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CHARACTERIZATION OF AN S-LAYER-LIKE PROTEIN AND SELECT
ANTIMICROBIAL FACTORS OF THE NOVEL SPECIES *LACTOBACILLUS*
COLEOHOMINIS.

by

NICOLE ANN MARIE AYALA
B.S. University of Wisconsin- Madison, 2008

THESIS

Presented to the Graduate Faculty of

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CHARACTERIZATION OF AN S-LAYER-LIKE PROTEIN AND SELECT
ANTIMICROBIAL FACTORS OF THE NOVEL SPECIES *LACTOBACILLUS*
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A Thesis

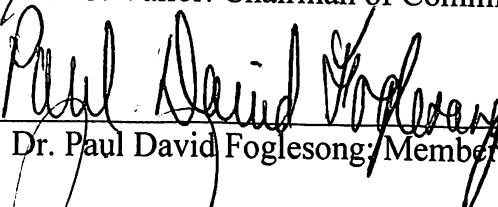
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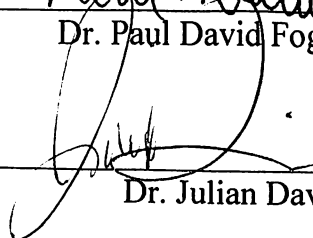
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Dr. Paul David Foglesong: Member



Dr. Julian Davis: Member

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ABSTRACT

Characterization of an S-layer-like Protein and Select Antimicrobial Factors of the Novel Species *Lactobacillus coleohominis*.

Nicole Ann Marie Ayala, B.S. University of Wisconsin- Madison

Lactobacilli in the vaginal tract have been reported be protective against infections with vaginal pathogenic microorganisms through the production of antimicrobial factors, competition for adherence to vaginal epithelium, and co-aggregation with pathogens to aid in their clearing. In addition, the antimicrobial factors produced by certain species of lactobacilli have even been shown to interrupt the replication or alter the function of select viruses. Vaginal lactobacilli have been investigated for their antimicrobial characteristics, but little is known about the novel species *L. coleohominis*, recently described in vaginal isolates (Nikolaitchouk et al. 2001). It was hypothesized that *L. coleohominis*, found in the vaginal microenvironment, would have similar characteristics to *L. iners*, a less protective inhabitant of the vagina. The goal of this thesis research was to initially characterize the antimicrobial factors of *L. coleohominis*, investigate a possible S-layer protein, and later compare the characteristics of *L. coleohominis* to other vaginal lactobacilli. The initial characterization involved analysis of cell and colony morphology, aggregation patterns, and lactic acid production. Further studies included reporting the reaction in litmus milk, investigating whether bacteriocins are produced, analyzing a possible S-

layer protein gene through molecular means, and examining cell surface extracts through SDS-PAGE, UV-vis spectroscopy, intrinsic fluorescence spectroscopy, and Fourier transform infrared spectroscopy. Results indicated that *L. coleohominis* exhibited strong similarities to *L. iners* in regards to cell and colony morphology, aggregation patterns, and characteristics of cell surface proteins. These results suggest that *L. coleohominis* is likely a weak colonizer and would not provide the best protection against pathogenic microorganisms.

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INTRODUCTION

A probiotic is “a live microorganism which, when administered in adequate amounts, confers a health benefit on the host,” (Spurbeck et al. 2010). Of the major human probiotics, many of them are in the genus *Lactobacillus* or *Bifidobacterium* (Pridmore et al. 2008). *Lactobacillus* is the largest genus of the *Clostridium* subphylum with over 80 known species (Nikolaitchouk 2001). Taxonomically, lactobacilli are classified in the kingdom *Eubacteria*, phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae* (Leeber et al. 2008).

Lactobacilli are Gram-positive, non-spore-forming, facultatively anaerobic, catalase-negative rods (Boris et al. 1997). They metabolize carbohydrates to make lactic acid as a major end-product (Falsen et al. 1999). They can grow in temperatures ranging from 5° C to 45° C, but they have strict growth requirements and need additional nutrients supplemented into their media such as amino acids, fatty acids, salts, peptides, nucleic acids, and other components (Vodnar et al. 2010). Lactobacilli are found naturally in plants, milk, meat, and mucosal surfaces of many mammals (Goh et al. 2010). They can be found in the infant gastrointestinal tract, but they do not colonize the female urogenital tract until later in life, when the host reaches reproductive age (Leeber et al. 2008). Some species of lactobacilli have been found to cause food spoiling (Avall et al. 2005), while other species have been used as additives in foods to prevent spoiling.

Lactobacilli in the gastrointestinal tract have the ability to modulate or focus the immune system. This ability has encouraged researchers to use lactobacilli as a vector for delivery of live vaccines (Martinez et al. 2000). *Lactobacillus acidophilus* is an important part of the normal gastrointestinal microbiota (Muriana et al. 1987). This species is helpful because it stimulates the production of IgA, maintains the gastrointestinal microbial balance, alleviates lactose intolerance by assisting in lactose digestion, and alleviates cold-like symptoms in children (Goh et al. 2010). Because of these health benefits, lactobacilli are being added to commercial foods such as yogurt.

In the vaginal tract, lactobacilli are beneficial due to their production of antimicrobial compounds. These include lactic acid, which lowers the pH of the environment; hydrogen peroxide, which is a powerful oxidizer; and bacteriocins, which attack the membrane, prevent the proton motive force, or inhibit DNA and/or RNA synthesis (Mossie et al. 1979), (Bruno et al. 1993), (Venema et al. 1995). They are also protective due to competition for adherence to host tissues and stimulation of the immune system (i.e. macrophages and lymphocytes) (Ruiz et al. 2009).

One of the natural, antimicrobial compounds of lactobacilli are bacteriocins which are proteinaceous, toxic compounds that are produced by bacteria to inhibit the growth of related microorganisms. They often kill other microorganisms in order to eliminate competition for nutrients (Tobasco et al. 2009). Researchers are suggesting to use them for replacing antibiotics (Ruiz et al. 2009), preserving food (Vera et al. 2007), and eliminating household molds (Yang et al. 2004). Bacteriocins are produced

by a limited number of species of lactobacilli, and they are not produced by all strains within each species. Bacteriocin-producing strains tend to have a greater ability to protect against pathogenic microorganisms, however, some researchers have suggested that bacteriocin production must be induced, or triggered, by some other means in order to promote their production; therefore, the mechanisms of induction are an area of great interest in lactobacilli research.

An important characteristic of several bacterial species is their ability to co-aggregate with other species which aids in the clearing of pathogenic microorganisms. They may also self-aggregate, or form clumps with members of the same species. Self-aggregation has been shown to increase conjugation (Reniero et al. 1992), prevent IgA attacks on lactobacilli, and allow lactobacilli to form a protective barrier against pathogenic microorganisms (Goh et al. 2010).

Gram-positive bacteria have a thick cell wall that is composed of peptidoglycan and surrounded by proteins, polysaccharides, and lipoteichoic acids. In most lactobacilli, the proteins that are on the outermost part of the cell wall are called S-layer proteins. They form a paracrystalline lattice that is uniform in distribution around the cell (Leeber et al. 2008). S-layer proteins have been reported to constitute about 10% of the total cell protein mass (Boot et al. 1993). They form a porous structure, causing 70% of the bacterial cell surface to be porous (Avall et al. 2005). S-layers are 5-15 nm thick with a smooth outer surface and a complex structured inner surface. They are attached to the cell surface by charge interactions and may be removed using

guanidine hydrochloride and concentrated salts such as lithium chloride (Turner et al. 1997).

The major function of the S-layer proteins of lactobacilli appears to confer adherence. There are two commonly accepted theories as to why adherence is so important. One involves the adherence of lactobacilli to host tissues. If they adhere to the tissues of the female reproductive tract, this would inhibit the adherence of other, opportunistic pathogens from adhering and colonizing. The other theory is that lactobacilli adhere to pathogenic bacteria to prevent them from infecting the host tissue and clear that species from the area.

The vaginal tract may serve as an entrance for opportunistic microorganisms. In women of reproductive age, the vagina is typically protected by a mutualistic colonization of lactobacilli, a genus initially described in the late 19th century by German gynecologist Albert Doderlein (Conti et al. 2009). In many women, lactobacilli do not maintain their colonization or diminish in numbers, due to douching, smoking, drug use, use of antibiotics, and risky sexual behavior (Hawes et al. 1996). This gives way to colonization of opportunistic pathogens such as *Chlamydia* and *Neisseria gonorrhoeae*. During pregnancy, a diminished number of lactobacilli can lead to infections of the amniotic fluid and, in some cases, preterm birth. It is unknown whether the occurrence of disease in the reproductive tract is due to the depletion of lactobacilli which allows pathogenic microorganisms to invade, or whether the pathogenic microorganisms are the cause of the reduced levels of lactobacilli. The most common species of lactobacilli found in the vaginal tract are *L.*

iners, *L. crispatus*, *L. gasseri*, and *L. jensenii* (Leeber et al. 2008). The presence of *Lactobacillus crispatus* in the vaginal tract is indicative of a stable vaginal flora while the presence of *Lactobacillus gasseri* or *Lactobacillus iners* is indicative of an unstable vaginal microenvironment (Verstraelen 2009).

Bacterial vaginosis (BV) is a common infection of the urogenital tract in women. Some scientists believe that *Gardnerella vaginalis*, a Gram-variable bacterium, is the primary cause of BV (Zozaya et al. 2010), while other scientists believe that BV is not caused by one particular microorganism, rather, an imbalance in the vaginal microbiota leaves the vagina vulnerable to infection with multiple opportunistic pathogens. The imbalance is likely due to the reduction or complete absence of lactobacilli or a decrease in production of antimicrobial substances by the lactobacilli which open them up for attack by pathogens (Conti et al. 2009). The symptoms of BV include odorous discharge, preterm birth, and post-operative complications. BV can also predispose women to sexually transmitted diseases.

BV has been associated with human papillomavirus (HPV) and herpes simplex virus type 2 (HSV-2) infections. That is, when BV is present, lactobacilli numbers are reduced or eliminated, and the incidence of HPV and HSV-2 increases. Several factors contribute to the antiviral abilities of lactobacilli. The hydrogen peroxide that is produced by *Lactobacillus acidophilus* has been demonstrated to alter the function of human immunodeficiency virus type 1 (HIV-1), and the cell-free supernatant of certain species of lactobacilli has the ability to inhibit replication of HSV-2.

Lactobacilli adhere to host tissues to block viral entry, and produce proteins and compounds that inhibit viral replication (Conti et al 2009).

There also exists a correlation between HIV and the species of lactobacilli present in the urogenital tract. As the concentration of select species of lactobacilli increases, the HIV viral load tends to decrease (Conti et al. 2009). *L. crispatus* and *L. jensenii* both produce hydrogen peroxide, remain continuously present throughout a woman's reproductive life-cycle, and seem to provide the most protection against HIV (Rabe 2003). Women colonized with *L. iners*, however, show a higher risk for the development of sexually transmitted infections (Zozaya 2010).

Another common disease that affects the urogenital tract of both men and women worldwide is gonorrhea, caused by the bacterium *Neisseria gonorrhoeae*. This bacterium has developed resistance to each new antibiotic that is used to treat it. Lactobacilli can secrete proteins or molecules that interfere with the virulence of the pathogen. *Lactobacillus jensenii*, a vaginal isolate, has been studied directly for its ability to inhibit colonization of *Neisseria gonorrhoeae* in the vaginal tract. A surface protein has been isolated and demonstrated to prevent the adherence of *N. gonorrhoeae* to host tissues. This bacterium or its extracted surface proteins may be used to replace the current, failing antibiotics (Spurbeck et al. 2010).

Different strains of lactobacilli have different properties, and it is difficult to generalize characteristics of all of the strains of a specific species (Leeber et al. 2008). This project focused on multiple clinical strains of *L. gasseri*, *L. crispatus*, *L. jensenii*, *L. acidophilus*, *L. iners*, and *L. coleohominis*.

Lactobacillus coleohominis was recognized as its own species by Natalia Nikolaitchouk and colleagues at the University of Goteborg, Sweden in 2001 (Nikolaitchouk et al. 2001). It is a Gram-positive facultative anaerobe that is rod-shaped, catalase-negative, and oxidase-negative, which is consistent with the *Lactobacillus* genus; but it differs genetically to the extent that it is classified as its own, distinct species. It has recently been cultured from human reproductive tracts, but not much is known about it.

Lactobacillus iners, formerly known as *Lactobacillus* strain 1086, is a species that has been found in the vaginal flora of women who have bacterial vaginosis. This suggests that *L. iners* does not have the ability to protect against disease-causing microorganisms while maintaining colonization (Rabe et al. 2003). *Lactobacillus iners* was first described as its own species by Falsen et al. in 1999. It appears to colonize the vaginal tract when the vaginal health is in a transitional stage from healthy to non-healthy and vice versa. Also, *L. iners* alters its colonization due to hormonal changes. That is, differing levels of certain hormones will promote or inhibit the colonization of *L. iners* (Jacobsohn et al. 2008).

Lactobacillus crispatus is recognized for its ability to adhere to collagen by way of an S-layer protein, and its ability to bind to specific human cells, such as vaginal epithelial cells, erythrocytes, and sperm cells. The S-layer protein of *L. crispatus*, known as CbsA, has been fully characterized and has been expressed in the non-adhering species of bacteria *Lactococcus lactis* by transformation (Avall et al. 2003).

L. crispatus CTV05 has been used in clinical studies as a vaginal suppository to promote the growth of this species in the vaginal tract (Hawes et al. 1996).

Due to its use as a probiotic in human food products, the complete genome of *Lactobacillus acidophilus* has been sequenced (Goh et al. 2009). *L. acidophilus* is commonly found in the mammalian gastrointestinal tract and is the most extensively studied species of lactobacilli (Toba et al. 1995). Both *L. acidophilus* 4356 and *L. crispatus* CTV05 served as the representative standards for this project.

The goal of this thesis research was to initially characterize the antimicrobial factors of the newly identified species of lactobacilli, *Lactobacillus coleohominis*, and investigate a possible S-layer protein. Later studies included the comparison of *L. coleohominis* to other, previously characterized species of vaginal lactobacilli. To study antimicrobial activity, the aggregation patterns, production of lactic acid, and production of bacteriocins were examined. In addition, it was sought to study a possible S-layer protein by molecular means. The cell surface proteins were then extracted and examined through SDS-PAGE, UV-vis spectroscopy, intrinsic fluorescence spectroscopy, and Fourier transform electron microscopy. The results from these experiments further elucidate the role of lactobacilli in promoting vaginal health.

CHAPTER 1

Aggregation patterns, lactic acid production, and bacteriocin production of vaginal lactobacilli.

1.0. Introduction

Lactobacilli are considered probiotics because they provide health benefits in the gastrointestinal and vaginal tracts. Some health benefits include lowering serum cholesterol levels, stimulating immunoglobulin production, and binding mutagenic compounds (Boot et al. 1996). Certain species of lactobacilli can induce regulatory T-cells and prevent damage to the immune system by pathogenic microorganisms (Konstantinov et al. 2008). In the vaginal tract, lactobacilli are beneficial due to their production of antimicrobial lactic acid, hydrogen peroxide, and bacteriocins.

In 1780, C.W. Scheele, a Swedish scientist discovered that lactic acid is produced by bacteria in sour milk. Lactic acid appears in two forms, the D (-) form and the L (+) form. The D (-) form is harmful to humans in high levels, so bacteria that produce the L (+) form are used to enhance foods (Vodnar et al. 2010). Lactic acid bacteria (LAB), like lactobacilli, have the ability to produce lactic acid through fermentation (Avall et al. 2005). There are three different categories of lactobacilli based on the fates of lactic acid production. The *homofermentative* group produces fermentation end-products consisting of over 85% lactic acid. The *heterofermentative* group produces fermentation end-products consisting of about 50% lactic acid. The *facultatively heterofermentative* group will produce lactic acid as a fermentation end-product only under certain conditions (Vodnar et al. 2010). *L. acidophilus*, *L.*

crispatus, *L. gasseri*, and *L. jensenii* have all been classified as homofermentative, but it is not yet been determined which group *L. iners* and *L. coleohominis* are found in (Toit et al. 2001). LAB are useful because they produce an acidic environment, as low as pH 4.0, which inhibits the growth of many human pathogens (Vodnar et al. 2010).

Bacteriocins are antimicrobial proteins that are secreted by certain species of lactobacilli to inhibit the growth of closely related microorganisms. The bacteriocins produced by lactobacilli can be divided into 4 classes, I-IV. Class I, the lantibiotics, contains small membrane-active peptides that have the amino acid lanthionine. Class II consists of small, heat-stable, non-lanthionine, membrane-active proteins. Class III consists of large, heat-labile proteins. To this date, members of class III have only been found in lactobacilli. Class IV consists of complexes, often containing lipid or carbohydrate side-chains (Alpay et al. 2003). Another class of bacteriocins, class V, has circular, unmodified structures, but class V bacteriocins are not known to be produced by lactobacilli (Pascual et al. 2008).

There are several modes of action for bacteriocins, depending on the type of bacteriocin that is produced. Lantibiotics have been reported to attack the cytoplasmic membrane in order to destroy the membrane potential. Non-lantibiotics typically inhibit the synthesis of DNA and RNA (Venema et al. 1995). Some types of bacteriocins interrupt the proton motive force which dissipates the energy required for essential life functions. The proton motive force is directly involved in movement of the flagella and in drug excretion systems that protect cells from drugs (Bruno et al. 1993).

The genes encoding bacteriocins have not been found to be plasmid associated (Muriana et al. 1987). More current research has evaluated the exact genetic locus and sequence of genes that encode bacteriocins (Klaenhammer et al. 2008). Bacteriocin production is regulated through a mechanism similar to quorum sensing. The bacteria secrete an autoinducer molecule, sense its concentration, and produce bacteriocins when the concentration is at a certain level. In order to induce bacteriocin production, the bacteriocin-producing microorganisms must be co-cultured with live cells at comparable concentrations (Tobasco et al. 2009). Some bacteria require the combination of two or more bacteriocins in order to inhibit growth of competitor microorganisms (Diep et al. 1996). These bacteriocin complexes that are formed likely require the use of an autoinducer.

Analyzing the aggregation patterns of lactobacilli is an important way to observe whether aggregation is taking place. Aggregation involves the ability of microorganisms to come together and form clumps. They can self-aggregate with other members of their species or can co-aggregate with members of different species.

An aggregation-promoting factor (APF) is a protein that has been identified to aid in aggregation. It has been initially characterized in the vaginal isolate *L. gasseri* (Boris et al. 1997). Its mode of action has not been discovered, and there is no known APF receptor, but it appears to associate with the S-layer, and it contributes to cell shape (Goh et al. 2010). APF has been found in the supernatant as well as in the cell membrane of stationary cells, and it can be extracted using LiCl (Jankovic et al. 2003). *Lactobacillus acidophilus* has been previously described to co-aggregate with other

species of bacteria such as *Staphylococcus aureus*. APFs of *L. acidophilus* promote survival in the presence of bile which may assist them in colonizing the small intestine.

For the initial characterization of the lactobacilli, Gram stains were performed, colony morphology was examined, and their ability to self-aggregate was studied. Further research involved analyzing the lactic acid production, relative culture pH, reaction in litmus milk, and their ability to kill other species of bacteria that may colonize the vaginal tract.

1.1. Materials and Methods

1.1.1. Bacterial strains and media.

The clinical strains used for this thesis were obtained from the laboratory of Dr. Sharon Hillier at the Magee Women's Research Institute; University of Pittsburgh Medical Center. The lactobacilli taken from clinical subjects included *Lactobacillus coleohominis*, *Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, and *Lactobacillus jensenii* (Table 1.1). Indicator strains that were used in the bacteriocin assay included *Escherichia coli*, *Candida albicans*, *Streptococcus thermophilus*, *Lactococcus lactis*, *Staphylococcus aureus*, and *Enterococcus faecalis* (Presque Isle Cultures, Erie, PA) (Table 1.2). All bacteria and yeast were grown in Man-Rogosa-Sharpe (MRS) broth. All lactobacilli cultures were grown at 37° C for 72 hours in the presence of 5% CO₂. Indicator cultures were grown according to specified growth conditions (Table 1.2). Subcultures were created in

MRS broth. Streaked plates were examined for colony morphology, cells were Gram stained for microscopic examination of cell morphology and purity of culture.

Species	Strain Number	Abbreviation
<i>L. coleohominis</i>	HUZ 427 484-9	Coleo 484-9
<i>L. coleohominis</i>	HUZ 427 515-3	Coleo 515-3
<i>L. coleohominis</i>	HUZ 602 245-3	Coleo 245-3
<i>L. coleohominis</i>	HUZ 602 269-1R	Coleo 269-1R
<i>L. iners</i>	HUZ 602 276-2	Iners 276-2
<i>L. iners</i>	HUZ 602 238-1	Iners 238-1
<i>L. iners</i>	HUZ 602 248-14	Iners 248-14
<i>L. iners</i>	HUZ 602 245-2	Iners 245-2
<i>L. acidophilus</i>	4356	Acid 4356
<i>L. jensenii</i>	HUZ 602 293-a	Jens 293-a
<i>L. jensenii</i>	HUZ 602 295-4	Jens 295-4
<i>L. gasseri</i>	HUZ 602 300-2	Gas 300-2
<i>L. gasseri</i>	HUZ 602 264-1	Gas 264-1
<i>L. crispatus</i>	HUZ 602 277-1	Crisp 277-1
<i>L. crispatus</i>	CTV05	Crisp CTV05

TABLE 1.1. Lactobacilli strains from the University of Pittsburgh Medical Center, Magee Women's Research Institute.

Species	Catalog #	Growth Conditions
<i>Escherichia coli</i>	336	37° C, 24 h, aerobic
<i>Candida albicans</i>	925	37° C, 24 h, aerobic
<i>Streptococcus thermophilus</i>	521	37° C, 48 h, CO ₂
<i>Lactococcus lactis</i>	525A	37° C, 48 h, CO ₂
<i>Staphylococcus aureus</i>	4651	37° C, 24 h, aerobic
<i>Enterococcus faecalis</i>	522A	37° C, 48 h, CO ₂

Table 1.2. Bacterial strains from Presque Isle Cultures (Erie, PA).

1.1.2. Culture aggregation patterns.

Each strain of lactobacilli was grown to stationary phase in polystyrene tubes and in glass tubes. The growth and aggregation patterns of the cultures were recorded after the initial growth from a frozen stock and after four passes.

1.1.3. Detection of lactic acid through HPLC.

Stationary phase cultures of lactobacilli were centrifuged at 3,000 x g for 10 min. and the supernatants were collected. The supernatants were heated to 95° C for 15 min., and centrifuged a second time. Each supernatant was run through a C18 reversed-phase column using a phosphate buffer (50 mM Na₂HPO₄ dissolved in water and pH adjusted to 7.4 using 1 M HCl) with a flow rate of 1 ml per min. A photodiode array (PDA) detector was used to sense the chemicals as they passed through the column, and a chromatogram was created using the Lab Solutions™ program. The column was stored in 10% methanol between uses.

1.1.4. Relative pH of the cultures.

Lactobacilli were grown to stationary phase. The cultures were vortexed briefly to create uniform turbidity and the pH was determined using a pH meter calibrated at 4.0 and 7.0.

1.1.5. Reaction in litmus milk.

Lactobacilli were grown to stationary phase in litmus milk medium. Reactions in the litmus milk were analyzed immediately after incubation.

1.1.6. Bacteriocin assay.

This protocol was based on the previously published bacteriocin assay by Barefoot et. al. 1983. Each *Lactobacillus* strain was plated for isolated colonies on MRS agar and grown to stationary phase. Each indicator culture was grown at 37° C to stationary phase in the specified conditions listed in Table 1.2. The indicator strains were prepared by adding 250 µl of a 1:10 dilution to 10 ml of 0.5% soft MRS agar. The soft agar was poured over MRS agar plates and allowed to solidify. Colonies of different lactobacilli species were stabbed through the soft agar into the MRS agar, and separate plates were incubated at 37° C overnight in both aerobic and CO₂ conditions. The plates were then examined for zones of clearing around the stab points.

1.2. Results

1.2.1. Colony and cell morphologies.

Each species of lactobacilli was examined for colony morphology. Figure 1.1 illustrates the colony morphologies of different species of lactobacilli. *L. acidophilus* and *L. crispatus* produce faint colonies that are almost transparent. *L. jensenii* 293-a, *L. iners* 276-2, *L. coleohominis* 245-3, and *L. coleohominis* 515-3 produce medium sized colonies with a dense center and fading edge. *L. coleohominis* 269-1R produces large colonies with an irregular edge and dense center. *L. coleohominis* 484-9 and *L. iners* 248-14 produce smaller colonies with a smooth edge and dense center. *L. jensenii* 295-4 and *L. iners* 238-1 produce extremely small colonies with dense centers and fading edges.

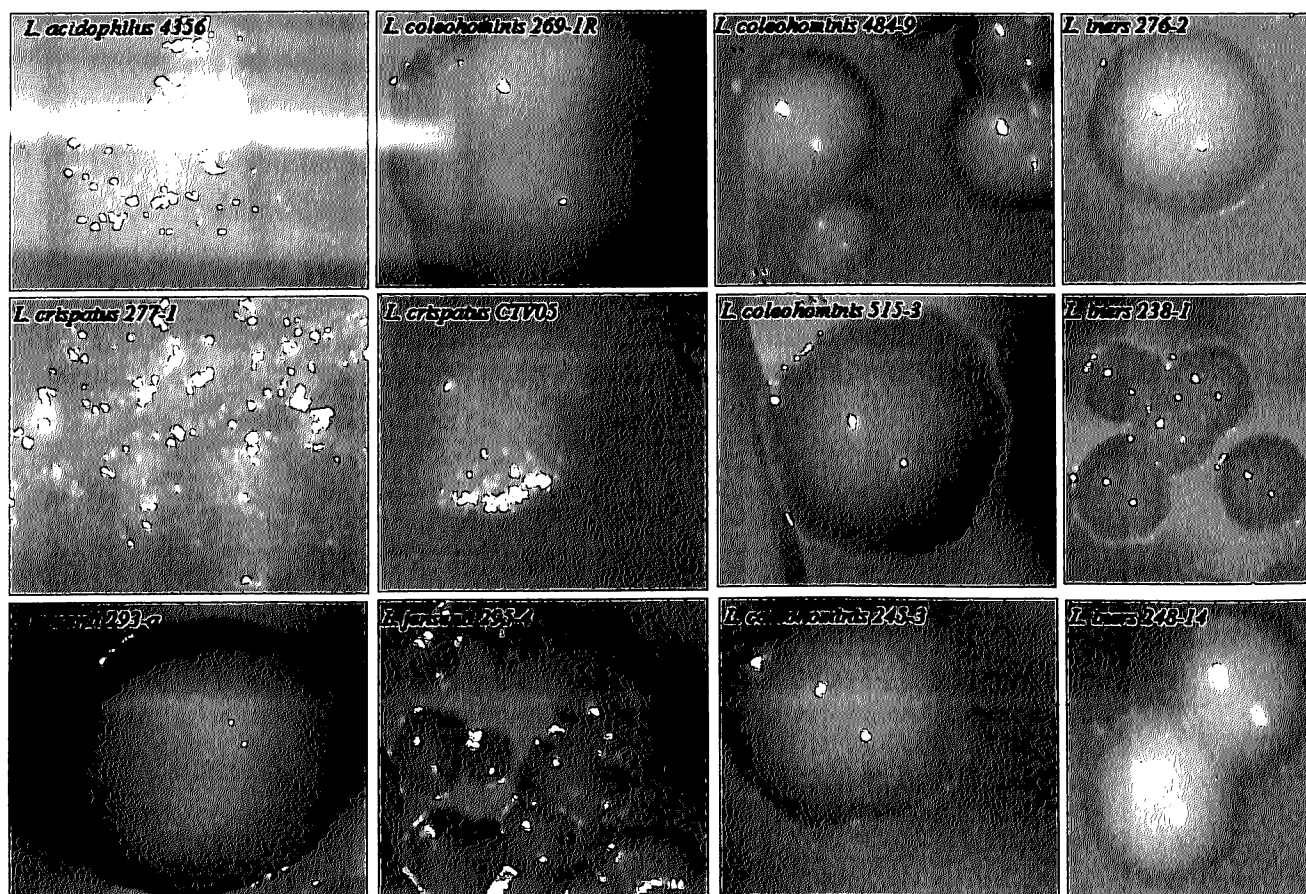


Figure 1.1. Colony morphology of lactobacilli.

Figures 1.2 and 1.3 show the Gram stains of select species of lactobacilli. All of the lactobacilli are Gram-positive. *L. acidophilus* and *L. crispatus* are large, long rods. *L. gasseri* are long rods that appear to be connected in strings from end-to-end. *L. jensenii* are smaller rods that form a bent vibrio or spirilla shape. *L. coleohominis* and *L. iners* are short, fat rods that look very similar.

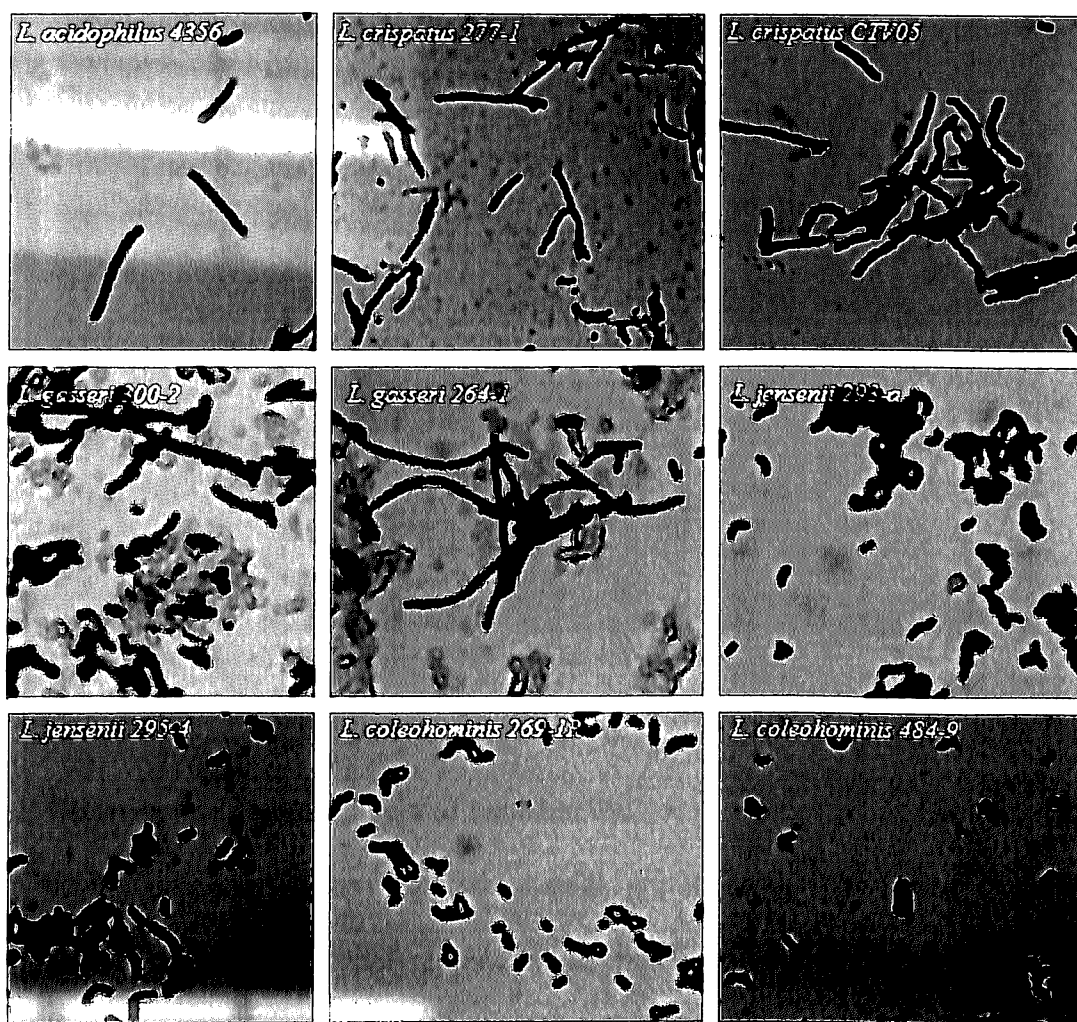


Figure 1.2. Gram stains of select species of lactobacilli.

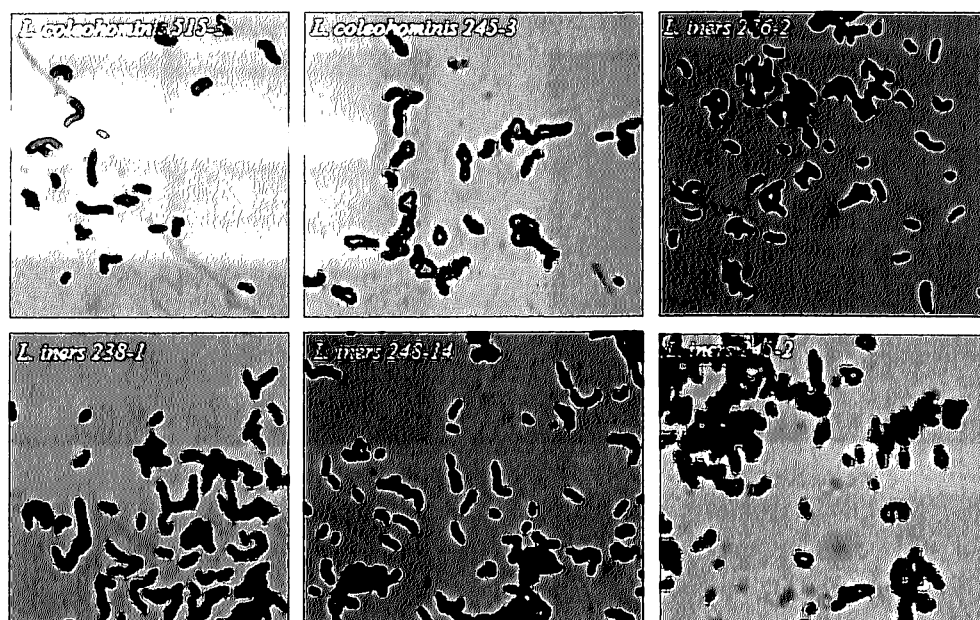


Figure 1.3. Gram stains of select species of lactobacilli.

1.2.2. Differences in aggregation of lactobacilli.

The aggregation patterns of lactobacilli are illustrated in Figure 1.4. When lactobacilli were grown in polystyrene tubes from the original frozen stock (tube number 1 of each species), *L. acidophilus* began growth as a sediment at the bottom of the tube, *L. crispatus* began growth as a self-aggregated, folded structure on the bottom of the tube, *L. gasseri* began growth as a lattice up the side of the tube, and *L. iners*, *L. coleohominis*, and *L. jensenii* began growth as uniform turbidity. When lactobacilli were grown in glass tubes from the original frozen stock (tube 2 of each species), all species showed self-aggregated clumps, some of which adhered to the side of the tube. After the fourth passage in the polystyrene tubes (data not shown) and in glass tubes (tube number 3 of each species), all lactobacilli lost the aggregation phenotype and formed sediment on the bottom of the tubes.

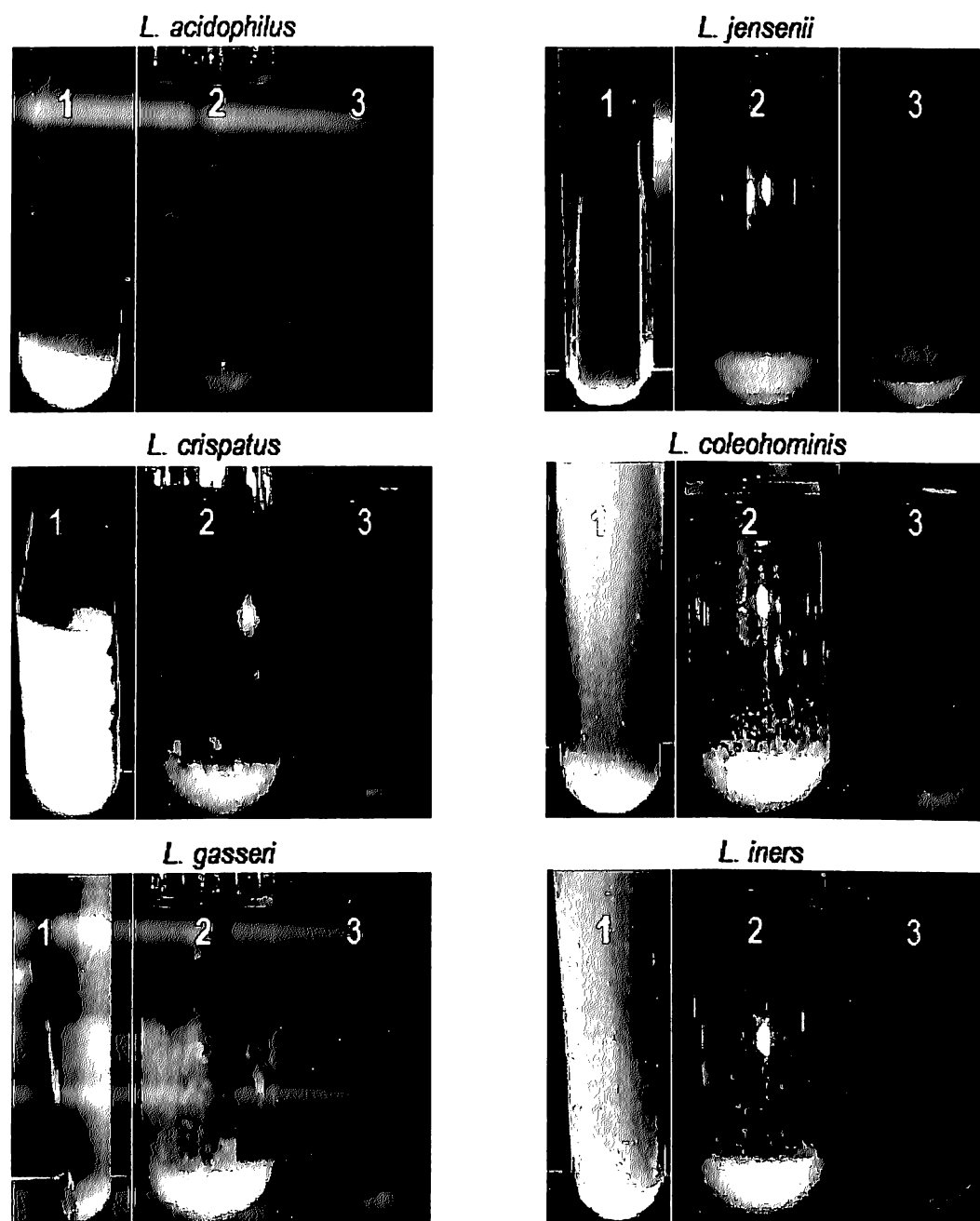


Figure 1.4. Aggregation patterns of lactobacilli in MRS. Differences in aggregation patterns of lactobacilli grown in polystyrene tubes from frozen stock (1), in glass tubes from frozen stock (2), and in glass tubes after 4 passes (3).

1.2.3. Assessment of lactic acid production by HPLC and relative pH.

A purified sample of lactic acid emerged from the C18 column at around 5- 5.5 minutes which resulted in the formation of a peak on the chromatogram. Peaks of lactobacilli supernatants were observed in the chromatograms of Figures 1.5, 1.6, and 1.7. Pure lactic acid forms a peak at around 5- 5.5 min. and pure MRS media did not have a peak at 5- 5.5 min. (data not shown). As seen in Figures 1.5, 1.6, and 1.7, each strain of lactobacilli produced a lactic acid peak.

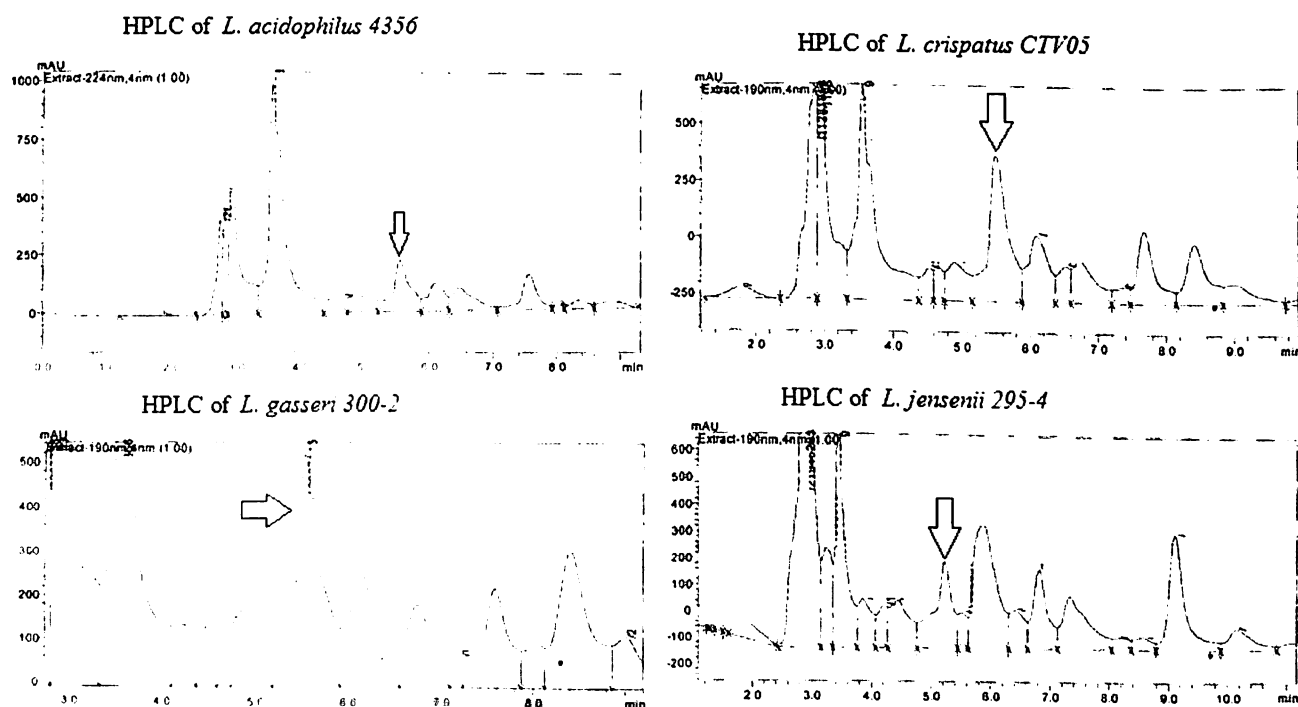


Figure 1.5. HPLC chromatograms showing lactic acid production by various species of lactobacilli.

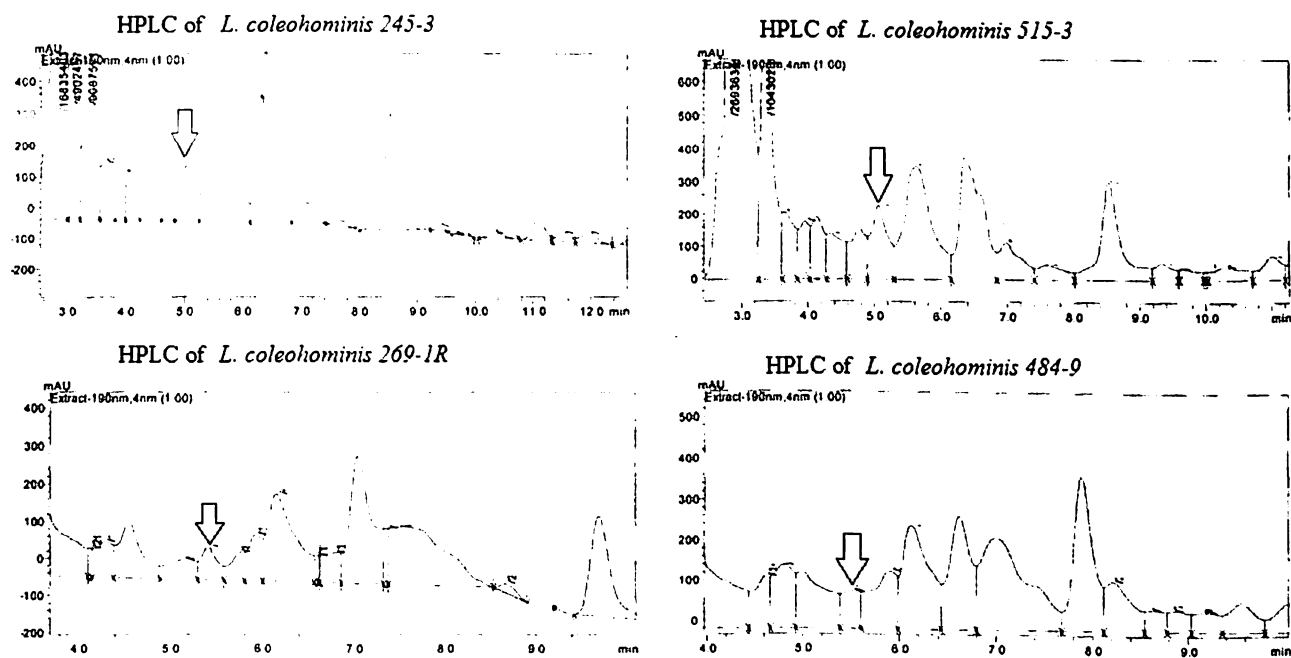


Figure 1.6. HPLC chromatograms showing lactic acid production by *L. coleohominis*.

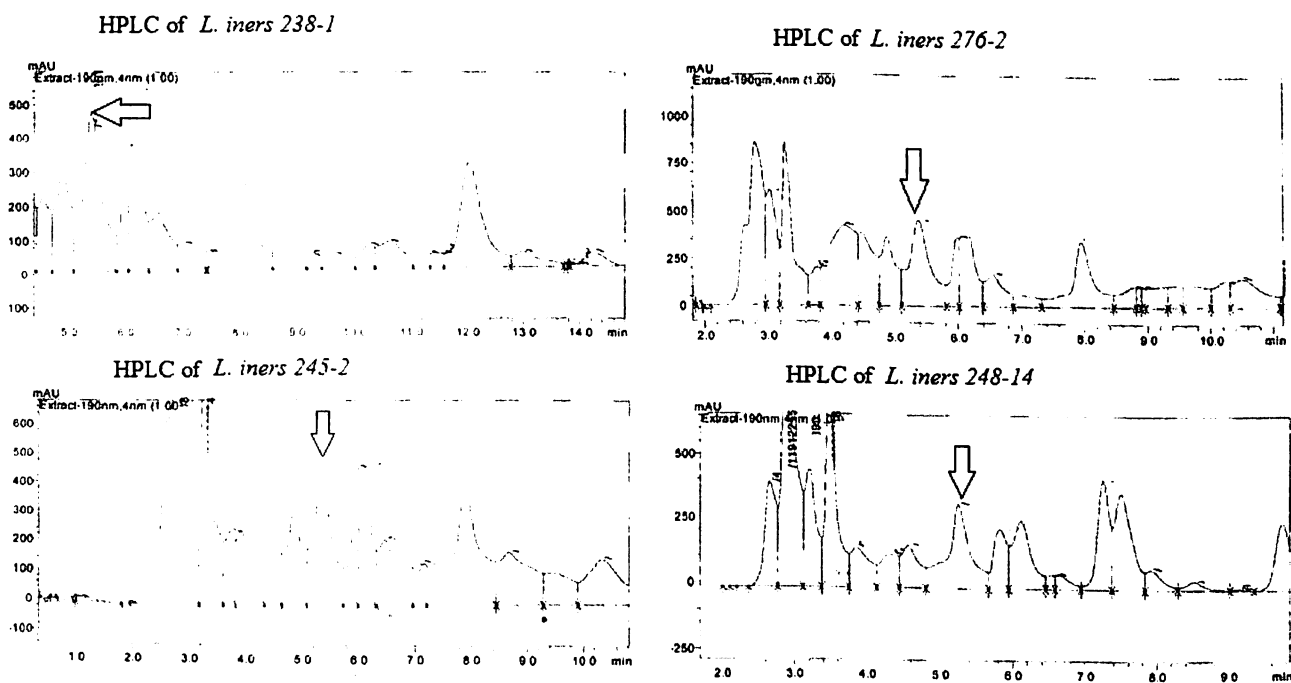


Figure 1.7. HPLC chromatograms showing lactic acid production of *L. iners*.

When the relative pH was tested, each species of lactobacilli created a similar acidic environment, illustrated in Table 1.3. Original MRS media had a pH of 6.8, so all species tested were able to decrease the pH of the media to an average of 4.04.

Strain	pH
<i>L. acidophilus</i> 4356	4.30
<i>L. crispatus</i> 277-1	4.01
<i>L. crispatus</i> CTV05	4.04
<i>L. gasseri</i> 300-2	4.10
<i>L. gasseri</i> 264-1	4.04
<i>L. jensenii</i> 293-a	4.04
<i>L. jensenii</i> 295-4	3.98
<i>L. coleohominis</i> 269-1R	4.08
<i>L. coleohominis</i> 484-9	4.12
<i>L. coleohominis</i> 515-3	3.98
<i>L. coleohominis</i> 245-3	3.98
<i>L. iners</i> 276-2	3.97
<i>L. iners</i> 248-14	3.98
<i>L. iners</i> 238-1	3.99
<i>L. iners</i> 245-2	4.02

Table 1.3. Relative pH of lactobacilli.

1.2.4. Reaction in litmus milk.

Litmus milk was inoculated with select species of lactobacilli to compare reactions across species. As seen in Figure 1.8, most species of lactobacilli fermented lactose, lowered the pH, and coagulated casein as seen by an acid clot formation. *L. coleohominis* 484-9 failed to grow in litmus milk, and *L. crispatus* CTV05 and *L. coleohominis* 269-1R appeared to have a higher pH than the other strains. *L. jensenii*,

L. iners, and *L. coleohominis* appeared to form a similar acid clot, while *L. acidophilus*, *L. crispatus*, and *L. gasseri* formed a smaller clot.

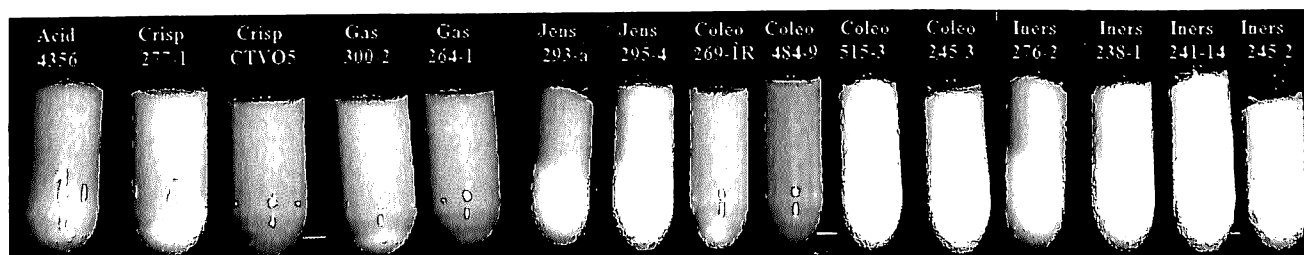


Figure 1.8. Litmus milk cultures of lactobacilli.

1.2.4. Bacteriocin production.

Of the strains tested, they were not able to inhibit the growth of any of the indicator cultures. This indicates a negative result for bacteriocin production for all strains. Result of using *S. aureus* as an indicator culture is shown in Figure 1.9.

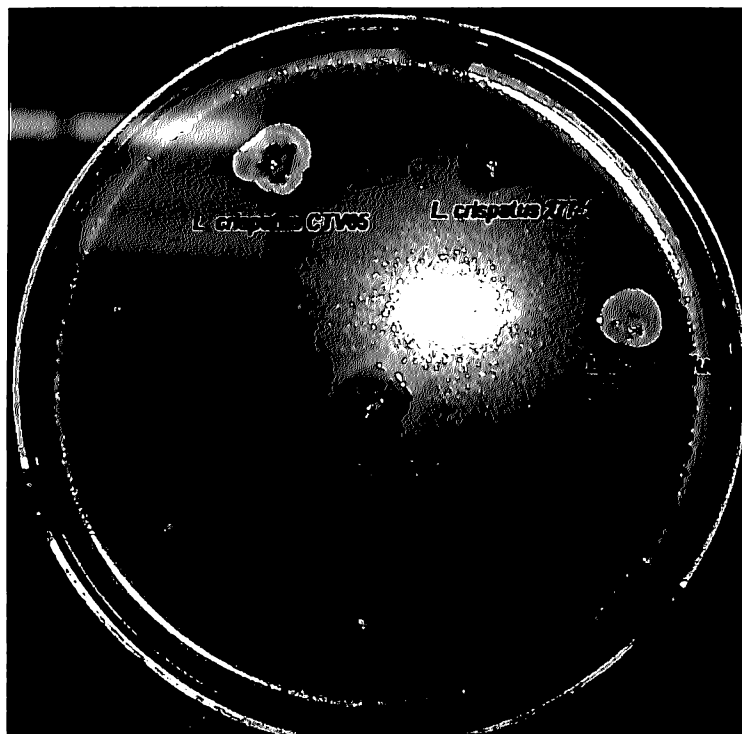


Figure 1.9. Results of bacteriocin assay. Colonies of select species of lactobacilli stabbed into a soft agar overlay of *S. aureus*.

1.3. Discussion

The aggregation patterns of vaginal lactobacilli allow us to infer how these microorganisms would grow on human tissue. Comparing different species of vaginal lactobacilli allows us to determine which species may be better at colonizing the female reproductive tract and, therefore, provide more protection. A colony typically arises from a single bacterial cell, and all cells within a colony are suggested to be clones of one another. The more divisions that have occurred during the growth of the colony, the larger and more dense the colony will become. So, when looking at colony

morphology, larger, more dense colonies are typically indicative of more robust growth. In this study, *L. acidophilus* and *L. crispatus* appeared to have almost transparent colonies, while *L. jensenii* and *L. coleohominis* seemed to have larger colonies. The transparent colonies, however, seemed to be spread out over a large surface area. *L. iners* had dense colonies, but they were smaller in comparison to the other species which is indicative of poor growth.

The edges of the colony can indicate possible aggregation or motility of the species. Since lactobacilli are characterized as being non-motile, the density of the colony is likely due to self-aggregation. Colonies with perfectly round and smooth edges are typically indicative of tightly aggregating species of bacteria, while colonies that are thin and spread out or colonies that have rough edges may be indicative of their ability to grow outward and colonize larger areas. *L. acidophilus* and *L. crispatus* create thin, transparent colonies, but they cover a larger surface area. *L. jensenii* and *L. coleohominis* have larger colonies with dense centers and fading, rough edges which indicates robust growth and medium aggregation. *L. iners* create colonies with perfectly smooth edges which indicates tight self-aggregation. The edges of the colony may also be indicative of the type of surface protein on the bacterial cell surface. S-layer proteins of lactobacillus have varying shapes (Smit et al. 2001) and these shapes may correlate with the type of colony that they produce.

Gram stains differentiate bacteria into two broad classes based on the peptidoglycan in their cell walls. They also show cell shape, aggregation of the cells, and purity of the culture. All of the Gram stains in Figures 1.2 and 1.3 (p. 17-18) show

one cell type per stain which confirms the purity of the cultures obtained. Longer rods expose more surface area to the environment. This allows more opportunities for exchanges with the exterior of the cell and may allow for more nutrients to be brought into the cell. Shorter, fatter rods limit the amount of surface area exposed to the environment. *L. acidophilus*, *L. crispatus*, and *L. gasseri* are all long rods while *L. jensenii*, *L. coleohominis*, and *L. iners* are short, fat rods. *L. crispatus*, *L. jensenii*, and *L. gasseri* appear to self-aggregate. *L. gasseri* self-aggregates from end-to-end forming long chains of bacterial cells. *L. crispatus* and *L. jensenii* form random aggregation patterns. *L. iners* and *L. coleohominis* show some clumping, but there are many free-floating or separated cells in the stain. Tight clumping or true positive aggregation would not allow the cells to separate during the staining procedure.

Different species of bacteria have different responses to stress. When bacteria are frozen, they become dormant. When they are revived from a frozen stock in broth, they begin to build strength and return to their normal state of growth through the expression of stress factors. In Figure 1.4 (p. 19), tube one, lactobacilli grown in polystyrene tubes show varying results. *L. acidophilus* begins growth by forming sediment on the bottom of the tube, *L. crispatus* forms self-aggregated folds of cells, *L. gasseri* forms a lattice up the side of the tube, and *L. coleohominis*, *L. iners*, and *L. jensenii* all begin with uniform turbidity. The different aggregation patterns in polystyrene tubes could be due to the non-specific binding of surface proteins of dead cells within the media to the sides of the tubes or the lactobacilli binding to the dead cells to form a structure. After four passes in the polystyrene tubes, they lose their

ability to aggregate and form uniform turbidity, and all strains form sediment on the bottom of the tubes (data not shown).

When grown from the original frozen stock in glass tubes, all strains exhibited tight, self-aggregation, with clumps that both adhered to the sides of the glass and settled to the bottom of the tube (Figure 1.4, tube 2, p. 19). This suggests that when coming from a weakened state, lactobacilli form aggregated clumps. The aggregated clumps are likely due to the binding of live cells to dead cells, hydrophobic interactions of the surface protein, or initial production of an aggregation promoting factor. As of yet, an APF has not been described for *L. coleohominis* or *L. iners*, but it has been described for *L. crispatus*, *L. acidophilus*, and *L. gasseri* (Goh et al. 2010). After four passes in glass tubes, all species lose their ability to aggregate and they form sediment on the bottom of the tube (Figure 1.4, tube 3, p. 19). This suggests that several passes make them alter gene expression to stop transcription of stress factors, which may include the APF gene. DNA and protein analysis are required to verify the existence of a possible APF gene and subsequent protein.

Lactic acid production allows lactobacilli to produce an acidic environment which prevents the growth of many pathogenic microorganisms. As seen in Figures 1.5, 1.6, and 1.7, all species tested in this project produce lactic acid. The varying degree of lactic acid produced is important to gauge which species may be more protective. The more lactic acid produced, the lower the pH, and the more protection exhibited by that species. The results indicate that each strain produces a similar acidic environment.

The litmus milk test is a common test performed on lactic acid bacteria. It is useful in identifying the ability of bacteria to produce acid from lactose and digest the protein casein. Lactose fermentation produces acid which turns the medium pink in color due to the presence of the pH indicator azolitmin. The accumulation of acid may cause the protein casein to aggregate in the form of an acid clot. In most of the species of lactobacilli in this project, this was the case. *L. crispatus* CTV05 and *L. coleohominis* 269-1R appeared to have extremely low acid production in comparison with the other species. This may be due to a lessened growth of the microorganism. *L. coleohominis* 484-9 did not grow in litmus milk, likely due to its weak growth in normal media.

Bacteriocins are produced by a small number of strains in select species of lactobacilli. Finding the strains that produce bacteriocins can be achieved through DNA analysis and bacteriocin assays. A true positive in a bacteriocin assay would result in the complete absence of indicator culture growth surrounding a colony of *Lactobacillus*. Of the strains tested in this project, none of them produced bacteriocins. Strains of *L. acidophilus* have been previously identified to produce bacteriocins, but *L. acidophilus* 4356 was not classified as a bacteriocin producing strain (Alpay et al. 2003). Further steps could be to determine whether they have the genes for bacteriocin production and then analyzing different bacteriocin inducer molecules to promote bacteriocin production.

In comparing *L. coleohominis* to other species of vaginal lactobacilli based on aggregation patterns, cell and colony morphology, and lactic acid production, *L.*

coeleohominis is more closely related to *L. jensenii* and *L. iners*. *L. coeleohominis* exhibits weak growth patterns, low lactic acid production, and an intermediate ability to colonize large areas. These results indicate that *L. coeleohominis* is weakly protective and is not the best candidate for clinical trials for protecting women against vaginal infections. Further data must be collected to determine whether *L. coeleohominis* remains continuously colonized throughout a woman's reproductive cycle, whether *L. coeleohominis* is present in the vaginal flora of women with vaginal infections, and whether any strains of *L. coeleohominis* are known to produce bacteriocins.

CHAPTER 2

Determination of a CbsA homolog and characterization of the cell surface proteins in select species of lactobacilli.

2.0. Introduction

S-layer proteins are found on the surface of most lactobacilli. The most commonly characterized S-layer protein is CbsA, a collagen-binding protein expressed by *Lactobacillus crispatus*, the predominant species in the vaginal tract (Toba 1995). The transfer of the S-layer protein gene CbsA of *L. crispatus* to *L. casei* through transformation has allowed the non-adhering *L. casei* to adhere to collagen (Martinez et al. 2000).

Some researchers suggest that bacteria contain multiple genes encoding S-layer protein genes in their DNA so that they can undergo antigenic variation or express the different S-layer proteins in response to different environmental factors. *Lactobacillus brevis* has three S-layer protein genes. One gene is expressed when *L. brevis* grown under aerobic conditions and results in the formation of rough colonies. Another gene is expressed when the lactobacilli are grown under anaerobic conditions and results in the formation of smooth colonies. The third gene is not expressed (Jakava et al. 2002). In this project, primers were designed and PCR amplifications were performed to locate a possible CbsA homolog on the chromosomal DNA of *L. coleohominis* and *L. crispatus*.

The S-layer protein is typically a single protein type or glycoprotein subunit that covers the entire outer surface of the cell (Vidgren et al. 1992). In some bacteria, more

than one kind of S-layer protein is found. These S-layers are superimposed onto each other forming one layer (Avall et al. 2005).

There are two conserved regions on the S-layer proteins across different species of lactobacilli; the N-terminus which is conserved in 30 amino acids and has been shown to direct the secretion of the protein and the C-terminus which is conserved in 123 amino acids and has been shown to anchor the protein to the cell surface (Jakava et al. 2007). S-layer proteins are composed of various protein or glycoprotein subunits. The subunits are attached to each other and to the cell membrane by non-covalent bonds, and the whole protein follows an oblique, hexagonal, or square symmetry (Avall et al. 2008). S-layer proteins of many species of bacteria and archaea are typically acidic and hydrophobic, containing almost 40% hydrophobic amino acids. The S-layer proteins of lactobacilli, however, are highly basic (Smit et al. 2001). S-layer proteins of lactobacilli range from 25-71 kDa, but other species of bacteria have S-layer proteins as large as 200 kDa. *L. acidophilus* ATTC 4356 has two S-layer proteins: SlpA with a size of 43.6 kDa and SlpB with a size of 44.9 kDa. *L. crispatus* has CbsA with a size of 43.9 kDa. *L. gasseri* has an Apf1 and Apf2 protein both being 29.8 kDa in size for one strain and 26.3 kDa in another strain (Avall et al. 2005). The S-layer protein of *L. brevis* is 46 kDa (Vidgren et al. 1992).

S-layer proteins of lactobacilli have been isolated using LiCl, guanidine HCl, urea, and sodium dodecyl sulfate polyacrylamide gel electrophoresis. When the S-layers are removed, they spontaneously reassemble on solid surfaces (Callegari et al. 1998). Removal of the S-layer protein with LiCl causes a loss in the ability to

autoaggregate, but does not affect their adherence to human colon adenocarcinoma cell line (Caco-2) cells (Avall et al. 2005).

The S-layer proteins of different species of bacteria and archaea perform different functions. Some of these include maintaining cell shape, protecting the cell against harmful environmental factors, controlling the transfer of nutrients, aiding in surface recognition, assisting in conjugation (Jakava et al. 2007), promoting cell adherence to host tissue (Vidgren et al. 1992), causing virulence of the bacteria (Avall et al. 2005), acting as phage receptors (Boot et al. 1993), or shielding underlying phage receptors from invading phage (Boot et al. 1996). In lactobacilli, the primary function of the S-layer protein is adherence.

In order to elucidate the primary and secondary structure of proteins, typical scans include UV-vis spectroscopy, intrinsic fluorescence spectroscopy, and Fourier transform infrared spectroscopy (FTIR). UV-vis spectroscopy can be used to visualize the relative amino acid composition by looking at signals from three amino acids; tryptophan, tyrosine, and phenylalanine. Phenylalanine absorbs at a wavelength of 245-270 nm, tyrosine at a wavelength of 265-285, and tryptophan at a wavelength of 265-295 (Lucas et al. 2006). The differences in absorption is due to the π -electrons on the aromatic components of these amino acids. The intensity or vertical height of the peak determines the amount of scatter in the sample, and the wavelength at which it absorbs allows us to determine which of the three aromatic amino acids is found in the highest concentration. When the absorbance is at the lower end of the range for a specific amino acid, that particular amino acid is more solvent exposed. When it is at

the higher end of the range, it means that that amino acid is more buried. When running multiple samples, the relative scatter and absorption can be determined. From this, estimations can be made regarding the most prevalent amino acid and how exposed or buried this amino acid is.

Intrinsic fluorescence spectroscopy (IFS) is similar to UV-vis spectroscopy in that it measures absorbance, but it differs in the way that the absorbance is measured. In IFS, UV light is sent into the sample at a given wavelength to excite a certain type of amino acid. The light is then emitted at a 90° angle at a lower energy, and the absorbance of the emission is recorded. When excited with ultraviolet light, proteins have the ability to emit luminescence. The amino acids of a protein that are capable of luminescence are the three aromatics; phenylalanine, tryptophan, and tyrosine. Tryptophan and tyrosine can be used as probes for intrinsic fluorescence, but tryptophan, as a probe, is more sensitive and better characterized. Also, there are typically fewer tryptophan residues in proteins than tyrosine residues, so focusing on tryptophan allows for the investigation of a smaller number of points in the structure of the protein. To target tryptophan, an excitation of 295 nm is used. The emission of fluorescence that is given off by the protein is at a lower energy, or longer wavelength, typically in the range of 330-350 nm. The wavelength at which they emit luminescence or fluoresce is determined by how solvent exposed or unexposed the amino acids are. Tryptophan residues that are more exposed to the solvent will have emission wavelengths closer to 350 nm, and those that are buried will have emission

wavelengths closer to 330 nm (Lakowicz 1991). In this project, IFS allows us to determine how folded or unfolded the protein is in lithium chloride and water.

Fourier transform infrared spectroscopy (FTIR) measures the vibrations of select groups of atoms within a protein. Certain groups of atoms give similar vibrational bands around the same frequency, even when they are found on different molecules. The vibrations are dependent on peptide bond angles which allows FTIR to aid in determining secondary structure. During FTIR, the infrared light is passed into the sample and reflects back. The frequencies at which the amide bonds absorb infrared light determine the protein's secondary structure.

The secondary structure of a protein is dependent on dihedral angles. These angles determine the spatial orientation of the peptide backbone and the presence of specific hydrogen bonds. The dihedral angle for an α -helix is $\phi \sim -60^\circ$. The α -helix typically forms a right-handed spiral and is, on average, eleven amino acids long. The dihedral angle for a β -sheet is $\phi \sim -130^\circ$. The β -sheet is stabilized by antiparallel or parallel hydrogen bonds. Turns involve the protein folding back onto itself. They are stabilized by hydrogen bonding at each end (Pelton et al. 2000).

When using FTIR, infrared light is sent into the sample and data is collected on the frequencies or wavenumbers as a function of absorbance within the infrared region of the spectrum. There are three major categories of bands that are investigated in FTIR; amide I bands, amide II bands, and amide III bands (Gallagher 1958). The amide I band is primarily due to the stretching and vibrating of the C=O carboxyl group (Dong et al. 1994). The amide II band involves the stretching and vibration of

the N-H bond (Gallagher 1958). The amide III band involves the stretching and vibration of the C-H bond. The amide III band is very weak and not readily correlated to protein structure. A different range of frequencies are correlated with different secondary structures based on the amide band that is being examined. The amide I band is the best characterized and is much more commonly used for protein analysis. The amide I frequencies of the protein's secondary structures are as follows: an α -helix produces a peak between 1650-1657 cm^{-1} , β -sheet between 1612-1640 cm^{-1} and a weak signal is found between 1670-1690 cm^{-1} , and turns are found between 1655-1675 cm^{-1} and 1680-1696 cm^{-1} (Pelton et al. 2000). Absorbance peaks, thus, allow for the estimation of protein secondary structure.

To analyze the cell surface proteins, the proteins were extracted and run on SDS-PAGE. Spectroscopy analysis of surface proteins was performed by UV-vis spectroscopy, intrinsic fluorescence spectroscopy, and Fourier transform infrared spectroscopy to determine relative primary and secondary structure.

2.1. Materials and Methods

2.1.1. Bacterial strains and media.

Strains, media, and growth conditions follow that of chapter 1. Species used in chapter 2 included *Lactobacillus crispatus*, *Lactobacillus iners*, and *Lactobacillus coleohominis*.

2.1.2. DNA isolation.

Strains of lactobacilli were grown to stationary phase. DNA isolation was done using the UltraClean® Microbial DNA Isolation Kit by Mo Bio Laboratories, Inc. and

stored at 4° C until used. DNA samples were run on a 1% agarose gel and visualized using ethidium bromide to determine quality and relative quantity.

2.1.3. Primer design and PCR amplification.

Primers were made by the University of Texas Health Science Center based on previously published sequences (Sillanpaa et al. 2000). The primers used included the CbsA forward A-11-2931 (5' ACTCATGGATCTTGCTTTGAAG 3') and the CbsA reverse A-11-2932 (5' GACTTAATTAAAAGTTTGAAGCC 3'). 1 and 2 µl of chromosomal DNA from *L. crispatus* 277-1, *L. crispatus* 280-21, *L. crispatus* CTVO5, *L. coleohominis* 269-1R, *L. coleohominis* 484-9, and *L. coleohominis* 515-3 was PCR amplified using Taq polymerase (Sigma). The PCR system used was the PxE O.S. Thermal Cycle by Thermo Electron Corporation (Waltham, MA). It was programmed at 93 °C for 1 min for the initial denaturation, followed by 39 cycles of 95 °C for 30 sec. (denaturation), 58 °C for 1 min (annealing), 72 °C for 1 min. 30 sec. (elongation), followed by a 10 min. final elongation step at 72 °C and a holding temperature of 4 °C. PCR amplifications were run on a 1% agarose gel and visualized using ethidium bromide.

2.1.4. Extraction of the cell surface proteins.

Extractions were performed according to the previously published methods by Turner et al. 1997 with slight modifications. Lactobacilli were grown to stationary phase. The cells were pelleted at 7,000 x g for 15 min. and the supernatants were discarded. The cells were washed with 35 ml of 0.15% NaCl and pelleted at 7,000 x g for 15 minutes. Then, the cells were resuspended in 0.5 ml of extraction solution (5 M

LiCl) and allowed to incubate on ice for 15 min. After incubation, the cells in solution were transferred to a 1.5 ml microcentrifuge tube and centrifuged at 16,000 x g for 5 min. The cells and supernatants were stored at 4° C. For larger protein quantities, multiple extractions were run on individual strains and supernatants were pooled at the end of the extractions. To quantify the concentration, a bicinchoninic acid (BCA) protein assay was performed on the cell surface extracts and whole cell pellets. To bring the samples to a quantifiable range, the supernatants were diluted 1:10, and the cell pellets were diluted 1:100.

2.1.5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.

Samples of 5 M LiCl extracted supernatants from *L. coleohominis* 515-3, *L. coleohominis* 245-3, *L. coleohominis* 269-1R, *L. crispatus* CTVO5, and *L. iners* 276-2 were run on a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. 15 µl of each sample was mixed with an equal volume of Laemmli sample buffer supplemented with 5% β- mercaptoethanol, boiled for 3 min., cooled, and run on the gel at 200 V for 45 min. The gels were stained with 1% Coomassie blue for visualization of the bands, and destained with a destaining solution (70% distilled water, 20% methanol, and 10% glacial acetic acid) for 2 h.

2.1.6. UV-vis spectroscopy.

UV-vis was performed at the Feik School of Pharmacy at the University of the Incarnate Word. UV-vis spectra were collected on a Shimadzu UV-2450 UV-vis spectrophotometer (Koyoto, Japan) with the corresponding UVProbe software. Supernatants from the 5 M LiCl extractions of *L. coleohominis* 515-3, *L. coleohominis*

245-3, *L. coleohominis* 269-1R, *L. crispatus* CTVO5, and *L. iners* 276-2 were diluted separately in 5 M LiCl and in water to a final protein concentration of 25 µg/ml. The spectra of the samples were collected from 200-700 nm and data was analyzed in Microsoft Excel.

2.1.7. Intrinsic fluorescence spectroscopy.

IFS was performed at the Feik School of Pharmacy at the University of the Incarnate Word. IF spectra were collected on a Jasco FP-6500 spectrofluorometer (Easton, MD) using the corresponding Spectra Manager software. Supernatants from the 5 M LiCl extractions of *L. coleohominis* 515-3, *L. coleohominis* 245-3, *L. coleohominis* 269-1R, *L. crispatus* CTVO5, and *L. iners* 276-2 were diluted separately in 5 M LiCl and in water to a final concentration of 100 µg/ml. Fluorescence spectra was collected at an excitation wavelength of 295 nm to target tryptophan residues, and emission was monitored from 300-500 nm. Fluorescence spectra of the appropriate dilutions of 5 M LiCl in water were subtracted from sample spectra diluted in water, and pure 5 M LiCl was subtracted from sample spectra diluted in 5 M LiCl.

2.1.8. Fourier transform infrared spectroscopy.

FTIR was performed at the Feik School of Pharmacy at the University of the Incarnate Word. FTIR spectra were collected on a Thermo Scientific Nicolet 6700 FTIR (Madison, WI) in attenuated total reflectance (ATR) mode using the corresponding Omnic software. Supernatants from the 5 M LiCl extractions of *L. coleohominis* 515-3, *L. coleohominis* 245-3, *L. coleohominis* 269-1R, *L. crispatus* CTVO5, and *L. iners* 276-2 were concentrated using Pierce® Concentrators by Thermo

Scientific (Madison, WI) to an approximate concentration of 10 mg/ml. Neat samples were pipetted onto a diamond attenuated total reflectance (ATR) cell and the background was collected before each sample. For each measurement, 256 spectra were collected at 4 cm⁻¹ resolution. When the samples were complete, a scan of 5 M LiCl was subtracted from each spectra. Further investigation of the spectra involved performing Fourier self-deconvolution (FSD) to identify the relevant peaks of the spectra. The FSD was compared across strains at a bandwidth of 42.6, and an enhancement of 2.9. Spectra and FSDs were overlaid on a single graph and analyzed for similarities.

2.2. Results

2.2.1. CbsA homolog.

The resulting gel electrophoresis from the PCR amplified segments revealed a 200 bp band for all strains of *L. crispatus* that were tested. The primer was designed to amplify a region of 274 bp. *L. coleohominis* revealed a band at approximately 490 bp. Secondary bands appeared for *L. coleohominis* 484-9 at approximately 400 bp. These bands could be indicative of a second S-layer protein gene in the chromosomal DNA or non-specific binding of the primer.

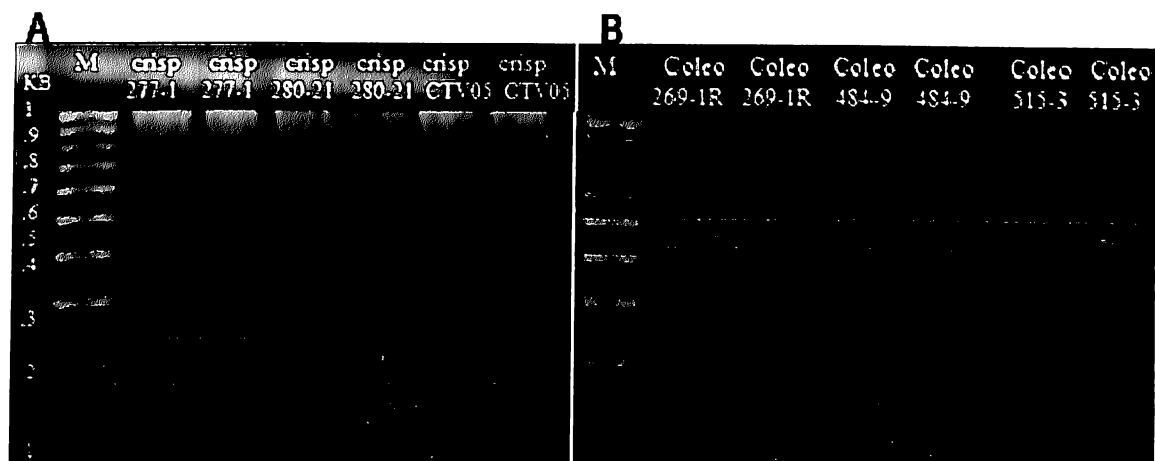


Figure 2.1. PCR amplifications of the CbsA homolog in *L. crispatus* (A) and *L. coleohominis* (B). The marker used was a 100 bp marker (Sigma).

2.2.2. Extraction of surface proteins.

After the cells were extracted using 5 M LiCl, they were plated for viability. The 5 M LiCl extraction led to cell death and no strains exhibited any growth. Protein concentrations for the different extraction solutions and strains are listed in Table 2.1.

	<i>L. crispatus</i> CTV05	<i>L. coleohominis</i> 515-3	<i>L. coleohominis</i> 245-3	<i>L. coleohominis</i> 269-1R	<i>L. iners</i> 276-2
5M LiCl	1.311	1.509	0.969	1.018	1.217

Table 2.1. List of BCA concentrations (in mg/ml) for five cell extracts of lactobacilli using 5 M LiCl as the extraction solution. Extracts resulting from an extraction of 15 ml cultures.

2.2.3. SDS-PAGE of surface proteins.

The SDS-PAGE picture in Figure 2.2 revealed several protein bands of interest with sizes of 12 kDa, 30 kDa, 37 kDa, and 65 kDa. These bands were much more prevalent in *L. coleohominis* 269-1R and *L. crispatus* CTV05.

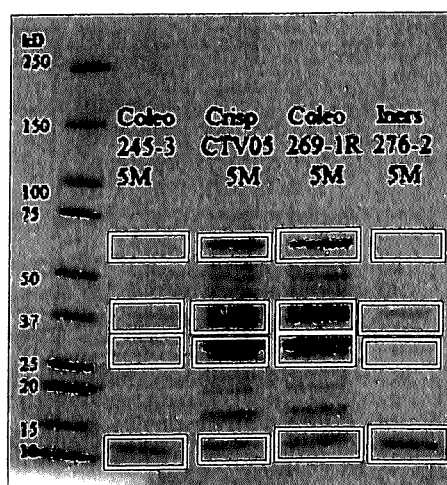


Figure 2.2. SDS-PAGE of extracted surface proteins using 5 M LiCl.

2.2.4. UV-vis Spectroscopy.

Analysis of the spectra in Figure 2.3 showed all peaks within the range of phenylalanine which suggests that phenylalanine is the most prevalent detectable amino acid in the cell surface proteins. The intensities of the peaks or peak heights indicate that the samples contain more precipitates or aggregates that scatter light when diluted in water than in 5 M LiCl. *L. crispatus* CTV05 diluted in water appears to have a much higher scatter than all other strains. According to the absorption

maxima (Table 2.2), the phenylalanine residues seem to be slightly more exposed in water. *L. coleohominis* 245-3 has phenylalanine residues that are slightly more exposed in water as well. *L. coleohominis* 515-3 and *L. coleohominis* 269-1R both have phenylalanine residues that are slightly more exposed in 5 M LiCl, and *L. iners* 276-2 has similar exposure, regardless of the diluent.

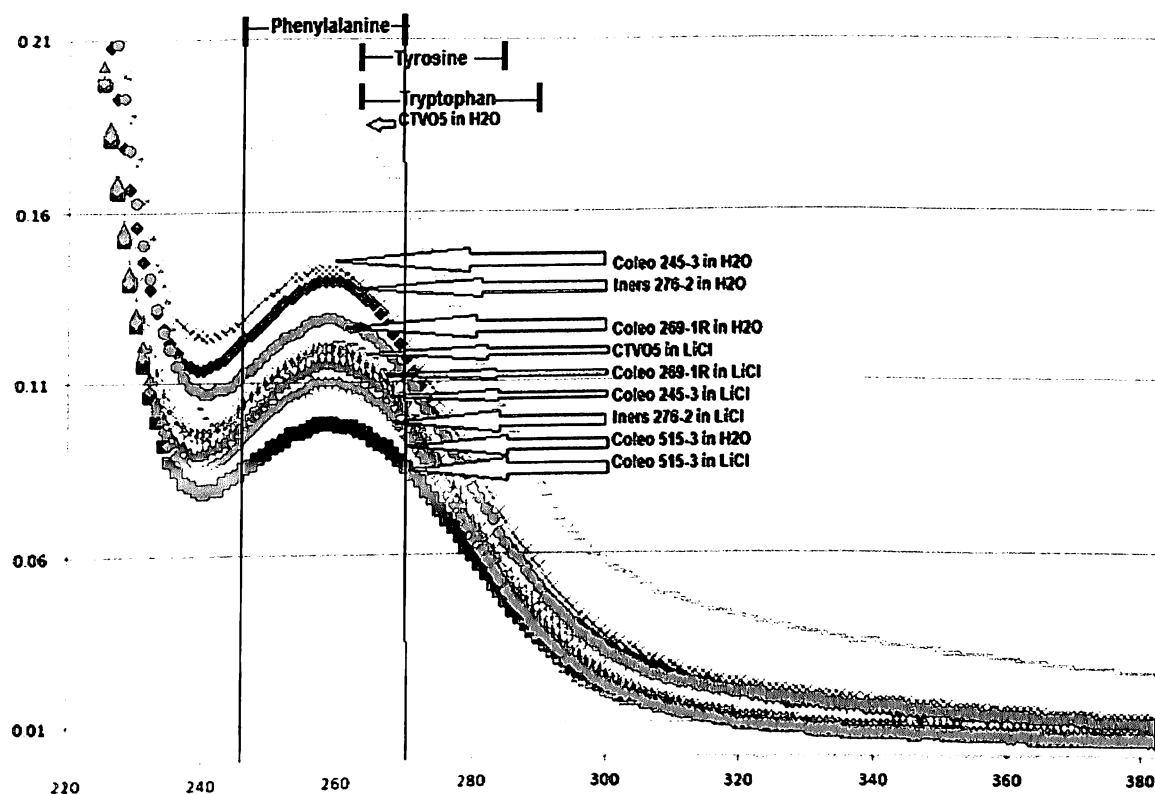


Figure 2.3. UV-Vis spectra of all 5 cell extracts diluted in 5 M LiCl and in water.

Wavelength ranges of amino acid residue absorbance are: phenylalanine 245-270 nm, tyrosine 265-285 nm, and tryptophan 265-295 nm.

Species	Abs. in H ₂ O	Abs. in LiCl
<i>Crisp CTVO5</i>	0.118	0.112
<i>Coleo 245-3</i>	0.143	0.115
<i>Coleo 515-3</i>	0.108	0.097
<i>Coleo 269-1R</i>	0.129	0.120
<i>Iners 276-2</i>	0.140	0.110

Table 2.2. Absorbance maxima at the peak of all 5 cell surface extracts diluted in 5 M LiCl and in water.

Figure 2.4 shows the amount of scatter in the sample at a wavelength of 330 nm, before the absorbance of the protein is detected. This shows how aggregated or precipitated the protein is before the absorption of the aromatic amino acids is observed. For all samples, there is more scatter of light in water than in 5 M LiCl. This suggests that the proteins aggregate or precipitate the most in the presence of water which could be indicative of hydrophobicity.

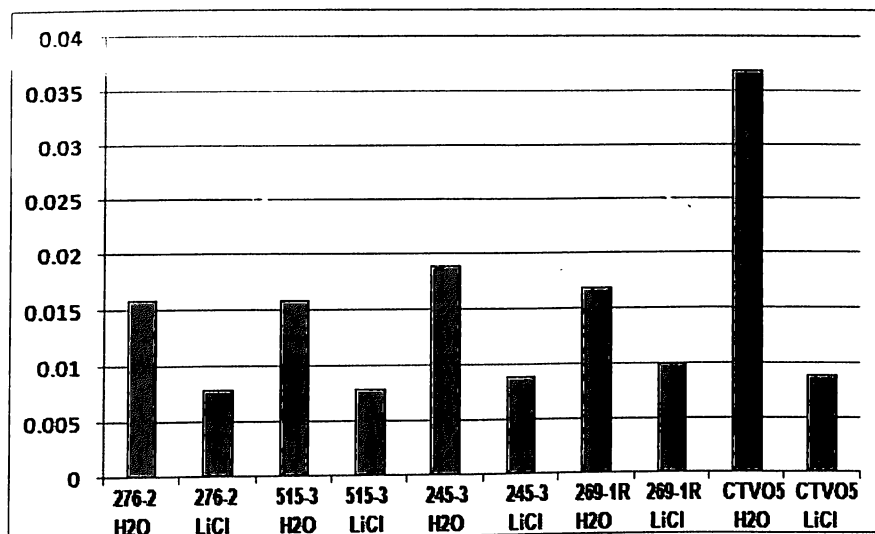


Figure 2.4. Comparison of the amount of scatter at a wavelength of 330 nm in each sample diluted in 5 M LiCl and in water.

2.2.5. Intrinsic Fluorescence Spectroscopy.

The intensity of the peak, or peak height, allows us to infer the relative quantity of tryptophan residues in the sample. Since all samples were equalized to the same concentration, *L. crispatus CTVO5* appears to have the highest number of tryptophan residues and *L. coleohominis 515-3* appears to have the lowest number of tryptophan residues. In IFS, the larger the number, the more exposed the tryptophan is, and the lower the number, the more buried it is. According to the data in Table 2.3, the tryptophan residues in *L. crispatus CTVO5* appears to be slightly more exposed in water than in lithium chloride. In *L. coleohominis 515-3*, *L. coleohominis 245-3*, and *L. coleohominis 269-1R*, the tryptophan residues appear to be slightly more exposed in

lithium chloride than in water. In *L. iners* 276-2, tryptophan exposure appears to be unaffected by the diluent.

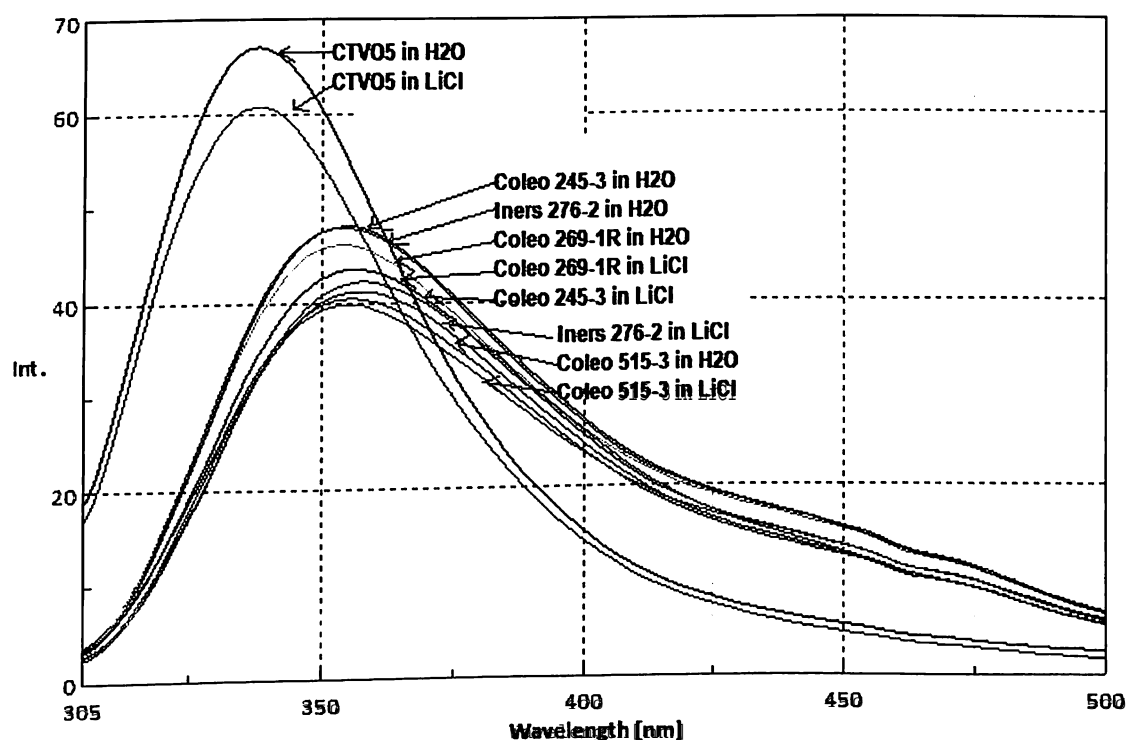


Figure 2.5. Fluorescence spectra of all 5 cell surface extracts diluted in 5M LiCl and in water.

	<i>Crisp CTV05</i>	<i>Coleo 245-3</i>	<i>Coleo 515-3</i>	<i>Coleo 269-1R</i>	<i>Iners 276-2</i>
	Int/ λ	Int/ λ	Int/ λ	Int/ λ	Int/ λ
5M LiCl	60.76 / 337.38	42.35 / 356.45	39.95 / 358.05	43.63 / 356.07	40.91 / 355.40
H ₂ O	67.72 / 338.23	48.25 / 353.40	40.53 / 354.73	46.06 / 353.37	48.25 / 355.40

Table 2.3. IFS. Peak intensities and wavelengths of IFS of lactobacillus surface

protein extractions using 5 M LiCl and H₂O as diluents.

2.2.6. FTIR.

The interpretations of the frequencies or wavenumbers of the Fourier self-deconvolution listed in Table 2.4 suggest that there exists a great number of β -sheets in the cell surface proteins of all strains examined in this study. Significant β -sheet content has been observed in well-characterized S-layer proteins (Fahmy et al. 2006). The amide I frequency ranges are: $1650\text{--}1657\text{ cm}^{-1}$ for α -helices, $1612\text{--}1640\text{ cm}^{-1}$ and $1670\text{--}1690\text{ cm}^{-1}$ for β -sheets, and $1655\text{--}1675\text{ cm}^{-1}$ and $1680\text{--}1696\text{ cm}^{-1}$ for turns (Pelton et al. 2000).

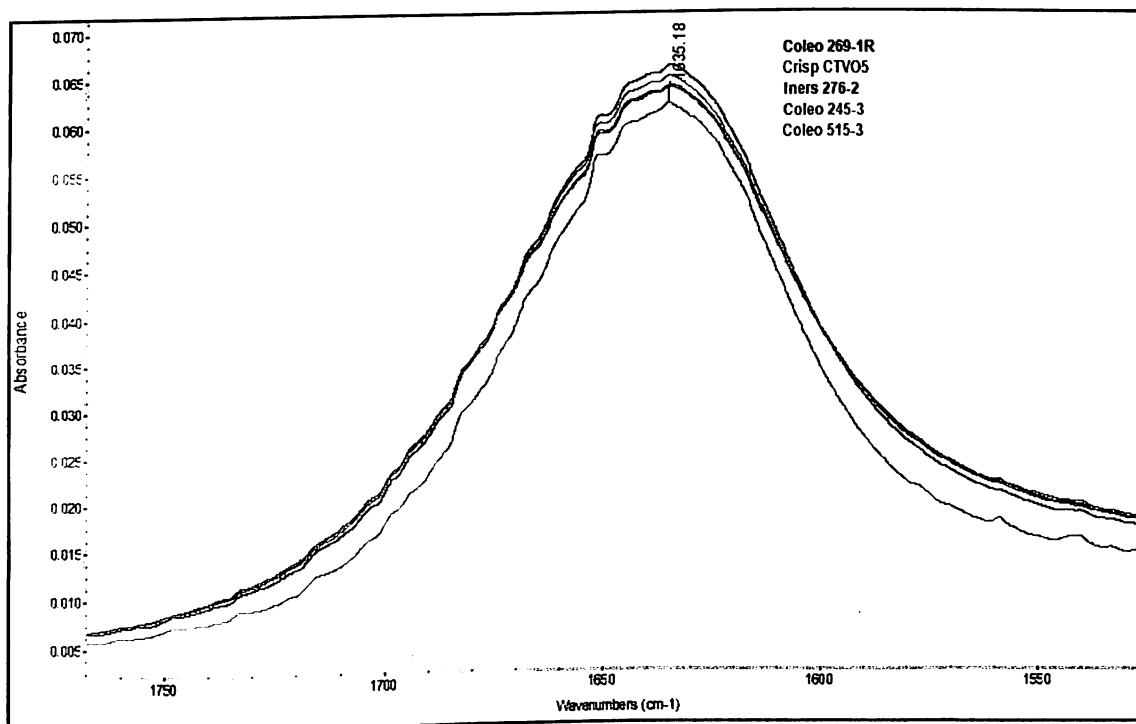


Figure 2.6. FTIR spectra of cell surface proteins of all 5 strains.

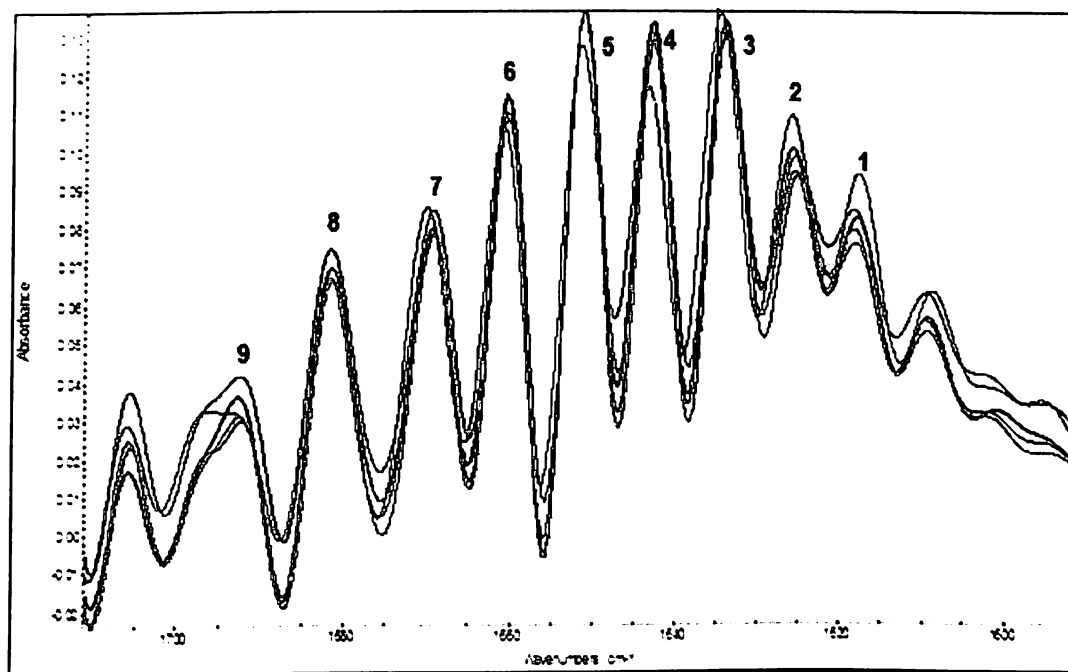


Figure 2.7. FSD of cell surface proteins of all 5 strains. Peak numbers correspond to Table 2.4.

Peak #	<i>Coleo 245-3</i>	<i>Coleo 269-1R</i>	<i>CTVO5</i>	<i>Coleo 515-3</i>	<i>Iners 276-2</i>	Interpretation
1	1618.22	1618.07	1618.23	1621.67	1620.40	β -sheet
2	1625.45	1625.10	1625.33			β -sheet
3	1633.89	1633.76	1633.80	1634.15	1632.38	β -sheet
4	1642.68	1642.64	1642.63			Unordered
5	1651.11	1651.15	1651.08	1649.22	1647.25	α -helix
6	1660.26	1660.32	1660.26	1661.28		Turn
7	1669.26	1669.34	1669.32	1670.51	1665.21	Turn
8	1681.68	1681.52	1681.59	1682.19	1680.79	Turn
9				1694.31	1695.94	Turn

Table 2.4. Peak locations in wavenumbers and interpretations of peak location for all

5 strains. Peak numbers correspond to Figure 2.7.

2.3. Discussion

The chromosomal DNA amplifications by PCR utilized primers that were originally designed to amplify a 274 bp region of the S-layer protein gene, *CbsA*, in *L. crispatus*. Results indicate that these primers recognized at least one site in the genome of *L. coleohominis* and led to the amplification of a target that was approximately 490 bp in length. The size difference from the amplified region in the genome of *L. crispatus*, which is about 200 bp, could be due to the possibility that the primers exhibited non-specific binding and amplified a larger section of DNA, or that there was a true *CbsA* homolog that either contained additional base pairs or was interrupted by another gene. The additional band found at about 400 bp in *L. coleohominis* 484-9 is indicative of non-specific binding of the primers. Further studies require designing non-degenerate primers to perform nested PCR for targeted amplification, purifying the PCR products for sequencing, and comparing homology of these sequences to previously sequenced S-layer genes.

SDS-PAGE was performed on the proteins that were extracted from the surface of the bacterial cell. These proteins were loosely attached by charge attractions and extracted using 5 M LiCl. In preparation of the samples for SDS-PAGE, the samples were mixed 1:1 with sample loading buffer containing 5% β -mercaptoethanol and boiled for 3 minutes. This denatured the protein and made it easier for the protein to run into the gel. The CbsA S-layer protein has been previously determined to be 43.9 kDa (Avall et al. 2005). Multiple protein bands were visualized at 12 kDa, 30 kDa, 37 kDa, and 65 kDa. These bands could be segments of one protein that dissociated

during the preparation of the samples, or they could be different proteins. Further steps would be to excise the protein bands, perform sequence characterization of peptides by GC mass spectral analysis, and compare these sequences to other proteins to look for homology.

In previous studies, the S-layer proteins of lactobacilli have been found to be highly hydrophobic, containing almost 40% hydrophobic amino acids (Smit et al. 2001). CbsA of *L. crispatus* has 36.8% hydrophobic amino acids, SlpA of *L. acidophilus* has 36.4% hydrophobic amino acids, and Apf1 of *L. gasseri* has 32.9% hydrophobic amino acids. The analysis of the cell surface proteins via UV-vis and IFS suggest that the surface proteins that were extracted exhibit similarities to known S-layer proteins. When the UV-vis spectra were analyzed at a wavelength of 330 nm (Fig. 2.4), it was found that there was a much higher amount of scatter when the proteins were diluted in water as opposed to lithium chloride. This scatter is typically due to aggregation of the protein. The aggregation of these proteins in water is indicative of hydrophobicity. In IFS, the tryptophan residues of *L. coleohominis* were slightly more exposed in lithium chloride than in water. This would suggest that the protein begins to fold onto itself or aggregate in water which further supports the hydrophobicity of the protein.

The FTIR results indicate a large number of β -sheets in the cell surface proteins of all strains tested in this project. This is consistent with previous studies on S-layer protein secondary structure (Smit et al. 2001), and suggests that the cell surface proteins were S-layer-like proteins.

When comparing *L. coleohominis* to both *L. crispatus* and *L. iners*, it appears that *L. coleohominis* exhibits high similarities to *L. iners*. Case in point, when observing the UV-vis spectra and IF spectra. In the UV-vis spectra, the intensity of the peak of *L. crispatus* is much higher than that of *L. iners* and *L. coleohominis*, and it has a much higher amount of scatter at 330 nm. This suggests that the cell surface proteins of *L. crispatus* are much more hydrophobic than those of *L. iners* and *L. coleohominis*. This is also evident in the IFS. The intensity of the peak for *L. crispatus* is much higher suggesting a larger number of tryptophan residues than what is found in *L. coleohominis* and *L. iners*.

SUMMARY AND CONCLUSIONS

The purpose of this project was to characterize the novel species *Lactobacillus coleohominis* and analyze a possible S-layer protein. *Lactobacillus coleohominis* is a Gram-positive, short rod. It has a large colony with a dense center and fading or rough edges which may be indicative of slight aggregation or a similar surface layer protein as *L. jensenii*. It forms an acid clot in litmus milk, and has been shown to produce lactic acid, creating a similar environmental pH as that of *L. jensenii* and *L. iners*. It begins colonization from a weakened, frozen state by being turbid, but eventually forms sediment after multiple subcultures. This may be indicative of a change in gene expression of the surface layer protein to express a more hydrophobic protein as previously reported for *L. brevis* (Jakava et al. 2002).

When the genome of *L. coleohominis* was probed by PCR for an S-layer protein gene, a 490 bp region was amplified, but additional research and sequencing of the PCR amplifications is necessary to determine whether it was truly a CbsA homolog. When cell surface proteins were extracted with 5 M LiCl and visualized, four bands appeared at 12 kDa, 30 kDa, 37 kDa, and 65 kDa. The cell surface proteins of *L. coleohominis* appeared to be the same approximate size as those of *L. crispatus* and *L. iners*. Further studies should address the sequence of the protein bands and a search for homology to any bacterial S-layer proteins.

The S-layer-like protein of *L. coleohominis* is quite similar to that of *L. iners* with respect to UV-vis spectroscopy, intrinsic fluorescence spectroscopy, and Fourier transform infrared spectroscopy. It appears to be hydrophobic in nature and aggregates

when diluted in water, and it contains a significant proportion of β -sheets which is consistent with S-layer proteins from other lactobacilli.

In conclusion, *L. coleohominis*, although distinct in its own way, is very similar in aggregation patterns and surface layer proteins as that of *L. iners*. Further studies should address the role of *L. coleohominis* colonization in the vaginal tract in vitro and the interaction of extracted cell surface proteins with host receptors.

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