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**Vaginal lactobacilli protect against  
*C. albicans* and *C. trachomatis* infections**

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## **ABSTRACT**

A normal vaginal microbiota, dominated by lactobacilli, is crucial for the prevention of several urogenital and sexually transmitted infections, including *Candida* and *Chlamydia* (Gupta et al., 1998; Spurbeck and Arvidson, 2008; Parolin et al., 2015; Nardini et al., 2016; Foschi et al., 2017; Ñahui Palomino et al., 2017). This aspect is strengthened by the demonstration that in case of bacterial vaginosis, a clinical condition characterized by the depletion of lactobacilli, a higher risk of STI transmission and acquisition is reported (Taha et al., 1998; Martin et al., 1999; Wiesenfeld et al., 2003; Abbai et al., 2015).

The protective role of lactobacilli against urogenital pathogens is exerted through different mechanisms including the production of various antibacterial compounds (lactic acid, hydrogen peroxide, bacteriocins and biosurfactants), the competitive exclusion for epithelial adhesion, and the immunomodulation (Kaewsrichan et al., 2006; Borges et al., 2014; Parolin et al., 2015; Younes et al., 2018). *Lactobacillus* spp. interaction with the vaginal epithelial cells is the first step in the formation of the biological barrier against colonization of opportunistic and pathogenic organisms. The blockage of undesirable microorganisms adherence by lactobacilli may take place by exclusion, competition, and displacement mechanisms (Coman *et al.*, 2015; Osset *et al.*, 2001; Verdenelli *et al.*, 2014).

In this project, some lactobacilli strains, previously isolated from healthy vaginal swabs by Parolin et al., (Parolin et al., 2015) were tested in order to evaluate their protective role against *C. albicans* and *C. trachomatis* infections. Specifically, the first part of this thesis regarded the study of lactobacilli protection versus *C. albicans* infection, and *L. crispatus* BC1 and BC2, *L. gasseri* BC9 and BC11, and *L. vaginalis* BC15 were employed. Two mechanisms of action at the basis of the protective role of lactobacilli against *C. albicans* were identified. The first mechanism was those exerted by *L. crispatus* BC1 and *L. gasseri* BC9 which interacting with HeLa cell plasma membrane caused a modification of polar lipids organization and increased membrane fluidity. The second mechanism was exerted by *L. crispatus* BC2, *L. gasseri* BC11 and *L. vaginalis* BC15 which acted modulating

$\alpha 5\beta 1$  exposure on HeLa plasma membrane. Both mechanisms resulted in the inhibition of *C. albicans* adhesion to HeLa cells.

The second part of the present thesis aimed to identify vaginal Lactobacilli strains able to interfere with *C. trachomatis* infection process. Specifically, *L. crispatus* BC4 and BC5, *L. gasseri* BC14 and *L. vaginalis* BC17 were tested, and *L. crispatus* BC5 was chosen as model strain because was the most active strain in counteract *C. trachomatis* adhesion to HeLa cells. Importantly, through siRNA silencing of ITGA5 gene, we demonstrated that *C. trachomatis* needs of  $\alpha 5$  integrin subunit for its adhesion and internalization into HeLa cells. Furthermore, our results showed that *L. crispatus* BC5 was able to protect from *C. trachomatis* infection by means of a dual mechanism. On the one hand, *L. crispatus* BC5 interaction with HeLa caused an increase of plasma membrane fluidity and a reduction of  $\alpha 5$  integrin exposure on cell surface, thus making this protein less available for *C. trachomatis* binding and internalization. On the other hand, *L. crispatus* BC5 directly interacted with *C. trachomatis*, grabbing it and thus preventing its binding to  $\alpha 5$  integrin. Interestingly, both in *L. crispatus* BC5 lysate and BS5, through western blot was identified a protein similar to  $\alpha 5$  integrin which could be responsible for *C. trachomatis* binding.

In conclusion, this study allowed a deeper understanding on the mechanisms underlying the protection against pathogenic microorganisms, in the specific case *C. albicans* and *C. trachomatis*. Thanks to their characteristics and to protective effects against pathogens, lactobacilli herein studied, with particular emphasis on *L. crispatus* BC5, could be good candidates for their use as probiotic agents promoting woman's vaginal health.

## **CHAPTER 1**

### **INTRODUCTION**

# **1.1 Vaginal microbiota**

## **1.1.1 Vaginal microbiota of healthy woman**

The human body hosts microorganisms that inhabit surfaces and cavities exposed or linked to the external environment. Each body site contains ecological communities of microbial species that exist in a mutualistic relationship with the host. The varieties of organisms present are highly dependent on the predominant environmental conditions and host factors and hence vary from site to site. Moreover, they vary between individuals and over time (Costello et al., 2009).

The vaginal mucosal ecosystem consists of a stratified squamous non-keratinized epithelium overlaid by a mucosal layer continuously lubricated by cervicovaginal fluid (CVF). Together, these form a physical and biochemical barrier against extraneous invading microorganisms. CVF is an acidic medium containing a large assortment of antimicrobial molecules such as antibodies (IgA and IgG), mucins, b-defensins, surfactant proteins etc., which in addition promotes the confinement of exogenous microorganisms (Aldunate et al., 2015; Witkin 2015; Witkin et al.; 2016). Besides the presence of the molecules mentioned above, the vaginal tract is also colonized by microorganisms, recognized as the vaginal microbiota (VM). These microorganisms, in addition to a complex synergism among secretion's proteins and peptides, epithelial, and immune cells, perform a crucial role in the defense of female genital tract against infectious and inflammatory processes (Valenti et al., 2018). The species that inhabit the vaginal tract can change based on intrinsic host factors such as stage of life cycle, immune responses, hormone levels, nutritional status and disease states. The vaginal microbiota can also be altered by external factors such as environmental exposures, microbial interspecies competition or commensalism, and hygiene behaviors (Bolton et al., 2008).

The human vaginal microbiota was first studied in 1892 by Döderlein, who considered the VM as homogenous and consisting only of gram-positive bacilli (Döderlein 1892). Over time this concept has been modified by researcher that have found the microbiota of asymptomatic woman to be composed of a variety of anaerobic and aerobic microorganisms. Döderlein's bacilli are currently



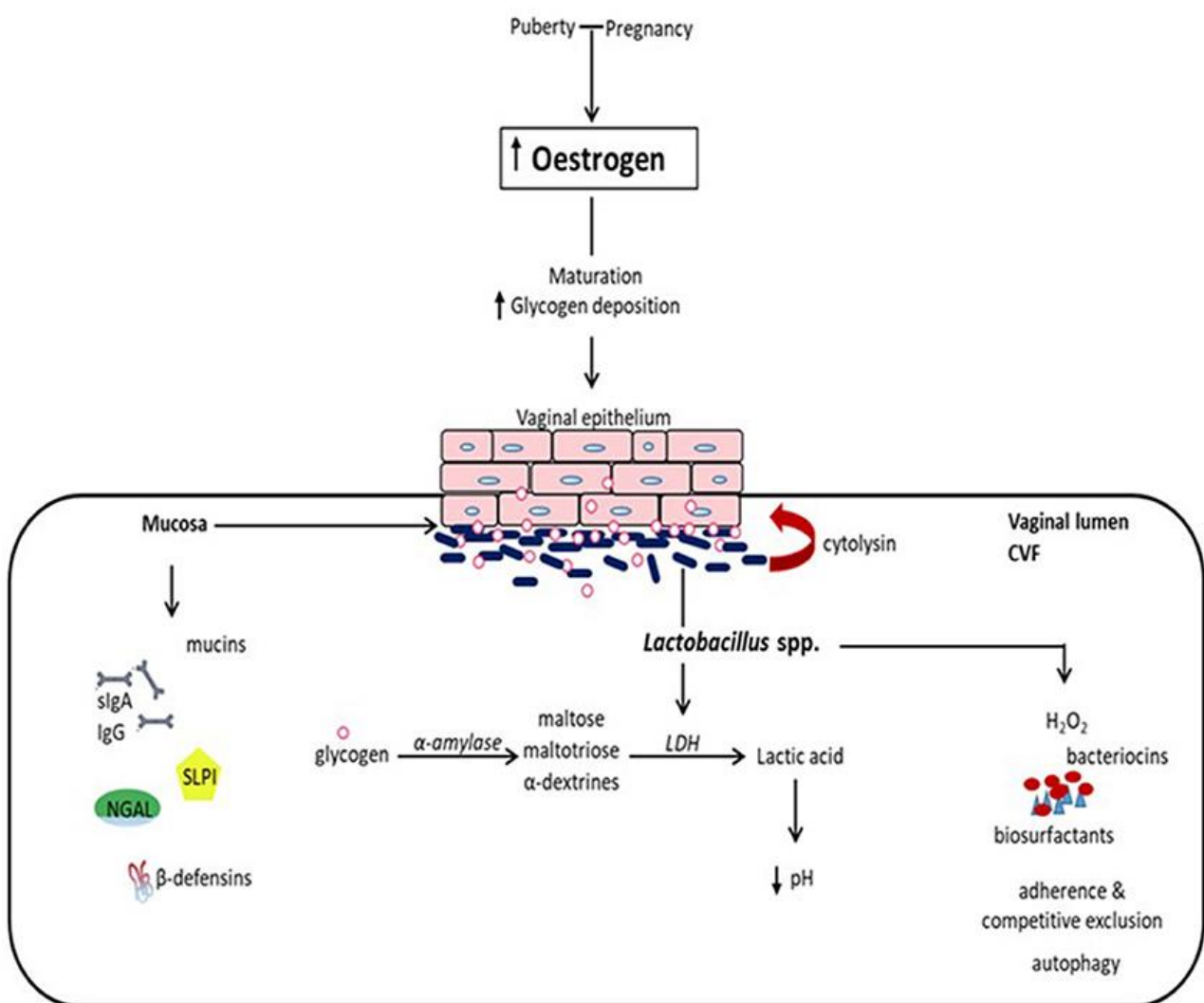
know to be members of *Lactobacillus* spp. Lactobacilli are the most prevalent and often numerically dominant microorganisms, at  $10^7$ – $10^8$  CFU/g of vaginal fluid in healthy premenopausal women (Boris et al., 2000; Farege et al., 2010). The most frequently lactobacilli species isolated from healthy woman are *L. iners*, *L. crispatus*, *L. gasseri*, *L. jensenii*, followed by *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. casei*, *L. vaginalis*, *L. delbrueckii*, *L. salivarius*, *L. reuteri*, and *L. rhamnosus* (Cribby et al., 2008). Other microbial species can be found in vaginal microbiota to a lesser extent, including *Staphylococcus*, *Ureaplasma*, *Corynebacterium*, *Streptococcus*, *Peptostreptococcus*, *Gardnerella*, *Bacteroides*, *Mycoplasma*, *Enterococcus*, *Escherichia*, *Veillonella*, *Bifidobacterium* and *Candida* (Hyman 2005; Larsen 2001; Marrazzo 2002; Redondo-Lopez 1990).

Recent progresses in DNA sequencing techniques have enabled the classification of the vaginal microbiota into five community state types (CSTs) among which CST-I, -II, - III and -V are dominated by *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* respectively, while CST-IV is dominated by mixed anaerobes similar to those found in Bacterial Vaginosis (BV) (Smith et al., 2017; Ravel et al., 2011). Multiple studies have shown that the vaginal microbiota may differ between women with different ethnicity and geographical location, with Blacks and Hispanics that harbor more aerobic bacterial species (CST-IV) and show higher vaginal pH (Ravel et al., 2011; MacIntyre et al., 2015).

### **1.1.2 Alteration of vaginal microbiota composition**

As mentioned above, microbial communities inhabiting different human body sites can vary based on intrinsic host factors such as the stage of life cycle, the nutritional status and hormonal levels or can be altered by external factors such as environmental exposures, microbial interspecies competition or commensalism and hygiene behaviors (Bolton et al., 2008). Age is one of the factors that most affects changes in the vaginal ecosystem. In fact, the vaginal microbial ecosystem undergoes significant changes during the different stages of the woman's life, changes that are directly influenced by estrogen levels. The vaginal tract is colonized within 24 hours of a female child's birth

and remains colonized until death (Farage et al., 2006; Farage et al., 2010). During puberty, the rising levels of estrogen promote the maturation and the accumulation of glycogen in the vaginal epithelial cells. Glycogen is metabolized by human  $\alpha$ -amylase to maltose, maltotriose and  $\alpha$ -dextrine, which in turn are metabolized to lactic acid by *Lactobacillus* species (**Figure 1.1**), which become predominant during this stage of woman life. This creates an acidic environment which favours lactobacilli growth at the expense of other anaerobic microorganisms (Aldunate et al., 2015; Marchesi et al., 2015).



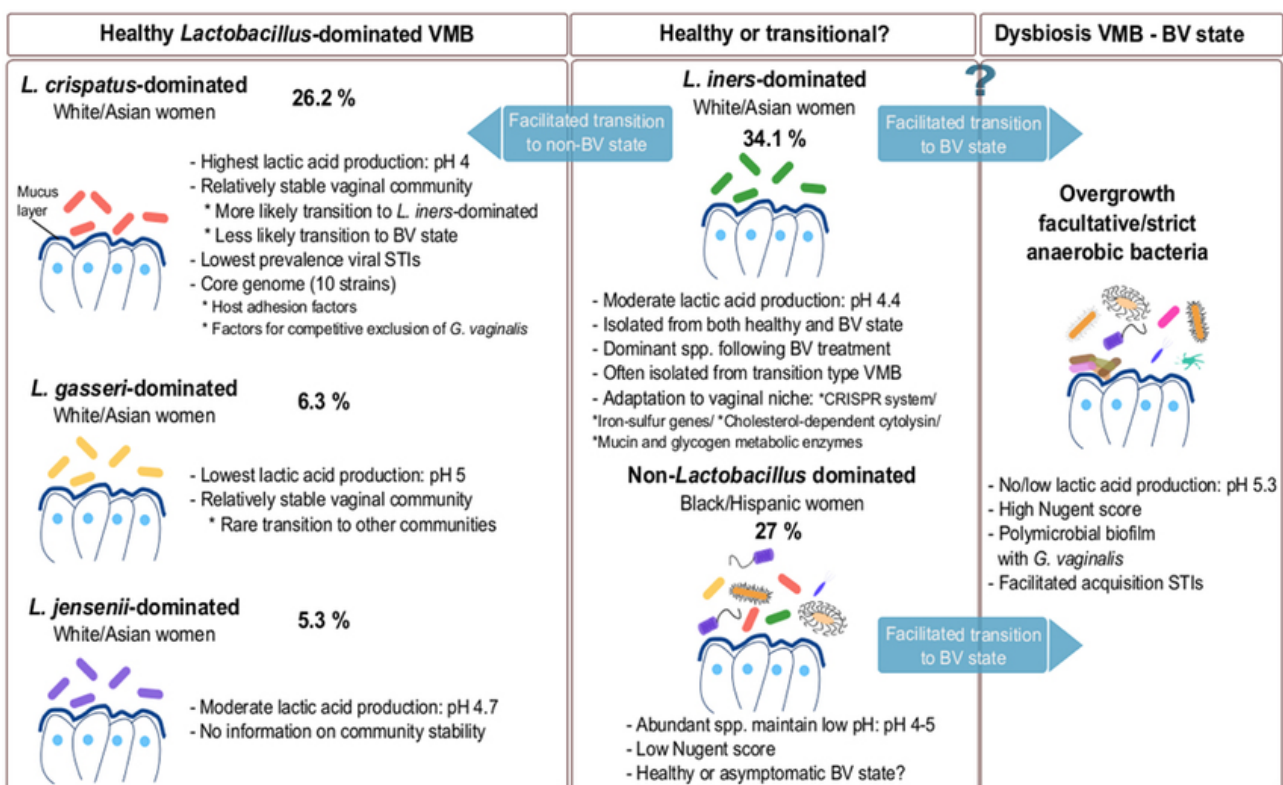
**Figure 1.1 Eubiotic effect of estrogen and *Lactobacillus* species in the vaginal milieu**  
(Amabebe-Anumba 2018).

During the menopause there is a drastic reduction in estrogen production and glycogen content in the vaginal epithelium drops as well, leading to a depletion of lactobacilli. The decrease in the number of lactobacilli results in an increase of pH values that promotes the growth of pathogenic microorganisms (Farage et al., 2010). Even during pregnancy, there is a change in the microbiota due to the high level of estrogen and to increased vaginal glycogen deposition, which enhances the proliferation of Lactobacilli- dominated microbiota (Witkin, 2015). Among the external factors that can induce alterations in the normal composition of the vaginal microflora, hygiene behaviors and sexual activity can represent disturbing factors (Schwebke et al., 1999). Antibiotics, in addition to eradicating pathogens, also affect non-pathogens and disrupt the natural microbiota. Therefore, the use of probiotics containing vaginal lactobacilli could be useful to restore a vaginal microflora dominated by *Lactobacillus* genus (Reid et al., 2011; Mastromarino et al., 2002).

### 1.1.3 Vaginal dysbiosis

The vaginal microbiota (VMB) dominated by lactobacilli play a pivotal role in protecting against several pathogenic microorganisms. In the state of mucosal health, the microorganisms constituting the vaginal microbiota coexist in a perfect balance and the resulting homeostasis derives from a deep and complex interaction between them. The rupture of homeostasis, and in particular the lack of lactobacilli, contribute to creating a micro-environment suitable for the growth of pathogenic microorganisms (Petrova et al., 2015) (**Figure 1.2**). There seems to be an association between absence (or low concentrations) of vaginal lactobacilli and the development of Bacterial Vaginosis (BV). BV is one of the most common vaginal disorders that affect fertile, premenopausal and pregnant women, resulting in millions of health care visits worldwide each year. BV is a complex, polymicrobial disorder characterized by the disruption of the vaginal microbial niche, resulting in a reduction of lactobacilli. This leads to an overgrowth of strict or facultative anaerobic bacteria such as *Gardnerella* spp., *Atopobium* spp., *Prevotella* spp., *Mobiluncus* spp., as well as other taxa such as *Clostridium* spp., *Megasphaera* spp., *Leptotrichia* spp (Verstraelen et al., 2004; Fredricks et

al., 2005; Tamrakar et al., 2007). Bacterial vaginosis is associated with high pH, a decrease in antimicrobial activity of the vaginal fluid, and local impairment of the multiple innate immune pathways (Dover et al., 2008). A typical feature of BV is the absence of inflammation. In BV, there is only a slight increase in interleukin I and a low production of interleukin 8, which prevents the attraction of inflammatory cells like macrophages and neutrophils (Donati et al., 2010).



**Figure 1.2 Composition of VMB during healthy and dysbiotic states** (Petrova et al., 2015).

Important to remark is that in around 50% of women BV is asymptomatic, but when symptomatic, BV is characterized by homogeneous malodorous vaginal discharge and vaginal discomfort. (Sweet, 2000). Clinical diagnosis of BV requires three out of four features: the presence of clue cells on microscopy; a homogenous discharge adherent to the vaginal walls; pH of vaginal fluid higher than 4.5; and a "fishy" amine odour of the vaginal discharge before or after addition of 10% KOH. Another

method of diagnosis is Gram staining that uses Nugent's criteria and allows to categorise the vaginal flora into three categories: normal, intermediate, and flora consistent with bacterial vaginosis (Joesoef and Schmid, 2005). Most of the time, BV is a non-inflammatory condition and can have important clinical sequelae. For example, it has been reported that changes in the VMB are associated with various vaginal and urinary tract infections (Harmanli et al., [2000](#); Koumans et al., [2002](#)). It has also been shown that BV facilitates the acquisition of sexually transmitted infections such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, HIV and HSV-2 (Martin et al., [1999](#); Cherpes et al., [2003b](#); Wiesenfeld et al., [2003](#)). Furthermore, BV may result in increased rates of early pregnancy loss and preterm delivery (Eckert et al., [2003](#); Verstraelen et al., [2005](#)).

#### **1.1.4 Role of lactobacilli in promoting vaginal health**

Vaginal microbiota is commonly dominated by *Lactobacillus* spp. in approximately 70% of women. Lactobacilli are a group of Gram-positive, facultative anaerobic bacteria producing lactic acid as product of glycogen fermentation (Axelsson 2004; Aguirre-Collins 1993).

In healthy pre-menopausal woman, the most frequently isolated species are *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii* and *Lactobacillus iners*. Residing at the port of entry of bacterial and viral pathogens, the vaginal lactobacilli can create a barrier against pathogen invasion. Indeed, mainly thanks to products of their metabolism secreted in the cervicovaginal fluid, lactobacilli can play an important role in the inhibition of bacterial and viral infections. Therefore, a *Lactobacillus*-dominated microbiota appears to be a good biomarker for a healthy vaginal ecosystem (Petrova 2015).

Several are the mechanisms by which lactobacilli stabilize the vaginal microbiota conferring protection against potential pathogens (**Figure 1.3**). These include: 1) the production of antimicrobial compounds such as hydrogen peroxide, lactic acid, bacteriocin-like substances and biosurfactant, 2) the capability to adhere and compete for adhesion sites in the vagina, and 3) the capability to co-aggregate (Borges et al., 2013).

## 1) Production of antimicrobial compounds

**Lactic acid.** Lactobacilli produce lactic acid through the fermentation of glucose released by vaginal epithelial cells. The production of lactic acid by lactobacilli can contribute to pH acidification, which is also maintained by the secretion of organic acids by the vaginal epithelial cells themselves. The vaginas of reproductive-aged women typically have a pH of approximately 4–4.5, and it is likely that this degree of acidity strictly limits the microbiota to acidophilic species such as *Lactobacillus* spp. However, pH may have more subtle effects than simply to provide an unfavorable environment for certain species of microorganisms. Furthermore, in vitro studies have shown that acidification by lactobacilli growth can inhibit the proliferation of pathogen microorganisms, such as *C. albicans*, *Escherichia coli*, *G. vaginalis*, *Mobiluncus* spp. and other bacteria cultured from vaginal specimens obtained from women with bacterial vaginosis (Boris et al., 2000).

**Hydrogen peroxide.** H<sub>2</sub>O<sub>2</sub> is an oxidizing agent, which is toxic to catalase-negative bacteria such as most anaerobic microorganisms. Some studies suggest that H<sub>2</sub>O<sub>2</sub> is produced by 95 % of *L. crispatus* and 94 % of *L. jensenii* vaginal isolates (Antonio et al., 1999); furthermore, approximately 80 % of the strains of vaginal origin produces H<sub>2</sub>O<sub>2</sub> (Aroutcheva et al., 2001) and only 6% of women with bacterial vaginosis contain H<sub>2</sub>O<sub>2</sub>-producing *Lactobacillus* species in their vagina (Eschenbach et al., 1989). Other in vitro studies also demonstrate the involvement of the peroxidase system in the inhibition of *Neisseria gonorrhoeae* in an acidic environment by a complex effect from H<sub>2</sub>O<sub>2</sub>, acid production and bacteriocin-like compounds (Zheng et al., 1994).

**Bacteriocins and biosurfactants.** Bacteriocins are antimicrobial peptides or proteins, produced by almost all genera of lactobacilli. Bacteriocins have several mechanisms of action, including cytoplasmic membrane pore formation, interference with cellular enzymatic reactions and nuclease activity (Gillor et al., 2005). However, only few bacteriocins from vaginal isolates of *Lactobacillus* spp. have been identified. Some examples are the bacteriocin produced by a strain of *L. salivarius* with activity against *E. faecalis*, *E. faecium* and *N. gonorrhoeae* (Ocaña et al., 1999), or a small bacteriocin, produced by *L. fermentum* strain L23, which displayed a wide inhibitory spectrum

including both Gram-negative and Gram-positive pathogenic strains and two species of *Candida* spp. (Pascual et al., 2008). Bacteriocins show inhibitory effects similar to those of antibiotics, although they are different as regards their synthesis, mechanism of action, toxicity, and resistance mechanisms (Li et al., 2005; Kaur et al., 2013). Antibiotic-resistant microorganisms are usually not resistant to bacteriocins. Furthermore, toxicity studies have shown that antimicrobial peptides produced by lactobacilli do not irritate the vaginal epithelium and have no effects on vaginal lactobacilli viability (Li et al., 2005; Stoyancheva et al., 2014).

Another characteristic of lactobacilli is the capability of producing biosurfactant substances. In a study of 1996, fifteen strains were found to produce biosurfactant. The substance or substances adsorbed to surfaces and inhibited the initial adhesion of *E. faecalis* by 70%. The crude substance was analyzed and was found to contain proteins and carbohydrates (Velraeds et al., 1996).

Numerous physiological functions of biosurfactants have been described. Biosurfactants can, among other things, enable microorganisms to grow on water-immiscible compounds by lowering the surface tension at the phase boundary, cause emulsification, and stimulate adhesion of microbial cells to organic substrates (Fiechter, 1992). It has been shown that biosurfactant activity is resistant to trypsin and pepsin, and sensitive to  $\alpha$ -amylase and lysozyme, and resistant to 75° C degree heating.

Among these substances, surfactin, produced by *L. acidophilus* and *L. fermentum*, has shown a particularly effective inhibitory activity against *Enterococcus faecalis*, *Escherichia coli*, and *Staphylococcus epidermidis*, as well as *Candida albicans* (Velraeds et al., 1998).

In this perspective, the antiadhesive molecules produced by certain lactobacilli look promising for application to many human sites where pathogens can attach, colonize, and confer disease.

## **2) Adhesion to the epithelial vaginal cells.**

One of the protective mechanisms exploited by the lactobacilli is their capability to adhere and compete for adhesion sites in the vaginal epithelium, preventing in this manner the colonization by a pathogen. Cell adhesion is a multistep process that involves the contact of the bacterial cell membrane and interacting surfaces (Kos et al., 2003)

Several factors such as hormonal changes (particularly estrogen), vaginal pH, and glycogen content can affect the ability of lactobacilli to adhere to epithelial cells and colonize the vagina (Cribby et al., 2008). Lactobacilli inhabiting a healthy urogenital tract exclude the colonization of pathogenic bacteria by occupying or masking (by steric hindrance) their potential binding sites in the mucosa. The blockage of urogenital pathogens adherence by lactobacilli can occur through exclusion, competition for receptor sites and displacement of adhered pathogens. Several studies have shown the capability of *Lactobacillus* spp. to adhere to epithelial vaginal cells to form a biological barrier against colonization by pathogenic bacteria (Coudeyras et al., 2008; Zárate et al., 2006). Multiple components of the bacterial cell surface seem to participate to this process. It has been reported that the factors responsible for adherence to epithelial vaginal cells seemed to be glycoproteins and carbohydrates (Boris et al., 1998).

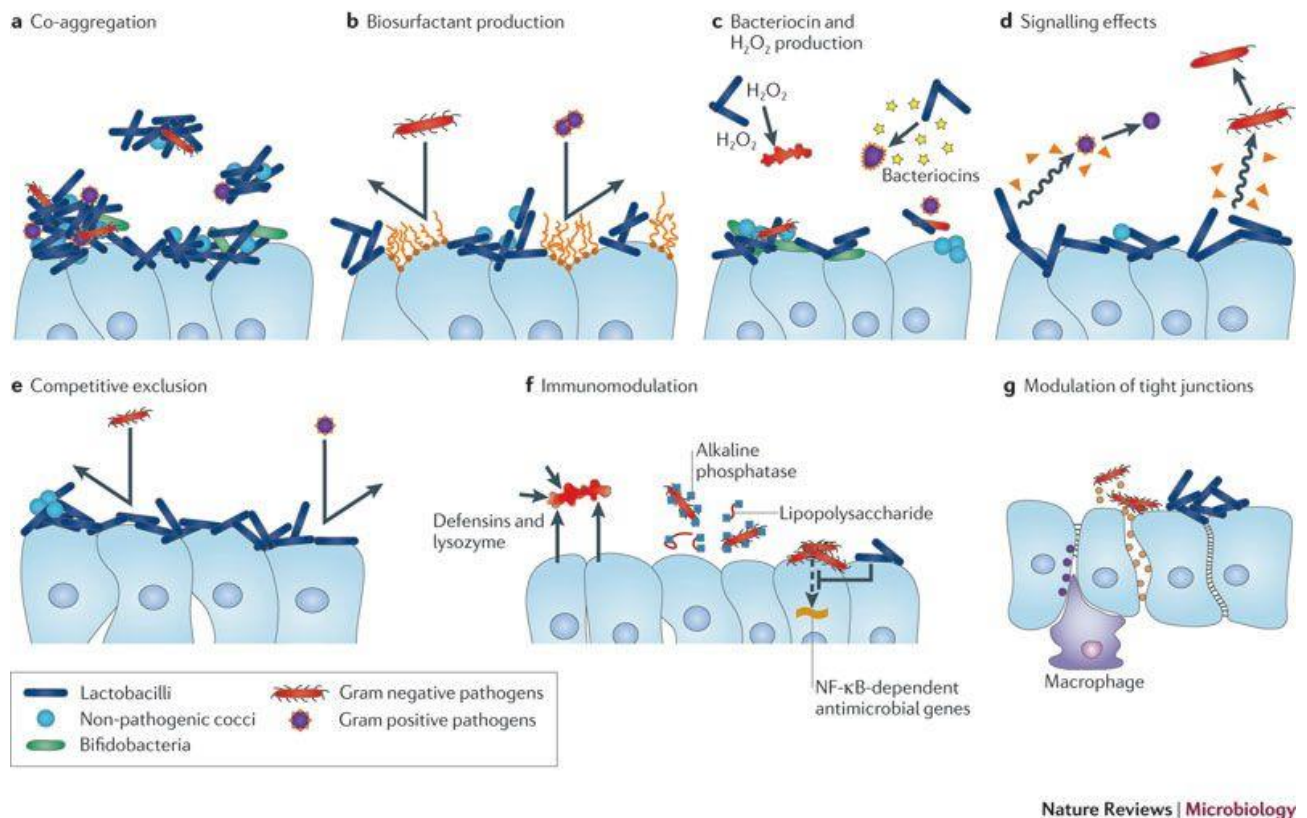
Furthermore, it has been shown as some lactobacilli strains were able to significantly reduce *C. albicans* adhesion by exclusion, competition and displacement experiments (Parolin et al., 2015).

### **3) Co-aggregation with pathogenic bacteria**

Coaggregation is a process by which genetically distinct bacteria become attached to one another via specific molecules (Rickard et al., 2003). Cumulative evidence suggests that such adhesion influences the development of complex multispecies biofilms. Coaggregation has been observed amongst bacteria isolated from biofilms in the mammalian gut, the human urogenital tract and potable-water-supply systems, indicating that the adhesion of genetically distinct strains could be a widespread phenomenon (Handley et al., 2001). Lactobacilli can form coaggregates and bind to pathogens, and this results in a return to homeostasis, since the coaggregation creates a hostile biochemical micro-environment around a pathogen and prevents it from continuation of growth and domination of the niche. The ability of lactobacilli to coaggregate with other bacteria probably influences the structure and stability of the urogenital flora (Reid et al., 1990). It has been shown that certain species of *Lactobacillus*, including *L. acidophilus*, *L. gasseri* and *L. jensenii* are able to co-aggregate with *E. coli*, vaginal staphylococci and *C. albicans* (Boris et al., 1998; Ekmekci et al., 2009). Furthermore,



*Lactobacillus reuteri* RC-14 has shown the ability to penetrate mature *E. coli* biofilms and kill the *E. coli* upon coaggregation and integration with the biofilm (McMillan et al., 2011).



**Figure 1. 3: Possible mechanisms contributing to restoration of the microbiota.** (Reid et al., 2011)

### 1.1.5 Lactobacilli: potential use as vaginal probiotics

Probiotics have been defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2006). Many studies provided evidence of the beneficial functions of the human microbiota, and prompted the selection of bacterial strains, recognized as probiotics, with health-promoting capacities for the treatment of conditions in which the microbiota, or its optimal functioning, is perturbed (Mastromarino et al., 2013). A remarkable interest from women was shown on the potential use of LAB for maintaining normal urogenital health

(Anukam et al., 2007). A probiotic may act indirectly through treating and preventing recurrent BV or directly by secreting substances (e.g., hydrogen peroxide, bacteriocins, lactic acid) that block sexually transmitted infection (Bolton et al., 2008) (**Figure 1.4**).



**Figure 1.4: Requirements in the choice of a probiotic** (Konings et al., 2000)

Since antimicrobial treatment of urogenital infections is not always effective, and problems remain linked to bacterial and yeast resistance, recurrent infections, and side-effects, it is not surprising that alternative remedies are of interest to patients and their caregivers. Indeed, lactobacilli probiotics can be used over a long period of time without adverse effects, making them an attractive alternative to antibiotics, particularly in addressing the problem of high recurrence rates (Cribby et al 2008).

Since lactobacilli can ascend passively from the rectum to the vagina, probiotics can be administered either vaginally or orally, which can be a significant breakthrough in being able to deliver probiotics in foods and dietary supplements (Reid, 2008). Through oral administration, the time required to affect the vaginal tract is clearly longer than direct vaginal instillation, and will depend on viability of the strains as they pass through the stomach and gut. In addition, the load of lactobacilli that can be delivered this way is obviously lower than via vaginal administration. However, an advantage of the oral approach may be the ability of the lactobacilli to reduce the transfer of yeast and pathogenic bacteria from the rectum to the vagina, which could potentially lower the risk of infection (Cribby et al., 2008). Vaginal dosage forms available around the world include creams, gels, tablets, capsules, films, tampons, rings, and douches. While the majority of vaginal drugs so far have been in the form of gels, there is a growing interest in alternative dosage forms such as rings, tablets, and films (Garg et al., 2010).

Several clinical trials have been performed to assess whether specific strains of lactobacilli are able to colonize the vaginas of women with symptomatic or asymptomatic BV, to reduce the colonization of pathogens, and to improve symptoms of BV when they are present (Falagas et al., 2007). Two types of experimental approaches have been employed in these studies using probiotics for treatment of BV. In the first, BV therapy was carried out using only probiotics. In the second, probiotics were administered following a conventional antibiotic therapy. Two studies (Anukam et al., 2006b; Mastromarino et al., 2009) used a combination of different species of lactobacilli with different biological properties on fertile non-pregnant women. *L. rhamnosus* gR-1 and *L. fermentum* RC-14 were the strains used in the first study (Anukam et al., 2006b). *L. rhamnosus* gR1 adheres strongly to uroepithelial cells and inhibits adhesion and growth of uropathogens (Reid et al., 1987). *L. fermentum* RC-14 produces biosurfactant compounds (Velraeds et al., 1998) and significant amounts of hydrogen peroxide, adheres to uroepithelial cells and inhibits pathogen binding (Reid and Bruce, 2001). Cure of BV was based on a Nugent score  $\leq 3$  at 30 days. A BV cure rate of 65% was achieved after probiotic treatment compared to 33% of the metronidazole therapy.

The second study used a product containing a combination of three strains of lactobacilli: *L. brevis* CD2, *L. salivarius* FV2 and *L. plantarum* FV9 (Mastromarino et al., 2009). The strains were able to temporarily colonize the human vagina (Massi et al., 2004), reduce vaginal proinflammatory cytokines IL-1 $\beta$  and IL-6 (Hemalatha et al., 2012) and showed inhibitory activity towards HSV-2 replication in cell cultures (Conti et al., 2009; Mastromarino et al., 2011). The double-blind, placebo-controlled trial (Mastromarino et al., 2009) used both the Amsel criteria and Nugent scores to assess BV cure as recommended by the FDA. The intravaginal probiotic-treated group (*L. brevis* CD2, *L. salivarius* FV2 and *L. plantarum* FV9) obtained a BV cure rate of 50% compared to 6% in the placebo-treated group with the combined test methods, whereas a 67% vs 12% cure rate was obtained when considering only the Amsel criteria. In a randomized double-blind placebo-controlled study, vaginal capsules containing *L. gasseri* LN40, *L. fermentum* LN99, *L. casei* subsp. *rhamnosus* LN113 and *P. acidilactici* LN23 ( $10^8$  and  $10^9$  viable cells for capsule) were administered for 5 days to 95 women after conventional treatment of BV and/or vulvovaginal candidiasis. Probiotic strains were present 2–3 days after administration in 89 % of the women. After one menstruation, 53 % were colonized by at least one *Lactobacillus* strain. Nine percent were still colonized 6 months after administration. The probiotic supplementation resulted in less malodorous discharge, and a trend towards higher clinical cure rate, compared with the placebo group (Ehrström et al., 2010). Some studies evaluated the use of lactobacilli for the prevention of recurrent urinary tract infections (UTI). Among these, it has been reported a study with nine patients inserted with vaginal suppositories containing the strain *L. crispatus* GAI 98322 ( $1.0^8$  cfu per suppository) every 2 days for 1 year. A significant reduction in the number of recurrences was noted, without any adverse complication (Uehara et al., 2006).

## 1.2 *Candida albicans*

### 1.2.1 Vulvovaginal candidiasis (VVC)

Vulvovaginal candidiasis is an infection of the vulva and the vagina caused by fungi belonging to *Candida* genus, and is estimated to be the second most common cause of inflammation after bacterial vaginosis (Spence, 2010). The most common pathogen is *Candida albicans*, which is isolated in 85 to 90% of all VVC cases (Sobel, 1997).

### 1.2.2 *Candida albicans*: pathogenicity mechanisms

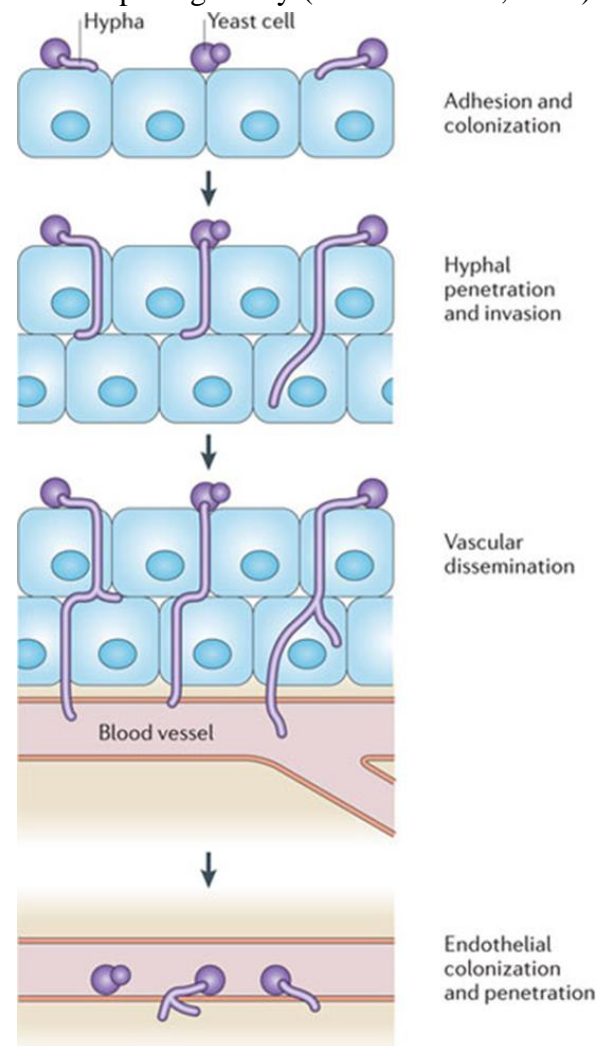
*C. albicans* is one of the very few fungal species causing disease in humans. It is a member of the healthy microbiota, asymptotically colonizing the gastrointestinal (GI) tract, reproductive tract, oral cavity, and skin of most humans (Achkar and Fries, 2010; Ganguly and Mitchell, 2011; Kennedy and Volz, 1985; Kumamoto, 2002; Kumamoto, 2011). In individuals with healthy immune systems, *C. albicans* is often harmless, and kept in balance with other members of the local microbiota. However, alterations in the host microbiota caused by lactobacilli depletion and antibiotic use, changes in the host immune response that can occur during stress, infection by other microbe, or immunosuppressant therapy, or variations in the local environment such as shifts in pH or nutritional content, can enable *C. albicans* to overgrow and cause infection (Nobile and Johnson; 2015).

*Candida* spp are the fourth most common cause of hospital-acquired systemic infections in the United States with crude mortality rates of up to 50% (Pfaller and Diekema, 2010). *C. albicans* can cause two major types of infections in humans: superficial infections, such as oral or vaginal candidiasis, and life-threatening systemic infections.

*Candida albicans* is a polymorphic fungus that can grow as an ovoid budding yeast (also named blastospores), as elongated ellipsoid cells that remain attached at a constricted separation site (pseudohyphae), or as parallel-sided true hyphae (Berman and Sudbery, 2002) (**Figure 1.5**). The fungus can also form chlamydospores, spore-like structures, produced under distinct conditions, of as yet unknown biological function (Martin et al., 2005; Citiulo et al., 2009), and undergo phenotypic

switching between white and opaque morphologies, an event which is important for mating and biofilm formation (Miller and Johnson, 2002; Daniels et al., 2006). The transition between yeast and hyphal growth, termed dimorphism, is tightly regulated by a network of signal transduction pathways in response to environmental stimuli. For example, at low pH (< 6) *C. albicans* cells predominantly grow in the yeast form, while at a high pH (> 7) hyphal growth is induced (Odds, 1988). Furthermore, it has been proposed that both growth forms are important for pathogenicity (Jacobsen et al., 2012).

The hyphal form has been shown to be more invasive than the yeast form (Berman and Sudbery, 2002), while the smaller yeast form is believed to represent the form primarily involved in dissemination (Saville et al., 2003). One of the most important virulence factors of *C. albicans* is the adhesion to the host cells, which is mediated by a specialized set of proteins, the adhesins. The best studied *C. albicans* adhesins are the agglutinin-like sequence (ALS) proteins and particularly the Als3, which is an hypha-associated adhesion especially important for the adhesion (Zordan et al., 2012; Phan et al., 2007; Murciano et al., 2012). Adhesion and hyphal formation are strictly related; indeed, the contact of *C. albicans* to abiotic surfaces or to host cells stimulates hyphal formation and the simultaneous induction of hypha-associated adhesins. In addition, *C. albicans* invades the



**Figure 1.5: *Candida albicans* tissue invasion.**

(Gow et al., 2011)

epithelial cells by means of two distinct processes: induced endocytosis and active penetration (Zakikhany et al., 2007; Dalle et al., 2010; Wächtler et al., 2011). Induced endocytosis is a host-driven process activated mainly by interaction between the *C. albicans* invasin, Als3, and host E-

cadherin. This process results in clathrin dependent uptake, similar to the internalin-E-cadherin dependent internalization of *Listeria monocytogenes* (Zakikhany et al., 2007; Phan et al., 2007; Moreno-Ruiz et al., 2009). A further important virulence factor of *C. albicans* is its capability to form biofilms on abiotic (e.g. catheters) or biotic surfaces (e.g. mucosal cell surfaces). Biofilms form in a sequential process including adherence of yeast cells to the substrate, proliferation of the yeast cells, formation of hyphal cells in the upper part of the biofilm, accumulation of extracellular matrix material and, finally, dispersion of yeast cells from the biofilm complex (Finkel and Mitchell, 2011). It has been reported that mature biofilms are much more resistant to antimicrobial agents and host immune factors in comparison to planktonic cells (Finkel and Mitchell, 2011; Fanning and Mitchell, 2012). The production of proteolytic enzymes, toxins and phospholipases also contributes to increasing the pathogenicity and the virulence of the fungus. For example, the presence of aspartyl proteinases produced by pathogenic *Candida* spp. has been found in vaginal secretions of women with symptomatic vaginitis, but not in women with asymptomatic colonization (De Bernardis 1990; Al-Hedaithy 2002).

### **1.2.3 VVC: etiology, predisposing factors and incidence**

As mentioned above, *C. albicans* is the most common cause of VVC. The second most common pathogen identified in women with VVC is *C. glabrata*, which is isolated in 7 to 16% of cases (Achkar and Fries, 2010). Lactobacilli are an important element of vaginal microflora because their production of lactic acid keeps the vaginal pH low and prevents overgrowth of other pathogens (Achkar and Fries, 2010; Ronnqvist et al., 2006). Predisposing factors for VVC include diabetes mellitus, use of antibiotics, oral contraception, pregnancy and hormone therapy (Fidel, 2004). The use of antibiotics causes a change in vaginal microflora, which increases colonization with *Candida* spp. Colonization with *C. albicans* is increased from approximately 10% to 30%, and VVC is diagnosed in 28 to 33% of cases (Sobel, 2007).

VVC can be distinguished into uncomplicated and complicated cases. Uncomplicated cases are sporadic episodes of mild infections caused by *C. albicans* (Sobel et al., 1998). Complicated cases include VVC caused by other species of *Candida*, cases of severe infection, VVC during pregnancy, or VVC associated with other medical conditions such as immunosuppression or diabetes. Another form of complicated infection is the recurrent VVC (RVVC) which is defined as four or more episodes of VVC per year (Achkar and Fries, 2010; Berek et al., 2012; Peters et al., 2014). About 75% of all women suffer at least once in their lifetime from vulvovaginal candidiasis (VVC), with 40–50% experiencing at least one additional episode of infection (Hurley and De Louvois, 1979; Sobel, 2007). A small percentage of women (5–8%) suffer from at least four recurrent VVC per year (Foxman et al., 1998), and *C. glabrata* and other non-*C. albicans* forms are isolated in 10 to 20% of these cases (Mitchell, 2004; Peters et al., 2014). However, it is difficult to evaluate the exact incidence of VVC because of the high rate of self-treatment with over-the-counter medications. Moreover, the diagnosis is frequently based entirely on signs and symptoms without any tests to confirm the diagnosis (Achkar and Fries, 2010).

#### **1.2.4 VVC: clinical presentation, diagnosis and treatment**

The most common symptoms of VVC infection are burning pain and pruritus of the vulva with discomfort that can lead to dysuria and dyspareunia in more severe cases (Anderson et al., 2004). The vaginal pH in women with VVC is usually lower than 4.5 (Achkar and Fries, 2010). Clinical signs are edema and erythema of vulva and vagina associated with an abnormal vaginal discharge that may appear watery, cheese-like, or minimal (Anderson et al., 2004). The diagnosis is most frequently made clinically and microscopic examination of the discharge is also helpful. Indeed, mycelia can be seen under microscopy in 50 to 80% of cases. The whiff test is used to distinguish between VVC and bacterial vaginosis, and is performed by adding 10% potassium hydroxide to the vaginal discharge. In bacterial vaginosis, an amine-like odor is released following this reaction. Conversely, the test is negative in cases of VVC (Berek, 2012).



Antifungal treatment is essential to relieve symptoms and to avoid reinfection. Both oral and topical antifungal preparations are available, with fewer side effects for the topical preparations (Sobel, 2014). Treatment of VVC depends on whether the patient has uncomplicated or complicated VVC (Pappas et al., 2009). In cases of uncomplicated VVC, the recommended therapy is a single oral dose of fluconazole 150 mg or, alternatively, a topical azole such as clotrimazole, terconazole or miconazole for 7 days. In the cases of complicated VVC instead, short-term antifungal therapy would not be effective and therefore long-term treatment are required (Sobel, 1997). In recurrent candidiasis, when the infection is caused by *C. albicans*, a regimen with antifungal drug is recommended for 14 days, followed by a 6-month of maintenance therapy with fluconazole (150 mg once a week), itraconazole (10 to 50 mg a day) or others. Alternatively, 500 mg vaginal ovules containing, for example, clotrimazole can be administered topically once a week.

Treatment in pregnant women is indicated to alleviate the uncomfortable symptoms. A topical antifungal (clotrimazole or miconazole) is suggested for 7 days; on the contrary, oral treatment is not recommended in the first trimester of pregnancy since some cases of abnormalities of the face, bones, skull and heart have been reported following exposure of the baby to high doses (Sobel 2014).

### **1.2.5 Lactobacilli and VVC**

Several are the studies highlighting that the presence of lactobacilli in the vaginal ecosystem of healthy women represent an important barrier for vulva-vaginal infections caused by *Candida* spp.

In vitro studies have demonstrated that *L. rhamnosus*, *L. casei*, and *L. acidophilus* significantly reduced levels of *Candida albicans* biofilms at the initial colonization phase and the later maturation phase of biofilm development, showing an activity strain-specific against *Candida* (Matsubara et al., 2016). Through exclusion, competition and displacement experiments the interference exerted by lactobacilli toward the *C. albicans* adhesion to HeLa cells has been investigated. Most *Lactobacillus* strains significantly reduced yeast adhesion through several mechanisms. In particular, *L. crispatus*

BC2, *L. gasseri* BC10 and *L. gasseri* BC11 appeared to be the most active strains, indicating that anti-candida activity was a strain specific feature (Parolin et al., 2015).

The lactobacilli may be able to inhibit *Candida* growth by competing for nutrients and producing lactic acid and other organic acids that lower pH. This creates an unsuitable environment, and can lead to up-regulation of stress-related genes in *Candida* (Kohler et al., 2012).

Multiple studies have demonstrated that probiotics may be beneficial to patients with acute VVC treated with standard antifungals by improving vaginal symptoms. Comparing women with acute VVC receiving only antifungal therapy to women with acute VVC receiving antifungal therapy and vaginal probiotic containing *L. acidophilus*, *L. rhamnosus*, and *L. delbrueckii* subsp. *bulgaricus* it has been found that the probiotic group had greater improvement in clinical complaints (Kovachev and VatchevaDobrevska, 2015).

Moreover, a significant increase in resolution of symptoms, including vaginal burning and itching in women with acute VVC by using *L. plantarum* after antifungal therapy compared to women using antifungal therapy alone has been reported (De Seta et al., 2014). Similarly, women with acute VVC using a probiotic containing *L. rhamnosus* and *L. reuteri* after antifungal therapy showed significantly less vaginal discharge compared to women receiving antifungals alone (Martinez et al., 2009). In addition, it has been demonstrated that probiotics are helpful in decreasing recurrence of VVC infection. Indeed, women receiving vaginal *L. plantarum* showed a threefold-reduced risk of recurrence of VVC three months after single-dose clotrimazole therapy, compared to women receiving clotrimazole alone (Palacios et al., 2016).

### 1.3 *Chlamydia* infections

*Chlamydia* spp. are important causes of human disease for which no effective vaccine exists. Chlamydiae are Gram-negative bacteria, aerobic, obligate, intracellular pathogens of several organisms, including humans. Because they are unable to synthesize their ATP, they have to use their host cell's energy resources. The genus *Chlamydia* involves in total nine species: *C. trachomatis*, *C. muridarum*, *C. pneumoniae*, *C. pecorum*, *C. suis*, *C. abortus*, *C. felis*, *C. caviae*, and *C. psittaci*. These species exhibit major differences in host range, tissue tropism, and disease pathology (Stephens et al., 2009). Certainly, among these, the most important species is *C. trachomatis*, which infects exclusively the human being, causing ocular and genital infections. Based on the antigenic properties of the major outer membrane protein MOMP, *C. trachomatis* has been classified into different serovars (Stephens et al., 1987; Baehr et al., 1988; Gomes et al., 2007).

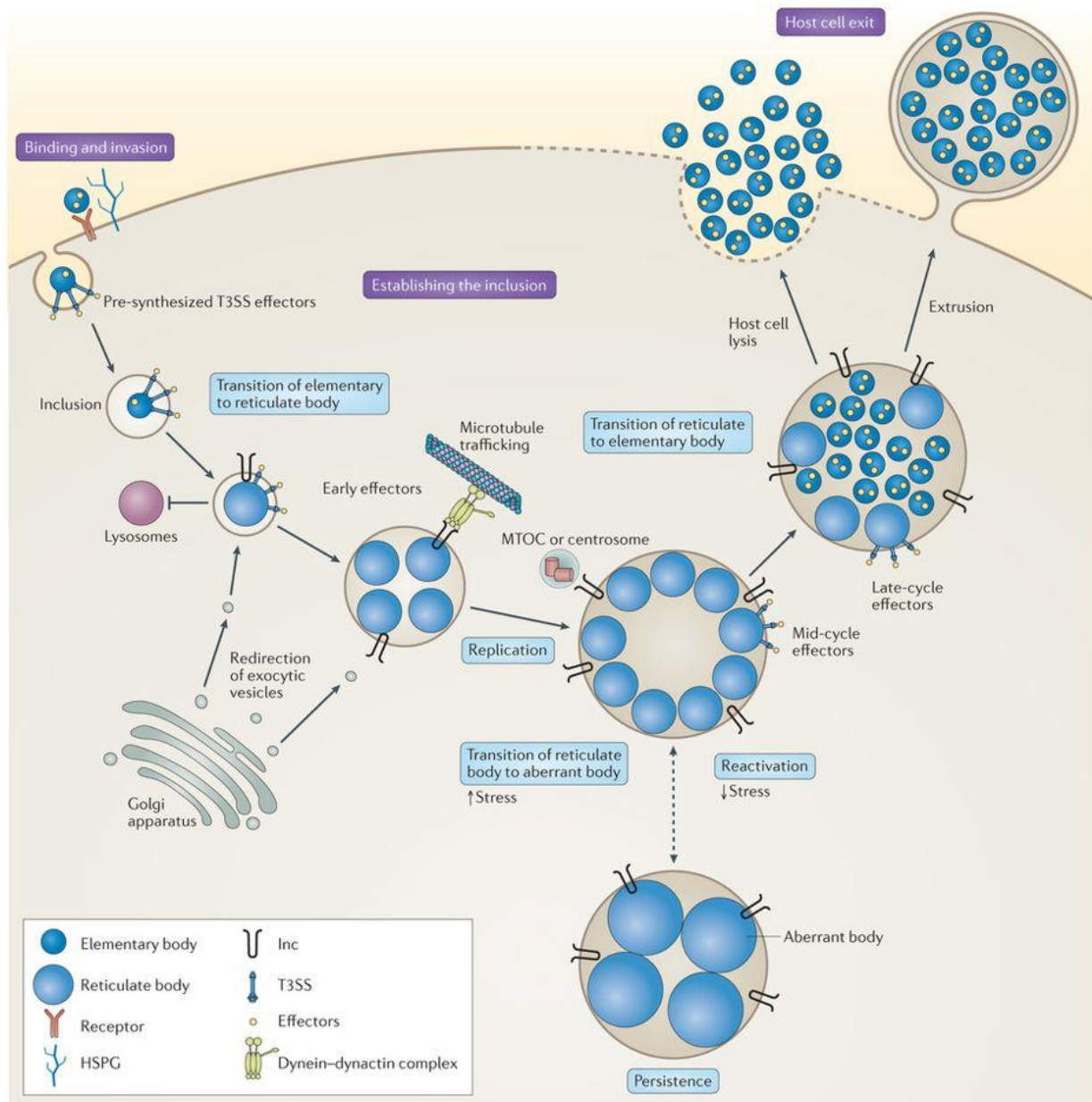
Serovars A-C are the etiologic agents of trachoma, which is the leading cause of infectious blindness worldwide (Stocks et al., 2014). With an estimate of 131 million new cases per year, *C. trachomatis* serovars D-K are the main bacterial cause of sexually-transmitted infections (STI). Remarkably, up to 70–80% of genital tract infections with *C. trachomatis* are asymptomatic and about 15- 40% can ascend to the upper genital tract and lead to serious complications such as pelvic inflammatory disease, ectopic pregnancy, and infertility in women (Malhotra et al., 2013). Furthermore, *C. trachomatis* serovars D-K can cause inclusion conjunctivitis in adults, and perinatal infections such as ophthalmia neonatorum and chlamydial pneumonia in infants (Rönnerstam et al., 1985; Schachter et al., 1986; Stenberg and Mardh, 1991; Darville, 2005; Hammerschlag, 2011). Finally, the more invasive serovars L1-L3 are the cause of a less frequent form of STI, the lymphogranuloma venereum (LGV), which is a systemic illness characterized by inguinal lymphadenopathy and/or severe proctitis/proctocolitis (Herring and Richens, 2006; White, 2009). *C. trachomatis* infection also facilitates the transmission of HIV and is associated with cervical cancer (Malhotra et al., 2013).

### 1.3.1 The developmental cycle

*Chlamydiae* displays an exclusive biphasic developmental cycle, characterized by the alternation between extracellular, infectious elementary bodies (EBs), and intracellular, non-infectious reticulate bodies (RBs). EBs enter mucosal cells and differentiate into RBs in a membrane bound compartment, called inclusion. After several cycles of replication by binary fission, RBs differentiate back to EBs, which are finally released from the host by cell lysis and/ or extrusion, and ready to infect neighbouring cells (Bastidas et al., 2013). EBs and RBs show distinct morphologic and functional features. Elementary bodies have a spore-like cell wall that is stabilized by a set of proteins cross-linked by disulphide bonds. These proteins form the outer membrane complex, which confers resistance to osmotic and physical stress (Nelson, 2012). EBs can survive in the extracellular environment, and once were considered metabolically inactive. However, recent studies indicated that EBs have high metabolic and biosynthetic activity depending on D-glucose-6-phosphate as a source of energy (Omsland et al., 2014). Indeed, EBs contain a plenty of proteins that are required for central metabolism and glucose catabolism (Saka et al., 2011), which might be used for the huge energy required for entry in the host cell and drive differentiation into RBs. During this transition, the reduction of cross-linked complexes provides the membrane fluidity that is required for replication (Nelson, 2012). RBs are able to acquire nutrient and actively replicate (Bastidas et al., 2013); specifically, they express proteins that are involved in the generation of ATP, protein synthesis and nutrient transport, such as V-type ATP synthases, ribosomal proteins and nucleotide transporters (Saka et al., 2011).

*Chlamydia trachomatis* developmental cycle (**Figure 1.6**) requires a finely regulated expression of stage-specific factors. The cycle (reviewed by Elwell et al., 2016) begins with the binding of the EBs to the host cell which involves several bacterial ligands and host receptors. On the contact, pre-synthesized T3SS (type III secretion system) effectors are injected into the host cell and some of them initiate cytoskeletal rearrangements to facilitate internalization and initiate mitogenic signalling to

establish an anti-apoptotic state. Thus, EBs are internalized into the inclusion. After 6-8 hours post-infection, when the transition to reticulate body occurs, early genes are transcribed. Early effectors, the newly secreted inclusion membrane proteins (Incs), remodel the inclusion membrane, redirect to the inclusion the exocytic vesicles that are in transit from the Golgi. The nascent inclusion is transported, probably by an Inc protein, along microtubules to the microtubule-organizing centre (MTOC) or centrosome. Next, about 8–16 hours post-infection, mid-cycle genes are expressed, which comprise effectors that mediate nutrient acquisition and maintain the viability of the host cell. The bacteria divide by binary fission, replicating exponentially, and the inclusion considerably expands. At late stages, 24–72 hours post-infection, RBs differentiate into EBs in an asynchronous manner, which might be stimulated by their detachment from the inclusion membrane. Late-cycle genes encode the outer membrane complex and the DNA binding histone H1-like and H2-like proteins, Hc1 and Hc2, which condense DNA and switch off the transcription of many genes. Some late-cycle effectors are then packaged in the newly formed elementary bodies to be discharged in the next cycle of infection. Elementary bodies exit the host through lysis or extrusion (reviewed by Elwell et al., 2016). However, under certain condition, such as nutrient deprivation, exposure to penicillin, IFN  $\gamma$ , and other stressing conditions, *Chlamydia* enters into a persistent state (Byron 2012), during which the microorganism slows down the DNA replication, continuing genes transcription, but stops to divide. This persistent state is usually associated with the presence of enlarged, aberrant RBs. Nevertheless, *Chlamydia* is able to revert into infectious EBs once the stress condition is removed (Ouellette et al., 2006; Muramatsu et al., 2016).



**Figure 1.6: The life cycle of *Chlamydia trachomatis* (Elwell et al., 2016).**

### 1.3.2 Chlamydial strategies for host cell invasion

Chlamydiae can invade most cultured cells, suggesting that the receptors that aid host invasion are ubiquitous or that multiple receptors can be used. Binding is supposed to be a two-step process for some species, involving a first reversible interaction between the EB and the host cell mediated by heparan sulfate proteoglycans (HSPGs), followed by high-affinity irreversible binding to a secondary receptor (Dautry-Varsat et al. 2005). In addition to heparan sulfate (Chen et al. 1996; Wuppermann

et al. 2001), other receptors have been proposed to mediate *Chlamydia* entry into the host cell. These include the mannose receptor, the mannose 6-phosphate receptor, and the estrogen receptor (Cocchiaro and Valdivia 2009). Cell surface-exposed protein disulfide isomerase (PDI) has also been shown to play an important role in EB attachment and entry (Abromaitis and Stephens 2009). The diversity in binding and internalization mechanisms between species probably contributes to differences in tropism for specific hosts and tissues.

On top of that, numerous bacterial adhesin and ligands have been proposed to promote the invasion and their use may vary depending on both the host-cell type and the chlamydial species (Cocchiaro and Valdivia 2009). Adhesins include the lipopolysaccharide (LPS) in *C. trachomatis*, which is proposed to bind to the cystic fibrosis transmembrane conductance regulator (CFTR) (Hegemann, 2012; Ajonuma et al., 2010). Others ligands are the major outer membrane protein (MOMP; also known as CT681), which binds to the mannose receptor and the mannose 6-phosphate receptor18, and CT017 (also known as Ctad1) in *C. trachomatis*, which binds to  $\beta$ 1 integrin (Stallmann et al., 2015). Also the polymorphic membrane protein (Pmp) family in *C. trachomatis* and *C. pneumoniae* mediates adhesion (Becker and Hegemann, 2014). Indeed, it has been shown that Pmp21 (also known as Cpn0963) protein of *C. pneumoniae* binds to the epidermal growth factor receptor (EGFR) and functions as both an adhesin and an invasin (Möllerken et al., 2013).

*C. trachomatis* also binds to ephrin receptor A2 (EPHA2) to activate downstream signalling (Subbarayal et al., 2015), whereas apolipoprotein E4 may act as a receptor for *C. pneumoniae* enhancing the attachment of EBs to host cells (Gerard et al., 2008). Finally, PDI, a component of the oestrogen receptor complex, is implicated in the attachment and entry of many *Chlamydia* spp. (Davis et al., 2002; Mehrlitz and Rudel, 2013).

All these indicates that multiple redundant strategies likely exist to ensure chlamydial entry, and the path is dependent on the *Chlamydia* species or features of the host cell type being invaded (Cocchiaro and Valdivia 2009).

### 1.3.3 Immunopathogenesis

*C. trachomatis* is a strong immunogen, which stimulates both humoral and cell mediated immune responses. In addition to the immunogenic antigens, the outcome of chlamydial infection depends on interaction and balance of cytokines secreted by the activated lymphocytes. Interferon gamma (IFN- $\gamma$ ) has been described as a single most important factor in host defense against *Chlamydia*, while disease susceptibility has been linked to enhanced expression of Interleukin- 10 (IL-10)<sup>17</sup>. Immune system changes or disturbances induced by *C. trachomatis* may favour its own survival in the infected host, and induce persistent infections (Malhotra et al., 2013).

*C. trachomatis* infection can be distinguished in primary infection or chronic recurrence/re-infection.

**Primary infection.** As mentioned above, the first defensive line against *Chlamydia* infections is the mucosal barrier of the female genital tract. During primary infection the mucosal cells secrete numerous pro-inflammatory chemokines and cytokines, including IL-1, IL-6, IL-8, GM-CSF and TNF- $\alpha$  (Malinverni 1996; Morton 1999). The release of these molecules induces vasodilatation, increased endothelial permeability, activation and influx of neutrophils, monocytes and T-lymphocytes, and elevated expression of adhesion molecules. The initial amplification of *C. trachomatis* seems to be counteracted by neutrophils, which possibly limit the spread within the female genital tract (Rasmussen et al., 1997). T helper cells (Th1) also play an important role during early phase of infection. Indeed, upon *Chlamydia* antigen-induced activation, T helper secrete IFN- $\gamma$ , necessary for the infection regression. IFN- $\gamma$ , in turn, increases the potential of various phagocytes to destroy *Chlamydia* and stimulates the secretion of other cytokines, including IL-1, which by stimulating the secretion of IL-2 by Th1 cells, causes increased replication of cytotoxic lymphocytes and natural killer cells (Witkin et al., 2000). Thus, the primary infection leads to a local inflammatory reaction caused by penetration and reproduction of the bacteria in the epithelial cells and to IgA secretory antibody production. In most cases the host's reaction to the primary infection is transient and does not cause tissue damage (Zdrodowska-Stefanow et al., 2003). The host's immune response



is crucial for the resolution of the infection; however, as mentioned above, *Chlamydia* has found the way to neutralize the immune system, remaining in a state of latency and causing persistent infections.

**Chronic infection, recurrence/re-infection.** Chronic infection, associated with persistence of *Chlamydia* in the host cells, and recurrent infection or reinfection are more dangerous. Chronic infection is characterized by the maintenance of a microorganism in the host cell. The inflammation is induced in less time and with increased intensity, and a rapid immune response by previously sensitized lymphocytes occurs (Choroszy-Król et al., 2012). A delayed hypersensitivity reaction or rarely type 3 hypersensitivity reactions (Arthus reaction) is observed in long term or recurrent stimulatory action of chlamydial antigens (Paavonen, 1996). Processes that occur during these reactions cause tissue damage, fibrosis and cicatrization within the affected organs. Irreversible consequences like pelvic inflammatory disease (PID) leading to infertility, ectopic pregnancy, chronic pelvic pains and chronic urethritis may occur. Lack of treatment or improper therapeutic strategies can result in chronic infection. Furthermore, dietary factors like insufficient supply of tryptophan, L-isoleucine, and cysteine in diet, as well as certain cytokines, like  $\text{INF-}\gamma$ ,  $\text{TNF-}\alpha$ , may affect the outcome of the infection (Zdrodowska-Stefanow et al., 2003).

### 1.3.4 Epidemiology

*C. trachomatis* is the leading cause of bacterial sexually transmitted infection (STI) in the world. Moreover, in endemic areas, mostly in Africa and the Middle East, *C. trachomatis* also causes trachoma, a leading cause of preventable blindness worldwide. Furthermore, *C. trachomatis*, as well as other genital infections, can promote the sexual transmission of viruses such as HIV (Fleming and Wasserheit, 1999). The World Health Organization estimated that in 2016 there were 376 million new infections (more than 1 million per day) of the four curable STIs – chlamydia, gonorrhoea, syphilis and trichomoniasis. Among these, 127 million of new cases were caused by *Chlamydia*. STI prevention and control has increased spread public health benefits. Left untreated, some STIs increase

the risk of HIV transmission during unprotected sexual contact and lead to complications, such as PID, infertility, ectopic pregnancy, miscarriage, fetal death and congenital infections (Taylor et al., 2018).

Most of the men and woman infected with *C. trachomatis* are often asymptomatic, therefore a significant proportion of the cases remain undiagnosed and can develop complications of infection. Rates of reported cases of chlamydia are highest among adolescents and young adults aged 15-24 years. In 2014, the rate among 15-19 year olds was 1804 cases per 100000 and the rate among 20-24 year old was 2484,6 cases per 100000 (Workowski et al., 2015).

*C. trachomatis* is transmitted via infected secretions and mucous membranes of urethra, cervix, rectum, conjunctivae and throat. In addition, a neonate can be infected by the infected mother during vaginal delivery (Manavi, 2006). Additional predictors of incidence chlamydial infection in young women include single marital status, having a new sex partner or concurrent partnerships, smoking and associated indicators of socioeconomic status, having gonorrhea or bacterial vaginosis, and presence of carcinogenic human papillomavirus (Crichton et al., 2015; Aghaizu et al., 2014; Batteiger et al., 2010; Hwang et al., 2014; Jorgensen et al., 2015).

### **1.3.5 Clinical symptoms of *Chlamydia trachomatis* infections**

Most persons who are infected with *C. trachomatis* are asymptomatic. However, when symptoms of infection are present, in women they most commonly include abnormal vaginal discharge, vaginal bleeding, and dysuria (Stamm et al., 1990).

Chlamydial infection in women can cause urethritis, cervicitis and salpingitis. Other clinical signs of *C. trachomatis* infection are mucopurulent endocervical discharge, easily induced endocervical bleeding, or edematous ectopy (Marrazzo et al., 2002). Untreated, infection may persist for up to 4 years (Molano et al., 2005) although spontaneous clearance of infection after diagnosis has been described (Geisler et al., 2013), suggesting development of some degree of protective immunity.

Untreated chlamydial infection leads to PID in 20–40% of infected women (Paavonen et al., 1999). PID is the result of post-infectious inflammation of female upper genital tract that includes salpingitis, endometritis and inflammation of fallopian tubes (Manavi, 2006). When untreated, PID can eventually lead to infertility, ectopic pregnancy and/or chronic pelvic pain. Chlamydial PID can manifest as pelvic or lower abdominal pain associated to cervical motion tenderness or uterine or adnexal tenderness at the physical exam (Workowski et al., 2015) but even upper genital tract infection may be asymptomatic (Wiesenfeld et al., 2012).

*C. trachomatis* genital tract infection can also negatively impact pregnancy. A previous chlamydial infection is associated to a high risk of ectopic pregnancy (Bakken et al., 2007). *C. trachomatis* infection has been associated with spontaneous abortion, stillbirth and preterm delivery (Liu et al., 2013; Hollegaard et al., 2007; Andrews et al., 2000). *C. trachomatis* can also be transmitted to a neonate during delivery through contact with infected cervix tissue and secretions. This causes infection of mucous membranes of the eye, oropharynx, urogenital tract, and rectum. The most common presentation is *C. trachomatis* conjunctivitis that develops 5-12 days after birth (Rours et al., 2008), but *C. trachomatis* also can cause a subacute, afebrile pneumonia with onset at ages 1-3 months (Rours et al., 2009). Furthermore, *C. trachomatis* has also been proposed as a possible risk factor for cervical cancer: a cofactor based on detection of chlamydial DNA in HPV-associated lesions (Paba et al., 2008), and the presence of anti-CT antibodies correlated with risk for squamous cell carcinoma (SCC) or invasive cervical cancer (ICC) (Paavonen et al., 2003). In addition, primary fallopian tube carcinomas have been described in patients with chronic PID (Zardawi 2014), and infertility is a known risk factor for epithelial ovarian cancer (reviewed by Salvador et al., 2009).

Infection with L1, L2 and L3 serovars of *C. trachomatis* cause Lymphogranuloma venereum (LGV). LGV infections are associated with urogenital ulceration and invasion of the lymphatic system in both men and women, which can result in bubo formation, fistulae, fibrosis and rectal stenosis (de Vrieze and de Vries, 2014). These infections are usually symptomatic and symptoms include

anorectal pain, discharge, tenesmus, rectal bleeding and constipation, often accompanied by fever (de Vries et al., 2013).

### **1.3.6 Treatment and prevention**

Uncomplicated genitourinary chlamydial infection in non-pregnant adolescents and adults should be treated with azithromycin 1g in a single dose or doxycycline 100 mg twice daily for seven days. Studies indicate that both treatments are equally effective (Workowski et al., 2015)

For pelvic inflammatory disease treatment, ofloxacin 400 mg twice daily for 2-week course and metronidazole 400 mg twice a day are recommended. Alternatively, doxycycline 100 mg twice a day can substitute for ofloxacin. Ceftriaxone should be added to the above regimen in case of gonococcal PID. Doxycycline and ofloxacin (Floxin) are contraindicated during pregnancy; therefore, the CDC recommends erythromycin base or amoxicillin for the treatment of chlamydial infection in pregnant women (Workowski et al., 2015).

Doxycycline (100 mg twice daily for 21 days) is the preferred treatment for LGV. An alternative treatment regimen includes erythromycin (500 mg four times daily for 21 days); azithromycin (1 g once weekly for three weeks) may also be used (CDC, 2006).

Since the majority of CT infections are asymptomatic, detection of infection often depend on screening. The 2010 CDC Sexually Transmitted Diseases (STD) Treatment Guidelines recommends annual CT screening in all sexually active women 25 years of age or younger, as well older women with risk factors. Benefits of CT screening in women have been showed in areas where screening programs have reduced rates of PID, and recent works suggest a continued decline in PID rates in the United States (reviewed by William, 2015). The method for the diagnosis and management of uncomplicated CT infection in adolescents and adults includes:

(1) use of nucleic acid amplification tests (NAATs) to assess CT infection;

- (2) treatment with CDC (Centers for Disease Control and Prevention)-recommended therapy to reduce complications and prevent transmission to others;
- (3) treatment of sexual partners to prevent reinfection and complications in both patients and partners;
- (4) repeat CT testing a few months following treatment to identify repeat infection;
- (5) a test of cure (TOC) in pregnant women at a minimum of 3 weeks following treatment to identify persisting or repeat infection so that repeat treatment can be provided punctually to reduce risk for maternal and neonatal morbidity (reviewed by William, 2015).

Since *C. trachomatis* is sexually transmitted, barrier methods of contraception, including condom use, are effective at preventing chlamydial transmission, however utilization rates are low (Bearinger et al., 2007).

Despite numerous attempts to develop a protective vaccine against *C. trachomatis* infections, effective vaccines are not yet available. Because MOMP is a highly abundant surface antigen, it has long been considered a promising candidate. Recent studies have shown that novel formulations delivering MOMP proteins through cationic liposomes, induced antibody, type-1 immunity and partial protection from infection in minipigs (Lorenzen et al., 2015) and significant protection against upper tract disease in mice (Olsen et al., 2015; Boje et al., 2016). In a pre-clinical study, intranasal immunization using MOMP in combination with Nanostat™, oil-in-water nanoemulsion in mice was performed and mice were then subsequently challenged intra-vaginally with chlamydia. In this study, 100 percent of mice receiving no treatment developed oviduct pathology (indicator of PID) versus just 20 percent of mice treated ( $p < 0.001$ ). A polyvalent vaccine, composed of MOMP with PMPs formulated with DDA/MPL adjuvants, reduces chlamydial shedding when tested in a transcervical *C. trachomatis* mouse model (Stary et al., 2015).

Because vaccines are the best form of prevention and protection against infections, including those from Chlamydia, further studies are needed in order to identify protective antigens.

### 1.3.7 Lactobacilli and *C. trachomatis* infections

The innate defence system of the female mucosal genital tract involves a complex interaction among the healthy vaginal flora, immune cells, and several proteins that defend the host from pathogens.

*Lactobacillus* spp. are the main host defence factor against pathogens, like *C. trachomatis*, within the cervico-vaginal ecosystem. As already broadly described, they are able to limit the growth of genital pathogens by means of different mechanisms, such as competitive exclusion, anti-microbial compound production (lactic acid, hydrogen peroxide, defensins, etc.), the immune system activation as well as the maintenance of a low vaginal pH (Mijac et al., 2005; Vielfort et al., 2008; Petrova et al., 2015). Women with lactobacilli poor microbiota show an increased susceptibility to sexually transmitted pathogens. Several studies indicate that abnormal vaginal flora lacking lactobacilli is associated with the acquisition of infections by *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* (reviewed by Nardis et al., 2013).

Gong et al. demonstrated that lactic acid and, hence, a low pH, were essential for the anti-chlamydial activity of predominant *Lactobacillus* species in the cervico-vaginal microbiota (Gong et al., 2014). Apart from this, studies reported the ability of different vaginal *Lactobacillus* strains such as *Lactobacillus brevis* or *Lactobacillus crispatus* to strongly inhibit early phases of *C. trachomatis* infection as well as its intracellular replication. In particular, *L. brevis* and *L. salivarius*, showed an adverse effect on chlamydial EBs, on chlamydial adsorption to epithelial cells, and on intracellular phases of chlamydial replication. Furthermore, *L. brevis* was significantly more effective than *L. salivarius*. Interestingly, *L. brevis* was able to inhibit the development of persistent forms of *C. trachomatis* induced by coinfection with herpes simplex virus type 2 (HSV-2) (Mastromarino et al., 2014). Several potential mechanisms interfering with *C. trachomatis* adhesion to host cell have been described.

Rizzo et al., explored the ability of *L. crispatus* to affect *C. trachomatis* infectivity. Importantly, they showed that *L. crispatus* and its supernatant inhibited the adhesion of *C. trachomatis* cells to human epithelial cells or macrophages, and inhibited *C. trachomatis* infectivity. In addition, *L. crispatus* had

no cytotoxic effect on the epithelial cells or macrophages, reduced the production of the pro-inflammatory cytokines, IL-6, IL-8, and TNF- $\alpha$ , and increased the production of the anti-inflammatory cytokine, IL-10. *L. crispatus* commonly resides in the urogenital microbiota of healthy women and these results suggest that increasing the presence of such microbes can play an important role in protecting the genitourinary tract against pathological conditions (Rizzo et al., 2015).

Nardini et al. performed a study, that included eight strains of *L. crispatus*, six strains of *L. gasseri*, three strains of *Lactobacillus vaginalis*, and lactic acid as a lactobacilli cellular metabolite, on the infectivity of *C. trachomatis*. All lactobacilli exerted a strong inhibitory effect, although, *L. crispatus* showed the highest effectiveness. Larger anti-chlamydial activity was correlated to increased cellular metabolites resulting in a lower pH, and the acidic conditions produced by lactic acid production were shown to be necessary for chlamydial inhibition. However, lactobacilli supernatants exhibited greater inhibition than only lactic acid, suggesting synergism with other lactobacilli metabolites (Nardini et al., 2016).

Although all these studies demonstrated the protective role of lactobacilli against Chlamydia, further efforts are needed to deeply understand the protective mechanisms exerted by lactobacilli and their great contribution to the health of the vaginal ecosystem.

## **CHAPTER 2**

### **AIMS OF THE RESEARCH**



A normal vaginal microbiota, dominated by lactobacilli, is crucial for the prevention of several urogenital and sexually transmitted infections, including *Candida* and *Chlamydia* (Gupta et al., 1998; Spurbeck and Arvidson, 2008; Parolin et al., 2015; Nardini et al., 2016; Foschi et al., 2017; Ñahui Palomino et al., 2017). This aspect is strengthened by the demonstration that in case of bacterial vaginosis, a clinical condition characterized by the depletion of lactobacilli, a higher risk of STI transmission and acquisition is reported (Taha et al., 1998; Martin et al., 1999; Wiesenfeld et al., 2003; Abbai et al., 2015).

*Candida albicans*, is the largely prevalent etiological agent of vulvovaginal candidiasis (VVC), which is a common infection among women associated with considerable morbidity and healthcare cost. Although the pathogenesis of VVC remains a controversial issue, it seems that when the balance between the microorganisms existing in the vaginal microbiota is disrupted, the overgrowth of *Candida* is facilitated. (Matthew E. Falagas et al., 2016).

*C. trachomatis* is the leading cause of bacterial sexually transmitted diseases with 127 million new cases per year, according to the most recent World Health Organization estimates. *Lactobacillus* spp. are the main host defense factor against pathogens like *C. trachomatis* within the cervico-vaginal ecosystem; in fact several studies reported the ability of different vaginal *Lactobacillus* strains, such as *Lactobacillus brevis* or *Lactobacillus crispatus*, to strongly inhibit early phases of *C. trachomatis* infection as well as its intracellular replication (Marisa Di Pietro et al., 2019).

The protective role of lactobacilli against urogenital pathogens is exerted through different mechanisms including the production of various antibacterial compounds (lactic acid, hydrogen peroxide, bacteriocins and biosurfactants), the competitive exclusion for epithelial adhesion, and the immunomodulation (Kaewsrichan et al., 2006; Borges et al., 2014; Parolin et al., 2015; Younes et al., 2018). *Lactobacillus* spp. interaction with the vaginal epithelial cells is the first step in the formation of the biological barrier against colonization of opportunistic and pathogenic organisms. The blockage of undesirable microorganisms adherence by lactobacilli may take place by exclusion,

competition, and displacement mechanisms (Coman *et al.*, 2015; Osset *et al.*, 2001; Verdenelli *et al.*, 2014).

In this contest, the present thesis aims to understand the mechanisms at the basis of Lactobacilli protection against pathogens with particular attention to *C. albicans* and *C. trachomatis* infection. In the first part of this work, we investigated the protective role of Lactobacilli against *C. albicans* and we selected five strains belonging to three representative vaginal species. Specifically, we chose *L. crispatus* BC1 and BC2, *L. gasseri* BC9 and BC11, and *L. vaginalis* BC15, that showed a good profile adhesion to HeLa cells. Furthermore, *L. crispatus* strains have showed to significantly reduce the adhesion of *C. albicans* to the cervical cell line in competition experiments (Parolin *et al.*, 2015). Lactobacilli exert beneficial effects on the cervical and vaginal mucosa not only through the microbial competition but also by modulating several important functional activities of the human epithelium, such as immune response, cell proliferation and apoptosis (Abedin-Do *et al.*, 2015; Mirmonsef *et al.*, 2011; Motevaseli *et al.*, 2013; Rizzo *et al.*, 2013; Rizzo *et al.*, 2015; Rose *et al.*, 2012). In this perspective, the aim of this work is to study the manner in which the interaction with the different Lactobacilli strains could modulate plasma membrane properties of HeLa cells. In particular, we investigated the effect of Lactobacilli interaction in modulating plasma membrane permeability and fluidity by using DAPI and Red Nile staining. Moreover, we assessed the role of Lactobacilli in modulating  $\alpha 5$  integrin subunit exposure and in peroxide production.

The purpose of the second part of the present thesis was to identify vaginal Lactobacilli strains able to interfere with *C. trachomatis* infection process. Specifically, we have chosen *L. crispatus* BC4 and BC5, *L. gasseri* BC14 and *L. vaginalis* BC17 to assess their capability, in counteract *C. trachomatis* interaction with HeLa cells through exclusion assay. In order to understand the rationale of the interaction between Lactobacilli, *C. trachomatis* and HeLa cells, *L. crispatus* BC5 was chosen as a model to study the molecular mechanism underlying the activity against Chlamydia, with particular interest in the modulation of plasma membrane properties. Furthermore, since the integrin family of

receptors is a major target of bacterial pathogens that colonize human tissues or invade specific cell types (Hoffmann et al., 2011; Hauck et al., 2012), we investigated the role of  $\alpha 5$  integrin subunit in *C. trachomatis* internalization in HeLa cells. In order to study this last important aspect, we followed two different experimental strategies. First, we checked whether *C. trachomatis* was able to infect HT29, a colon cancer cell line lacking the  $\alpha 5$  integrin subunit, and if a re-expression of this subunit was able to mediate *Chlamydia* entry into the same cell line. Subsequently, we verified whether after silencing the integrin  $\alpha 5$  subunit, *C. trachomatis* maintained its ability to enter and infect HeLa cells. Finally, all the experiments of the thesis aimed to gain a deep understanding of the close relationship between vaginal microbiota, host, and HeLa cells used as model of vaginal epithelium, in the prospect of employing lactobacilli as natural therapeutic agents to promote women's health.

## **CHAPTER 3**

### **MATERIAL AND METHODS**

### 3.1 Cell culture

HeLa, a human cervical carcinoma cell line, and HT29, a colon cancer cell line, were used in these experiments. Cells were seeded in plates (*Orange Scientific*) at a density of  $2 \times 10^5$  cells/cm<sup>2</sup> and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. The composition of the complete medium is reported in **Table 3.1**.

**Table 3.1:** Composition of cell complete medium.

RPMI 1640 ( <i>Lonza</i> )	89%
Fetal bovine serum (FBS) ( <i>EuroClone</i> )	10%
L-glutamine 200 mM ( <i>Sigma-Aldrich</i> ) in phosphate buffered saline (PBS)	1%

Cells were washed two times in Phosphate Buffer Solution (PBS) and subsequently, trypsin 0.115% (*Sigma-Aldrich*) in a solution of PBS-ethylenediaminetetraacetic acid (EDTA) (*Sigma-Aldrich*) 0.02% was added to split cells, incubated with cells for 5 minutes at 37°C and neutralized with complete medium. Cells were finally counted using a Burkert chamber. The composition of PBS is given in **Table 3.2**.

**Table 3.2:** Composition of phosphate buffered saline.

NaCl ( <i>Sigma-Aldrich</i> )	8 g/L
Na <sub>2</sub> HPO <sub>4</sub> ( <i>Sigma-Aldrich</i> )	1.15 g/L
KCl ( <i>Sigma-Aldrich</i> )	0.2 g/L
KH <sub>2</sub> PO <sub>4</sub> ( <i>Sigma-Aldrich</i> )	0.2 g/L

### 3.2 Cell treatments with *Candida albicans*

*Candida albicans* used in the present study, were kindly provided by Professor Marangoni (Microbiology Laboratory of Sant'Orsola-Malpighi University Hospital of Bologna).

*Lactobacillus crispatus* BC1 and BC2, *Lactobacillus gasseri* BC9 and BC11 and *Lactobacillus vaginalis* BC15 were previously isolated from vaginal swabs of healthy premenopausal women (Parolin *et al.*, 2015).

These strains were selected on the basis of their adhesive properties toward HeLa cells (1–11 lactobacilli/HeLa cell) (Parolin *et al.*, 2015). Lactobacilli were cultured in de Man, Rogosa and Sharpe (MRS) broth supplemented with 0.05% L-cysteine. Incubation was carried out in anaerobic jars supplemented with GazPack EZ for 18 hours at 37 °C, afterwards cultures were centrifuged at  $5,000 \times g$  for 10 min at 4 °C and cell pellets were washed in sterile saline.

HeLa cells were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> in plastic wells or on sterile glass coverslips for 48 h, treated with lactobacilli at a ratio of 1:100 (HeLa cell: *Lactobacillus*), for 1 h, at 37 °C and 5% CO<sub>2</sub> atmosphere. Afterwards, HeLa-bacteria co-cultures were washed 3 times with PBS to remove unbound bacteria. When indicated, HeLa cells were simultaneously treated with *C. albicans*, using the same ratio.

### 3.2.1 DAPI and Nile Red staining

Stock solutions of Nile Red (NR, 9-diethylamino-5H-benzo-[alpha]-phenoxazine-5-one; *Sigma-Aldrich*) and DAPI (4',6-diamidino-2-phenylindole; *Thermo Fisher Scientific*) were prepared in Dimethyl sulfoxide (DMSO) at the concentration of 1mM and 0.1mg/mL, respectively, and stored protected from light. HeLa cells were grown on glass coverslips and treated with lactobacilli, as described above. After three washes with PBS, NR staining was performed with a final dye concentration of 5μM for 5 min, at 37 °C; DAPI was used at the concentration of 5μg/mL for 15 min, at 37 °C. Cells were then fixed with 3% paraformaldehyde for 15 min, and repeatedly washed with 0.1M glycine/PBS and 1% BSA/PBS. Specimens were embedded in Mowiol and analyzed using a Nikon Coolscope II equipped with a Eclipse 90i microscope. A 20X objective was used.

### 3.2.2 Fluorescence anisotropy measurements

The plasma membrane fluidity of HeLa cells was estimated by means of the fluorescence anisotropy of the hydrophobic probe PA-DPH (1,6-diphenyl-1,3,5-hexatriene-4'-propionic acid; *Thermo Fisher Scientific*). HeLa cells were treated with lactobacilli, then washed 3 times with PBS and resuspended at a final concentration of  $3 \times 10^5$  cells/mL. The absorbance of the cell suspension was kept lower than 0.15 at the excitation wavelength of PA-DPH. A few microliters of PA-DPH stock solution were added to the cell suspension in order to obtain a final probe concentration of 1μM. Fluorescence anisotropy measurements were performed by using a PTI QuantaMaster fluorometer (*Photon Technology International*) equipped with a temperature-controlled cell holder and Polaroid HNP'B polarizers. Temperature was kept at 25 °C. Excitation and emission wavelengths were set at 360 nm and 430 nm, respectively. Fluorescence anisotropy (r) was calculated by using the equation:

$$r = (I_{VV} - I_{VH} \cdot G) / (I_{VV} + 2 \cdot I_{VH} \cdot G)$$

where  $I_{VV}$  is the fluorescence intensity measured with vertical excitation and vertical emission polarization filters and  $I_{VH}$  is the fluorescence intensity measured with vertical excitation and horizontal emission polarization filters.  $G$  is the grating factor used to correct for monochromators grating induced polarization and it was obtained as a ratio of the emission intensities using horizontally polarized excitation:  $G = I_{HV}/I_{HH}$ . (Calonghi *et al.*, 2017).

### 3.2.3 Immunocytochemical integrin staining

HeLa cells were grown on glass coverslips for 48h and then treated 1h with lactobacilli. Cells were washed 3 times with PBS and fixed in 500  $\mu$ L of paraformaldehyde 3% for 15 min in agitation. Glass slides were washed twice with 1 mL of PBS-glycine 0.1 M (*Sigma-Aldrich*), and washed twice again with 1 mL of PBS-bovine serum albumin (BSA) 1% (*Sigma-Aldrich*). Samples were first incubated with anti-human CD49e primary antibody (*BioLegend*) for 1 h in agitation at RT. Samples were washed again twice with 1 mL of PBS-bovine serum albumin (BSA) 1% and then incubated with anti-mouse Alexa 568-conjugated secondary antibody (*Thermo Fisher Scientific*) for 1 h at RT. Finally, after 2 washes with 1 mL of PBS-BSA 1%, specimens were embedded in Mowiol and analyzed by using a Nikon C1s confocal laser-scanning microscope, equipped with a Nikon PlanApo 60X, 1.4-NA oil immersion lens.

### 3.2.4 Detection of ROS generation

The Dichlorofluorescein diacetate (DCF-DA) fluorimetric assay (*Thermo Fisher Scientific*) was used to analyse the intracellular production of ROS.

HeLa cells were incubated with 5 $\mu$ M DCF-DA for 30 min at 37 °C, then washed twice with PBS and treated with lactobacilli for one hour at 37 °C. HeLa cells treated with Tert-buthyl-hydroperoxyde (TBH) 100 $\mu$ M were used as a positive control, untreated and unstained cells were used as negative



control. DCF-DA fluorescence was measured by using an EnSpire Multimode Plate Reader (PerkinElmer) at excitation and emission wavelengths of 485 nm and 535 nm, respectively.

### 3.3 Cell treatments with *Chlamydia trachomatis*

*Chlamydia trachomatis* (CT) used in the present study, were kindly provided by Professor Marangoni (Microbiology Laboratory, Sant'Orsola-Malpighi University Hospital of Bologna).

*Lactobacillus crispatus* BC4 and BC5, *Lactobacillus gasseri* BC14, and *Lactobacillus vaginalis* BC17, previously isolated from vaginal swabs of healthy premenopausal women (Parolin *et al.*, 2015, Nardini *et al.*, 2016), were used in this set of experiments. Lactobacilli strains were grown as described in paragraph 3.2.

#### 3.3.1 Adhesion assay

HeLa cells were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> on sterile glass coverslips for 48h. Cells were treated with lactobacilli CP of *L. crispatus*, BC4 and BC5, and *L. gasseri* BC14 applying a ratio of 1:100 (HeLa cell: Lactobacillus), for 1h, at 37 °C and 5% CO<sub>2</sub> atmosphere. Afterwards, HeLa-bacteria co-cultures were washed 3 times with sterile PBS and then were treated with  $5 \times 10^3$  CT EBs, for 1h, at 37 °C and 5% CO<sub>2</sub> atmosphere. Samples were washed 3 times with PBS, fixed in 3% paraformaldehyde for 10 min, and marked with a monoclonal antibody against the chlamydial membrane lipopolysaccharide antigen conjugated with fluorescein (Meridian), for 30 min at RT. Specimens were embedded in Mowiol and analyzed by using a Nikon C1s confocal laser-scanning microscope, equipped with a Nikon PlanApo 60X, 1.4-NA oil immersion lens. Chlamydia adhesion was assessed by counting the number HeLa cells positive to EBs attached in 10 random fields.

### **3.3.2 Effect of *L. crispatus* BC5 interaction on HeLa cells**

The effect of *L. crispatus* BC5 interaction on HeLa cells was evaluated by studying the lipid membrane organization, membrane fluidity and modulation of  $\alpha 5\beta 1$  integrin exposure.

The modulation of membrane lipid organization was studied using Red Nile staining as described in paragraph 3.2.1.

The effect of BC5 CP on plasma membrane fluidity of HeLa cells was estimated by means of the fluorescence anisotropy of the hydrophobic probe TriMethylAmmonium Diphenyl Hexatriene (TMA-DPH; *Thermo Fisher Scientific*) as described in paragraph 3.2.2.

The effect of *L. crispatus* BC5 CP on surface  $\alpha 5\beta 1$  integrin exposure was evaluated as described in **Paragraph 3.2.3.**

### **3.3.3 Exclusion assay with anti-CD49e antibody**

HeLa cells were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> on sterile glass coverslips for 48h. HeLa cells were first pre-exposed to IgG isotype (1:500 ) or anti-CD49e antibody (10  $\mu$ g/mL) (*BioLegend*) for 1 h and then incubated with  $5 \times 10^3$  CT EBs for 48 h at 37 °C and 5% CO<sub>2</sub>. HeLa cells infected with  $5 \times 10^3$  CT EBs were used as controls. After 48h of incubation, samples were fixed in 3% paraformaldehyde for 10 min, permeabilized in ethanol and stained with the fluorescein-conjugated chlamydial membrane lipopolysaccharide antibody (*Meridian*). Specimens were analysed by confocal microscopy as described above. Number of IFUs/ field were counted by using Image J.

### 3.3.4 Re-expression of $\alpha 5$ integrin subunit in HT29

HT29 were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> on petri dish. After 24h of adhesion, cells were treated with 5-Azacytidine (AZA) 10 $\mu$ M for 72h at 37 °C and 5% CO<sub>2</sub>. Afterwards, cells were trypsinized and counted. 5x10<sup>6</sup> cells were centrifuged for 10 min at 250 g and washed twice with 1 mL of PBS by centrifugation at 3,000 g for 3 min. For the RNA extraction and purification, the RNeasy Mini Kit (*Qiagen*) was used. Once extracted, RNA was quantified by NanoDrop spectrophotometer (*Thermo Fisher Scientific*) and employed to generate cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (*Roche*). cDNA obtained was used to analyze the levels of transcripts by quantitative Real-Time PCR (qRT-PCR). The LightCycler FastStart DNA Master SYBR Green I kit (*Roche*) and the LightCycler 2.0 Instrument (*Roche*) were employed. Primers (*Sigma-Aldrich*) used and qRT-PCR conditions are listed in **Table 3.3**. After the qRT-PCR, the presence of singles amplicons at the expected size was verified on a 1.8% agarose gel.

**Table 3.3:** List of primers and conditions for quantitative Real-Time PCR.

PRIMER	SEQUENCE	T <sub>annealing</sub>	T <sub>fluorescence</sub>	AMPLICON
B-ACTIN FW	GCCACACGCAGCTCATTGTAGA	65°C	65°C	272 bp
B-ACTIN REV	GCCCTCGTCGTCGACAACGG			
ITGA5 FW	GGCAGAAGGCAGCAATGGTG	60°C	65°C	303 bp
ITGA5 REV	AGGCATCTGAGGTGGCTGGGA			

In order to evaluate  $\alpha 5$  integrin subunit protein expression, HT29 were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> on sterile glass coverslips for 24h. Samples were treated or not with 5-azacytidine 10 $\mu$ M for 72h and subsequently fixed and stained with anti-human CD49e primary antibody (*BioLegend*) as described is **Paragraph 3.2.3**.

To evaluate *C. trachomatis* capability to infect HT29, cells treated or not with 5-azacytidine 10 $\mu$ M for 72h were subsequently incubated with  $5 \times 10^3$  CT EBs for 48 h at 37 °C and 5% CO<sub>2</sub>. After 48h of incubation, samples were fixed, permeabilized, and stained with the fluorescein-conjugated antibody against chlamydial membrane lipopolysaccharide (*Meridian*). Speciemment were analysed by confocal microscopy as described above and Chlamydia infection evaluated counting the number of IFUs/ field by using Image J.

### 3.3.5 $\alpha 5$ integrin subunit silencing

siRNA silencing was applied after 72 h of HeLa adhesion, when cells were 70% confluent. The specific siRNA against *ITGA5* gene and the scramble siRNA used in this thesis are described in **Table 3.4**.

**Table 3.4:** Custom validated siRNA used for the experiments.

Validated Silencer Select siRNA ( <i>Thermo Fisher Scientific</i> )	s7549
Silencer Select Negative Control siRNA ( <i>Thermo Fisher Scientific</i> )	#1

Both specific siRNA against ITGA5 gene (siRNA) and Negative Control siRNA (scramble) were dissolved in RNase-free water up to a 10  $\mu$ M stock. siRNA stock was first diluted 1:100 in OptiMEM (*Thermo Fisher Scientific*). The RNAiMAX (*Thermo Fisher Scientific*) solution was diluted 1:50 in OptiMEM, too. The diluted solution of siRNA was gently mixed to an equal volume of the diluted RNAiMAX, and incubated at RT for 20 minutes. Finally, the siRNA-RNAiMAX solution was diluted 1:5 in OptiMEM medium and added drop by drop to cells. siRNA treatment was applied at the final concentration of 10nM either for 48h or 72h. After treatment, total protein extraction was performed and  $\alpha$ 5 integrin subunit expression level was assessed by Western blotting, as described below.

### **3.3.6 Total protein extraction**

HeLa treated either not with siRNA or scramble 10nM as described in paragraph 3.3.5, were washed twice with PBS and incubated with 500  $\mu$ L of Radioimmunoprecipitation assay buffer lysis (RIPA) and 500  $\mu$ L of HNTG buffer at 4°C for 15 minutes with agitation. After cell lysis, the solution was centrifuged for 20 minutes at 12,000 g and the supernatant containing the proteins was quantified using the Bio-Rad protein assay (*Bio-Rad*) based on the method of Bradford. The compositions of the RIPA lysis buffer and the HNTG buffer are described in *Table 3.5*.

**Table 3.5:** RIPA lysis buffer and HNTG buffer compositions for protein extraction.

<b>RIPA Buffer</b>	
Trizma Base - HCl, pH 7.4 ( <i>Sigma-Aldrich</i> )	50 mM
NaCl	150 mM
EDTA	1 mM
NaF ( <i>Sigma-Aldrich</i> )	1 mM
Sodium deoxycholate ( <i>Sigma-Aldrich</i> )	1%
Triton X-100 ( <i>Sigma-Aldrich</i> )	1%
Sodium dodecyl sulfate (SDS) ( <i>Sigma-Aldrich</i> )	0.1%
Sodium orthovanadate ( <i>Sigma-Aldrich</i> )	1 mM
Leupeptin, aprotinin, antipain, pepstatin A ( <i>Calbiochem</i> )	10 µg/mL
<b>HNTG Buffer</b>	
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 7.4 ( <i>Sigma-Aldrich</i> )	50 mM
NaCl	150 mM
Triton X-100	0.1%
Glycerol ( <i>Sigma-Aldrich</i> )	10%

### 3.3.7 Western blot

Proteins were analysed by SDS-PAGE and Western blotting on nitrocellulose membrane. To detect  $\alpha 5$  integrin subunit, 10 µg of total protein lysate for each sample (CTRL, siRNA and scramble (48h and 72h)) were resolved on a 7.5% polyacrylamide gel in running buffer at 200

V for 1 hour. Western blot was performed in transfer buffer at 100 V for 1 hour. The composition of running buffer and transfer buffer are given in **Table 3.6 and Table 3.7**, respectively.

**Table 3.6:** *Composition of running buffer for SDS-PAGE.*

Trizma Base	25 mM
Glycine ( <i>Sigma-Aldrich</i> )	192 mM
SDS	0.1%

**Table 3.7:** *Composition of transfer buffer for western blot.*

Trizma Base	25 mM
Glycine	192 mM
Methanol ( <i>Sigma-Aldrich</i> )	20%

After western blot, the nitrocellulose membrane was initially blocked by incubation with PBS-polyoxyethylene sorbitan monolaurate (TWEEN 20) 0.1% (*Sigma-Aldrich*) in agitation for 1 hour, then it was incubated with a rabbit anti-human  $\alpha 5$  integrin subunit (1:1000 in PBS Tween,

Cell Signaling Technology) or a rabbit anti  $\beta$ - actin (1:2000, Sigma Aldrich) antibodies. Immunoreactive bands were detected by using a horseradish peroxidase (HRP) conjugated secondary antibody (1:20,000 in PBS Tween, *GE Healthcare*) followed by WESTAR EtaC 2.0 (*Cyanagen*). Densitometry analysis of immunoreactive bands was done by Fluor-S Max MultiImager (*Bio-Rad*). Relative quantification of  $\alpha 5$  integrin subunit was performed by using  $\beta$ -actin signal as control. Mean of at least three independent analysis and Student's t-test were used to verify the significance of results (a p-value less than 0.05 was considered significant).

### **3.3.8 Evaluation of *C. trachomatis* infection capability upon $\alpha 5$ integrin subunit silencing in HeLa**

HeLa were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> on sterile glass coverslips for 72h. Cell were treated either not with siRNA or scramble 10nM for 72h as described in paragraph 3.3.5. Afterwards, samples were incubated with  $5 \times 10^3$  CT EBs for 48 h at 37 °C and 5% CO<sub>2</sub>. Cells were then washed three times with PBS, fixed in paraformaldehyde, and permeabilized in ethanol. Cells were stained for chlamydial membrane lipopolysaccharide and analyzed by confocal microscopy as described above.

### **3.3.9 *C. trachomatis* infectivity interference assay**

To study the capacity of both *L. crispatus* BC5 (BC5) cells and BS5 to interfere with the entry of CT EBs into HeLa cells, following experiments were performed. HeLa were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> on sterile glass coverslips for 48h. Cells were treated as described in **Table 3.8**.



**Table 3.8:** *Samples description.*

<b>CTRL</b>	Control cells (no treatment)
<b>BC5</b>	Cells first treated with <i>L. crispatus</i> BC5 CP for 1h, and then incubated with CT EBs for 48h.
<b>CD49e-BC5</b>	Cells treated 1h with <i>L. crispatus</i> BC5 pre-incubated with CD49e antibody for 1h, and then incubated with CT EBs for 48h.
<b>CT+anti-CD49e</b>	Cells treated 48h with CT EBs pre-incubated 1h with anti-CD49e antibody.
<b>BS5</b>	Cells treated with Released surface-associated components of <i>L.crispatus</i> BC5 (BS5) for 1h and then incubated for 48h with CT EBs.
<b>BC5 w/o BS5</b>	Cells treated 1h with BC5 deprived of BS5, and then incubated for 48h with CT EBs.
<b>CT+ BS5</b>	Cells treated 48h with CT EBs pre-incubated 1h with BS5.

After treatment, samples were washed three times with PBS, fixed in paraformaldehyde, and permeabilized in ethanol. Cells were stained for chlamydial membrane lipopolysaccharide and analyzed by confocal microscopy as described above. The number of IFUs was counted in 30 randomly chosen 200× microscopic fields. Results were expressed as the percentage (median percentage  $\pm$  median absolute deviation) of CT infectivity, comparing the number of IFUs of the single experiments with the control.

### 3.3.10 Released surface-associated components extraction

40 mL of an overnight culture of *L. crispatus* BC5 were inoculated in 400 mL of MRS broth and allowed to grow for 24 h under anaerobic conditions. Cell pellet was harvested by centrifugation (10,000g, 10 min) and, after 2 washes in sterile water, re-suspended in 150 mL of PBS. The suspension was then gently stirred at RT for 2 h in order to release the cell-bound BS5, and centrifuged again. Afterwards the supernatant was filtered through a 0.22 µm pore size filter, and BS5 was purified by dialysis against demineralized water in a Cellu-Sep© membrane (molecular weight cutoff 6,000–8,000 Da; Spectra/Por 2 dialysis membrane (*Spectrum Laboratories Inc.*) for 24 h at RT. Finally, the purified BS5 was lyophilized at 0.01 atm and –45 °C (*Christ Freeze Dryer ALPHA 1–2*). 1.6 mg of BS5 powder were dissolved in 1 mL of sterile PBS and filtered through a 0.22 µm pore size filter (Giordani B et al, 2019). The final concentration of use was 0.05 mg/mL.

### 3.3.11 Cytotoxicity assay

In order to evaluate the BS5 cytotoxicity, HeLa were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> on 96 wells plate for 24h. Afterwards, the media was eliminated and fresh media (200 µL) containing BS5 in the concentration range of 0.05, 0.1, 0.2, 0.4, 0.8, 1.0 and 1.2 mg/mL were added, followed by incubation for 24h. Once the incubation period was completed, media containing BS5 was gently removed and Alamar Blue was added in each well diluted 1:10 in sterile PBS in a final volume of 100 µL. Samples were incubated for 4h at 37°C and 5% CO<sub>2</sub>. Absorbance was measured at wavelengths of 570 nm and 600 nm using the spectrophotometer (*Uvikon*). The percent difference in reduction between treated and control cells in cytotoxicity assays was calculated as follow:

$$\% \text{ difference between treated and control cells} = \frac{(\text{O2} \times \text{A1}) - (\text{O1} \times \text{A2})}{(\text{O2} \times \text{P1}) - (\text{O1} \times \text{P2})} \times 100$$

Where:

O1 = 80,586, molar extinction coefficient (E) of oxidized alamarBlue (Blue) at 570 nm\*

O2= 117,216, E of oxidized alamarBlue at 600 nm\*

A1 = absorbance of test wells at 570 nm

A2 = absorbance of test wells at 600 nm

P1 = absorbance of positive control well (not treated cells plus alamarBlue) at 570 nm

P2 = absorbance of positive control well (not treated cells plus alamarBlue) at 600 nm

### 3.3.12 Western blot of proteins from bacterial cells

Two-hundred  $\mu\text{L}$  of *L. crispatus* BC5 suspension ( $5 \times 10^8$  *Lactobacillus* CFU/mL) were pelleted and treated with 180  $\mu\text{L}$  of enzymatic lysis buffer (20 mM tris-HCl, 2 mM EDTA, 1.2% Triton X-100, 20 mg/mL lysozyme) for 30 min at 37 °C. Afterwards, proteins were analysed by SDS-PAGE and Western blotting on nitrocellulose membrane. To detect  $\alpha 5$  integrin subunit, 10  $\mu\text{g}$  of *L. crispatus* lysate and 15  $\mu\text{g}$  of BS5 were resolved on a 10% polyacrylamide gel in running buffer at 200 V for 1 hour. Western blot was performed in transfer buffer at 100 V for 1 hour. The composition of running buffer and transfer buffer are given in **Table 3.6 and Table 3.7**, respectively.

### **3.3.13 Statistical analysis**

In the experiments regarding NR and DAPI fluorescence intensity quantification, PA-DPH anisotropy, and intracellular ROS production, a paired Student's t test was used to determine whether treated and untreated samples were significantly different. P values below 0.05 were considered significant and evidence of population differences.

## **CHAPTER 4**

### **RESULTS AND DISCUSSION**

## 4.1 Role of lactobacilli in the prevention of *C. albicans* infection

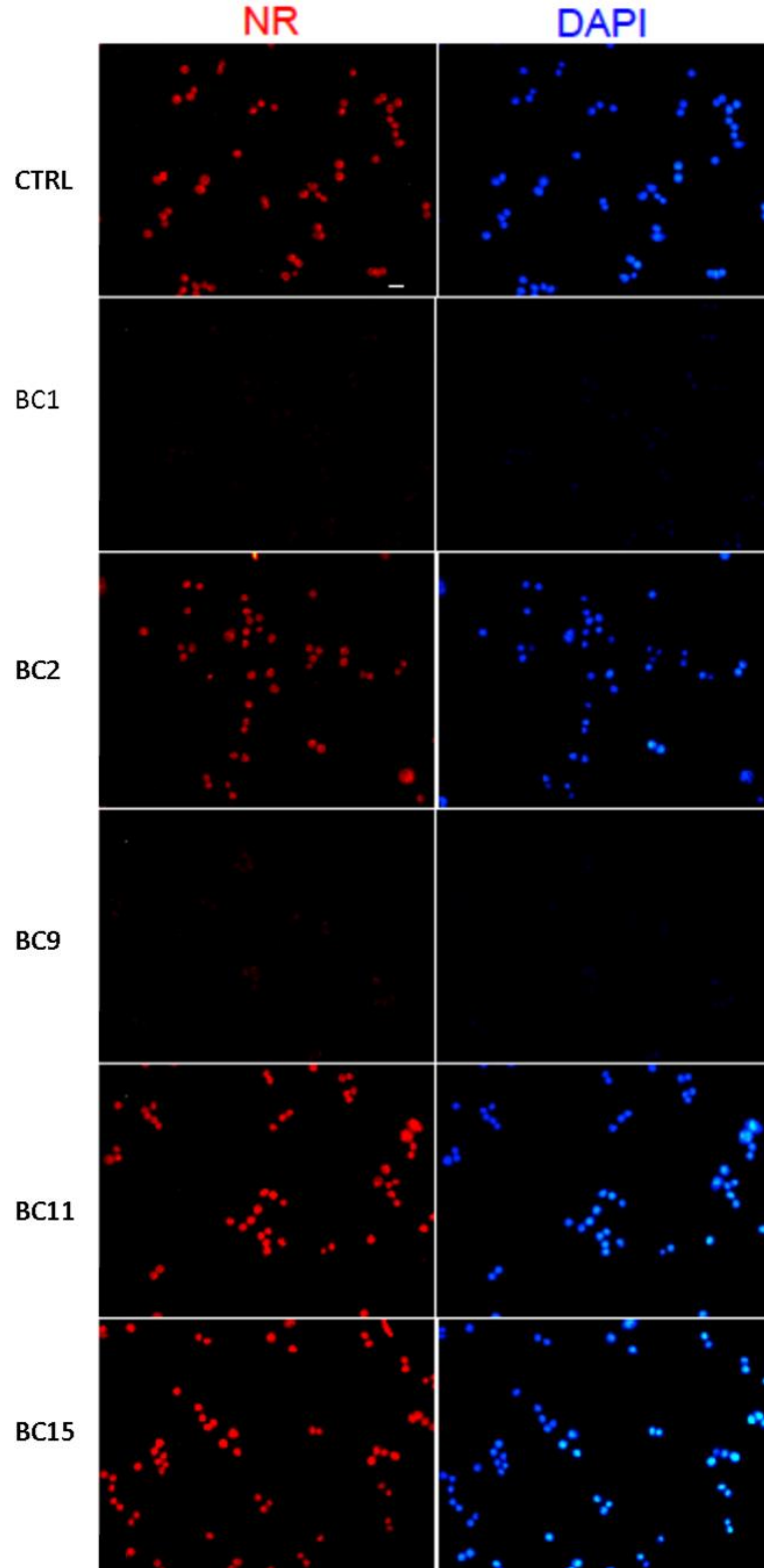
In this first part of the chapter, results regarding the study of the protective role of lactobacilli against *C. albicans* are described. *L. crispatus* BC1 and BC2, *L. gasseri* BC9 and BC11 and *L. vaginalis* BC15 were selected on the basis of their good adhesion profile to HeLa cells, a cervical cell line, according to the results published by Parolin et al., in 2015. The effects exerted by the different *Lactobacillus* strains of vaginal origin on the physical properties of the plasma membrane in HeLa were studied, and two putative mechanisms at the basis of lactobacilli protective role were identified. Results herein described have been published in the paper “*Interaction of vaginal lactobacillus strains with HeLa cells plasma membrane*” (N. Calonghi, G. Frisco et al., Beneficial Microbes 2017).

### 4.1.1 Modulation of HeLa plasma membrane properties by lactobacilli

In order to evaluate the effect of lactobacilli strains on plasma membrane properties, lipid bilayer organization of HeLa cells was firstly investigated. For this experiment, HeLa were seeded on sterile glass coverslips and incubated for 1h with *L. crispatus* BC1 and BC2, *L. gasseri* BC9 and BC11 and *L. vaginalis* BC15. After the treatment, HeLa cells were stained with the NR dye, which acts as a hydrophobic probe, exhibiting different fluorescence depending on the relative hydrophobicity of the surrounding environment. Specifically, NR is able to fluoresce intensely in the presence of polar lipids such as phospholipids, showing excitation and emission maximum at 549 nm and 628 nm, respectively (Greenspan and Fowler, 1985). Samples were analysed by confocal microscopy and the results are shown in the left panel of **Figure 4.1**. The interaction of HeLa cells with *L. crispatus* BC1 and *L. gasseri* BC9 strongly decreased NR emission fluorescence, indicating a reduced exposure of membrane polar lipids. The treatment with *L. crispatus* BC2 caused a weaker reduction of NR fluorescence, whereas *L. gasseri* BC11 and *L. vaginalis* BC15 did not affect membrane lipid organization. Nile Red Fluorescence quantification of HeLa control and treated with *L. crispatus* BC1 and BC2, *L. gasseri* BC9 and BC11 and *L. vaginalis* BC15 is shown in **Figure 4.2**.

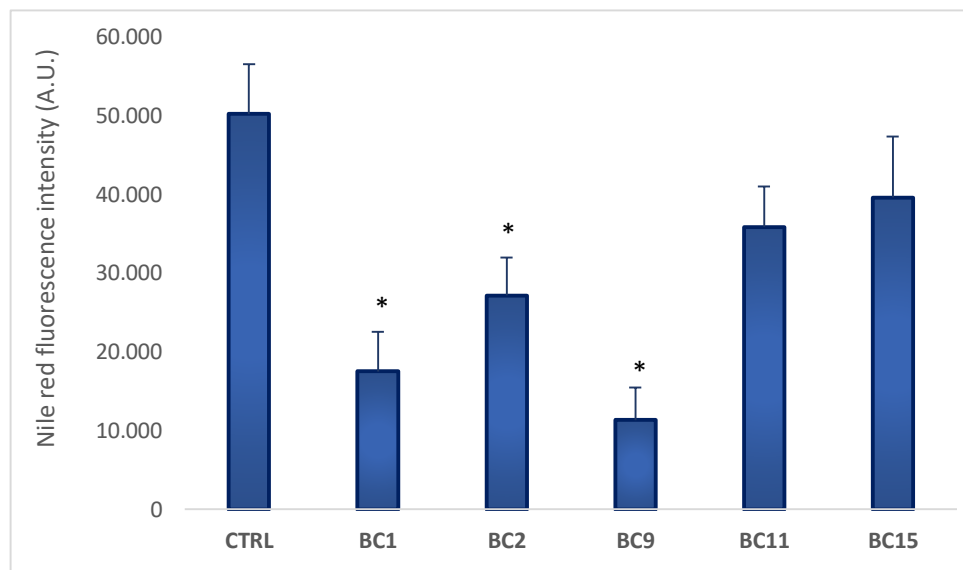
Afterwards, HeLa plasma membrane permeability was evaluated. Indeed, HeLa cells were also counterstained with DAPI which, being hydrophobic, is able to spontaneously cross the plasma membrane and bind to nuclear DNA. Results regarding DAPI staining are shown in the right panel of **Figure 4.1**. Upon the interaction with *L. crispatus* BC2, *L. gasseri* BC11 and *L. vaginalis* BC15 HeLa cells remained permeable to DAPI. On the contrary, DAPI fluorescence resulted strongly decreased after *L. crispatus* BC1 and *L. gasseri* BC9 treatment, indicating that the interaction of this two Lactobacilli strains with cell plasma membrane prevented DAPI internalization and its binding to HeLa nuclear DNA. DAPI fluorescence quantification of HeLa control and treated with *L. crispatus* BC1 and BC2, *L. gasseri* BC9 and BC11 and *L. vaginalis* BC15 is shown in **Figure 4.3**

Treatment with *L. crispatus* BC1 and *L. gasseri* BC9 have led to changes in lipid organization and permeability that could be not attributed simply to lactobacilli adherence capability to HeLa cells. According to Parolin et al., adhesiveness properties are strains specific rather than species-specific. Thus, such effects seem to be related to a specific activity of some lactobacilli strains, which, despite a low adherence to HeLa cells, are able to perturb the organization of plasma membrane and its functionality. This observation is in agreement with the results of Parolin *et al.* (2015), showing no correlation between the adhesiveness level of lactobacilli and their ability to counteract the adhesion of *Candida*. Indeed, the most active strains in reducing pathogen adhesion were not the most adhesive strains, suggesting that the inhibitory effects are not purely due to steric encumbrance and saturation of the adhesion sites, but rather to changes affecting the epithelial cells surface (Parolin *et al.*, 2015).

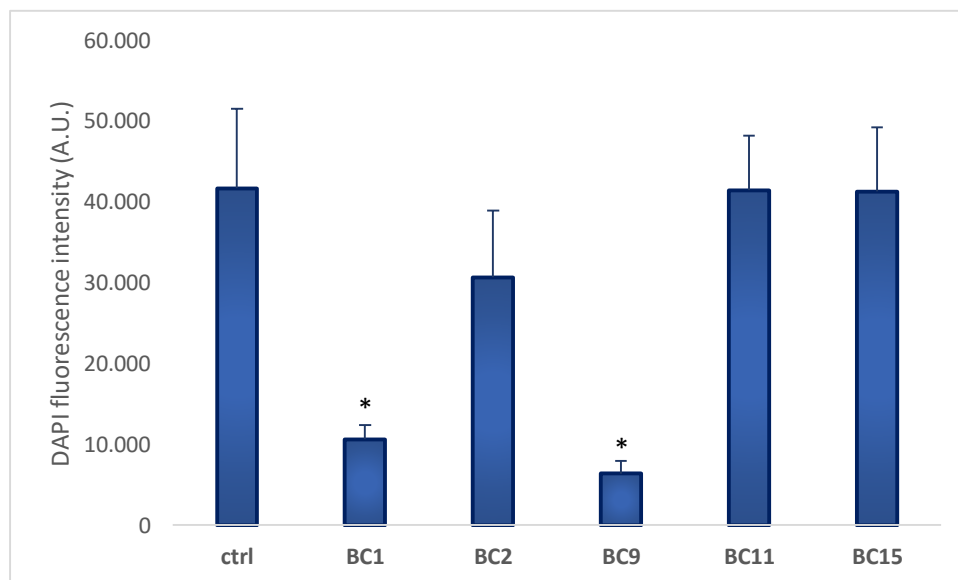


**Figure 4.1.** *Confocal microscopy of NR lipid staining and DAPI nuclear staining in control and Lactobacillus treated HeLa cells. HeLa cells were incubated with Lactobacillus strains for 1 hour, then stained with NR (left panel) and DAPI (right panel). (CTRL) Control HeLa cells (bar = 50  $\mu$ m); HeLa cells treated with *L. crispatus* BC1, *L. crispatus* BC2, *L. gasseri* BC9, *L. gasseri* BC11, *L. vaginalis* BC15.*



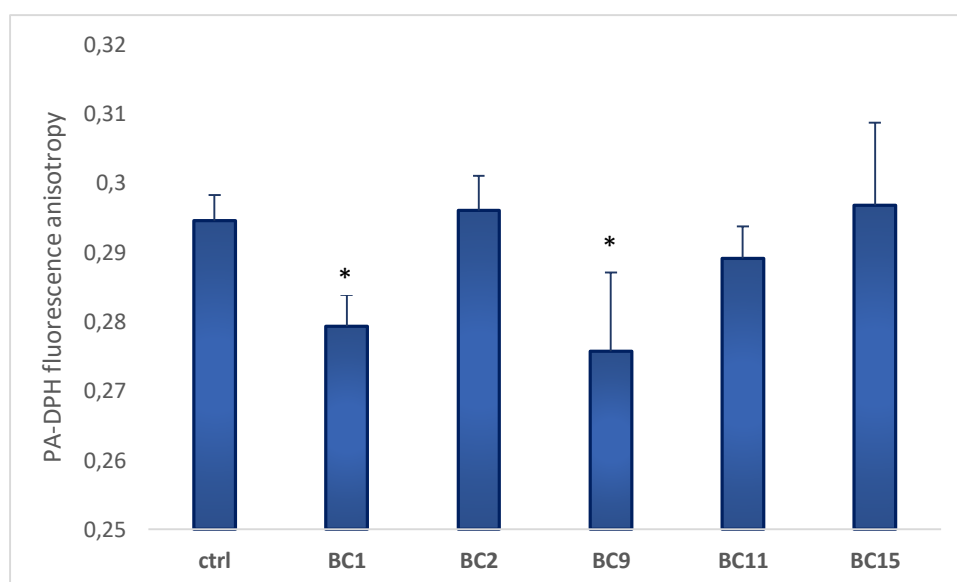


**Figure 4.2. Quantification of Nile Red fluorescence intensity in control and lactobacilli treated HeLa cells.** NR fluorescence intensity of HeLa cells alone (CTRL) or treated with the indicated *Lactobacillus* strains, was calculated by image densitometry using ImageJ (\*  $p < 0.05$  respect to control).



**Figure 4.3. Quantification of DAPI fluorescence intensity in control and lactobacilli treated HeLa cells.** DAPI fluorescence intensity of HeLa cells alone (CTRL) or treated with the indicated *Lactobacillus* strains, was calculated by image densitometry using ImageJ (\*  $p < 0.05$  respect to control).

Finally, the effect of lactobacilli strains on plasma membrane fluidity was studied by means the fluorescence anisotropy of PA-DPH probe. This probe allows to monitor lipid organization of the fatty acyl chains thanks to its capability to locate within the plasma membrane bilayer and to fluoresce, with a polarized fluorescence correlated to its rotational diffusion in the membrane (Trotter and Storch, 1989). The PA-DPH fluorescence anisotropy of HeLa cells in response to the interaction with different lactobacilli strains is reported in **Figure 4.4**. The interactions of *L. crispatus* BC1 and *L. gasseri* BC9 with HeLa cell membranes significantly reduced the PA-DPH fluorescence anisotropy as compared to the untreated cells, while no significant differences were observed upon interaction with *L. crispatus* BC2, *L. gasseri* BC11, and *L. vaginalis* BC15 strains.



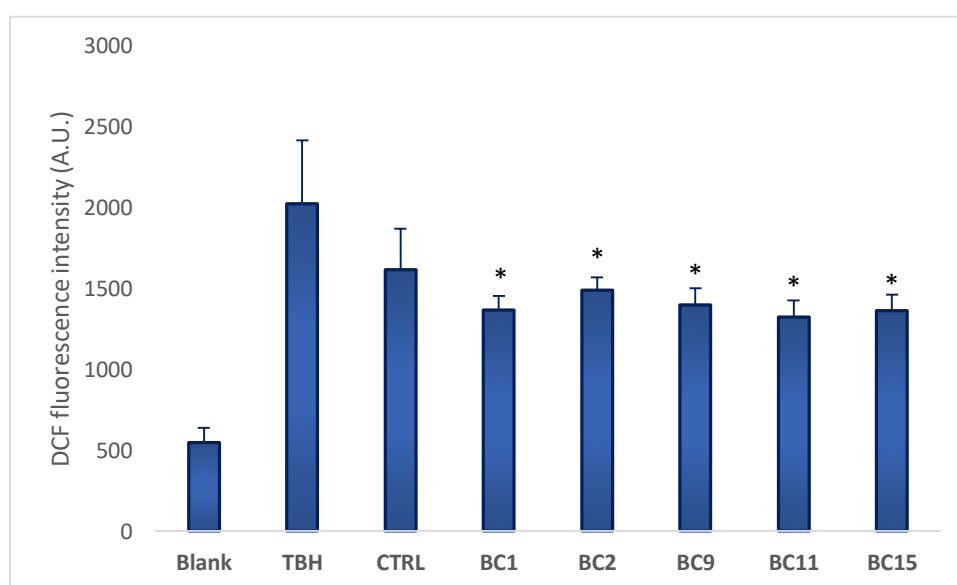
**Figure 4.4. Quantification of DA-DPH fluorescence anisotropy in control and lactobacilli treated HeLa cells.** DAPI fluorescence intensity of HeLa cells alone (CTRL) or treated with the indicated *Lactobacillus* strains, was calculated by image densitometry using ImageJ (\*  $p < 0.05$  respect to control).

PA-DPH fluorescence anisotropy followed a trend similar to both NR and DAPI fluorescence: indeed, *Lactobacillus* strains BC1 and BC9 treated HeLa cells showed the lowest NR fluorescence intensity and a significantly reduced internalization of DAPI as compared to untreated cells. The reduction of PA-DPH fluorescence anisotropy resulting from the interaction of lactobacilli with the plasma

membrane of HeLa indicates an increase in membrane fluidity that can be strictly correlated with membrane polar lipid organization and permeability changes. All these data suggest a modification of the HeLa cells membrane properties due to interactions with *L. crispatus* BC1 and *L. gasseri* BC9.

#### 4.1.2 Effect of lactobacilli on ROS production in HeLa cells

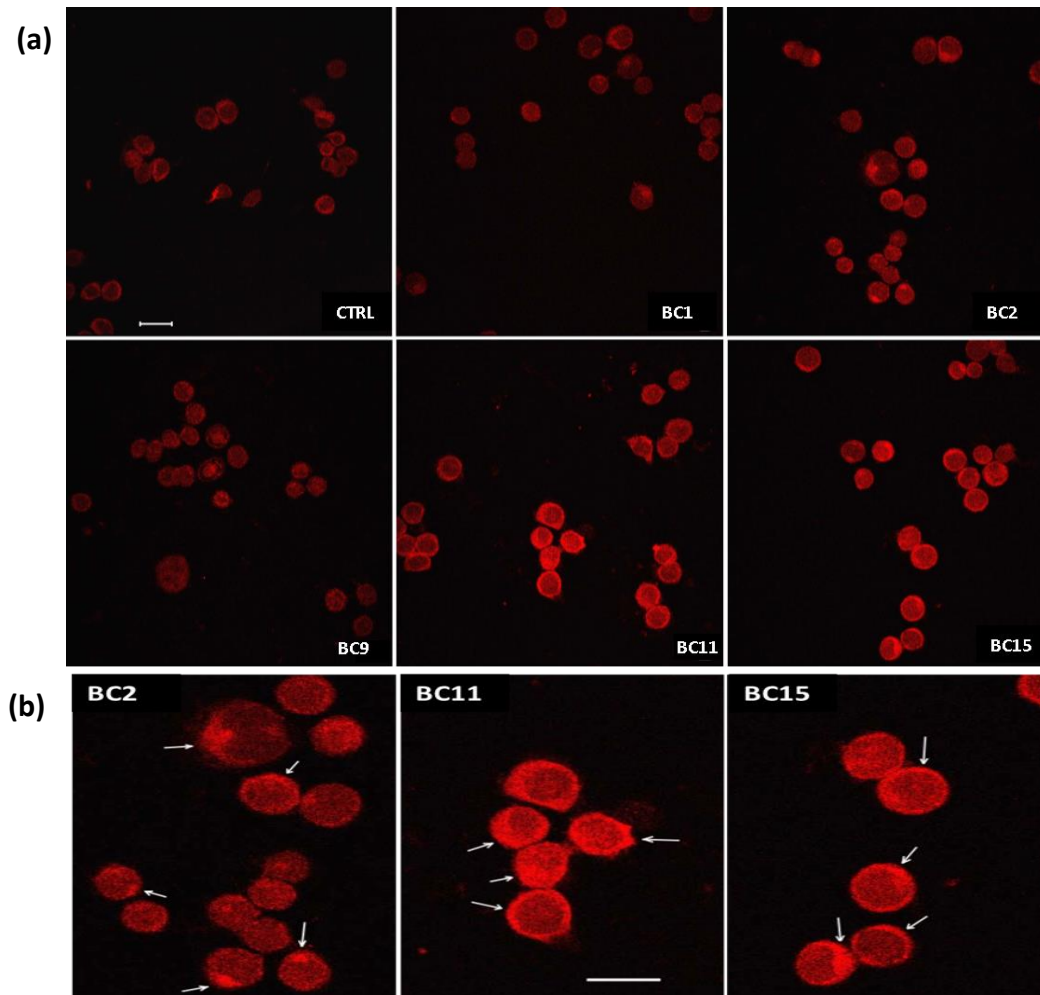
In order to study effects of lactobacilli on ROS production, cellular oxidative stress was measured by means of the cell-permeant probe DCF-DA, commonly used to detect free radical/ROS production in cells. HeLa treated 1h with *L. crispatus* BC1 and BC2, *L. gasseri* BC9 and BC11 and *L. vaginalis* BC15 were analyzed, and HeLa cells exposed to tert-buthyl-hydroperoxide (TBH) were used as a positive control for the DCF-DA assay; untreated and unstained cells were used as negative control. As shown in Figure 4.5, all *Lactobacillus* strains significantly decreased HeLa ROS content.



**Figure 4.5 Analysis of intracellular ROS production in control and *Lactobacillus* treated HeLa cells.** ROS production was measured by DCF-DA fluorescent assay. Blank: untreated and unstained cells; TBH: tert-buthyl-hydroperoxide treated cells; control HeLa cells (CTRL) or treated with the indicated *Lactobacillus* strains. (\*  $p < 0.05$  respect to control).

### 4.1.3 Effect of lactobacilli on surface $\alpha 5\beta 1$ integrin exposure

In light of the results obtained so far, *L. crispatus* BC1 and BC2, *L. gasseri* BC9 and BC11 and *L. vaginalis* BC15 were also tested to investigate their role in modulating  $\alpha 5\beta 1$  integrin exposure. In fact, as it is known, membrane fluidity affects the free movement of phospholipid molecules and proteins in the bilayer to modulate various biological functions like ion transport, cell signaling and cell growth (Park *et al.*, 2005). Thus, the question was addressed whether *Lactobacillus* strains interaction with HeLa plasma membrane could lead to changes on integrin exposure. HeLa cells stained for surface  $\alpha 5\beta 1$  integrin were analyzed by confocal microscopy and results are shown in **Figure 4.6**.



**Figure 4.6.** Confocal microscopy of  $\alpha 5\beta 1$  integrin staining in control and *Lactobacillus* treated HeLa cells. (a) (CTRL)Control HeLa cells (bar = 50  $\mu\text{m}$ );HeLa cells treated with *L. crispatus* BC1, *L. crispatus* BC2, *L. gasseri* BC9, *L. gasseri* BC11, *L. vaginalis* BC15. (b) Enlargement of  $\alpha 5\beta 1$  integrin staining in BC2, BC11 BC15 treated HeLa cells. Arrows indicate clustering of  $\alpha 5\beta 1$  integrin. (bar = 50  $\mu\text{m}$ )

The interaction of *L. crispatus* BC1 and *L. gasseri* BC9 with HeLa cells did not change the expression of  $\alpha 5\beta 1$  integrin neither its localization, despite they significantly increased plasma membrane fluidity. On the contrary, *L. crispatus* BC2, *L. gasseri* BC11 and *L. vaginalis* BC15 stimulation, which did not affect membrane lipid organization and anisotropy, greatly changed the  $\alpha 5\beta 1$  integrin exposure. Indeed, BC2, BC11 and BC15 strains in addition to promoting integrin exposure, modified its organization on the plasma membrane, leading to the protein clustering (**Figure 4.6 b**).

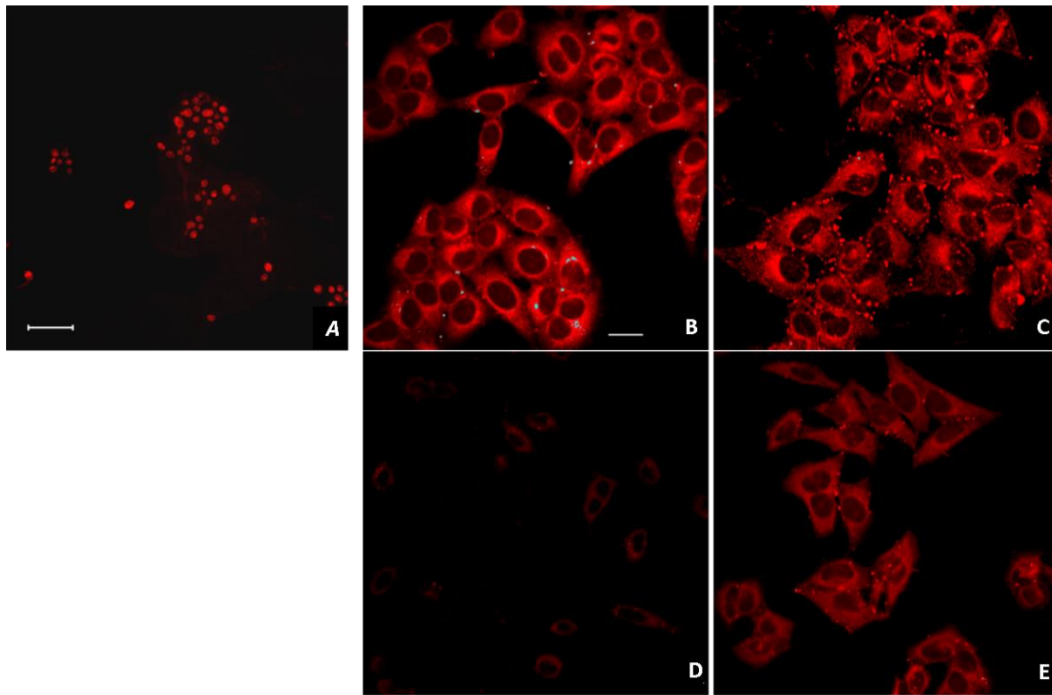
#### **4.1.4 Interaction of lactobacilli with HeLa cells prevented *C. albicans* adhesion**

All the results shown so far have led to identify two mechanisms of action for the different strains of lactobacilli analyzed: the first one was exerted by *L. crispatus* BC1 and *L. gasseri* BC9, whose interaction with HeLa plasma membrane caused a significant increase in membrane fluidity by modulating lipid organization of the bilayer. The second one was exerted by *L. crispatus* BC2, *L. gasseri* BC11, and *L. vaginalis* BC15, whose stimulation of HeLa plasma membrane led to a modulation of  $\alpha 5\beta 1$  integrin exposure and clustering formation.

In order to verify if the two mechanisms of interaction of lactobacilli with HeLa plasma membrane could have a role in the prevention of *C. albicans* adhesion to HeLa cells, *L. crispatus* BC1 and BC2 were chosen as representative strains of the two groups of lactobacilli here identified. In this experiment HeLa were treated simultaneously with *L. crispatus* BC1 or BC2 and *C. albicans* for 1h. *Candida* adhesion was visualized by means of NR, which is able to stain yeast lipid particles (Verstrepen *et al.*, 2004), and HeLa membrane phospholipids too.

**Figure 4.7 (A)** shows the NR staining of *C. albicans* cells adherent to HeLa cells, and **Figure 4.7 (B)** the modulation of the yeast adhesion to epithelium driven by *L. crispatus* BC1 and BC2. The images acquired by confocal microscopy confirm the ability of both *L. crispatus* strains to compete with *C. albicans* adhesion to HeLa cells. Since no significant difference was observed between the reductions

induced by *L. crispatus* BC1 and BC2 the two mechanisms are equally efficient to prevent *Candida* adhesion.



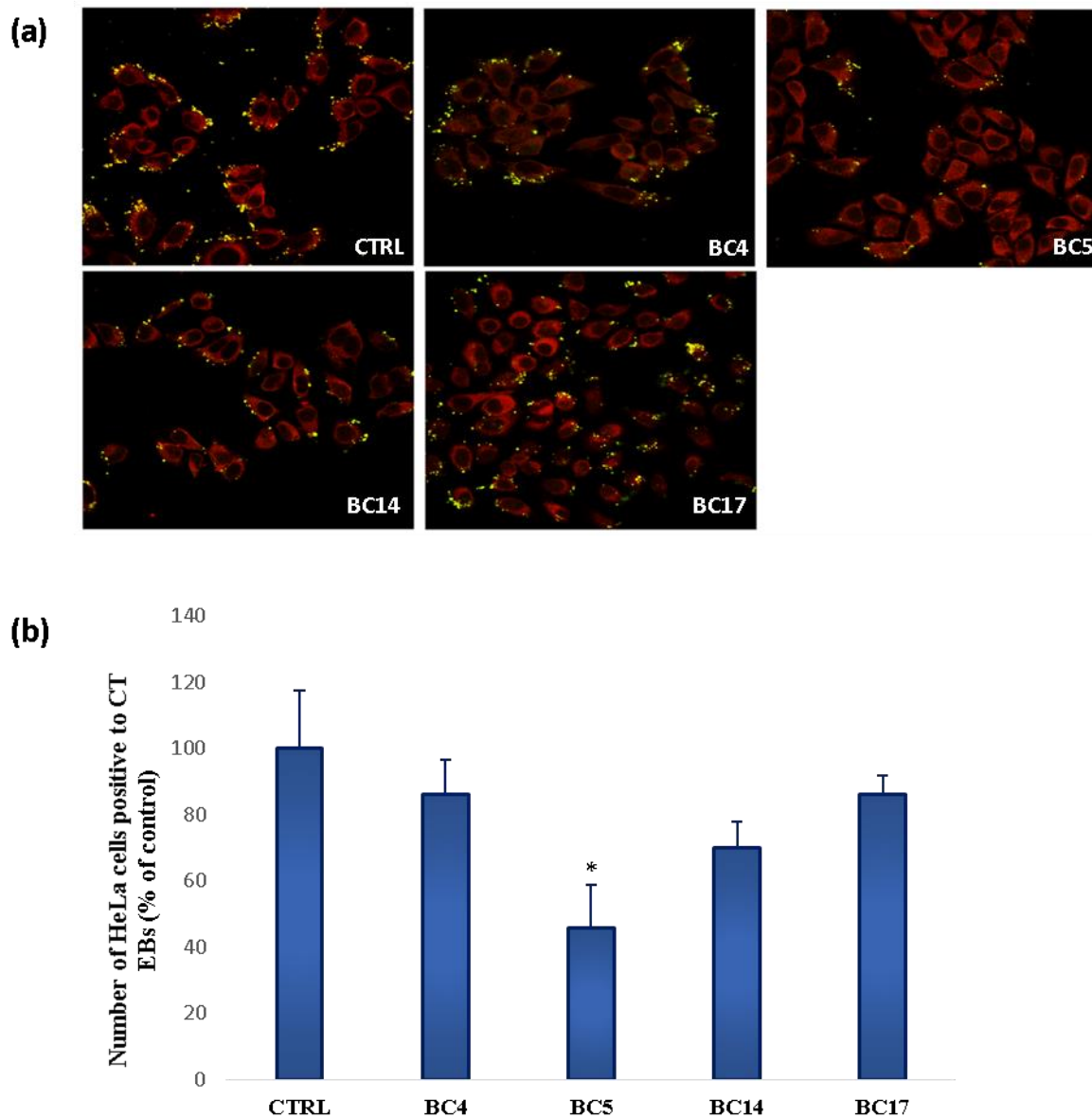
**Figure 4.7. Confocal microscopy of NR lipid staining of *C. albicans* adherent to HeLa cells, after 1 hour of coincubation with *Lactobacillus* strains.** (A) NR staining of *C. albicans*. (B) Control HeLa cells (bar = 50  $\mu\text{m}$ ); (C) HeLa cells treated with *C. albicans*. HeLa cells treated with *C. albicans* and *L. crispatus* BC1 (D), *L. crispatus* BC2 (E).

## 4.2 Role of lactobacilli in the prevention of *C. trachomatis* infection

In this second part of the chapter, *L. crispatus* BC4 and BC5, *L. gasseri* BC14 and *L. vaginalis* BC17 were employed in order to investigate their protective role in counteract *C. trachomatis* infection of HeLa cells, chosen as vaginal epithelial model. Specifically, the potential mechanism of protection was investigated in *L. crispatus* BC5, chosen as the model strain. Results described herein have been published in the paper “*Lactobacillus crispatus* BC5 Interferes with *Chlamydia trachomatis* Infectivity Through Integrin Modulation in Cervical Cells” (G. Frisco et al., *Frontiers in Microbiology* 2018).

### 4.2.1 Interference of lactobacilli with *C. trachomatis* adhesion to HeLa

In order to evaluate the role of lactobacilli in the prevention of *C. trachomatis* adhesion to HeLa, cells were incubated with lactobacilli CP of *L. crispatus* BC4 and BC5, *L. gasseri* BC14 and *L. vaginalis* BC17 for 1h and then with CT EBs for an additional 1h (exclusion mechanism). Specimens were stained for chlamydial membrane lipopolysaccharide antigen and images acquired by confocal microscopy are shown in **Figure 4.8 (a)**. *Chlamydia* adhesion was assessed by counting the number of HeLa cells positive to CT EBs and quantification is reported in **Figure 4.8 (b)**. *L. crispatus* BC5 was the most active strain in counteract *Chlamydia* adhesion to HeLa cells, whereas no significative differences were observed upon *L. crispatus* BC4, *L. gasseri* BC14 and *L. vaginalis* BC17 treatment.



**Figure 4.8 Interference of lactobacilli with *C. trachomatis* adhesion to HeLa cells.** (a) Confocal microscopy of HeLa cells treated or not (CTRL) for 1h with the indicated lactobacillus strains and incubated for 1h with CT EBs. (b) Quantification of HeLa cells positive to CT EBs. Representative micrographs are shown; results were expressed in percentage compared to control taken as 100%. Bars represent mean values and error bars represent standard deviations. Statistical significance was calculated vs. control (\* $P \leq 0.01$ )



### 4.2.2 *L. crispatus* BC5 modulate HeLa cells plasma membrane

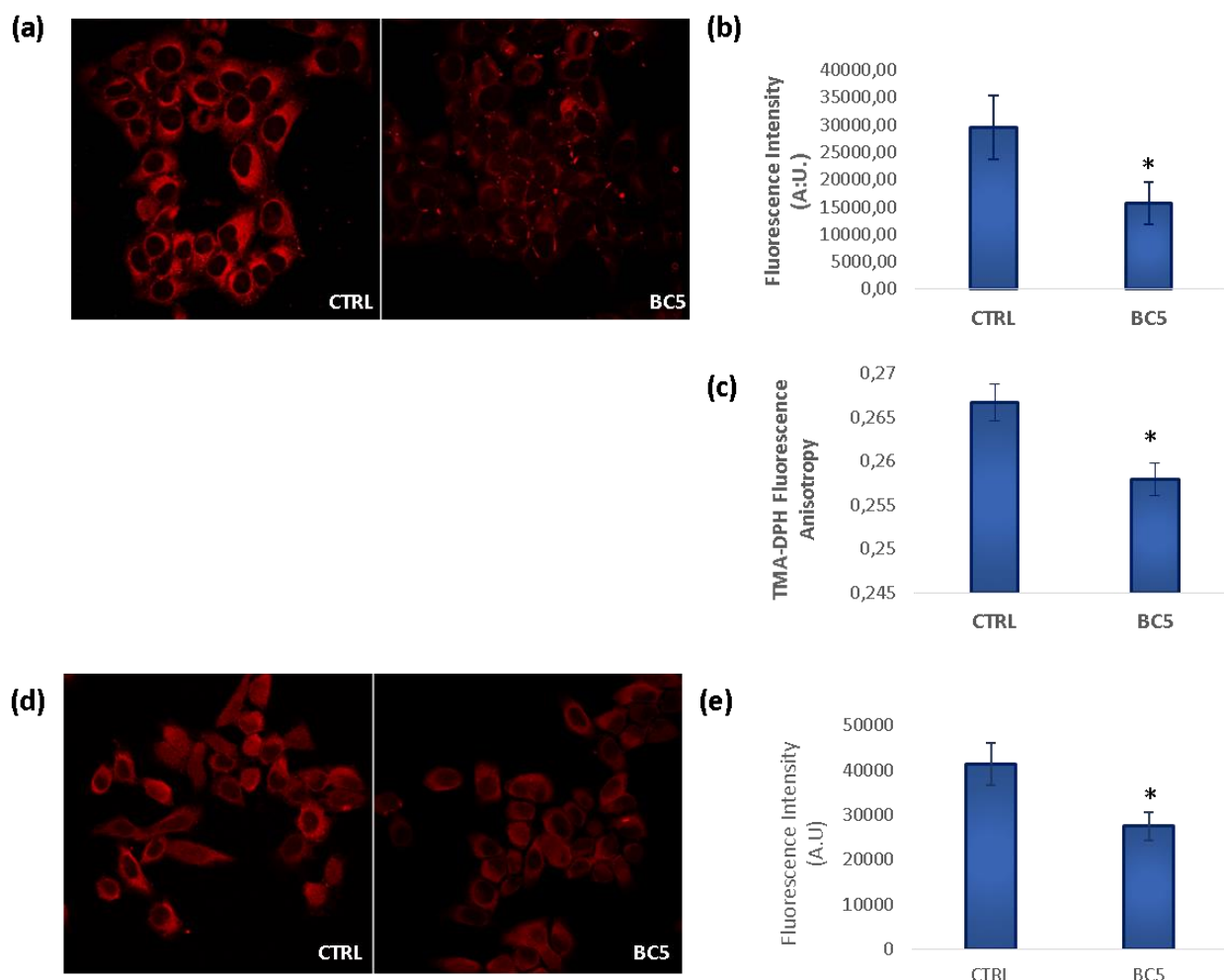
Since *L. crispatus* BC5 was the most active in counteract *C. trachomatis* adhesion to HeLa cells through the exclusion mechanism, it was chosen as a model strain for all the subsequent experiments. It was evaluated if 1h-treatment of HeLa cells with *L. crispatus* BC5 CP would be able to induce modifications at the HeLa plasma membrane level. The effect of *L. crispatus* BC5 on plasma membrane modulation was investigated in terms of lipid organization, membrane fluidity and modulation of protein exposure.

For the investigation of membrane lipid organization, HeLa cells were stained with the lipid dye NR and analyzed by confocal microscopy. Results are reported in **Figure 4.9(a)**. As shown in **Figure 4.9(b)**, *L. crispatus* BC5 interaction with HeLa caused a decrease in NR emission fluorescence, indicating a reduced exposure of polar membrane lipids.

In order to evaluate if *L. crispatus* BC5 interaction with HeLa cells could affect the physico-chemical characteristics of plasma membrane, the steady-state fluorescence anisotropy of TMA-DPH was measured both in control cells and in HeLa pre-incubated 1h with *L. crispatus* BC5. As reported in **Figure 4.9(c)**, the treatment with *L. crispatus* BC5 CP induced a significant decrease in TMA-DPH anisotropy.

Finally, it was investigated whether *L. crispatus* BC5 pre-treatment could alter integrin exposure on HeLa plasma membrane by immunostaining of CD49e. Images acquired by confocal microscopy are shown in **Figure 4.9 (d,e)**. The pre-incubation of HeLa cells with *L. crispatus* BC5 significantly reduced the exposure of  $\alpha 5$  integrin subunit on the plasma membrane.

The results here reported, led to elucidate the biochemical mechanism at the basis of *L. crispatus* BC5 interaction with HeLa cells plasma membrane. This interaction induced modifications at the plasma membrane level that are strain-specific. In particular, *L. crispatus* BC5 acted by increasing membrane fluidity and altering lipid composition, as well as  $\alpha 5\beta 1$  integrin exposure.

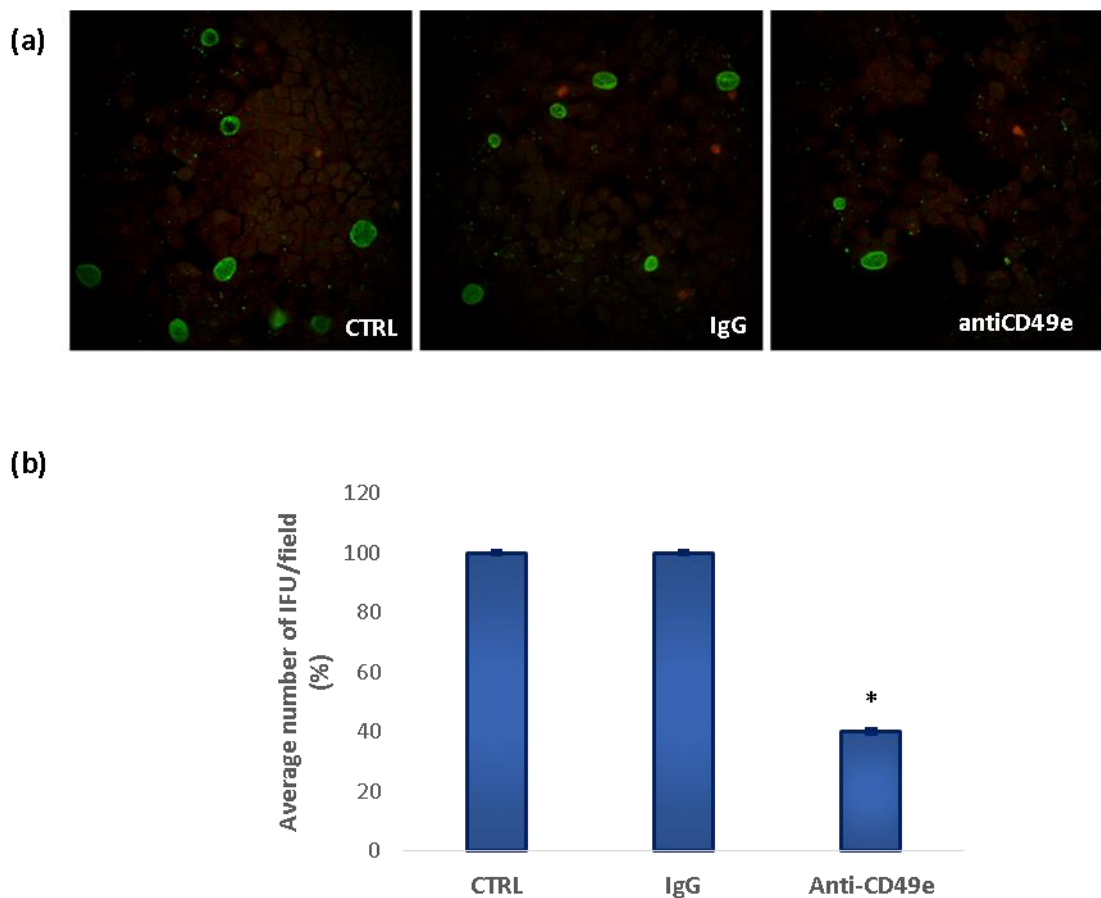


**Fig.4.9. Effect of *L. crispatus* BC5 on Membrane lipid organization, fluidity and  $\alpha 5$  integrin exposure of HeLa cells.** (a,b) HeLa cells incubated or not with *L. crispatus* BC5 for 1 h and then stained with NR. (c) Steady-state fluorescence anisotropy of TMA-DPH of control and *L. crispatus* BC5 treated HeLa cells. (d, e) HeLa cells incubated or not with *L. crispatus* BC5 for 1 h and then stained with anti-CD49e antibody. Fluorescence intensity was quantified by using Image J. Representative micrographs are shown. Experiments were repeated at least 3 times with similar results. Results are expressed as mean values  $\pm$  s.e.m. \*  $P \leq 0.001$ .

#### 4.4.3 Role of $\alpha 5$ integrin subunit in *C. trachomatis* internalization.

It is known that numerous pathogens exploit integrins expressed on target cell in order to mediate adhesion and cell internalization (Hoffmann et al., 2011; Hauck et al., 2012).. Since  $\alpha 5\beta 1$  integrin exposure was altered upon the interaction of *L. crispatus* BC5, it was examined if  $\alpha 5$  integrin subunit could be involved in *C. trachomatis* internalization into HeLa. Thus, an anti-CD49e blocking

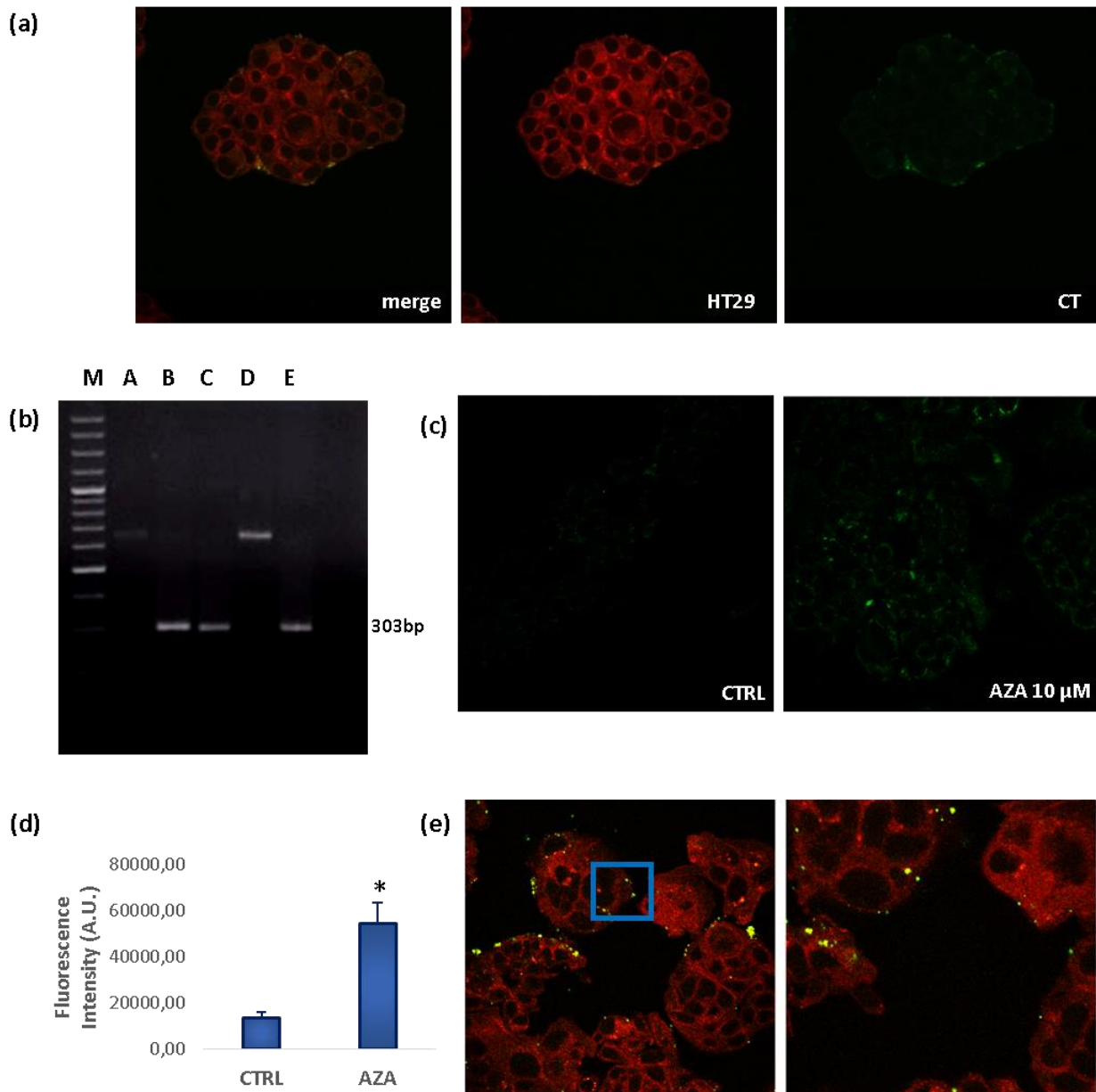
antibody was used to mask  $\alpha 5$  integrin subunits exposed on HeLa cell surface, and then CT infectivity was evaluated. The specificity of  $\alpha 5$  integrin subunit blocking was verified by using IgG isotype. As shown in **Figure 4.10 (a, b)**, the CT infectivity was reduced by approximately 60% when anti-CD49e blocking antibody was used. On the contrary, the pre-incubation with IgG isotype did not affect *C. trachomatis* infectious process. (Frisco et al., 2019)



**Figure 4.10. An  $\alpha 5$  integrin blocking antibody prevents HeLa infection by *C. trachomatis*.** (a, b) HeLa cells were treated or not with an anti- $\alpha 5$  integrin antibody for 1 h, then incubated with CT EBs for 48 h. *C. trachomatis* infectivity was evaluated as number of IFUs/microscopic fields. Results were expressed in percentage compared with control taken as 100%. Bars represent median values, error bars represent median absolute deviations. Statistical significance was calculated vs control. \*  $P \leq 0.01$ .

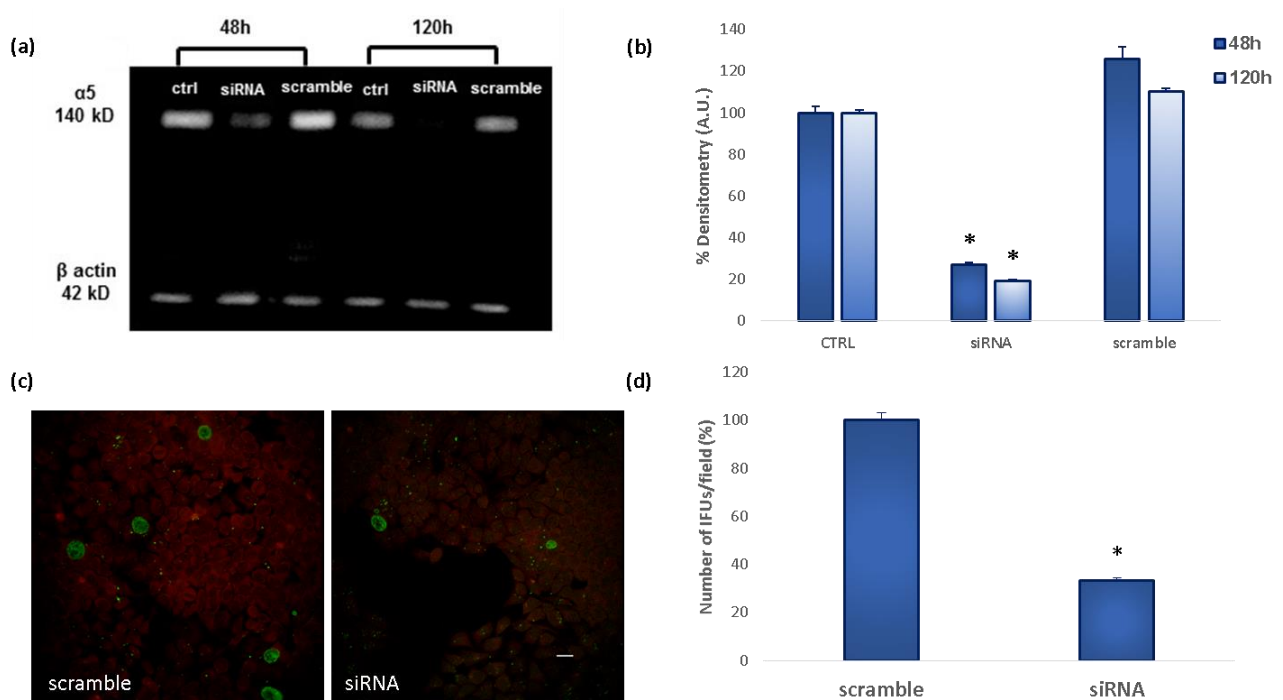
To further assess the role of  $\alpha 5$  integrin subunit on CT infection, HT29, a human colon carcinoma cell line that constitutively lacks  $\alpha 5$  subunit but does express the  $\beta 1$  subunit (Kempermann et al., 1997;

Schmidt et al., 1998) were incubated 48h with CT EBs. Image acquired by confocal microscopy (**Figure 4.11a**) showed how the absence of  $\alpha 5$  integrin subunit inhibits *C. trachomatis* internalization. In order to allow  $\alpha 5$  integrin re-expression, HT29 were treated with a Histone Deacetylase Inhibitor (HDACi), sodium butyrate (NaBu), or with a hypomethylating agent, 5-Azacytidine (5-AZA). HDACs are enzymes that remove the acetyl groups from histone lysine residues. The process of deacetylation creates more positive charges on the histones and thus increases the interaction of the positively charged N termini of histones with the negatively charged phosphate groups of DNA. These interactions transform DNA into a more condensed form, making it less accessible to the cell's transcriptional machinery (Hong et al., 1993; Nagy et al., 1997). The cytosine analogue 5-azacytidine is one of the currently most advanced drugs for epigenetic cancer therapies. This compound acts as a DNA methyltransferase inhibitor and has shown substantial potency in reactivating epigenetically silenced tumor suppressor genes *in vitro* (Stresemann et al., 2008). On this ground, HT29 were treated with NaBu 5mM or 5-AZA 10  $\mu$ M for 72 h. After the treatment, mRNA and protein expression levels of  $\alpha 5$  integrin subunits were verified by RT-PCR and confocal microscopy, respectively. Results reported in **Figure 4.11(b, c)** show that only treatment with 5-AZA induced a re-expression of  $\alpha 5$  integrin subunit in HT29 cells at both mRNA (**b**) and protein (**c**) levels. Thus, HT29 re-expressing  $\alpha 5$  integrin subunit were subsequently incubated for 48h with CT EBs and CT infectivity was evaluated. As evidenced by confocal images reported in **Figure 4.11 (e)**, CT was able to infect HT29.



**Figure 4.11. Role of  $\alpha 5$  integrin subunit on CT infectious process.** (a) HT29 were incubated for 48h with CT EBs. Specimens were stained for chlamydial antigen (b) ITGA5 gene expression. Lane M: GeneRuler DNA Ladder; Lane A: cDNA of HT29 CTRL; lane B and C: cDNA of HT29 treated with 5-AZA diluted 1:10 and 1:100, respectively; Lane D: cDNA of HT29 treated with NaBu; Lane E: cDNA of HeLa positive CTRL. (c, d) Confocal microscopy of  $\alpha 5$  integrin subunit in HT29 control and treated with 5-AZA and fluorescence intensity quantification. Representative micrographs are shown. Experiments were repeated at least 3 times with similar results. Results are expressed as mean values  $\pm$  s.e.m. \*  $P \leq 0.001$ . (e) Confocal microscopy of HT29 treated with 5-AZA and subsequently incubated for 48h with CT EBs. Specimens were stained as described above.

To further confirm the involvement of  $\alpha 5$  integrin in CT infectious process in HeLa cells, a siRNA silencing was performed to knockdown endogenous  $\alpha 5$  integrin subunit expression (Frisco et al., 2019). Validated siRNAs were used for the analysis: a specific anti-ITGA5 and a correspondent negative siRNA were chosen (scramble). A silencing time course was assessed by quantification of  $\alpha 5$  integrin subunit protein 48-120 h after silencing with siRNA 10 nM. Total protein lysates were resolved in SDS-PAGE and  $\alpha 5$  integrin subunit protein was identified by a specific antibody in western blot. By normalization on the amount of  $\beta$ -actin, quantification of  $\alpha 5$  integrin subunit in the specific siRNA sample was compared with the relative scramble sample (**Figure 4.12 a, b**). Since  $\alpha 5$  integrin subunit expression was already greatly reduced after 48 h of siRNA treatment, this time point was chosen as optimal. Following silencing, cells were incubated with CT EBs for 48h, stained for chlamydial antigen and finally CT infectivity was evaluated. Transfection of cells with  $\alpha 5$  integrin-specific siRNA reduced CT infection by 64%, whereas transfection of cells with scramble did not affect CT infection (**Figure 4.12 c, d**). These results demonstrate that  $\alpha 5$  integrin subunit plays an essential role in *C. trachomatis* adhesion and internalization in HeLa cells.



**Figure 4.12. ITGA5 gene silencing prevents *C. trachomatis* infection of HeLa cells.** (a, b) Western blotting of α5 integrin subunit expression in control, ITGA5 siRNA and scramble HeLa cells. Quantification of α5 integrin subunit was normalised on β-actin. Bars represent mean values based on three independent experiments, error bars represent standard deviations. (c, d) HeLa cells treated with siRNA or scramble were infected with CT EBs, and stained for chlamydial antigen. Bar, 20 μm. Results were expressed in percentage compared with scramble, taken as 100%. Bars represent median values, error bars represent median absolute deviations. Statistical significance was calculated vs control. \*  $P \leq 0.01$

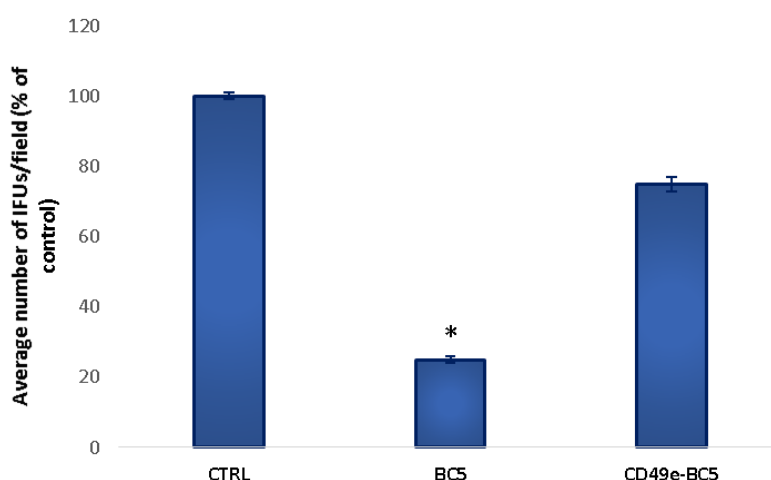
### 4.3. Understanding *L. crispatus* BC5 mechanism of action

Once the role of  $\alpha 5$  integrin subunit in promoting CT infection has been demonstrated, it remained to elucidate what was the mechanisms by which *L. crispatus* BC5 exerted its protective effect.

First, it was investigated whether the protective mechanism could be ascribed to a direct interaction between *L. crispatus* BC5 and  $\alpha 5$  integrin subunit or, alternatively, to a direct interaction between lactobacillus and *Chlamydia*.

#### 4.3.1 The pre-incubation with the antibody anti-CD49e inhibited the protective effect of *L. crispatus* BC5

In order to elucidate *L. crispatus* BC5 mechanism of action, BC5 CP was pre-treated 1h with CD49e antibody and then employed to counteract CT infection. CT infectivity was evaluated by counting the number of IFUs/field and results are reported in **Figure 4.13**. Notably, the pre-incubation of *L. crispatus* BC5 with anti- CD49e antibody led to a loss of protective capability.

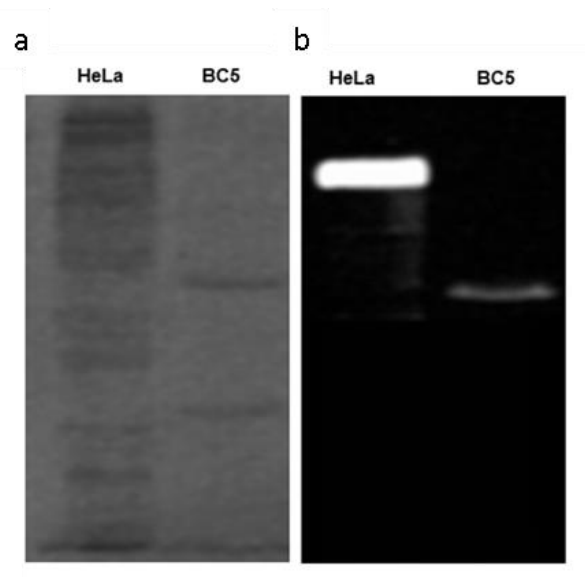


**Figure 4.13** CD49e antibody inhibited *L. crispatus* BC5 protective role. HeLa cells were treated with *L. crispatus* BC5 CP not treated or pre-treated with an anti- CD49e antibody for 1 h. HeLa/lactobacilli co-culture were then incubated with CT EBs for 48 h (exclusion). HeLa cells incubated CT EBs for 48 h were used as control. *C. trachomatis* infectivity was evaluated as number of IFUs/microscopic fields. Results were expressed in percentage compared with control taken as 100%. Bars represent median values, error bars represent median absolute deviations. Statistical significance was calculated vs control. \*  $P \leq 0.01$ .



In order to understand the meaning of these results, a western blot was performed . HeLa cells lysate and *L. crispatus* BC5 lysate were resolved by 10% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane and then immunoblotted with an anti-CD49e antibody. The anti-CD49e antibody recognized a specific band for  $\alpha 5$  integrin subunit in HeLa cells lysate of 140 KDa, but also a specific band in BC5 lysate of about 50 KDa.

The results reported in **Figure 4.14** indicated that the mechanism of protection from *C. trachomatis* infection could not be ascribed to a direct interaction between *L. crispatus* BC5 and  $\alpha 5$  integrin subunit.

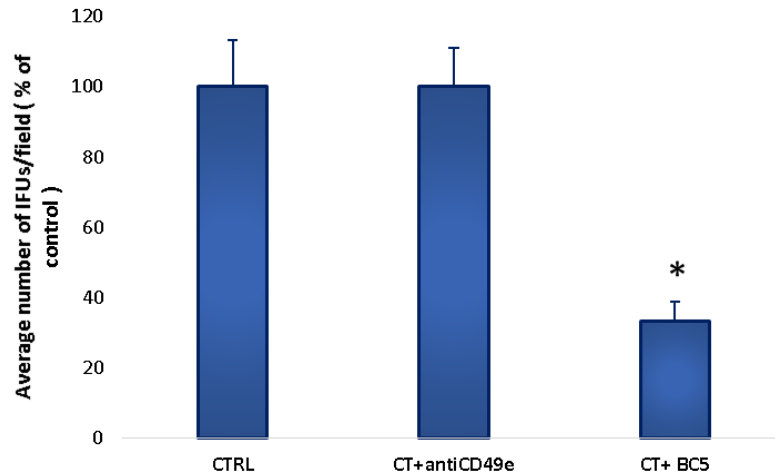


**Figure 4.14. SDS-PAGE and Western blot.** HeLa cells lysate (CTRL) and BC5 lysate were separated by SDS-PAGE (a) and the western blot membrane (b) was labeled with anti- CD49e antibody.

#### 4.3.2 *L. crispatus* acts by sequestering *Chlamydia*

In order to verify whether the protective mechanism of *L. crispatus* BC5 could be ascribed to a direct interaction with *C. trachomatis*, CT EBs were first incubated 1h with anti-CD49e or with *L. crispatus* BC5 CP, and then incubated with HeLa cells for 48h. The results reported in **Figure 4.15** showed as the pre-incubation of CT EBs with *L. crispatus* BC5 CP reduced CT infectivity by approximately 67%. On the contrary, the pre-incubation of CT EBs with anti-CD49e did not affect CT infectivity.

Thus, these results indicated that *L. crispatus* BC5 interacted directly with *C. trachomatis*, sequestering it and preventing its binding to integrin.



**Figure 4.15 The pre-incubation of CT EBs with *L. crispatus* BC5 inhibited *C. trachomatis* infection.**

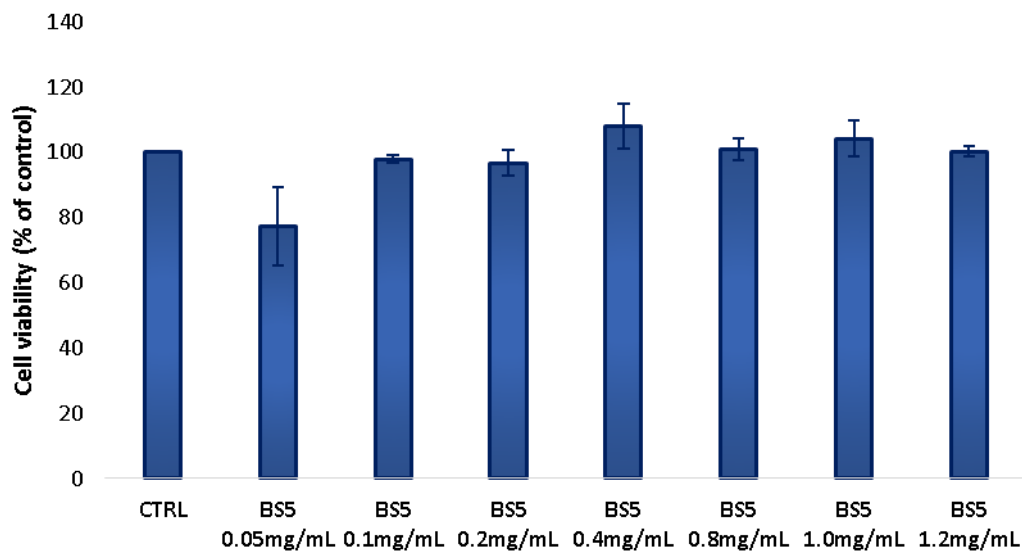
*HeLa* cells were incubated for 48h with CT EBs pre-treated 1h with anti- CD49e antibody or pre-treated 1h with *L. crispatus* BC5 . *HeLa* incubated with not treated CT EBs for 48 h were used as control. *C. trachomatis* infectivity was evaluated as number of IFUs/microscopic fields. Results were expressed in percentage compared with control taken as 100%. Bars represent median values; error bars represent median absolute deviations. Statistical significance was calculated vs control. \*  $P \leq 0.01$ .

### 4.3.3 Effect of released surface-associated components of *L. crispatus*

#### BC5 on *C. trachomatis* infection

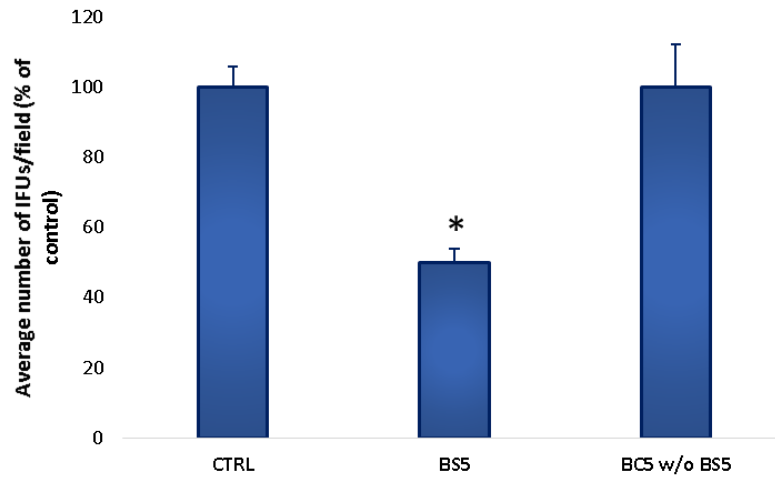
It is known that among the released surface- associated components there are amphiphilic compounds such as biosurfactants, produced by microorganisms, which can be localized on their cell surface, or secreted extracellularly. It is known that these molecules exert several functions including the capability to act as antimicrobial, antiadhesive and antibiofilm agents (Gudiña et al., 2013, Sambanthamoorthy et al., 2014, Giordani et al., 2019). In this perspective, released surface-associated components were isolated from *L. crispatus* BC5 (BS5). In order to verify its cytotoxic

effect, BS5 was administered to HeLa cells for 24h, in a range between 0.05 mg/mL and 1.2 mg/mL. Cells viability was evaluated by using Alamar blue assay. The results reported in **Figure 4.16**, showed as BS5 had no cytotoxic effect on HeLa for all the tested amounts.



**Figure 4.16 Cytotoxicity assay.** The effect of BS5 isolated from *L. crispatus* BC5 on cells viability was evaluated by means Alamar blue absorbance in control and treated HeLa cells. Results were expressed in percentage compared with control taken as 100%. Bars represent mean value; error bars represent standard deviations. Statistical significance was calculated vs control. \*  $P \leq 0.05$ .

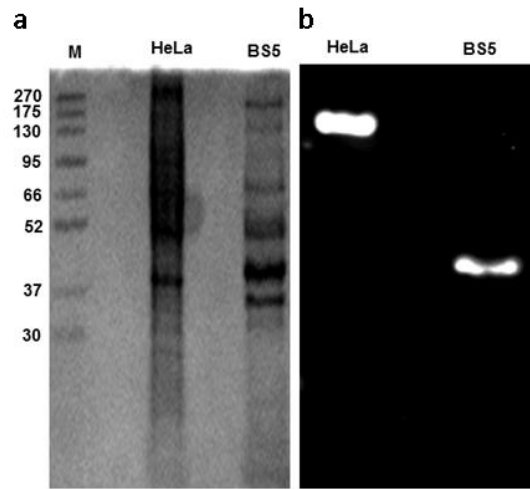
Afterwards, HeLa cells were incubated with BS5 (0.05 mg/mL) or with BC5 CP deprived of BS5 for 1h at 37°C, and subsequently incubated with CT EBs for 48h. CT infectivity was evaluated by counting the number of IFUs/field and results, reported in **Figure 4.17**, showed as the treatment with BS5 significantly reduced *C. trachomatis* infection by approximately 50%, while BC5 deprived of BS5 lost its protective effect. These results demonstrated that BS5 was an important component in conferring protection from *C. trachomatis* infection, since *L. crispatus* BC5 deprived of BS5 lost its protective capability.



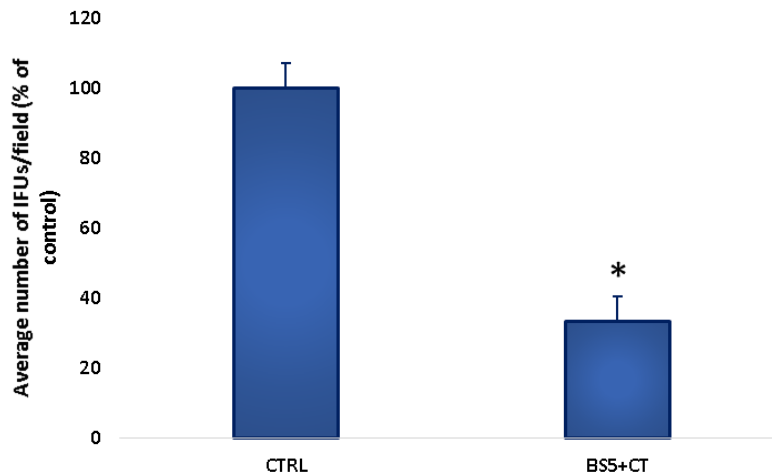
**Figure 4.17 BS5 prevents *C. trachomatis* infection of HeLa cells.** Control HeLa cells, HeLa treated with 0.05 mg/mL BS5 or with BC5 deprived of BS5 were infected with CT EBs. *C. trachomatis* infectivity was evaluated as number of IFUs/microscopic fields. Results were expressed in percentage compared with scramble, taken as 100%. Bars represent median values; error bars represent median absolute deviations. Statistical significance was calculated vs control. \*  $P \leq 0.01$ .

As before, in order to investigate the mechanism by which the BS5 isolated from *L. crispatus* BC5 could protect from *C. trachomatis* infection, a western blot was performed. HeLa cells lysate and BS5 were resolved by 10% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane and then immunoblotted with an anti-CD49e antibody. Results are showed in **Figure 4.18**. Similar to the experiment previously described in **Paragraph 4.3.1**, the anti-CD49e antibody recognized also in BS5 a specific band of about 50 KDa. As well as for *L. crispatus* BC5, BS5 mechanism of protection from *C. trachomatis* infection could not be ascribed to a direct interaction with  $\alpha 5$  integrin subunit. Afterwards, it was investigated whether BS5 could bind directly to *C. trachomatis* as well. CT EBs were first incubated 1h with BS5 (0.05 mg/mL) and then incubated with HeLa cells for 48h. The results reported in **Figure 4.19** showed that the pre-incubation of CT EBs with BS5 reduced CT infectivity by approximately 67%. In conclusion, these results indicated

that BS5, as well as *L. crispatus* BC5, interacted directly with *C. trachomatis*, sequestering it and preventing its binding to integrin.



**Figure 4.18. SDS-PAGE and Western blot.** HeLa cells lysate(CTRL) and BS5 were separated by SDS-PAGE (a) and the western blot membrane (b) was labeled with anti- CD49e antibody.



**Figure 4.19. The pre-incubation of CT EBs with BS5 inhibited *C. trachomatis* infection.** HeLa cells were incubated for 48h with CT EBs pre-treated 1h with BS5. HeLa incubated with not treated CT EBs for 48 h were used as control. *C. trachomatis* infectivity was evaluated as number of IFUs/microscopic field. Results were expressed in percentage compared with control taken as 100%. Bars represent median values; error bars represent median absolute deviations. Statistical significance was calculated vs control. \*  $P \leq 0.01$ .

## **CHAPTER 5**

## **CONCLUSIONS**

The vaginal microbiota, dominated by lactobacilli, play a pivotal role in protecting against several pathogenic microorganisms, including *C. albicans* and *C. trachomatis*. In the state of mucosal health, the microorganisms constituting the vaginal microbiota coexist in a perfect balance and the resulting homeostasis derives from a deep and complex interaction between them. The rupture of homeostasis, and in particular the lack of lactobacilli, contribute to creating a micro-environment suitable for the growth of pathogenic microorganisms (Petrova et al., 2015). The protective role of lactobacilli against urogenital pathogens is exerted through different mechanisms including the production of various antibacterial compounds (lactic acid, hydrogen peroxide, bacteriocins and biosurfactants), the competitive exclusion for epithelial adhesion, and the immunomodulation (Kaewsrirachan et al., 2006; Borges et al., 2014; Parolin et al., 2015; Younes et al., 2018). *Lactobacillus* spp. interaction with the vaginal epithelial cells is the first step in the formation of the biological barrier against colonization of opportunistic and pathogenic organisms. The blockage of undesirable microorganisms adherence by lactobacilli may take place by exclusion, competition, and displacement mechanisms (Coman *et al.*, 2015; Osset *et al.*, 2001; Verdenelli *et al.*, 2014).

In this project, some lactobacilli strains, previously isolated from healthy vaginal swabs by Parolin et al., (Parolin et al., 2015) were tested in order to evaluate their protective role against *C. albicans* and *C. trachomatis* infections. Specifically, the first part of this thesis regarded the study of lactobacilli protection versus *C. albicans* infection, and *L. crispatus* BC1 and BC2, *L. gasseri* BC9 and BC11, and *L. vaginalis* BC15 were employed. Two mechanisms of action at the basis of the protective role of lactobacilli against *C. albicans* were identified. The first mechanism was those exerted by *L. crispatus* BC1 and *L. gasseri* BC9 which interacting with HeLa cell plasma membrane caused a modification of polar lipids organization and increased membrane fluidity. The second mechanism was exerted by *L. crispatus* BC2, *L. gasseri* BC11 and *L. vaginalis* BC15 which acted modulating  $\alpha 5\beta 1$  exposure on HeLa plasma membrane. Both mechanisms resulted in the inhibition of *C. albicans* adhesion to HeLa cells.

The second part of the present thesis aimed to identify vaginal Lactobacilli strains able to interfere with *C. trachomatis* infection process. Specifically, *L. crispatus* BC4 and BC5, *L. gasseri* BC14 and *L. vaginalis* BC17 were tested, and *L. crispatus* BC5 was chosen as model strain because was the most active strain in counteract *C. trachomatis* adhesion to HeLa cells. Importantly, through siRNA silencing of ITGA5 gene, we demonstrated that *C. trachomatis* needs of  $\alpha 5$  integrin subunit for its adhesion and internalization into HeLa cells. Furthermore, our results showed that *L. crispatus* BC5 was able to protect from *C. trachomatis* infection by means of a dual mechanism. On the one hand, *L. crispatus* BC5 interaction with HeLa caused an increase of plasma membrane fluidity and a reduction of  $\alpha 5$  integrin exposure on cell surface, thus making this protein less available for *C. trachomatis* binding and internalization. On the other hand, *L. crispatus* BC5 directly interacted with *C. trachomatis*, grabbing it and thus preventing its binding to  $\alpha 5$  integrin. Interestingly, both in *L. crispatus* BC5 lysate and in BS5, through western blot was identified a protein similar to  $\alpha 5$  integrin which could be responsible for *C. trachomatis* binding.

In conclusion, this study allowed a deeper understanding on the mechanisms underlying the protection against pathogenic microorganisms, in the specific case *C. albicans* and *C. trachomatis*. Thanks to their characteristics and to protective effects against pathogens, lactobacilli herein studied, with particular emphasis on *L. crispatus* BC5, could be good candidates for their use as probiotic agents promoting woman's vaginal health.



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