.44	0.41	0.41	0.38	0.36
L. gasseri 5 (control)	0.6	0.55	0.50	0.46	0.43	0.41	0.38	0.35	0.33

TABLE (7)

T= 44, pH= 6.2, Anaerobic conditions

No. of sample	**Zero min.**	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**150 min.**	**180 min.**	**210 min.**	**240 min.**
L.crispatus 1	0.6	0.59	0.57	0.55	0.53	0.52	0.52	0.51	0.50
L.crispatus 2	0.6	0.59	0.57	0.55	0.54	0.53	0.52	0.51	0.51
L.crispatus 3	0.6	0.59	0.58	0.57	0.56	0.55	0.54	0.53	0.53
L.crispatus 4	0.6	0.58	0.56	0.54	0.53	0.53	0.52	0.51	0.52
L.crispatus 5	0.6	0.59	0.57	0.55	0.54	0.54	0.53	0.52	0.51
L.crispatus 6	0.6	0.58	0.56	0.55	0.54	0.53	0.52	0.51	0.51
L.crispatus 7 (control)	0.6	0.58	0.56	0.54	0.52	0.51	0.51	0.50	0.49
L. gasseri 1	0.6	0.57	0.54	0.51	0.48	0.45	0.43	0.41	0.40
L. gasseri 2	0.6	0.56	0.52	0.48	0.45	0.43	0.41	0.39	0.38
L. gasseri 3	0.6	0.56	0.53	0.49	0.46	0.44	0.42	0.40	0.39
L. gasseri 4	0.6	0.56	0.51	0.48	0.45	0.43	0.42	0.41	0.41
L. gasseri 5 (control)	0.6	0.55	0.50	0.46	0.42	0.41	0.39	0.38	0.37

Table 8

T =44, pH=5, Anaerobic conditions

No. of sample	**Zero min.**	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**150 min.**	**180 min.**	**210 min.**	**240 min.**
L.crispatus 1	0.6	0.59	0.57	0.55	0.53	0.52	0.51	0.50	0.49
L.crispatus 2	0.6	0.58	0.56	0.54	0.52	0.50	0.49	0.49	0.48
L.crispatus 3	0.6	0.59	0.57	0.55	0.54	0.54	0.53	0.52	0.51
L.crispatus 4	0.6	0.59	0.57	0.55	0.53	0.51	0.50	0.50	0.49
L.crispatus 5	0.6	0.59	0.57	0.55	0.53	0.51	0.51	0.50	0.49
L.crispatus 6	0.6	0.58	0.55	0.54	0.53	0.52	0.51	0.50	0.50
L.crispatus 7 (control)	0.6	0.58	0.56	0.54	0.52	0.50	0.49	0.47	0.48
L. gasseri 1	0.6	0.56	0.53	0.49	0.46	0.44	0.42	0.40	0.39
L. gasseri 2	0.6	0.55	0.50	0.46	0.42	0.41	0.39	0.38	0.37
L. gasseri 3	0.6	0.55	0.52	0.48	0.45	0.43	0.41	0.39	0.38
L. gasseri 4	0.6	0.56	0.51	0.47	0.44	0.41	0.39	0.38	0.38
L. gasseri 5 (control)	0.6	0.55	0.50	0.46	0.42	0.41	0.39	0.38	0.37

Table 9

T =44, pH=8, Anaerobic conditions

No. of sample	**Zero min.**	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**150 min.**	**180 min.**	**210 min.**	**240 min.**
L.crispatus 1	0.6	0.59	0.58	0.57	0.56	0.56	0.55	0.55	0.55
L.crispatus 2	0.6	0.59	0.58	0.57	0.56	0.55	0.55	0.54	0.54
L.crispatus 3	0.6	0.59	0.59	0.58	0.58	0.57	0.57	0.57	0.57
L.crispatus 4	0.6	0.59	0.59	0.58	0.57	0.57	0.56	0.55	0.55
L.crispatus 5	0.6	0.59	0.58	0.58	0.57	0.57	0.56	0.56	0.56
L.crispatus 6	0.6	0.59	0.58	0.58	0.57	0.56	0.56	0.55	0.55
L.crispatus 7 (control)	0.6	0.59	0.58	0.57	0.56	0.55	0.54	0.54	0.54
L. gasseri 1	0.6	0.58	0.55	0.52	0.50	0.48	0.46	0.45	0.44
L. gasseri 2	0.6	0.57	0.54	0.51	0.49	0.47	0.45	0.44	0.43
L. gasseri 3	0.6	0.58	0.55	0.52	0.49	0.47	0.46	0.45	0.44
L. gasseri 4	0.6	0.58	0.55	0.53	0.51	0.49	0.47	0.46	0.45
L. gasseri 5 (control)	0.6	0.58	0.54	0.51	0.48	0.46	0.45	0.44	0.43

Table 10

T =37, pH = 6.2, Aerobic conditions

No. of sample	Zero min.	30 min.	1 hr.	1:30 min.	2 hr.	2:30 min.	3 hr.	3:30 min.	4 hr.
L.crispatus 1	0.6	0.59	0.56	0.53	0.50	0.48	0.47	0.46	0.45
L.crispatus 2	0.6	0.56	0.56	0.53	0.50	0.49	0.48	0.47	0.46
L.crispatus 3	0.6	0.59	0.56	0.53	0.51	0.49	0.46	0.44	0.43
L.crispatus 4	0.6	0.56	0.54	0.52	0.50	0.49	0.48	0.47	0.46
L.crispatus 5	0.6	0.58	0.55	0.52	0.50	0.48	0.45	0.44	0.43
L.crispatus 6	0.6	0.59	0.57	0.55	0.53	0.51	0.48	0.46	0.45
L.crispatus 7 (control)	0.6	0.57	0.54	0.51	0.49	0.47	0.44	0.43	0.42
L. gasseri 1	0.6	0.56	0.51	0.48	0.45	0.43	0.42	0.41	0.40
L. gasseri 2	0.6	0.56	0.53	0.50	0.47	0.45	0.44	0.43	0.42
L. gasseri 3	0.6	0.55	0.52	0.48	0.45	0.43	0.41	0.39	0.38
L. gasseri 4	0.6	0.56	0.53	0.49	0.46	0.44	0.42	0.40	0.39
L. gasseri 5 (control)	0.6	0.56	0.50	0.47	0.44	0.41	0.41	0.38	0.36

Table 11

T =39 (hyperthermia), pH =6.2, Anaerobic conditions

No. of sample	Zero min.	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.	210 min.	240 min.
L.crispatus 1	0.6	0.56	0.52	0.49	0.47	0.45	0.44	0.43	0.42
L.crispatus 2	0.6	0.59	0.56	0.53	0.51	0.49	0.46	0.44	0.43
L.crispatus 3	0.6	0.57	0.54	0.51	0.48	0.45	0.43	0.41	0.40
L.crispatus 4	0.6	0.58	0.55	0.52	0.49	0.47	0.45	0.44	0.43
L.crispatus 5	0.6	0.57	0.54	0.51	0.48	0.45	0.43	0.41	0.40
L.crispatus 6	0.6	0.57	0.53	0.49	0.47	0.45	0.44	0.42	0.42
L.crispatus 7 (control)	0.6	0.56	0.53	0.50	0.47	0.44	0.41	0.39	0.39
L. gasseri 1	0.6	0.56	0.51	0.46	0.41	0.38	0.36	0.36	0.35
L. gasseri 2	0.6	0.55	0.50	0.46	0.43	0.41	0.38	0.35	0.33
L. gasseri 3	0.6	0.56	0.50	0.46	0.44	0.40	0.36	0.33	0.33
L. gasseri 4	0.6	0.56	0.50	0.47	0.43	0.39	0.36	0.33	0.31
L. gasseri 5 (control)	0.6	0.56	0.51	0.46	0.42	0.39	0.36	0.33	0.30

Table 12

T =35 (hypothermia), pH =6.2, Anaerobic conditions

No. of sample	Zero min.	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.	210 min.	240 min.
L.crispatus 1	0.6	0.59	0.57	0.55	0.52	0.49	0.47	0.46	0.45
L.crispatus 2	0.6	0.58	0.54	0.51	0.49	0.47	0.45	0.45	0.44
L.crispatus 3	0.6	0.57	0.54	0.51	0.48	0.45	0.43	0.41	0.40
L.crispatus 4	0.6	0.57	0.55	0.52	0.50	0.48	0.47	0.45	0.44
L.crispatus 5	0.6	0.58	0.55	0.52	0.50	0.48	0.46	0.44	0.43
L.crispatus 6	0.6	0.58	0.54	0.51	0.49	0.47	0.45	0.44	0.44
L.crispatus 7 (control)	0.6	0.56	0.51	0.48	0.45	0.42	0.42	0.41	0.41
L. gasseri 1	0.6	0.56	0.51	0.46	0.41	0.38	0.36	0.36	0.35
L. gasseri 2	0.6	0.55	0.50	0.46	0.43	0.41	0.38	0.35	0.33
L. gasseri 3	0.6	0.56	0.50	0.46	0.42	0.38	0.35	0.33	0.32
L. gasseri 4	0.6	0.56	0.50	0.47	0.43	0.39	0.36	0.33	0.31
L. gasseri 5 (control)	0.6	0.56	0.50	0.45	0.40	0.36	0.33	0.31	0.30

Appending 2: Information of oligos primers according to the manufacture companies:

BioCorp
www.biocorp.ca
oligos@biocorp.ca
514 994-7265 Fax: 221-2157

Oligo Data

Suzan

M008603

01/10/2012

Iraqi Biotechnology Company

No. oligos: 8

	Oligo	*Length*	*Tm*	*GC%*	*Scale*	*Purity*	*Modifications*
1	L. crispatus F - QI8211 GAT AGA GGT AGT AAC TGG CCT TTA	24	53.9	41.7	50 nm	Standard	-
2	L. crispatus R - QI8212 CTT TGT ATC TCT ACA AAT GGC ACT A	25	52.7	36	50 nm	Standard	-
3	L. gasseri F - QI8213 AGC GAG CTT GCC TAG ATG AAT TTG	24	55.6	45.8	50 nm	Standard	-
4	L. gasseri R - QI8214 TCT TTT AAA CTC TAG ACA TGC GTC	24	52.2	37.5	50 nm	Standard	-
5	L. iners F - QI8215 ACA GGG GTA GTA ACT GAC CTT TG	23	55.2	47.8	50 nm	Standard	-
6	L. iners R - QI8216 ATC TAA TCT CTT AGA CTG GCT ATG	24	52.2	37.5	50 nm	Standard	-
7	L. jensenii F - QI8217 CCT TAA GTC TGG GAT ACC ATT	21	50.3	42.9	50 nm	Standard	-
8	L. jensenii R - QI8218 ACG CCG CCT TTT AAA CTT CTT	21	50.3	42.9	50 nm	Standard	-

Each oligo has been detritylated, deprotected and dried down in a single 1.5 ml centrifuge tube and may be resuspended in final volumes of 200 µl each for the 40 nmole scale and 600 µl for the 200 nmole. The concentration in picomoles/µl can be determined by diluting an aliquot of the resuspended oligonucleotide 100 fold (e.g. 5 µl into 500 µl) and measuring the OD_{260}. The concentration is calculated according to the following formula:

$$[OD_{260}/\text{length of oligo}] \times 100 \times \text{Dilution factor } (100)$$

The expected yield is from 75 to 125 picomoles per µl, less for OPC purified oligos and long oligos.

Thank-you for your order.

Chaque oligo a été détritylé, déprotégé et séché dans un tube de centrifugation de 1.5 ml. Nous recommandons de resuspendre les oligos synthétisés à l'échelle de 40 nm dans un volume final de 200 µl et 600 µl pour le 200 nmole. La concentration en picomoles/µl peut être déterminée en mesurant la densité optique (OD260) d'une dilution 1:100 (e.g. 5 µl dans 500 µl). La concentration est calculée selon la formule suivante:

[OD260/longeur de l'oligo] X 100 X Facteur de dilution (100)

Le rendement anticipé est de 75 à 125 picomoles par µl.

Merci de votre commande

Page 1 of 1

BioCorp
www.biocorp.ca
oligos@biocorp.ca
514 994-7265 Fax: 221-2157

Oligo Data

Suzan

M008603

01/10/2012

Iraqi Biotechnology Company

No. oligos: 8

	Oligo	*Len*	*ODunits*	*µg*	*pmoles/µl*	*µg/µl*	*Vol in µl to add*
1	L. crispatus F - QI8211	24	18.0	594	300	2.4	**750**
2	L. crispatus R - QI8212	25	20.0	660	320	2.6	**800**
3	L. gasseri F - QI8213	24	18.1	597	302	2.4	**754**
4	L. gasseri R - QI8214	24	14.1	465	235	1.9	**588**
5	L. iners F - QI8215	23	15.5	512	270	2.0	**674**
6	L. iners R - QI8216	24	11.8	389	197	1.6	**492**
7	L. jensenii F - QI8217	21	13.4	442	255	1.8	**638**
8	L. jensenii R - QI8218	21	13.8	455	263	1.8	**657**

Please spin down for a few seconds before opening for the first time.
If the oligos are resuspended in 250 µl then they will have the concentration indicated in columns 5 and 6.

You may also reuspend the oligos in the volume indicated in the last column in order to obtain a concentration of 100µM (100 picomoles/µl)

Complete resuspension may be accelerated by incubating at 65 degrees for 5-10 minutes.

Veuillez passer les oligos a la centrifuge pendant quelques secondes avant de les sortir de leur contenant
Si les oligos sont resuspendues dans 250 µl leur concentration sera indiquée aux colonnes 5 et 6.

Vous pouvez aussi resuspendre les oligos dans le volume indiqué dans la dernière colonne pour avoir une concentration de 100 µM (100 picomoles/µl).

Une complète resuspension peu être accelerée par l'incubation a 65 degres pendant 5-10 minutes.

الخلاصة

من ضمن 42 من الاناث البالغات اللاتي يعانين من الاصابات البكتيرية المهبلية، تم عزل عشر عزلات فقط من بكتريا *Lactobacilli*. بالإضافة الى عزلتين من نساء صحيحات (غير مصابات) والتي استخدمت كعزلات قياسية في هذه الدراسة.

لمنع حصول التلوث من قبل البكتريا المرضية المهبلية الاخرى، استخدمت طريقة العزل لأنواع بكتريا *Lactobacilli* من الاناث المصابات بالإصابات البكتيرية المهبلية وذلك بتغيير ظروف وسط النمو. هذا التغيير تضمن تغيير متبادل في درجة حموضة الوسط من 6.2 الى 4.0 ثم مرة اخرى الى 6.2.

نتائج التشخيص الجزيئي باستعمال 16S rRNA(الرنا الرايبوسومي) لهذه العشر عزلات لأنواع بكتريا *Lactobacilli* من النساء المصابات اظهرت ان ست عزلات شخصت على انها بكتريا *Lactobacillus crispatus*، بينما العزلات الاربع المتبقية شخصت بانها *Lactobacillus gasseri*. بالإضافة الى ذلك، عزلتا النساء الصحيحات شخصت ك*Lactobacillus crispatus* و *Lactobacillus gasseri*. ان بروتوكول التشخيص تضمن تطبيق استخدام بوادئ 16S rRNA امامي وعكسي خاصة, وان حجم الجين الناتج من التضاعف كان 156bp (لبكتريا *Lactobacillus crispatus*) و 322bp (لبكتريا *Lactobacillus gasseri*). لكلا البادئان، كانت درجة حرارة التميع المثلى هي 52 م°.

بينت نتائج التحليل العيني للتجمع الذاتي ان بكتريا *L.gasseri* اظهرت كتل كبيرة للتجمع الذاتي، مقارنة ب *L. crispatus* التي كشفت عن كتل اصغر لهذا التجمع.

أظهرت نتائج عوامل النمو المؤثرة على التجمع الذاتي باستعمال طريقة المقياس الطيفي الضوئي، بغض النظر عن حالة التهوية، الى ان اعلى نسبة من التجمع الذاتي حدثت عندما كانت درجة حموضة النمو5 وعند درجة حرارة 37 ^{o}م. إن ذلك اعطى قيمة 70% (لبكتريا *L. gasseri*) و 61% (لبكتريا *L. crispatus*). بينما النسبة الأدنى للتجمع الذاتي حدثت عندما كانت درجة الحموضة للنمو 8 وعند درجة حرارة نمو تبلغ 44 ^{o}م. لقد اعطت قيم هي, 27% (لبكتريا *L. gasseri*) و 8% (لبكتريا *L. crispatus*).

الظروف اللاهوائية اظهرت نسبة أعلى للتجمع الذاتي مقارنة بالظروف الهوائية. وقد اعطت قيم هي,50% (لبكتريا *L. gasseri*) عند الظروف اللاهوائية، (مقارنة ب 35% عند الظروف الهوائية)، بينما *L. crispatus*اعطت قيمة للتجمع الذاتي 30% عند الظروف اللاهوائية، (مقارنة ب 26% عند الظروف الهوائية).

مع الاخذ بنظر الاعتبار ظروف النمو، لم يلاحظ وجود أختلافات واضحة على نمو بكتريا *Lactobacilli* عند درجة الحرارة للهابرثيرميا والتي تتراوح بين (38- 39 ^{o}م) ودرجة الحرارة للهبوثيرميا والتي تتراوح بين (35- 36 ^{o}م) لبكتريا *L. gasseri* و*L. crispatus*.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
الجامعة المستنصرية
كلية العلوم

تأثير بعض الظروف البيئية على قابلية التجمع الذاتي لبكتريا *Lactobacilli spp.* المعزولة من الاناث المصابات بالبكتريا المرضية المهبلية

رسالة مقدمة إلى
مجلس كلية العلوم–الجامعة المستنصرية
وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة/أحياء مجهرية

من قبل الطالبة
سوزان عبد الرحيم حسن مهدي

بكالوريوس علوم حياة/أحياء مجهرية
2002

بإشراف

د. محمد فخري احمد
أستاذ مساعد

د. صفاء عبد الكريم عبد الرزاق
طبيب استشاري

1434 هـ/رمضان
2013 م/تموز