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Effects of sugars and lactic acid isomers on *Chlamydia trachomatis* infectivity, an *in vitro* study

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1.Abstract

The thesis investigates two different *in vitro* aspects of *Chlamydia trachomatis* (CT), a bacterium responsible for one of the most spread sexually transmitted infection (STI). First, since previous works suggest that sugars can have a beneficial effect on CT survival and virulence, the thesis analyzes the effect of different sugars on CT infectivity, elucidating some of the molecular mechanisms behind CT-sugar interaction. CT infectivity is investigated on HeLa cells after 2 hour-incubation of elementary bodies (EBs) with glucose, sucrose, or mannitol solutions (0.5, 2.5, 5.0 mM). The effect of sugars on EB membrane fluidity is investigated by fluorescence anisotropy measurement, whereas the changes in lipopolysaccharide (LPS) exposure are examined by cytofluorimetric analysis. By means of a Western blot, the phosphorylation state of Focal Adhesion Kinase (FAK) in HeLa cells infected with EBs pre-incubated with sugars it's explored. All sugar solutions significantly increase CT infectivity on epithelial cells, acting directly on the EB structure. Sugars induce a significant increase of EB membrane fluidity, leading to changes in LPS membrane exposure. Especially after incubation with sucrose and mannitol, EBs lead to a higher FAK phosphorylation, enhancing the activation of anti-apoptotic and proliferative signals in the host cells.

Secondly, the thesis explores the protective effect of two different Lactobacilli against CT infection: *Lactobacillus crispatus* and *Lactobacillus reuteri*. CT infectivity is evaluated after host cells were treated for 1 hour with diluted supernatant cell-free fraction or with the bacterial cells. Assessed that *L.crispatus* is more protective than *L.reuteri,* total lactic acid production is evaluated by HPLC. Subsequently, to understand the role of the two isomers of lactic acid in conferring protection, Lactate dehydrogenases activity is evaluated by resazurin assay and by LC-MS. Then, D-lactate dehydrogenase specific activity has been investigated by measuring NADH formation. Afterwards, addition of D or L-lactic acid to *L.reuteri* supernatant has been performed and their effect in promoting protection in the host cells assessed by infection experiments. Subsequently a metabolic analysis has been carried out by real-time measurement of HeLa mitochondrial respiration after treatment with diluted lactobacilli supernatants and with a D-lactic acid solution. Finally, histone acetylation and lactylation, as well as gene and protein expression of relevant targets of treated HeLa cells, have been investigated in order to understand the greater protection conferred by D-lactic acid. Results show that the D isomer is more efficient in conferring protection from CT infection, causing a shift in the host cell metabolic profile and a pattern of histone modifications that changes the expression of important targets, such as cell cycle regulators.

2.Introduction

2.1 Chlamydia trachomatis

2.1.1 General characteristics

Chlamydia trachomatis is a bacterium responsible for various diseases in humans, many of which are sexually transmitted. *C. trachomatis* belongs to the family of Chlamydiaceae, a group formed by oval

Gram-negative bacteria. These bacteria are immobile, obligate endocellular parasites of eukaryotic cells, which can be hosts of a vast set of organisms, including several mammals. Being obligate intracellular organisms (Figure 1), chlamydiae are entirely dependent on the host cell for metabolism. They can derive ATP and other biosynthetic intermediates to complete their own life cycle only by the host cell.

Initially, due to the small size and obligate parasitism, the chlamydiae they have been mistakenly considered to be large viruses. In the light of subsequent evidence, such as the presence of the cell membrane with a structure similar to Gram-negative bacteria, the simultaneous presence of both nucleic acids (DNA and RNA), the presence of ribosomes and the spectrum of sensitivity to numerous antibiotics, a real reclassification was carried out. That included them in all respects among the bacteria. Within this family, *Chlamydia trachomatis* and *Chlamydia pneumoniae* represent the two species of greatest medical interest, as they are responsible for infections affecting humans. Although these infections are very often asymptomatic or pauci-symptomatic, in some cases they may be associated with disabling complications with significant morbidity.

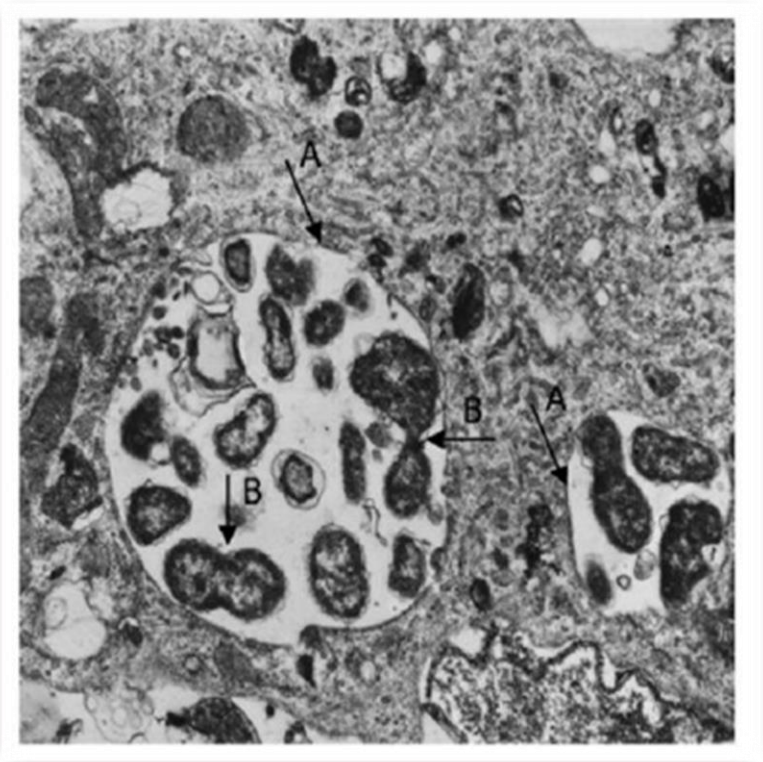

Figure 1: Electron microscope section of a cell infected with *C. trachomatis*. Inclusion membrane **(A)** and reticular bodies in active replication inside the inclusion **(B)** [1].

2.1.2 Life-cycle, structure and genomics

Chlamydia has a dimorphic life cycle characterized by the alternation of two different biological forms: the Elementary Body (EB) and the Reticular Body (RB). The elementary body has a spherical shape and represents the infecting form of Chlamydia, being able to initiate the infectious cycle. It has rather modest dimensions (0.25-0.35 μm in diameter), dense and compact cytoplasm, and is capable of surviving the extracellular environment. Its target is represented by cylindrical epithelial cells, of different organs and systems, such as the conjunctival, urethral and endocervical epithelium [2]. Although historically the EB has always been considered inert from the metabolic point of view, recent studies have identified limited metabolic activity in the elementary bodies of *Chlamydia*, mainly based on the consumption of sugars [3]. The reticular body, on the other hand, represents the actively replicating form that originates from elementary body inside the host cell, at the level of the endocytosis vacuoles (Figure 1). Its dimensions are larger than the EB (0.5-1 μm) and it is characterized by an intense metabolic activity at the same time as sustained replication.

This life cycle has a duration of about 48-72 hours and begins with anchoring and invasion of the host cell by the elementary body (Figure 2). The EB, once has penetrated into the cell through a phagocytosis mechanism induced by contact with the cell cytoplasmic membrane, resides within a phagosomal vacuole and in the arch of 4-5 hours undergoes a reorganization process with the conversion to RB. After about 8-12 hours, *Chlamydia* actively reproduces by binary fission inside the cell with the consequent increase in the volume of the endosome. These vesicles of cytoplasmic endocytosis, defined as 'inclusions', do not undergo lysosomal acidification or fusion and are easily observed under the optical microscope of preparations stained with Giemsa or by direct immunofluorescence, as in figures 2 and 3.

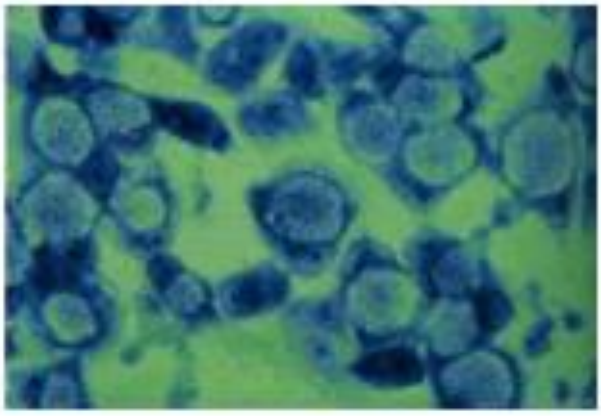

Figure 2: In vitro cultured cells, experimentally infected with C. trachomatis, viewed under a microscope optical normal after staining with the Giemsa method. Almost all cells have almost cytoplasm completely occupied by a roundish 'inclusion' with a reticular appearance that pushes the nucleus to the periphery of the cell.

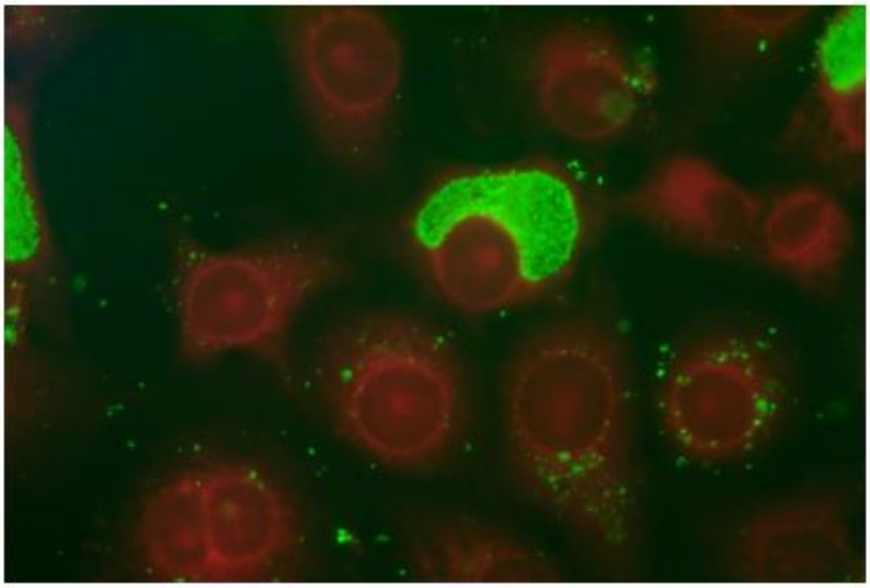

Figure 3: *C. trachomatis* inclusions highlighted by immunofluorescence. The nuclei of infected cells and uninfected cells appear red due to the presence of Blue Evans, a contrast dye. 400x magnification.

About 18-24 hours after infection, a dehydration and condensation process transform some RBs back into EBs. Finally, approximately 48-72 hours after infection, lysis of the vacuolar and cytoplasmic membrane occurs, with the consequent release of EBs in the external environment, ready to infect new neighboring cells and to initiate a new replication cycle. A summary representation of the life cycle of *Chlamydia* is present in **Figure 4**.

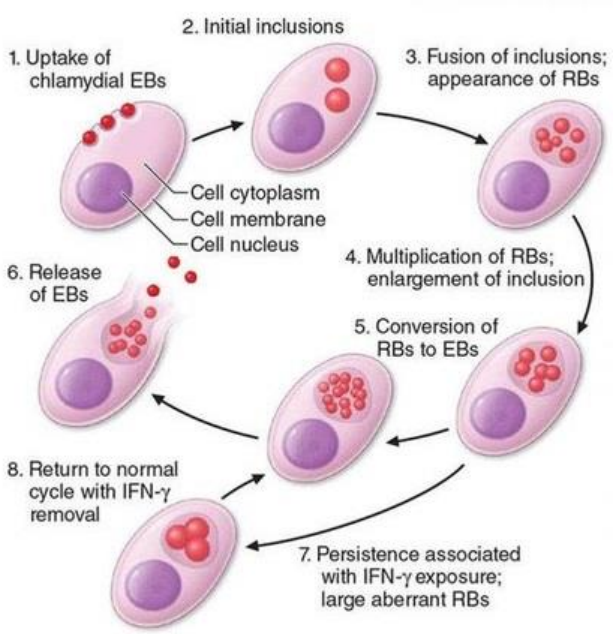

Figure 4: Chlamydia life cycle

As for the structure of the bacterial cell, *Chlamydia trachomatis* has an external membrane similar to that of Gram-negative bacteria, consisting of a lipopolysaccharide component (LPS) and a protein component represented by OMPs (Outer Membrane Proteins) (Figure 6). Although the cellular organization is similar to that of Gram-negative bacteria, the chlamydiae do not possess peptidoglycan in the cell wall, a property it confers the natural insensitivity to β-lactam antibiotics [4]. However, in the genome of chlamydiae all the genes necessary for the synthesis of peptidoglycan are present [5]. It has therefore been hypothesized that the peptidoglycan is degraded during the differentiation of the reticular body into an elementary body from amidase, whose gene is present in the chlamydia genome [7].

A peculiar feature of chlamydiae is the low toxicity of the component endotoxin of the LPS, which with reduced ability to activate the system of complement and stimulation of cytokine release. From

an evolutionary point of view, this low cytotoxicity is related to the life cycle of Chlamydia itself: a toxic action of LPS that interfere with the biosynthetic and metabolic capacity of the host cell it would be detrimental to obligate intracellular pathogens. The LPS of the chlamydia possesses some antigenic determinants in the polysaccharide portion common with other Gram-negative bacteria and between different species of chlamydiae (group or genus specific antigens).

Among the different OMPs proteins, the most significant are represented by:

• MOMP (Major Outer Membrane Protein): this protein alone represents 60% of all OMPs, and it has a high degree of homology between the different species of chlamydia. This protein, encoded by the omp1 gene, is essential to guarantee membrane integrity [8] [9], plays a key role in host cell adhesion and several accessory roles during successive stages of the replication cycle. Genus-specific, species-specific antigens are found on the MOMP and type-specific (serovar) to which antibodies are produced in the course of infections human [9].

• Large CrP and Small CrP: these are Cystein rich Proteins. They differ from each other for the molecular weight, respectively 60 KDa (large CrP) and 9 Kda (small CrP). They are synthesized only late in the development cycle. Great CrP forms a protein layer that is at the base of the lipopolysaccharide layer, while CrP Small acts as a connection between the two layers. The disulfide bridges that are established between cysteine residues of the CrPs are responsible for the strong membrane stiffness that allows the CE to survive in extracellular environments. In the intracellular phase this rigidity is lost with the conversion of the CE to CR, during which many disulfide bridges are reduced. The structure of the chlamydial outer envelopes appears to be related to the capacity of the chlamydiae to prevent fusion of the phagosome into which they are introduced with lysosomes, inside the parasitic cell.

The Chlamydia genome, considered one of the most modest at the prokaryotic level. Its bacterial chromosome is made up of about 1000 Kbp, and encodes about 600 proteins. However, it is deficient for genes encoding various enzymes involved in amino acid synthesis. This aspect reflects the obligate intracellular parasitism of Chlamydia and its auxotrophy against some amino acids, such as tryptophan. The degree of genomic similarity in the various *C. trachomatis* serotypes is extremely high, while the interspecies homology, as far as chromosomal DNA is concerned, is low [10] [11]. In particular, the region with the greatest genomic diversity appears to be the coding one for virulence factors, called “Plasticity Zone” [12]. In addition to the genome, some extra-chromosomal elements have been identified. The first plasmids they were described in 1980 by Lovett and colleagues. They have dimensions equal to 7.5 Kbp and are present in variable numbers, about 5-10 copies per bacterial cell. These plasmids have on average 8 “Open Reading Frames”

(ORFs) which code for different proteins each consisting of more than 100 amino acids, interspersed with short non-coding sequences [13] [14]. Its replication is closely linked to the bacterial replicative cycle, and is especially active during the division of reticular bodies. Chlamydia plasmids are non-conjugative and are not involved in bestowal antibiotic resistance; it is believed that they play an important role in the accumulation of glycogen [14]. Since the variations within the nucleotide sequence of *C. trachomatis* are <1%, plasmids are considered to be very conservative.

Chlamydiae are able to independently synthesize various organic substances a low molecular weight and can provide for macromolecular syntheses in an autonomous way, provided they have the appropriate precursors available. Metabolic defect main cause of their obligate endocellular parasitism is the absence of any enzymatic system for the production of energy (ATP), therefore they are forced to procure from the host cell of ATP and other cofactors (NAD, CoA, etc.) with which integrate, in terms of energy, their biosynthetic capacities [15] [16] [17] [18].

2.1.3 Classification

Chlamydia trachomatis strains are classified into 18 serovars (serotypes) or genovars (genotypes) according to the antigenic differences of the “Major outer membrane protein” (MOMP) or of the polymorphism of the ompA gene (omp1), coding for the MOMP protein itself [19]. The 'Major Outer Membrane Protein' is used as the basis for the classification of Chlamydia serotypes as it represents a species and subspecies-specific antigen, against which the antibody response is most concentrated during infections (immunodominant). Against the MOMP protein, organisms infected with *C. trachomatis* produce neutralizing antibodies [20], which are responsible for most part of the reactivity in antibody assay tests. Structurally the MOMP is characterized by 4 hydrophilic domains exposed outside the cell membrane, surrounded by 5 hydrophobic domains present on the inner surface (Figure 7).

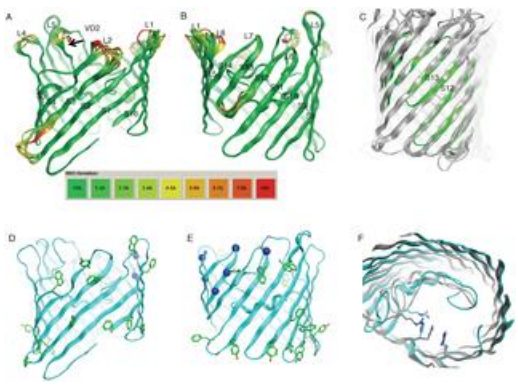

Figure 5: three-dimensional representation of the Chlamydia MOMP protein [21]

These 18 serovars can then be grouped into 3 different biovars based on their similarities in physiological and biochemical functions:

➢***Trachoma biovar***: includes serovars A-C. These serotypes are associated with "Trachoma endemic", an eye infection which, if not properly treated, can lead to blindness. This biovar is mostly present in developing countries.

➢***Genital Tract biovar***: includes D-K serotypes. These serotypes cause sexually transmitted infections and lead to diseases of the urogenital tract. In women, the infection can spread to the ascending genital tract (15-40% of cases) and give rise to complications such as infertility tubal congestion, pelvic inflammatory disease and increased risk of ectopic pregnancies.

➢***Lymphogranuloma Venereum (LGV) biovar***: includes serotypes L1-L3, associated with lymphogranuloma venereum disease. Compared to the manifestations of the classic LGV, typical of the countries tropical and sub-tropical, in industrialized countries the infection has been mostly characterized by a hemorrhagic proctitis, typical of homosexual male patients HIV-positive.

2.1.4 Pathogenesis

Infections caused by C. trachomatis mainly involve epithelial cells of the urogenital (urethra and cervix), rectal and ocular mucous membranes. Despite being obligate parasites with clear cytolytic potential (as evident in *in vitro* infected cell cultures), the evidence of a consistent toxic/cytolytic effect *in vivo*, are modest. In fact, at the level of the infected mucous membranes, there are only a little number of infected cells compared to the extent of the damage. On the other hand, in an acute infection, it is evident an intense infiltration of polymorphonuclear cells, T lymphocytes and B lymphocytes [22] with a consequent production of inflammatory cytokines. It is therefore thought that the host immune response to the infection is involved in the genesis of the damage to the mucous membranes. The innate immune response plays an important role in controlling infection chlamydia, although its action is short-lived and not serotype specific [23]. Humoral immune response is instead important in cases of reinfection.

The initial response to infection occurs within two days of infection, via a submucosal infiltration of neutrophils and a small number of monocytes. Later, in the infection site, there is great quantity of T lymphocytes, which play an important role in the control of the infection. At the same time, the

cells of the epithelium release pro-inflammatory factors as cytokines and chemokines, including IL-1, IL-8, IL-6 and GM-CSF [24]. Such factors attract, at the site of infection, numerous innate immune cells such as Natural killer (NK) [25], dendritic cells [26], and neutrophils [22]. Other cytokines produced during *C. trachomatis* infection, include IL-10 and TNFα.

NK cells, under stimulation of some ILs, produce Interferon-γ (IFN-γ), which has the task to promote the differentiation of CD4+ T lymphocytes into Th1. In addition to this function, the IFN-γ helps block chlamydia replication by inducing expression of the cellular enzyme indoleamine-2,3-dioxygenase (IDO), which transforms tryptophan into kynurenine. In this way the host cell is deprived of tryptophan and chlamydia no longer manages to use this amino acid, essential for its metabolism and subsequent replication. As a result, the bacterium assumes a non-infectious form metabolically less active, defined as "persistent", which is maintained until the bacterium does not find the conditions suitable for intracellular replication, which is further inhibited by the action of anti-Chlamydia antibodies. These antibodies are produced by plasma cells and are directed against the antigens of the bacterium: LPS, MOMPs and bacterial heat shock proteins (HSPs).

➔Lipopolysaccharide (LPS) is the major group-specific antigen; it is present on the surface of the elementary body and the reticular body during the life cycle of chlamydia. It has two main antigenic domains: one specific for chlamydia, the other completely similar to the LPS of other Gram-negative bacteria [27].

➔Major Outer Membrane Protein (MOMP) represents a species and sub antigen species-specific, against which the antibody response is most concentrated (immunodominant). Against MOMP, organisms produce antibodies neutralizers [20], which are responsible for most of the reactivity of the serum antibody dosage test.

➔Heat Shock Proteins, in particular HSP10 and HSP60 [28]. These proteins share high levels of homology with human cell HSPs. Production of antibodies directed against antigenic epitopes of these proteins could lead to cross-reactions against epithelial components of human tissues. It is thought that such cross-reactions are at the basis of possible chronic damage, following a *Chlamydia trachomatis* infection.

However, a high antibody titer does not appear to be closely related to healing, but is present in cases of infections with a high degree of systemic involvement (such as lymphogranuloma venereum) or associated with the development of complications (such as PID). In particular, antibodies produced against the MOMP protein appear to be prevalent in the acute stages of the

infection, while high levels of antibodies directed against HSP60 are found more frequently in case of recurrent infections [29].

24-48 hours after infection, infiltration of phagocytes occurs. Those phagocytes are polymorphonuclear cells (PMNs) [30], recalled by the activation of complement by the elementary bodies, or, alternatively, by antibodies (chemotactic action). Although with the resolution of the infection, there is an attenuation of the inflammatory response but chronic damage can be generated due to fibrotic phenomena and to persistent scar formation. Indeed, if in the acute phase there is local neoformation of lymphatic "follicles" with typical germinal centers, in the late phase of the infection formation of fibrosis and scarring occur at the level of the mucosa, with consequent loss of functional activity (with particularly serious consequences in ocular trachomatous lesions or in fallopian tube lesions). The host cell's immune response to primary Chlamydia infection, however, in most patients, it is temporal and not associated with the injury tissue [31]. If, on the other hand, a balance is reached between the host's defense mechanisms and the multiplication of the microorganism, the bacterium is not completely eliminated and can persist in the cell in a state of latency. These latent clinical forms are usually asymptomatic, often undiagnosed and untreated; they are a relevant source of infection transmission.

C. trachomatis in the host can therefore lead to persistent infections characterized by one incomplete development of the microorganism, with sporadic production of elementary bodies, but causing recurring infections. One more factor important in the defense mechanisms against chlamydia infection is represented by host cell redox system. In the early stages of the infection, production of reactive oxygen species (ROS) contributes to the elimination of the infectious agent. The concentrations of reactive oxygen species are decisive for the cell viability: low levels of ROS condition the replication mechanisms, while high ROS levels cause cell death. Studies in the literature show that the survival of an infectious agent within a host cell is linked to its own ability to induce a state of oxidative stress, while a reduced production of ROS promotes the genesis of a chronic infection [32]. In Figure 6, the immune response triggered by is schematically represented a Chlamydia infection.

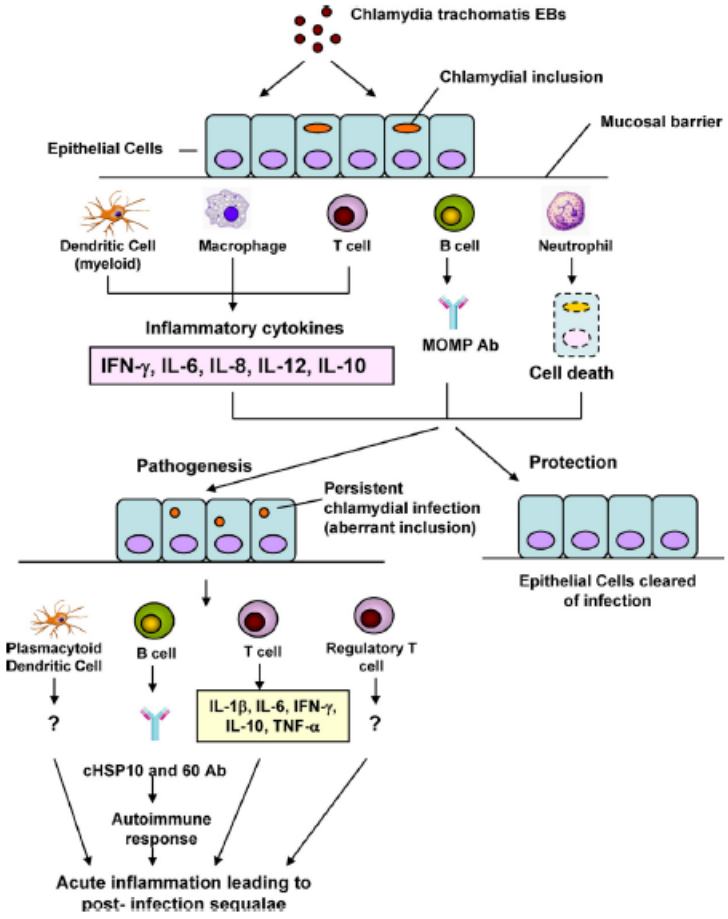

Figure 6: Schematic representation of the immune response to *C.trachomatis* infection [33].

2.1.5 Epidemiology

C. trachomatis is the agent responsible for the most sexually transmitted bacterial infection widespread in the world, with important sequelae both from a clinical and socioeconomic point of view. Data on the incidence of *C. trachomatis* genital infections vary widely between countries and reflect what are the habits and sex education of the population, but also the preventive, diagnostic and therapeutic measures in the country. In Europe, the incidence of *C. trachomatis* infections is around 200 new cases/100,000 inhabitants in one year [34]. However, this incidence is probably underestimated because asymptomatic infections remain undiagnosed, especially in women. The age group most affected is between 15 and 24 years, which overall represents more than 70% of cases, with a further peak observable between 20 and 24 (**Figure 7**). There are many risk factors that can lead to infection. The most relevant are represented by inappropriate sexual behavior, characterized by a high number of partners, by the lack of use of barrier contraceptives and by the early beginning of sexual activity.

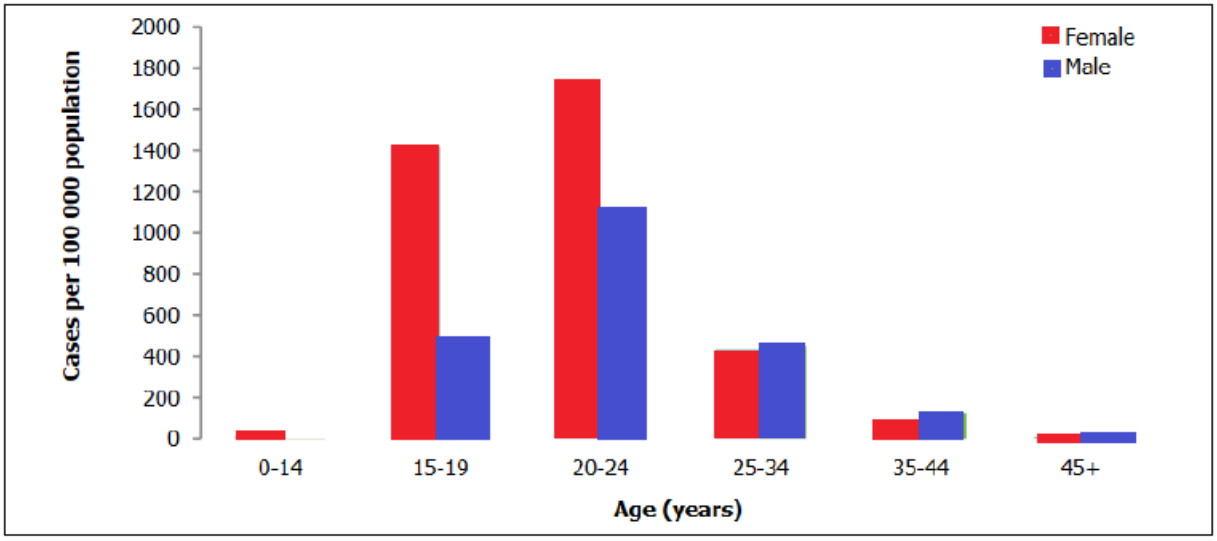

Figure 7: *C. trachomatis* cases by age category and gender [34].

The incidence is higher in the female population (Figure 7), but this data is probably due to the greater attention to screening among women of childbearing age rather than among men. In recent years there has been a progressive increase in cases of *C. trachomatis* infection mainly due to the adoption of more sensitive diagnostic methods and screening campaigns carried out in several countries to reduce the distant complications of this infection, such as the disease pelvic inflammatory disease, tubal infertility and the risk of ectopic pregnancy. As shown in Figure 8, the majority of infection cases (>80%) occurs by transmission during heterosexual intercourse, while only 9% do associated with homosexual relations between males. Finally, the percentage of neonatal infections, acquired during delivery from mothers with chlamydia cervical infections, is rather low (<1%).

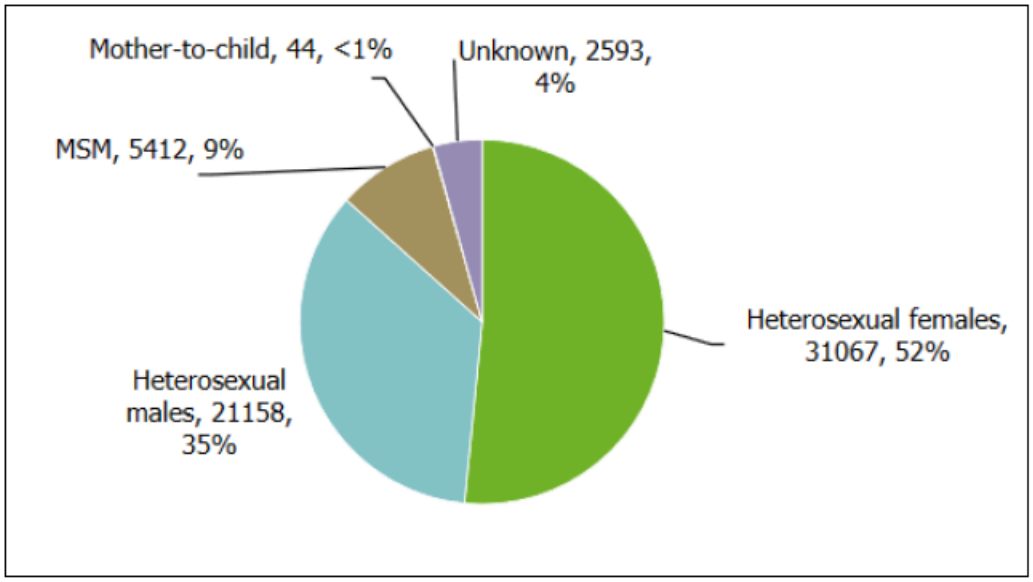

Figure 8: Cases of *C. trachomatis* infections according to sexual behaviour [34].

On the other hand, the situation is different for the L1-L3 serotypes of *Chlamydia trachomatis*, responsible for LGV. In the last 10 years, in fact, in industrialized countries (North America, Europe and Australia) numerous cases of rectal LGV have been recorded in male homosexuals (MSM or "men who have sex with men") especially in the age group aged between 25 and 45 (Figure 9) [35].

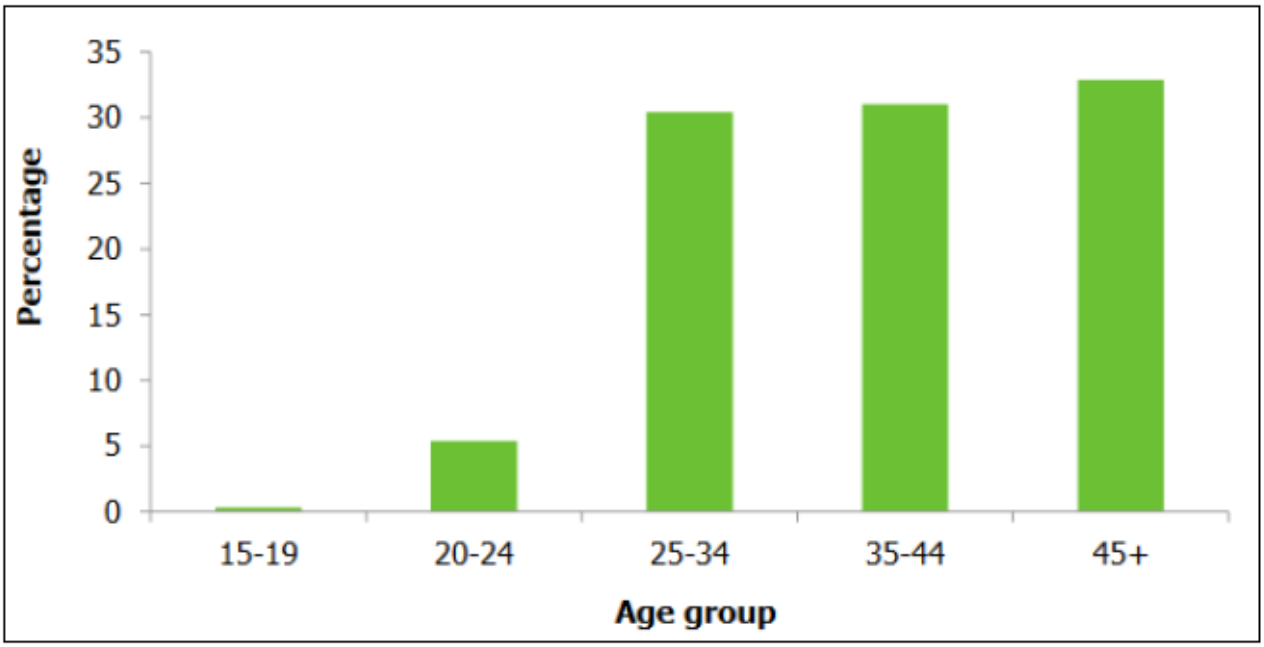

Figure 9: percentage of LGV cases by age group [35].

Despite the recognized importance of chlamydial infections in terms of incidence and in terms of severity of potential consequences, do not currently exist common screening programs in various European countries and in most of them, as in Italy, *Chlamydia trachomatis* is not among the infections subject to mandatory notification.

2.1.6 Clinical manifestations

C. trachomatis infections are characterized by different pathological characteristics, which involve many fields of medicine, such as dermatology, venereology, ophthalmology, gynecology-obstetrics and rheumatology [36]. The serotype and strain-specific virulence differences underlie the different clinical manifestations of *C. trachomatis* infections. The classification of *C. trachomatis* in the

different serotypes, previously described, yes translates into the various clinical forms of infection for which it is responsible.

D-K serotypes infections in adults mainly involve the uro-genital district, causing urethritis in men and cervicitis in women. Despite this, in both sexes, according to the type of sexual intercourse, other anatomical districts may be involved, such as the pharynx and the rectum.

In women, *Chlamydia trachomatis* uro-genital infection it is mainly characterized by cervicitis, possibly associated with urethritis, after an incubation period of approximately 2-3 weeks. Urogenital infections in women are often asymptomatic (up to 50-70% of cases) or characterized by mild symptoms e non-specific. When present, symptoms include the presence of mucus-purulent secretions cervicovaginal (leucorrhea), dysuria, pelvic pain, dyspareunia and postcoital bleeding. On physical examination of the endocervix, in about half of the cases, signs of local inflammation are observed, with mucus-purulent secretion and ease of al bleeding. There is also an inflammation of the epithelium columnar and sub-epithelial layer of the endocervix.

If left untreated, genital infection can persist for several months with the risk of complications and sequelae due to the resurgence of the upper genital tract infection. Indeed, endometritis can follow cervicitis with the potential to spread the infection up to the fallopian tubes (salpingitis) and to the ovaries, with risk of developing pelvic inflammatory disease (PID: Pelvic Inflammatory Disease). It is estimated that around 10% (9- 14%) of untreated chlamydial infections may progress to PID within 1 year and that such risk is higher in case of recurrent infections. PID is an inflammatory condition of the female upper genital tract affecting the endometrium (endometritis), the salpinges (salpingitis) and extends to the ovaries. In some cases, this inflammation can spread beyond the reproductive organs, causing pelvis-peritonitis, generalized peritonitis, perihepatitis (Fitz-Hugh-Curtis syndrome) and pelvic abscesses. The classic symptomatology of such pathology is characterized by widespread pain in the pelvic area, fever and leucorrhea [37]. The scarring of reproductive organs due to chronic inflammation in PID can lead to several sequelae such as tubal infertility (tubal fibrotic closure), chronic pelvic pain and increased risk of ectopic pregnancy. A direct consequence of female genital infections are neonatal infections; the transmission of chlamydia to the newborn occurs at the time of delivery. In 18-50% of cases, the newborn contracts an acute conjunctivitis called inclusion conjunctivitis (ophthalmia neonatorum) which usually resolved, without giving rise to consequences. Incubation is 5-12 days from birth and the palpebral fissure appears edematous and hyperemic with abundant purulent secretion. In in 3-13% of cases, the newborn undergoes interstitial pneumonia which usually it begins with a rhinitis which is followed by the appearance of a characteristic cough without feverish rise.

In males, the most frequent clinical manifestation is instead represented by urethritis mildly purulent, termed nongonococcal urethritis (NGU). Such inflammation causes pain and burning during urination, urethral mucoid discharge and pain widespread. Chlamydial urethritis is also referred to as "post gonococcal" (PGU) when there is both a co-infection with *Neisseria gonorrhoeae* since, being the period of incubation of chlamydial infection longer than that of gonorrhea, the infection from chlamydia appears after resolution with antibiotic treatment of the purulent framework caused by gonorrhea (to which chlamydia is insensitive). The urethral infection with *C. trachomatis* in humans, if untreated, can occasionally be complicated by epididymitis and less frequently from prostatitis [38]. Furthermore, in the male, cases of conjunctivitis (Para trachoma) may occur. Conjunctivitis, often unilateral, is also observed in subjects with asymptomatic urethral infection, and is usually the result of self-injection by fingers contaminated with traces of material from the urethra. The Para trachoma stands out easily from trachoma and evolves without complications, especially if adequately treated.

In both men and women, depending on the type of sexual intercourse, D-K serotypes can cause infections in the rectum (proctitis) or throat (pharyngitis). These demonstrations are often asymptomatic but contribute significantly to the spread of the infection. When present, symptoms include anal pain and mucous discharge in the case of proctitis, and pharyngeal pain and hoarseness during pharyngitis.

If the infecting agent is a serotype L1-L3 of *C. trachomatis*, there is a systemic pathological condition defined as lymphogranuloma venereum (LGV). The serotypes L1-L3 of *C. trachomatis* are more virulent than D-K serotypes. Unlike the other serotypes, which remain confined to the level of the mucous membranes, the L serovars are invasive, being able to penetrate the submucosal connective tissue and spread in the lymphatic vessels, until it reaches regional lymph nodes [39]. Two clinical pictures of LGV infection can be distinguished: "classic LGV" and the "new LGV". The "classic LGV" is a typical pathology of tropical and subtropical countries (Asia, Africa, South America and part of the Caribbean), very rare in industrialized countries. It is transmitted by sexual contact and affects both sexes. It manifests itself through the formation of transient genital ulcers, followed by the appearance of lymphadenopathy multilocular suppurative (buboes), characterized by swelling of the lymphatic pathways along entire inguinal ligament. The acute form of LGV is almost always accompanied by symptoms systemic, such as fever and leukocytosis. After a latency period of some years, they can manifest late complications including an elephantiasis of the genitals due to involvement lymphatic system and fistulas of the penis, urethra and rectum. The "new LGV", which has emerged in Europe in the last 10 years, is instead characterized by rectal infection

(proctitis) in homosexual males (MSM), often HIV-positive. Such infection is frequently symptomatic and presents with anal pain, bloody mucous secretions, tenesmus, diarrhea and regional lymphadenopathy. The transmission of the infection seems to be mostly related to the use of sex toys with transfer ano-anal of infectious pathological material. For a correct management and therapy of these infections, it is still necessary to resort to a molecular typing to identify the responsible *C. trachomatis* genotype.

2.1.7 Diagnosis

Given the low specificity of the symptoms of chlamydial infections, the history and clinical investigation are not sufficient to detect a possible infection. A laboratory diagnosis is therefore essential to confirm a suspected *Chlamydia trachomatis* infection. The diagnostic methods for these infections are based essentially on the direct search for the microorganism or its components (such as antigens or genome) in pathological materials. Diagnostic methods have evolved over the years starting from direct microscopic research, *in vitro* culture and enzyme immunoassays for antigen research, up to modern acid amplification techniques nucleic acids, currently considered the “gold standard”. The pathological material to be analyzed depends not only on the sex of the individual and on the site of infection, but also by the diagnostic method used. In case of urogenital infections, the samples that can be analyzed are generally represented by urethral swabs or urine for men; for women vaginal or cervical swabs are used. In the case of extragenital infections (pharyngitis and proctitis) it is possible to use a rectal or oropharyngeal swab instead. In the end, for suspected ocular infections (for example in the newborn or in the adult for self-injection) conjunctival swabs can be collected and analyzed.

Since their introduction, molecular tests have immediately shown great potential in terms of sensitivity and specificity, today they represent the "gold standard" diagnostic both in case of suspected *Chlamydia trachomatis* infection and as screening methods, in case of asymptomatic subjects. These tests are based on research of a specific portion of the microorganism's genome (DNA or RNA) in the pathological material. Since the sought-after target is represented by the DNA or RNA of the microorganism it is not necessary that bacterial viability is maintained until processing of the sample, making the management of the pre-analytical phase much easier. Common technologies used are the Strand Displacement Amplification (SDA), the Ligase Chain Reaction (LCR) and the Polymerase Chain Reaction (PCR; in particular real-time PCR), which have generally shown a sensitivity and specificity close to 100%. The usage of target sequences at plasmid localization was also allowed to increase the sensitivity of the test considerably, as they are

on average 5-10 copies of the plasmid for each individual bacterial cell. Thanks to their excellent sensitivity, the methods of nucleic acid amplification are also applicable to collected biological samples through much less invasive procedures, such as urine and "self-collected" vaginal samples, greatly favouring patient compliance [45]. In addition, the use of real-time multiplexes PCR allows to detect multiple pathogens simultaneously starting from the same pathological material. In this way, a diagnosis can be made quickly differential between the microorganisms that most often cause urethritis-cervicitis (e.g. *C. trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium*). A possible limitation in the use of molecular tests should be taken into consideration in the follow-up of infected patients undergoing antibiotic treatment. DNA research of chlamydia can in fact remain positive up to 3-4 weeks after the eradication of the pathogen, as the genome of dead microorganisms persists in biological fluids for some weeks. It is therefore recommended not to carry out follow-up investigations in treated patients before this period of time has elapsed, in order not to run the risk of false positives results [46]. Molecular techniques also make it possible to identify the genotype of Chlamydia responsible for the infection. This is possible through various methods, including amplification of the omp1 gene (gene encoding the MOMP protein), followed by sequencing or by RFLP (Restriction Fragment Length Polymorphism) technique [47]. Nowadays home-made or commercial real-time PCRs are also available for rapid differentiation between 'classic' genotypes (D-K) and genotypes responsible for lymphogranuloma venereal (L1-L3). Typing of Chlamydia serotypes has an important utility for several reasons:

(I) to identify the responsible strains of LGV which require a different antibiotic treatment compared to classic serotypes;

(II) to obtain data epidemiological studies on the distribution of genotypes in various populations;

(III) to identify the most circulating genotypes locally and worldwide in order to implement prevention strategies.

2.1.8 Pharmacological therapy

C. trachomatis infections are treated with the use of active antibacterial drugs, which cross the cell membrane to act intracellularly. Generally, most used antibiotics in these cases are macrolides and tetracyclines, which act by inhibiting the bacterial protein synthesis. Other effective antibacterial drugs fluoroquinolones. There are currently no scientific reports of the presence and circulation of strains of *C. trachomatis* resistant to macrolides and tetracyclines. On the contrary, drugs that act on the peptidoglycan synthesis (beta-lactams such as penicillins and cephalosporins) can never be used

for therapy, given the intrinsic resistance to this class of drugs due to lack of target in the bacterial structure. According to the main international guidelines, it is recommended to prescribe a therapy empirical to patients presenting with signs and symptoms associated with *C. trachomatis* without waiting for the result of the laboratory test. In case of a positive test diagnosis, to avoid further spread of the pathogen, therapy is prescribed also to sexual partners of infected people. The drugs recommended are the following:

First choice drugs:

- **Azithromycin**. It is highly active against *C. trachomatis*. It belongs to the class of Macrolides and has an inhibitory action on bacterial protein synthesis by binding with the 50S subunit of ribosomal RNA. Penetrates very well into most tissues, except in cerebrospinal fluid, and in phagocytes; its bioavailability is prolonged and is concentrated at the tissue level, especially within the cells. Moreover, the drug is released from tissues slowly (tissue half-life of 2-4 days). These properties of azithromycin allow a single single-dose administration for the treatment of chlamydial infections (azithromycin 1 g orally in a single dose). Azithromycin is a well-tolerated drug, with good levels of efficacy and compliance, which it can also be used during pregnancy. However, its use could help the emergence of resistance in other sexually transmitted microorganisms, possibly present at the same time as the chlamydial infection (e.g. *Treponema pallidum*, *Mycoplasma genitalium*, *Neisseria gonorrhoeae*).

- **Doxycycline**. It is a tetracycline, broad spectrum bacteriostatic antibiotic, which penetrates the microorganisms and blocks protein synthesis by binding to the 30S subunit of ribosomes bacterial. For uncomplicated chlamydial infections, the doxycycline regimen is 100 mg orally, 2 times a day for 7 days. It is a drug with good efficacy but it cannot be administered in pregnancy and is less tolerated by patients than azithromycin. In comparative studies, for classic urogenital chlamydial infections, the two regimens (azithromycin vs doxycycline) showed similar efficacy with cure rates around at 98%.

Second choice drugs:

- **Erythromycin**. It is part of the macrolides; is poorly tolerated by patients, both for the dosage regimen (500 mg orally, 4 times a day for 7 days) and for its frequency of gastrointestinal side effects.

- **Levofloxacin** and **Ofloxacin**. They are part of the Fluoroquinolones, subgroup of Quinolones, which work by blocking bacterial DNA synthesis.

As far as anorectal chlamydial infections are concerned, it is interesting to note as the reference drug is doxycycline. It has in fact proved to be slightly more effective than azithromycin in fighting this type of infections [48]. Furthermore, the use of the doxycycline appears to be associated with a lower risk of inducing infections persistent and recurrent, compared with azithromycin [48]. This aspect could be related to the fact that at the rectal level chlamydia finds a particularly suitable ecological niche for its replication with high 'bacterial loads' present here in progress infection [48]. In this context, the use of a single dose of azithromycin may not be sufficient to achieve complete elimination of the pathogen.

For *C.trachomatis* L serotypes infections (e.g. LGV proctitis), given the high virulence, invasiveness and lymphotropism of these serovars, are indicated 3 weeks of doxycycline therapy, as only one week of treatment may not be capable of completely eradicating the pathogen.

Particular attention should be paid to the choice of therapy for women in the state of pregnancy with *C. trachomatis* infection. In this regard, it is important to remember that the doxycycline is contraindicated throughout the second and third trimesters of pregnancy given the possible fetotoxic effect; currently, the safest therapy recommended for pregnant patients is the use of azithromycin in a single dose, with the recommendation to perform a post-treatment follow-up test 3-4 weeks after treatment [49] [50].

During treatment, abstention from sexual intercourse is indicated for 7 days after administration of the single dose regimen (azithromycin) or for the duration of the therapy in the case of a 7-day regimen (doxycycline). It is also necessary to evaluate the partner of the infected subject and eventually start an empirical treatment. If the patient has followed the recommended therapy regularly and no symptoms persist, or no clinical or anamnestic data are appreciated that could lead to suspicion of reinfection, it is not necessary to resort to the control test (“test of cure”); if not, it is necessary to repeat the diagnostic test. In this case, the molecular tests should not be repeated in any case before 3-4 weeks of therapy, due to the risk of false positive results caused by the longer stay of DNA rather than viable microorganism at the site of infection. In the case of adolescents, given the high rate of reinfection that is found, it is recommend to repeat a chlamydia diagnostic screening test after 3-4 months from the execution of the therapy to identify any new cases.

2.1.9 Prevention

Since *C. trachomatis* is sexually transmitted, barrier methods of contraception, including condom use, are effective at preventing chlamydial transmission, however utilization rates are low [51] especially in MSM.

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, has long been considered a promising candidate. Some studies have shown that novel formulations delivering MOMP proteins through cationic liposomes, may induce antibody type-1 immunity and partial protection from infection in minipigs [52] and significant protection against upper tract disease in mice [53][54]. 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 ($p<0.001$).

Vaccines are known to be the best form of prevention and protection against infections, Chlamydia infections included, more in deep studies are needed in order to identify protective antigens. In the same time an effective prevention strategy could be represented by the use of probiotics, since several studies have investigated the complex interactions between Lactobacilli species, the host cells and pathogens such as *Chlamydia trachomatis* [55].

2.2 Chlamydia trachomatis and sugars

C.trachomatis is an obligate intracellular bacterium that can proliferate thanks to the host cells infected. For the bacterium the host is essential as it supplies the nutrients necessary for its cellular metabolism. Through the metabolism of these substances, Chlamydia, in its RB form, is able to obtain energy that it allows it to grow, replicate and then leave the cell to infect other future hosts. There are many *in vitro* studies that demonstrate the metabolic adaptation of this pathogen to the host cell, as well as its metabolic and transcriptional activity [56] [57] [58].

In part based on experimental data supporting the “energy parasite” hypothesis presented by Molder in 1962 [2], the classical view of the EB is that of a metabolically “inert” entity [3]. This view has largely been retained for years, but in recent years some findings have challenged it.

Recently published data showing that the chlamydial EB can metabolize extracellularly [11], along with data showing that the chlamydial EB can respond to specific energy sources [12] [13], suggest that the simple model that the EB is metabolically inert after formation and is a passive entity waiting for contact with the next host cell may be an oversimplification of its role in pathogenesis. The data presented in the work of Grieshaber et al. [59] illustrate that EBs can remain infectious for at least 24 h and utilize multiple energy sources to maintain infectivity. They also proposed a model whereby the EB actively maintains infectivity in at least three distinct environments and phases of the chlamydial developmental cycle, namely, upon deposition at mucosal surfaces, within the inclusion after terminal RB-EB development, and within extrusions. They challenged the classical hypothesis of the EB cell type in chlamydial pathogenesis, that has been thought for years to be limited to the passive action of initiating the next round of infection through chance encounter with a susceptible host cell immediately after release from the host cell by lysis.

Another interesting work regarding EBs and substrates that they may come in contact with is the work by Foschi et al. [60] in which they compared the urine metabolome of *C. trachomatis* infected and uninfected women. Urine analysis was performed by means of nuclear magnetic resonance spectroscopy proton, known as H-NMR, which allowed to analyze and quantify the metabolites present in the urine of 119 premenopausal Caucasian women, divided in CT+ and CT- groups.

By H-NMR analysis, they were able to identify several metabolites whose concentrations were significantly higher in the urine samples of CT-positive subjects, including sucrose, mannitol, pyruvate and lactate. These results were comparable for all the CT serovars detected (D, E, F, G, K), thus indicating a common behaviour in presence of chlamydia, irrespective of the specific serovars.

The greater amount of sucrose, which is a biomarker of sugar consumption, and mannitol, a sugar alcohol often found in hard candies, fruits and vegetables, may increase CT infectivity. It is known that sucrose, at least, can act as stabilizer of some chlamydial proteins [61] but is possible that these sugars may also have a major effect directly on EBs, as we know that they are not inert.

2.3 Role of lactobacilli in promoting a healthy vaginal niche

Human vaginal microbiota is commonly dominated by *Lactobacillus* species in healthy women.

Lactobacilli are a group of Gram-positive, facultative anaerobic bacteria mainly producing lactic acid as product of glycogen fermentation [62] [63].

In healthy pre-menopausal women, the most frequently isolated species are *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii* and *Lactobacillus iners*. Residing at the port of entry of various bacterial and viral pathogens, the vaginal lactobacilli are important create a barrier against pathogen invasion. Indeed, thanks to some products of their metabolism secreted in the cervicovaginal fluid, lactobacilli play an important role in the inhibition of bacterial and viral infections [64].

Lactobacilli can stabilize the vaginal microbiota conferring protection against potential pathogens (Figure 11).

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 [65].

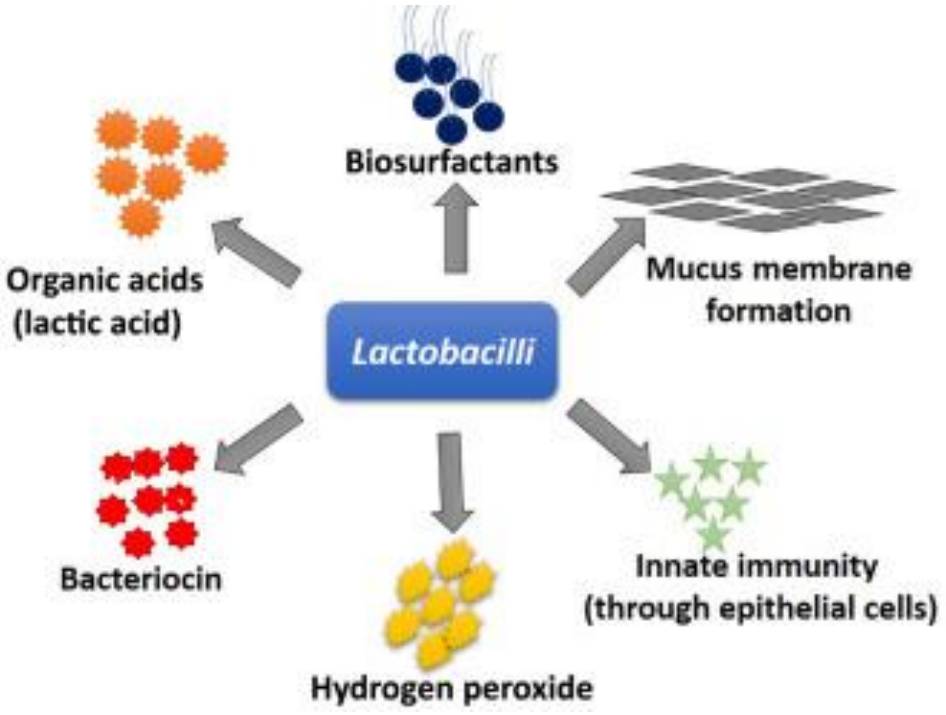

Figure 11: defensive mechanism and compounds exerted by lactobacilli against pathogens

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 limited the microbiota to acidophilic species such as *Lactobacillus* spp. However, pH may have more subtle effects than simply to provide a not suitable environment for several microorganism species. Furthermore, *in vitro* studies have shown that acidification by lactobacilli growth can inhibit pathogen proliferation: *C. albicans*, *Escherichia coli*, *G. vaginalis*, and *Chlamydia trachomatis* are known to be highly affected by lactic acid [66].

Hydrogen peroxide. H_2O_2 is an oxidizing agent, which is toxic to catalase-negative bacteria such as the vast majority of anaerobic microorganisms. Recent studies suggested that *L. crispatus* and *L. jensenii* are responsible for, respectively, 95 % and 94 % total H_2O_2 production in vagina [67]. Moreover, approximately 80 % of the strains of vaginal origin are able to produce H_2O_2 [68] and only 6% of women with bacterial vaginosis contained H_2O_2-producing *Lactobacillus* species in their vagina [69]. Other in vitro studies also demonstrated the involvement of the peroxidase system in the inhibition of *Neisseria gonorrhoeae* in an acidic environment by a complex effect from H_2O_2, acid production and bacteriocin-like compounds [70].

Bacteriocins and biosurfactants. Bacteriocins are antimicrobial peptides produced by almost every lactobacillus specie. Bacteriocins have several mechanisms of action, including cytoplasmic membrane pore formation, interference with cellular enzymatic reactions and nuclease activity [71]. However, only few bacteriocins from vaginal isolates of *Lactobacillus* spp. have been identified. Some examples are the bacteriocin produced by a strain of *L. salivarus* with activity against *E. faecalis*, *E. faecium* and *N. gonorrhoeae* [72], 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 that of antibiotics, although they are different as regards their synthesis, mechanism of action, toxicity, and resistance mechanisms [73][74]. 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 [73] [75].

Another characteristic of lactobacilli is the capability of producing biosurfactants substances.

Numerous physiological functions of biosurfactants have been described. Biosurfactants are able to allow microorganisms growth on water-immiscible compounds by lowering the surface tension at the phase boundary; to cause emulsification, and to stimulate adhesion of microbial cells to organic substrates [76]. It has been shown that biosurfactant activity is resistant to trypsin and pepsin, sensitive to -amylase and lysozyme, and resistant to 75^{o}C degree heating.

Among these substances, surlactine, produced by *L. acidophilus* and *L. fermentum,* had shown a particularly effective inhibitory activity against *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus epidermidis* and *Candida albicans* [77].

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 ability to compete for adhesion sites in the vaginal epithelium, preventing the colonization by a pathogen. Cell adhesion is a multistep process that involve the contact of the bacterial cell membrane and interacting surfaces [78].

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 [79]. Lactobacilli inhabiting healthy urogenital tract interfere with 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 or displacement of adhered pathogens. Several studies have shown the ability of *Lactobacillus* spp. to adhere to epithelial vaginal cells to form a biological barrier against colonization by pathogenic bacteria [80] [81]. Multiple components of the bacterial cell surface seem to participate to this process. Glycoproteins and carbohydrates have been reported to be the factors responsible for adherence to epithelial vaginal cells [82]. It has been shown as some lactobacilli strains were able to reduce *C. albicans* adhesion by exclusion, competition and displacement experiments [83].

Furthermore, some recent studies have shown that Lactobacilli can cause important modifications by adhesion in the epithelial plasma membrane [84]. Indeed, in the work of Parolin et al. is described how adhesive Lactobacilli can protect host cells from Chlamydia by altering lipid composition and α5 integrin subunit exposure.

3) Co-aggregation with pathogenic bacteria

Coaggregation is a process by which genetically distinct bacteria become attached to one another via specific molecules [85]. Cumulative evidence suggests that such adhesion influences the development of complex multispecies biofilms. Co-aggregation 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 [86]. Lactobacilli can form co-aggregates 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 co-aggregate with other bacteria probably influences the structure and stability of the urogenital flora [87]. 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* [82][88]. 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 [89].

2.4 Lactobacilli as potential vaginal probiotics

Probiotics have been defined as ‘‘live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host’’ (FAO/WHO, 2001). 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 [90]. A probiotic may act indirectly through treating and preventing recurrent bacterial vaginosis (BV) or directly by secreting substances (e.g., hydrogen peroxide, bacteriocins, lactic acid) that block sexually transmitted infection [91].

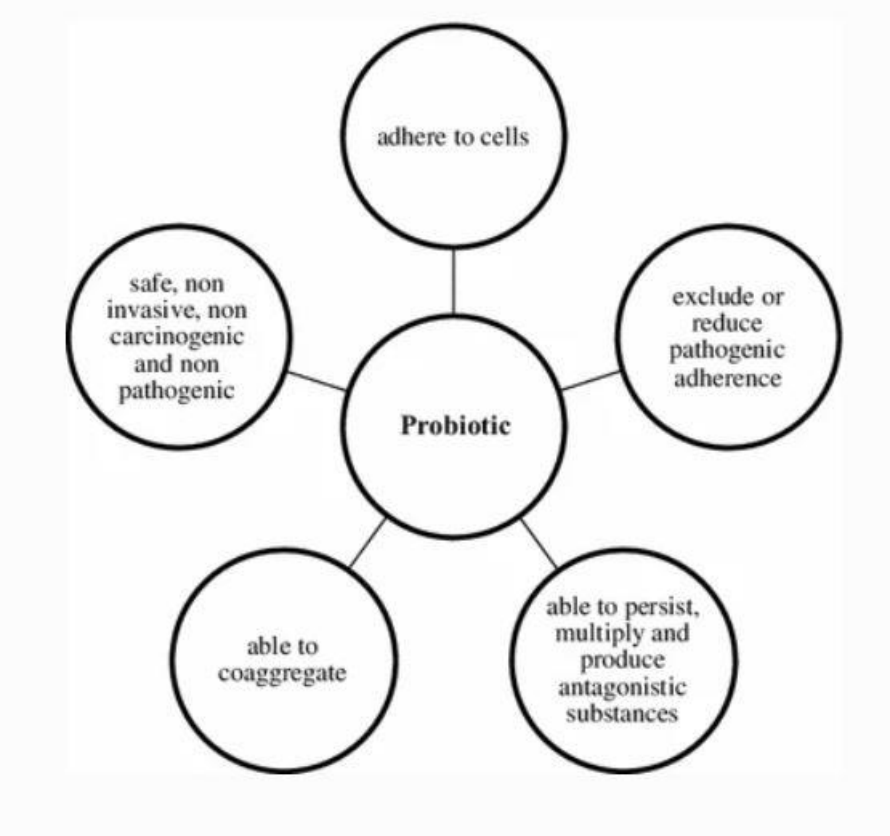

Figure 12. Requirements in the choice of a probiotic [65]

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 time without adverse effects, making them an attractive alternative to antibiotics, particularly in addressing the problem of high recurrence rates [82].

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 [92]. 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 [82]. 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 [93].

Several clinical trials have been performed to assess whether specific strains of lactobacilli, are able to colonize vaginas of women. In these studies authors have treated women with symptomatic or asymptomatic BV with probiotics to reduce the colonization of pathogens and to improve symptoms of BV when they are present [94]. Two different types of experimental approaches have been employed in these works 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. In both studies [95][96] combination of different species of lactobacilli with different biological properties on fertile non-pregnant women has been used.

In both studies, results were promising. In the study of Anukam et al. [95] a BV cure rate of 65% has been achieved after probiotic treatment compared to 33% of the metronidazole therapy; while in the study of Mastromarino et al. [96] the probiotic strains were able to temporarily colonize the human vagina resulted in less malodorous discharge, and a trend towards higher clinical cure rate, compared with the placebo group.

Moreover, 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 (1.08 cfu per suppository) every 2 days for 1 year. A significant reduction in the number of recurrences was noted, without any adverse complication [97]. Overall, these studies showed that lactobacilli are indeed real potential probiotics that can have an important role in therapy against urogenital infections.

Regarding *Chlamydia trachomatis* infection, some studies have explored beneficial effect from some lactobacilli species *in vitro* [55] [84] [98] but no clinical study has already been reported. In this context production of antimicrobial compounds such as lactic acid and biosurfactants are indicated as the main effector of CT EBs neutralization [99] [100] [101]. However, some studies have pointed out that certain species of lactobacilli may not be protective and also be associated with a microbiota susceptible to a higher incidence of CT infection [102]. Since production of lactic acid represents the main metabolic feature of all lactobacilli species [62] [63], different mechanisms other than EBs neutralization by external products may be involved in the protection conferred by some lactobacilli species against CT infection.

3.Aims of the research

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. Since the first findings, many features and mechanisms have been discovered about Chlamydia; however, several aspects are still unknown or not well understood. In this contest, the thesis investigates two different features about the bacterium and its interactions that are a novelty in the field.

First the thesis examines the effect of different sugars on CT infectivity in an *in vitro* model, elucidating some of the molecular mechanisms behind CT-sugar interaction, since previous works suggest that sugars, such as sucrose and mannitol, can have a beneficial effect on the bacterium survival and virulence [59] [60] [61]. To understand if sugars can increase infectivity, both CT EBs and the HeLa cells have been incubated with three different sugars at different concentrations and then infection after 48h has been evaluated through immunofluorescent inclusion count. Subsequently, membrane anisotropy measurements have been performed on sugar treated EBs and cells to evaluate if sugar can cause some modifications at membrane level; then CT EBs have been marked with Anti-LPS antibody and analysed by cytofluorimetry to assess a change in the expression of chlamydial LPS in EBs. Finally, an analysis on the protein expression of infected HeLa cells with sugar treated EBs was performed, to investigate modifications of the protein FAK, that is known to be important for anti-apoptotic and proliferation signalling [103][104] and during the internalization process of intracellular bacteria [105] [106] [107].

Secondly, the thesis explores the role of D (-) and L (+) isomer of lactic acid produced by two different lactobacilli in the prevention of CT infection in an *in vitro* HeLa cells model. It's assumed that the protective effect of vaginal microbiota is exerted through various mechanisms acting on both the extracellular and intracellular steps of the Chlamydia vital cycle. Among the various antimicrobial substances produced by lactobacilli, lactic acid is suggested to play various important roles in host defense. In particular, lactobacilli strains producing the D (-) isomer seems to exert a more powerful protection against sexually transmitted pathogens [108]. Since the chlamycidial effect of lactic acid is well established and the acidification properties of lactic acid does not seem to be related to the isomer present, the aim of the study is to explore the protective effects of the two lactic acid isomers against CT infection related to different nonexclusive mechanisms, as the shift in metabolism in the host cells, critical for both the EB infection and the later transformation in RB, as well as epigenetic modifications triggering different gene expression involved.

In the study two different lactobacilli have been considered: *Lactobacillus crispatus*, commonly found in human vaginal microbiota, described as one of the most protective lactobacilli specie against sexually transmitted pathogens [84] [98] [99] [100] [108] and producer of both lactic acid

isomers [109]; and *Lactobacillus reuteri,* commonly found in gastrointestinal microbiota and rarely in vagina, which has been used as vaginal probiotic in some studies [110] [111] and produces only L-lactic acid [112].

At first, lactic acid production by the two bacteria has been measured by High Performance Liquid Chromatography, and lactobacilli protection against CT infection has been analyzed through immunofluorescent inclusion count experiments. Then, bacterial lactate dehydrogenases presence has been assessed by both resazurin assay and Liquid Chromatography-Mass Spectrometry; moreover D-lactate dehydrogenase specific activity has been investigated by measuring NADH formation. Afterwards addition of D or L-lactic acid to *L.reuteri* supernatant has been performed and their effect in promoting protection in the host cells assessed by infection experiments. Subsequently a metabolic analysis has been carried out by real-time measurement of HeLa mitochondrial respiration after treatment with lactobacilli colture media and with a D-lactic acid solution. Finally, histone acetylation and lactylation, as well as gene and protein expression of relevant targets of treated HeLa cells, have been investigated in order to understand the greater protection conferred by D-lactic acid.

4.Materials and Methods

4.1 Chlamydia trachomatis isolation

Chlamydia trachomatis strain GO/86 (serovar D) was used for the experiments. This clinical strain was isolated from a patient with urethritis and belongs to the laboratory collection of the Microbiology Unit of S. Orsola- Malpighi Hospital of Bologna (Italy). The strain was initially propagated for about 2 weeks in LLCMK2 cells (ATCC® CCL-7TM). Afterwards, CT EBs purification was performed as follows: 24 plates with 6 wells of HeLa cells were prepared, then infected with a cell suspension containing the infected cells in which *C. trachomatis* has been propagated. After the infection, plates were centrifuged for 2 hours at 640 × g and incubate 48 hours at 37°C in an atmosphere with 5% carbon dioxide. Subsequently, cells were detached by manual scraping and resuspended in sucrose-phosphate-glutamate (SPG) solution. In order to completely break down the cellular structures and to allow the EBs to come out, cell suspension was vortexed with the addition of glass beads for 5 min and then sonicated three times for 10 seconds at medium power. Cell debris were removed by centrifuging at 500 × g for 10 min at 10°C, then supernatant was centrifuged at 40,000 × g for 1 hour at 4°C under Renografin density gradient. At the end of

this last step, Chlamydia EBs were isolated and stocked in freezer at – 80°C in 30 μL aliquots of SPG.

For the determination of the purified EBs quantity, 10 tubes containing a slide presenting a monolayer of HeLa cells were prepared. Every tube was infected with a different base 10 dilution of the purified Chlamydia, in a volume of 1 mL of culture medium with the addition of cycloheximide, a protein synthesis inhibitor which is used in order to favour the infection by the microorganism. After 48 hours of incubation at 37°C with 5% CO2, the slides with the infected monolayer were fixed with 1 mL of methanol and stained with a monoclonal antibody directed against Chlamydia LPS, conjugated to fluorescein (Meridian, Cincinnati, OH, USA). The slides were then incubated at 37°C in a humid environment for 20 minutes. Later, after a double wash in PBS, the slide was mounted with buffered glycerin solution pH 7.3, placing it upside down on a slide. For the observation of the cytoplasmic inclusions of Chlamydia a was employed fluorescence microscope (Nikon, Eclipse E600) at 20× magnification. Under microscopic observation, cytoplasmic inclusions of Chlamydia appear coloured bright green and are easily distinguishable from the uninfected cells that are coloured red due to the use of Evans Blue in the solution containing the antibody. This procedure was repeated three times. The number of inclusion forming units (IFU) per slide was then counted so as to trace the total number of IFU/mL.

4.2 Cell culture

The cell line mainly used in the experiments was HeLa, a human cervical carcinoma cell line. Cells were seeded in plates (Orange Scientific, Braine-l'Alleud, Belgium) at a density of $2x10^5$ cells/cm2 and incubated at 37°C in a 5% CO2 atmosphere. The composition of the complete medium is reported in **Table 1**.

Table 1: Composition of cell complete medium.

RPMI 1640 (*Lonza*)	89%
Fetal bovine serum (FBS) (*EuroClone*)	10%

L-glutamine 200 mM (*Sigma-Aldrich*) in phosphate buffered saline (PBS)	1%

Cells were washed two times in Phosphate Buffer Solution (PBS) and subsequently, trypsin 0.115% (Sigma-Aldrich, St. Louis, Missouri, United States) in a solution of PBS-ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, Missouri, United States) 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 Burker chamber. The composition of PBS is given in **Table 2**.

Table 2: Composition of phosphate buffered saline.

NaCl (*Sigma-Aldrich)*	8 g/L
Na2HPO4 (Sigma-Aldrich)	1.15 g/L
KCl (*Sigma-Aldrich*)	0.2 g/L
KH2PO4 (*Sigma-Aldrich*)	0.2 g/L

4.3 Evaluation of Chlamydia trachomatis infectivity after EBs incubation with sugar solutions

To assess the possibility that sugar solutions may directly enhance CT EB infectivity, infection experiments were performed after EBs incubation with sugar solutions.

Sugar solutions of glucose, sucrose, and mannitol were prepared from powder stocks (Sigma Aldrich, St. Louis, Missouri, United States) in sterile phosphate buffer saline (PBS) and then diluted to chosen final concentrations of 5 mM, 2.5 mM, and 0.5 mM. HeLa cells were seeded in 6-wells plates in 2 mL of complete medium and allowed to reach a total cell number of 5×10^5 approximately.

A total of 5×10^4 CT EBs (10 µL of a stock solution of 5×10^3 EBs/µL) were re-suspended in 1 mL of sugar solutions and then incubated for 2 h at 37 °C with 5% CO_2. A PBS sterile solution was used

as negative control. After the incubation, cell medium was removed and EB solutions were used to infect HeLa cells for 1 h. We chose to infect with a multiplicity of infection (MOI) = 0.1. At the end of the incubation, each plate was PBS-washed three times, and 2 mL of complete medium were added. No centrifugation steps were included, as well as no cycloheximide being added to the culture medium. Plates were then incubated at 37 °C with 5% CO_2 for 48 h.

All the experiments were conducted in triplicate.

CT infection was estimated by counting the number of IFUs by direct immunofluorescence, using a fluorescein-conjugated anti-chlamydial LPS monoclonal antibody (Meridian, Cincinnati, OH, United States). The number of IFUs was counted in 60 randomly chosen 40× microscopic fields.

4.4 Evaluation of CT Infectivity after HeLa incubation with Sugar Solutions

To exclude an effect of sugar solutions on HeLa cells, infection experiments after HeLa incubation with sugars were performed and analysed. HeLa cells (5×10^5 cells approximately) were incubated with sugar solutions previously prepared for 2 h, at 37 °C, with 5% CO_2. We chose to test only the highest concentration: 5mM, After the incubation, cell plates were washed twice with PBS, and infected with CT EBs at MOI = 0.1 for 1h. Next experimental steps were conducted as described in the previous paragraph: briefly, after 48 hours, cells were fixed and stained using the same fluorescein-conjugated anti-chlamydial LPS monoclonal; IFUs were then counted.

4.5 Fluorescence anisotropy measurements

The plasma membrane fluidity of CT EBs or of HeLa cells was estimated by means of the fluorescence anisotropy of the hydrophobic probe TMA-DPH [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluene sulfonate] ThermoFisher Scientific, Waltham, MA). TMA-DPH is a lipophilic fluorophore that penetrates the membrane hydrophobic core, orienting perpendicularly to the membrane plane [113]. In case of an increased fluidity of the membrane, the TMA-DPH probe rotates to a greater extent, leading to a depolarization of the fluorescence emission and a decrease in the fluorescence anisotropy.

HeLa cells and CT EBs were incubated with sugars solutions (glucose, sucrose, and mannitol 5 mM) for 2 h, at 37 °C, with 5% CO_2. Phosphate Saline Buffer solution treatment was used as a

control. HeLa cells and EBs were then washed 3 times with PBS and resuspended respectively at a final concentration of 3×10^5 cells/mL and 5×10^4 cells/mL.

The absorbance of the cell suspension was kept lower than 0.15 at the excitation wavelength of TMA-DPH. A few microliters of TMA-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, North Edison, NJ) equipped with a temperature-controlled cell holder and Polaroid HNPB 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:

$$r = (IVV - IVH \cdot G)/(IVV + 2 \cdot IVH \cdot G)$$

where IVV is the fluorescence intensity measured with vertical excitation and vertical emission polarization filters and IVH 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 = IHV/IHH. [114].

4.6 Flow cytometry

The flow cytometer is an instrument that detects the fluorescence intensity of each single cell and in relation to it allows the measurement of biophysical parameters and biochemicals, such as cells granulometry, which are then collected in the memory of a microprocessor e represented graphically. To test whether the change in fluidity induced by sugars can modify the membrane structure organization, EBs were incubated with PBS (control) or with sugar solutions at 5mM concentration for 2h, then stained with an anti-LPS antibody and analysed by flow cytometry. In particular, a total number of $2x10^5$ EBs were resuspended in 1 mL of the sugar solution for 2 h at 37 °C, with 5% CO_2. At the end of the incubation, EBs were labelled with FITC-conjugated anti-LPS monoclonal antibody for 1 hour at room temperature environment. Subsequently, they were ultra-centrifuged for 1 hour at 40,000 × g. The pellet, represented by the labelled EBs, was resuspended in sterile PBS and analysed using the S3e Cell Sorter flow cytometer (Bio-Rad, Hercules, CA, USA). As negative control for the antibody specificity, EBs incubated with FITC-conjugated anti-human IgG were analysed.

4.7 Cell Lysis and Western Blot Analysis

To evaluate modifications of intracellular signalling pathways during the early phase of CT infection, we performed an analysis of the phosphorylation state of two sites of FAK (tyrosine 925 (Y925) and serine 722 (S722)). FAK is a non-receptor tyrosine kinase involved in invasion mediated bacterial uptake and subsequent pro-inflammatory responses [11–13]. Cell lysis and Western blot experiments were performed as follows. HeLa cells were seeded in 6-wells plates in 2 mL of complete medium and allowed to reach a total cell number of 5×10^5 approximately. A total of 5×10^4 CT EBs (10 μL of a stock solution of 5×10^3 EBs/μL) were re-suspended in 1 mL of sugar solution (only 5 mM), and then incubated for 2 h, at 37 °C, with 5% CO_2. EBs diluted in PBS sterile solution, as well as sugar solutions without EBs were used as controls. After the incubation, the complete medium was removed and EB solutions were used to infect HeLa cells for 1 h. The MOI used for these experiments was always set at 0.1. Cells were washed with ice-cold PBS and lysed using a lysis buffer composed by: HEPES at pH 7.4 40 mM, glycerophosphate 60 mM, p-nitrophenyl phosphate 20 mM, 0.5 mM Na3PO4, 250 mM NaCl, 1% Triton X-100, 0.5 mM PMSF, and a solution of 10 mg/mL containing aprotinin, leupeptin, pepstatin and antipain (Sigma-Aldrich, St. Louis, Missouri, United States). Lysis was performed at 0 °C for 1 h. Once lysed, the cells were centrifuged at 12,000 g, for 20 minutes, at 4°C according to Parolin et al. [84] and protein concentration was determined by using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, United States). The proteins were resolved by SDS-PAGE and immunoblotted with a rabbit anti-human FAK pS722/pY925 (1:1000 in PBS), or with a mouse anti-tubulin (1:5000 in PBS) antibodies. Detection of immunoreactive bands was performed with a secondary antibody (1:10,000 in PBS Tween) conjugated with horseradish peroxidase (GE Healthcare, Milan, Italy), and developed with WESTAR EtaC 2.0 (Cyanagen, Bologna, Italy). Densitometry analysis was performed by Fluor-S Max MultiImager (Bio-Rad, Hercules, CA, United States). Relative quantification of FAK pS722/pY925 was done by using tubulin signal as a control. For each condition, densitometry arbitrary units (A.U.) were normalized by CT infectivity values.

4.8 Lactobacilli colture and lactic acid HPLC measurements

Lactobacilli strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (Becton Dickinson and Company, Sparks, MD, USA) supplemented with 0.05% l-cysteine, incubated at 37 °C under anaerobic conditions in an anaerobic jar (Sigma-Aldrich, St. Louis, Missouri, United States). The turbidity of 18-h Lactobacillus cultures was adjusted with sterile saline to 1.6 McFarland (McF), corresponding approximately to a cell concentration of 5×108 colony forming unit (CFU)/mL. Afterwards, the cultures were centrifuged at 5000×g for 10 min at 4 °C. Supernatants were filtered

through a 0.2 μm membrane filter to obtain stock Cell free supernatants (CFS). Cell pellets were washed and re-suspended in phosphate-buffered saline (PBS) to obtain stock suspensions of 1×10^9 CFU/mL for exclusion experiments. The pH values of CFS were measured by a pH Meter, after appropriate calibration, and then CFS Lactate concentration was measured by HPLC as described in the work by Liparulo et al. [115].

Briefly, for lactate acid determination the supernatants were diluted 1: 10 in the mobile phase and centrifuged at 14 000 g for 5 min at 4 °C. The supernatant was then injected in an HPLC system (Agilent 1100 Series System) equipped with a phenylic column (Agilent ZORBAX SB-Phenyl, 5 μm, 250 × 4.6 mm), using a mobile phase consisting of 50 mm KH_2PO_4, pH 2.4, at a flow rate of 0.8 mL·min−1. Absorbance at λ210 nm was monitored by a photodiode array detector. Lactate peak areas were evaluated by external standardization. All injections were performed in triplicate.

4.9 Evaluation of lactobacilli cell fraction on C. trachomatis infectivity

To study the capacity of the two lactobacilli to interfere with the CT infection of HeLa cells, exclusion experiments were performed [84]. HeLa cells were seeded at 2.5×10^5 cells/well in 6-well plates with coverslips in it in 2 mL of medium, and allowed to reach 95–100% of confluence (approximately 1×10^6 cells). Lactobacilli were incubated for 1 h at 37°C with 5% CO2 on HeLa cells. Two different HeLa cells: Lactobacilli ratio were tested: 1:100 and 1:200. Afterward, 3×10^6 CT EBs (0.01 mL of a stock solution of 3 × 108 EBs/mL) were added and further incubated for 1 h [multiplicity of infection (MOI) = 3]. HeLa infected with CT EBs without lactobacilli were used as controls. All the experiments were conducted in triplicate. No centrifugation steps nor cycloheximide addition to the culture medium were included. After 1h infection, media was discarded, cells were washed twice with PBS, fresh medium was added and cells were then incubated at 37°C with 5% CO_2 for 48 h. CT infection was evaluated by counting chlamydia inclusion forming units (IFUs) by direct immunofluorescence, using a monoclonal antibody against the chlamydial membrane lipopolysaccharide antigen conjugated with fluorescein (Meridian, Cincinnati, OH, United States), as previously described. Slides were observed under epi-fluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan) equipped with a super high pressure mercury lamp and Plan Fluor DLL 20, 40, 100× lenses. The number of IFUs was counted in 60 randomly chosen 100× microscopic fields. Results were expressed as the percentage (median percentage ± median absolute deviation) of CT infectivity, comparing the number of IFUs of the single samples with the control slides.

4.10 Assessment of CT Infectivity after HeLa incubation with L.crisptaus and L.reuteri cell-free diluted supernatants

To assess the effect of lactobacilli cell-free supernatants on HeLa cells, infection experiments were performed as follows. Supernatants from lactobacilli overnight coltures were diluted 1:100 to a final concentration of <5 mM with PBS. pH was constantly measured during the procedure by a pH meter (Beckman). Solutions were then filtered in order to avoid any contamination.

HeLa cells (3×10^5 cells approximately) were incubated with solutions for 1 hour, at 37 °C, with 5% CO_2. Complete medium was used as negative control. After the incubation, the cell plates were washed twice with PBS, and infected with CT EBs (MOI = 3) for 1h. No centrifugation steps nor cycloheximide addition to the culture medium were included. At the end of the incubation, each plate was PBS-washed three times, and 2 mL of complete medium were added. Plates were then incubated at 37 °C with 5% CO_2 for 48 h.

CT infection was estimated by counting the number of IFUs by direct immunofluorescence, using a fluorescein-conjugated anti-chlamydial LPS monoclonal antibody (Meridian, Cincinnati, OH, United States).

4.11 Resazurin reduction by Lactobacilli with or without lactic acid isomers

Appropriate amounts of subcultured *Lactobacilli* were mixed with PBS in a 96-well microplate to result in a final OD600nm of 0.1, with addition at 10 mM (D+) or (L-)- lactic acid. After incubation for 30 min at 37°C, 10 µL of resazurin was added in the final assay volume of 100 µl per well. Positive controls were also set up with the same resazurin addition in the 96-well microplate. Triplicate experiments were carried out for each test at 37°C. Absorbance was read after 15 min by a microplate reader (Infinite 200 PRO multimode plate reader, Tecan Group Ltd., Switzerland) at λex = 570 nm and λem = 600 nm.

For cell disruption, $5x10^8$ CFU/mL of the subcultured bacterial culture of *Lactobacilli* was diluted with PBS to OD600nm 0.1, and centrifuged at 4°C and 12,000 × g for 10 min and washed once using PBS buffer. Cell pellets were lysed in 500 µL of Enzymatic Lysis Buffer (20 mM Tris HCl pH 8, 2 mM sodium EDTA, 1.2% Triton X-100, 20 mg/mL lysozyme), incubated at 37°C for 30 min and then and then ultrasonicated (10 × 1 s; ……) to release the intracellular organelles and enzymes. The disrupted cells were then centrifuged (12,000 × g for 10 min at 4°C), and the

supernatant collected. An appropriate volume of supernatant was transferred into a 96-well microplate with (D+) or (L-)- lactic acid at 10 mM in the final assay volume of 90 µl per well. After incubation for 30 min at 37°C, 10 µL of resazurin was added in the final assay volume of 100 µl per well, and triplicates were carried out for each sample; the same experiments were also carried out without lactic acid addition. Finally, absorbance was measured using the method described above.

4.12 Lactate dehydrogenase-D activity

The Lactate dehydrogenase-D activity in bacterial lysates was measured by following the increase in absorbance due to the formation of NADH at 340 nm. The reaction buffer consisted of 50–100 µg of bacterial lysates, 25 mM potassium phosphate, 5 mM MgCl2, 5mM NAD^+, pH 7.2, 30 °C. The reaction started by adding 100 mM of D-Lactic Acid, total reaction volume was 1 mL.

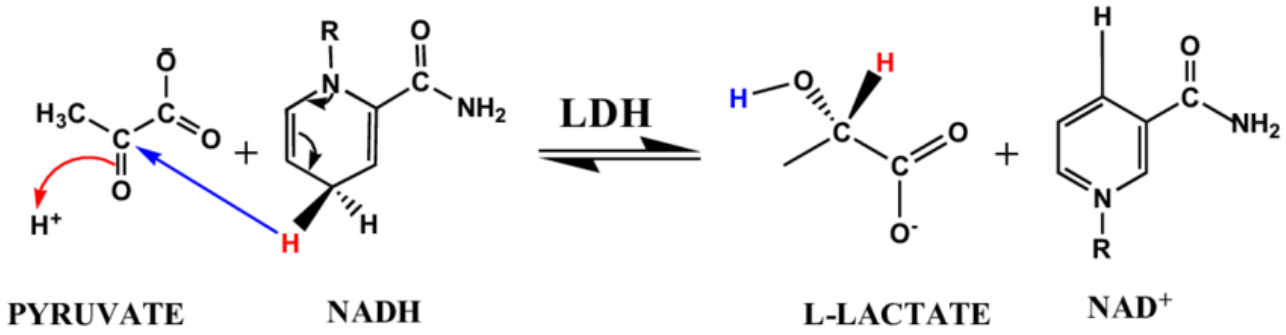

Figure 13: Lactate dehydrogenase activity [115A]

4.13 Liquid chromatography – Mass Spectrometry (LC-MS)

Sample preparation from Lactobacilli pellet is described as follows.

50 µL of ammonium bicarbonate buffer (50 mM, pH 8.0) were added to the lysate and briefly vortexed. 5 µL of dithiothreitol 0.1 M in ammonium bicarbonate were added and samples were incubated at 56°C for 30 min under agitation (400 rpm).

Afterwards 10 µL of a 10 mg/mL solution of iodoacetamide in ammonium bicarbonate 50 mM, pH 8 were added and samples were incubated for 40 min in the dark at room temperature. Finally, a 1 µL aliquot of a 1 mg/mL trypsin solution in HCl 2 mM was added and the sample was incubated

at37 °C, under gentle agitation (400 rpm) overnight (18 h). Digestion was stopped by adding 2 µL of a 10% aqueous solution of formic acid. 10 µL of tryptic digest were analyzed by LC-ESI-MS/MS.

Chromatographic separation of tryptic digests was carried out by using an ACQUITY Quaternary Solvent Manager (Waters, Manchester, UK) equipped with an autosampler. Analyses were performed on a C18 (Acquity, UPLC, BEH;100×2.1 mm i.d., 1.7 µm) column. Mobile phases A (water /FA, 100/0.1) and B (AcCN /FA, 100/0.1) were used to develop a gradient. The solvent gradient was set as follows: 5–50% B, 25 min; 50-80% B, 1 min. The column was equilibrated with initial conditions for 3 min before the next injection. Mass spectrometry analyses were performed on a Xevo G2-XS QTof (Waters, Manchester, UK) with Z-spray ion source. The ESI-QToF source temperature was set at 120°C, the desolvation temperature at 300°C, the capillary voltage at 3.0 kV, and the cone voltage at35 V. Peptide ions within a *m/z* 400–2000 survey scan mass range were analyzed for subsequent fragmentation. 2^+, $3^+$and 4^+ charged ions were selected for MS/MS analyses. From a single survey scan 20 ions were selected for subsequent fragmentation. Scan returned to mass survey mode after 15 s. Scan time was 0.2 s for the parent ion and 0.1 s for the MS/MS ions. Collision energy was selected using charge state recognition. The fragment ion spectra obtained from LC-ESI-MS/MS analyses were processed using Mascot Distiller 2.5.1.0 (Matrix Science, London, UK), a software program that reduces MS raw data to high-quality peak lists for database searching. LC-MS/MS data were analyzed by searching the human SWISSPROT database (2021_03, 565254 sequences; 203850821 residues; http://www.uniprot.org) selecting bacteria (eubacteria) as taxonomy and allowing only three missed cleavages. The precursor and fragment ion tolerance were 0.3 and 0.3 Da, respectively. Cysteine carbamidomethylation was selected as fixed modification while methionine oxidation was selected as variable modification.

4.14 Effect of L.reuteri cell-free enriched supernatant on C. trachomatis infectivity

To test if the addiction of the two Lactic acid isomers may have a major impact on HeLa cells in disfavouring Chlamydia infectivity, solutions from *L.reuteri* supernatant were prepared as follows. CFS were diluted 1:100 with PBS, then D-Lactate, or L-Lactate, from stock solution were added to a final concentration of 10mM; supernatant diluted 1:100 without any addiction was used as control solution for the following procedure.

HeLa cells were seeded at 2.5×10^5 cells/well in 6-well plates with coverslips in it in 2 mL of medium, and allowed to reach 95–100% of confluence (approximately 1×10^6 cells). Cell media was then discarded and cells were then treated for 1 hour with 1 mL of the three different solutions: S (1:1000 diluted supernatant only); D-LA 10mM (diluted supernatant with the addiction of D-Lactate 10 mM); L-LA 10mM (diluted supernatant with the addiction of L-Lactate 10 mM). After treatment cells were then washed with PBS twice and fresh medium was added. Infection and immunofluorescence staining were performed as described in the previous paragraphs.

4.15 HeLa Cells Energetic Profile

To assess the energetic profile of HeLa cells after diluted and enriched supernatant treatment, around 7.5×10^3 cells were seeded in every well of a 96-wells plate. Treatment for 1 hour with solutions was done 24 hours after the seeding. Cells were then washed twice with PBS and 180 µL of fresh medium were added, then the 96-wells plate was placed immediately in the Seahorse Agilent (Agilent Technologies, Santa Clara, CA, USA).

Evaluation of the energetic profile was made following the standard protocol of Agilent Seahorse XF Cell Mito Stress Test [116].

The Agilent Seahorse XF Cell Mito Stress Test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of cells on the Seahorse XFe and XF Extracellular Flux Analyzers (Agilent Technologies, Santa Clara, United States). It is a plate-based live cell assay that allows to monitor OCR in real time. The assay uses the built-in injection ports on XF sensor cartridges to add modulators of respiration into cell well during the assay to reveal the key parameters of mitochondrial function. The modulators included in this assay kit are Oligomycin, Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), Rotenone, and Antimycin [116].

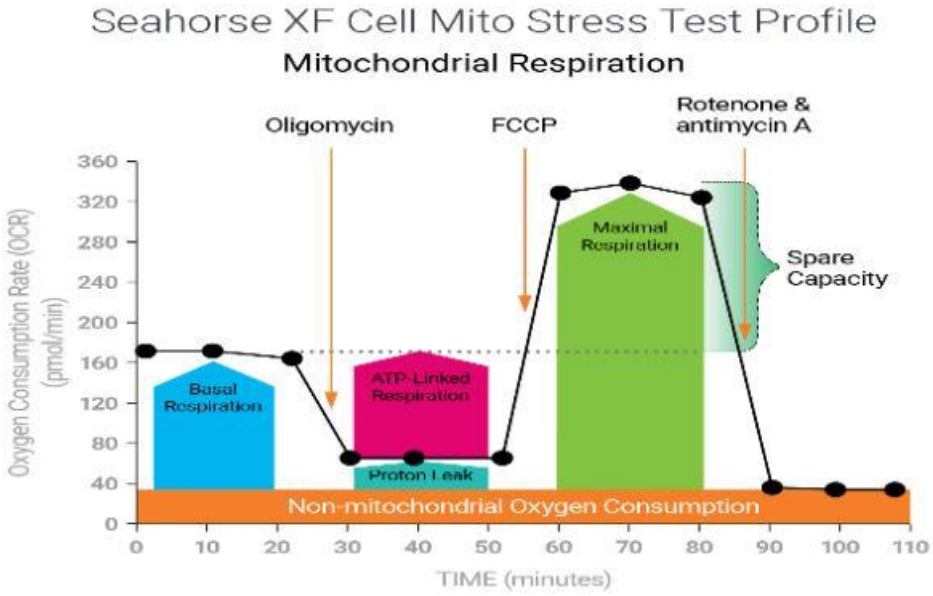

Figure 14: the injection sequence of the different modulators and the energetic parameters that can be obtained with the assay [116].

Oligomycin inhibits ATP synthase (complex V), and is injected first in the assay following basal measurements. It impacts or decreases electron flow through the Electron transport chain (ETC), resulting a reduction in mitochondrial respiration or OCR. This decrease in OCR is linked to cellular ATP production. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. It is the 2nd injection following Oligomycin. As a result, electron flow through the ETC is uninhibited, and oxygen consumption by complex IV reaches the maximum. The FCCP-stimulated OCR can then be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the ability of the cell to respond to increased energy demand or under stress.

4.16 Histone post-translational modification evaluation

To understand the effects of the supernatants on the HeLa cells histonic state, nuclear histone extraction and western blot were in order.

HeLa cells were seeded in dish and after 48 hours they were treated for 24 hours with *Lactobacillus crispatus* or *Lactobacillus reuteri* supernatant diluted 1: 100 in culture medium. Cells were harvested, washed with 10 mM sodium butyrate in PBS, and nuclei were isolated in according to

Amellem et al.[117]. Detergent-extracted fixed cells were prepared by resuspending cells in 1.5 ml low-salt detergent buffer (10 mM NaCl, 5 mM MgCl 2, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, 10 mM phosphate buffer (pH 7.4)). After 15 min 0.5 ml 4% paraformaldehyde were added to the extracted cells under vortexing. Nuclei were then fixed for 1 h and then washed twice in washing buffer (10 mM Tris, 0.15 mM NaCl, 2 mM Mg2Cl 2, and 0.1% Triton X-100 (pH 7.4)). All steps were carried out at 0°C.

The nuclear histones were subsequently extracted as described in the work of Calonghi et al. [118]. Briefly, the nuclear pellet was suspended in 0.4 M H_2SO_4 and incubated at 4 °C for 1 h. The suspension was centrifuged for 5 min at 14 000 × *g*, and the histones contained in the supernatant were precipitated overnight with 1 mL of acetone at −20 °C. The pellet was collected, air-dried and redissolved in 20 μL of water. Proteins were quantified using a protein assay kit (Bio-Rad, Hercules, CA, USA).

Histones were detected resolving samples on a 10% gel in MES buffer at 200 V for 40 minutes. Western Blot was performed in transfer buffer at 100 V for 1 hour. The nitrocellulose membrane was incubated with primary antibody specific for anti-acetylated lysines (Millipore, Billerica, MA) for 1 hour. After washes with PBS-TWEEN 20 0.1%, the membrane was incubated as before with secondary Horseradish Peroxidase-conjugated antibody (GE Healthcare, Milan, Italy). After washes with PBS-TWEEN 20 0.1%, antibody binding was detected by Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Milan, Italy). Densitometry analysis was performed by Fluor-S Max MultiImager (Bio-Rad, Hercules, CA, US), and relative quantification of histone acetylation signals was done by using densitometry and normalized on H1 signal as a control.

4.17 Quantitative Real-Time PCR

HeLa cells have been treated with *Lactobacillus crispatus* or *Lactobacillus reuteri* supernatant diluted 1: 100 in culture medium for 24 hours. Total RNA has been isolated by RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. 1 μg of RNA has been reverse-transcribed with RevertAid First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada) by using oligo(dT) primers. The cDNA has been analyzed by quantitative Real Time PCR (qRT-PCR), by employing the LightCycler FastStart DNA Master SYBR Green I Kit and the LightCycler 2.0 instrument (Roche Diagnostics, Manheim, Germany). Gene expression has been quantified by $\Delta\Delta C_T$ method, by using *β actin* as the housekeeping gene. The list of primers and conditions for quantitative Real-Time PCR. are report in **Table 3**, Amplicon specificity has been verified by first-derivative melting curve analysis and agarose gel electrophoresis.

Table 3: List of primers and conditions for quantitative Real-Time PCR.

PRIMERS	SEQUENCE
β actin FW *β actin REV*	CCAACCGCGAGAAGATGA CCAGAGGCGTACAGGGATAG
p21 WAF1 FW *p21 WAF1 REV*	CCTAAGAGTGCTGGGCATTTT TGAATTTCATAACCGCCTGTG
CCND1 FW *CCND1 REV*	GCCAACTGGTGTTTGAAAGTA TCCGGTGTGAAACATCTAAGA
HER-1 FW *HER-1 REV*	AGCGTGAGGATTCCCGTAGCTCT GAGGCAAAATGTCTACTCTCCAG GC
ITAG5 FW *ITAG5 REV*	GGCAGAAGGCAGCAATGGTG AGGCATCTGAGGTGGCTGGA

4.18 Western Blot

HeLa cells have been treated with *Lactobacillus crispatus* or *Lactobacillus reuteri* supernatant diluted 1: 100 in culture medium for 48 hours. Cells were washed twice with PBS and lysed for 1 h in lysis buffer (HEPES, pH 7.4 40 mM, glycerophosphate 60 mM, p-nitrophenyl phosphate 20 mM, Na3PO4 0.5 mM NaCl 250 mM, Triton X-100 1%, PMSF 0.5 mM, and 10 mg/mL each of aprotinin, leupeptin, pepstatin and antipain, (Sigma-Aldrich, St. Louis, Missouri, United States) at 0◦C. Cell lysates were centrifuged at 12,000 × g for 20 min. Supernatants were collected and protein concentration determined by using the Bio-Rad protein assay method (Bio-Rad, Hercules, CA, United States). The proteins were resolved on a 7.5% or 10% polyacrylamide gel and immunoblotted with a rabbit anti-human α5 integrin subunit (Cell Signaling Technology, Danvers, MA, United States), or a rabbit anti-EGFR (Biorbyt, Cowley Road, Cambridge, UK) or a rabbit anti-cyclin D1 (Millipore, Billerica, MA) or a rabbit anti-p21 or mouse anti- α-tubulin (Sigma-Aldrich, St. Louis, Missouri, United States) antibodies. Detection of immunoreactive bands was performed by using a rabbit or mouse HRP-conjugated secondary antibody (GE Healthcare, Milan,

Italy) followed by Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Milan, Italy). Densitometry analysis of immunoreactive bands was done by Fluor-S Max MultiImager (Bio-Rad). Relative quantification of bands was performed by using α-tubulin signal as control.

4.19 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software (GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Results are expressed as Means ± Standard Deviation (± SD) of a series of independent experiments. Data were analysed by ANOVA test, followed by post-hoc Multiple Comparison tests (i.e. Dunnett's test). $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.0001$ (***) were considered statistically significant.

5.Results

5.1 Effects of sugars on Chlamydia infectivity

5.1.1 Incubation of EBs with sugar solution increase CT infectivity

To assess the effect of sugars on *C.trachomatis* infectivity of, EBs were incubated with solutions of glucose, sucrose and mannitol respectively at the concentrations of 0.5, 2.5 and 5 mM, for 2 hours at 37°C. Control EBs were incubated in PBS. After incubation, EBs treated with the sugar solutions were used for infect HeLa cells.

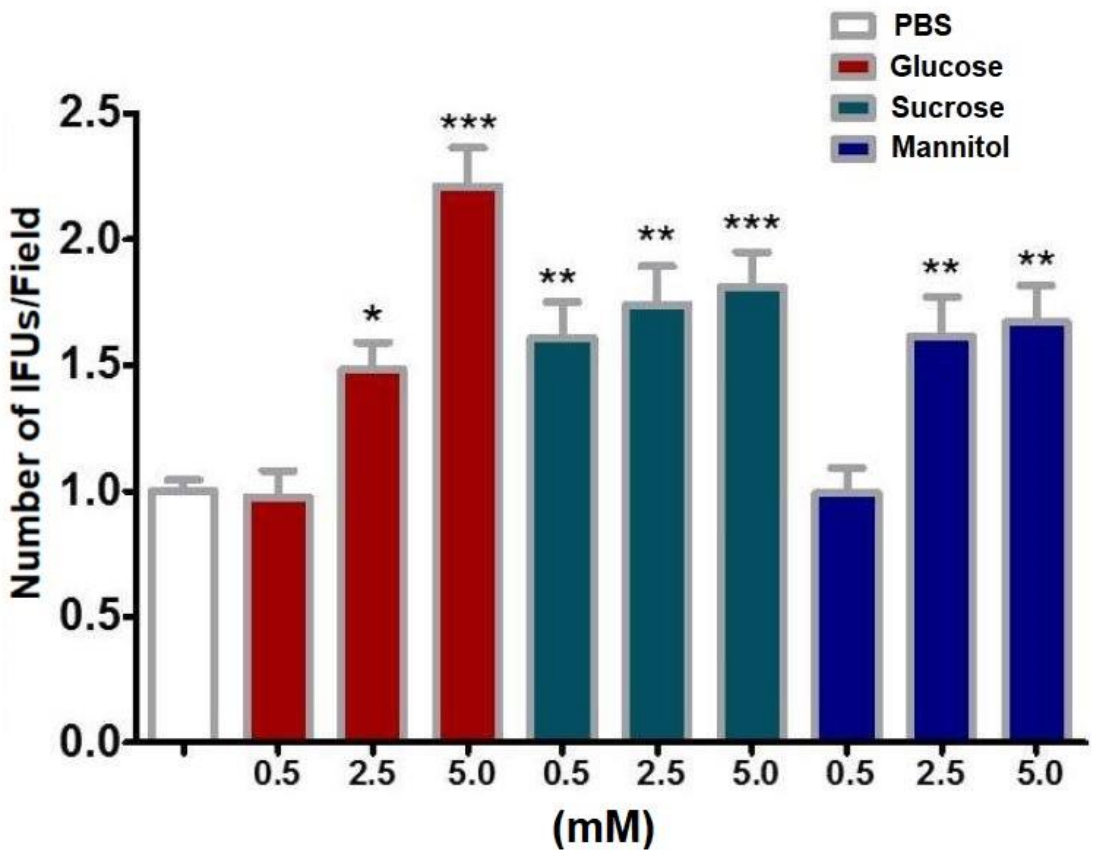

Figure 15: Evaluation of C. trachomatis (CT) infectivity after elementary bodies (EBs) incubation with sugar solutions. EBs were incubated with different sugar solutions (mannitol, sucrose, glucose; 0.5, 2.5 and 5 mM) for 2 h. Afterwards, HeLa cells were infected at a MOI = 0.1. CT infectivity was evaluated by counting the number of chlamydial IFUs. Results are given as Means ± SD of three independent experiments and are compared to control (PBS; EBs incubated in PBS with no sugars), taken as 1. The asterisks indicate a significant increase in CT infectivity (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$) compared to control. Statistical analysis was performed by ANOVA test, followed by Dunnett's multiple comparison.

As shown in **Figure 15**, sucrose is the only one sugar capable of increasing the infectivity of CT at all concentrations tested, while glucose and mannitol increase it at the highest concentrations, starting from 2.5 mM. Moreover, three important observations can be underlined:

1) Comparing the infective capacity of the incubated EBs at the highest sugar concentration (5 mM), glucose shows the greatest effect by increasing the inclusions of about 2.5 times compared to the control.

2) Globally, considering its significant effect even at the lowest concentration, sucrose exhibited the best activity in enhancing CT infectivity.

3) For all the sugars tested, a dose-response effect was noticed.

All subsequent experiments were carried out using sugar solutions at a concentration of 5 mM.

To test whether the increased infectivity of CT could be supported by an effect of the sugars on the host cells, HeLa cells were treated for 2 hours with the different sugar solutions at a concentration of 5 mM and subsequently infected with EBs.

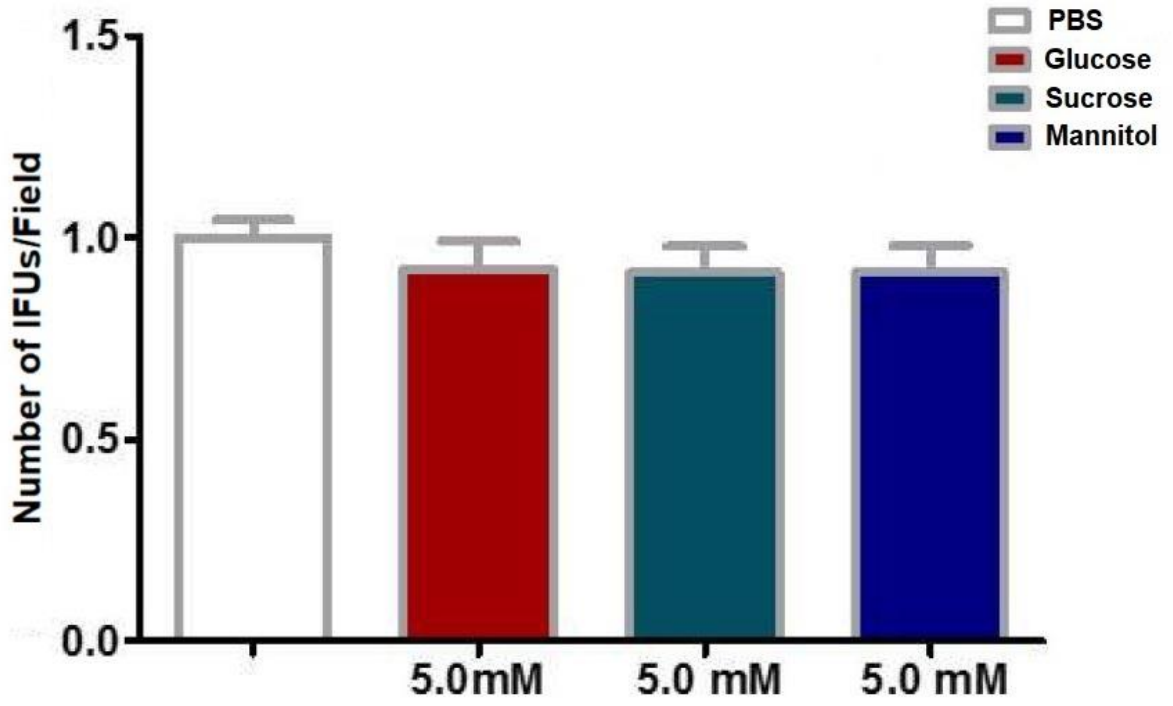

Figure 16. Evaluation of CT infectivity after HeLa incubation with sugar solutions. HeLa cells were incubated with different sugar solutions (mannitol, sucrose, and glucose 5 mM) for 2 h and then infected with CT EBs at a MOI = 0.1. CT infectivity was evaluated by counting the number of chlamydial IFUs. Results are given as Means ± SD of three independent experiments and are compared to control (PBS; HeLa incubated in PBS with no sugars), taken as 1. Statistical analysis was performed by ANOVA test, followed by Dunnett's multiple comparison.

As shown in **Figure 16**, it can be seen that sugars are non-inducing modifications in HeLa that can modify CT infections, and therefore there aren't significant differences between treated and control cells.

5.1.2 Sugars modify the membrane fluidity of EBs

To verify if the sugars could induce changes in membranes fluidity, static anisotropy measurements were performed both in HeLa and in EBs using TMA-DPH as a fluorescent probe. **Figure 17 (A)** shows significant anisotropy measure decrease in EBs treated for 2 hours with the solutions of glucose, sucrose and mannitol at a concentration of 5mM. On the contrary, in HeLa treated with the sugar solutions, there isn't any variation in the values of anisotropy as shown in **Figure 17 (B**). A

decrease in the anisotropy values indicates an increased fluidity of the membranes and this condition is often associated with modifications of the lipid bilayer structural organization.

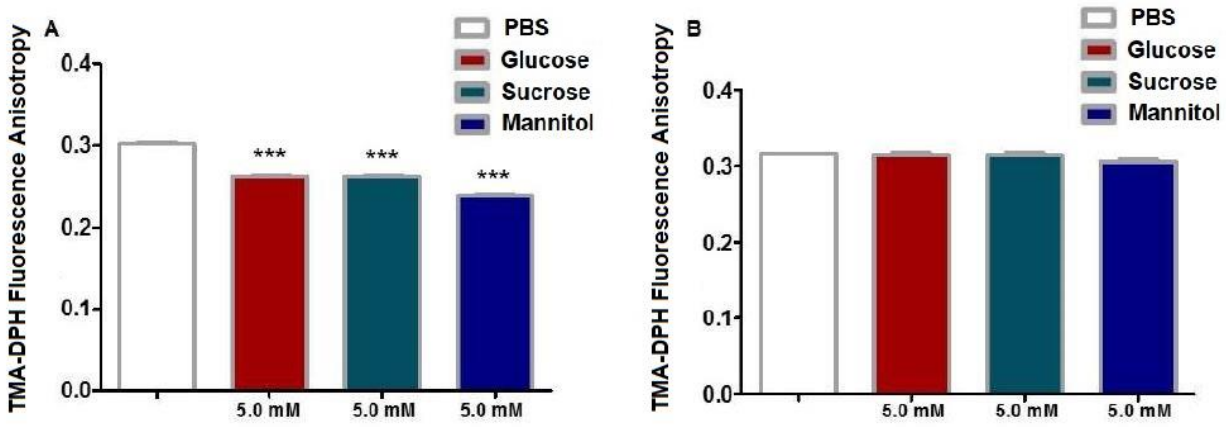

Figure 17. Evaluation of EBs and HeLa anisotropy after incubation with sugar solutions. EBs **(A)** or HeLa cells **(B)** were incubated with different sugar solutions (mannitol, sucrose, and glucose 5 mM) for 2 h. Afterwards, fluorescence anisotropy was evaluated by the hydrophobic probe TMA-DPH. Results are given as Mean ± SEM of three independent experiments and are compared to control (PBS; EBs or HeLa cells incubated in PBS without sugars). The asterisks indicate a significant modification in fluorescence anisotropy (***, $p < 0.0001$) compared to control. ANOVA test and Dunnett's multiple comparison were used for the statistical analysis.

To verify if the increase in the fluidity of the membranes of the EBs treated with the sugars could induce variations in the exposure of protein components, the EBs were labeled with fluorescent anti-LPS antibody and were tested using flow cytometry as a technique.

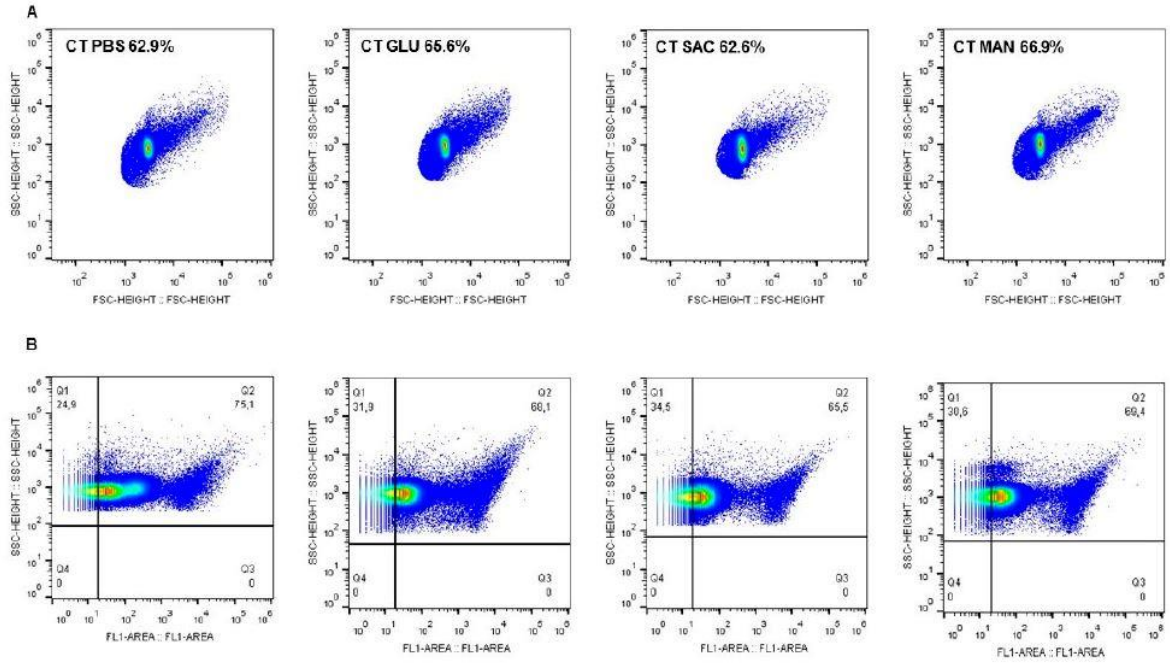

Figure 18: Quantification of labelled CT EBs by flow cytometry. **A)** Dot-plot of the size and granulometry of fluorescent CT EBs after incubation with PBS or glucose (5 mM), sucrose (5 mM), or mannitol (5 mM) for 2 h. **B)** Fluorescence of EBs LPS. A gate was drawn around the LPS-positive cells to calculate the percentages of labelled EBs. Four independent experiments showed similar results.

Figure 18 (A) shows the dot plot of the size and density of the fluorescent EBs after incubation with PBS or sugar solutions. **Figure 18 (B)** shows how the 75% of the control EBs expose the lipopolysaccharide in the membrane, while treatments with glucose, sucrose and mannitol reduce positivity about 10%, 13% and 8%, respectively. Overall, these data demonstrate that glucose, sucrose, and mannitol solutions increase the membrane fluidity of CT EBs and that this effect leads to a decrease in LPS exposure.

5.1.3 EBs treated with sugars induce the phosphorylation of FAK in infected cells

FAK is an intracellular kinase belonging to the non-tyrosine kinase family receptors. It is activated by integrins and can phosphorylates numerous targets intracellular cells that regulate cell migration, proliferation and differentiation. In particular, it has been shown that the phosphorylation of tyrosine 925 (Y925) is a prerequisite for anti-apoptotic activity [103], while phosphorylation of serine 722 (S722) is involved in the transduction of proliferation signals [104]. Furthermore, it is known from the literature that FAK plays an important role in the invasion and internalization of

various microorganisms, including *Yersinia enterocolitica*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Neisseria meningitidis* and *Salmonella typhimurium* [105][106][107]. Related to these observations, the phosphorylation status of FAK residues Y925 and S722 was evaluated in HeLa cells infected with EBs pre-incubated with sugar solutions. The cells were lysed and total protein content was analyzed by Western Blot.

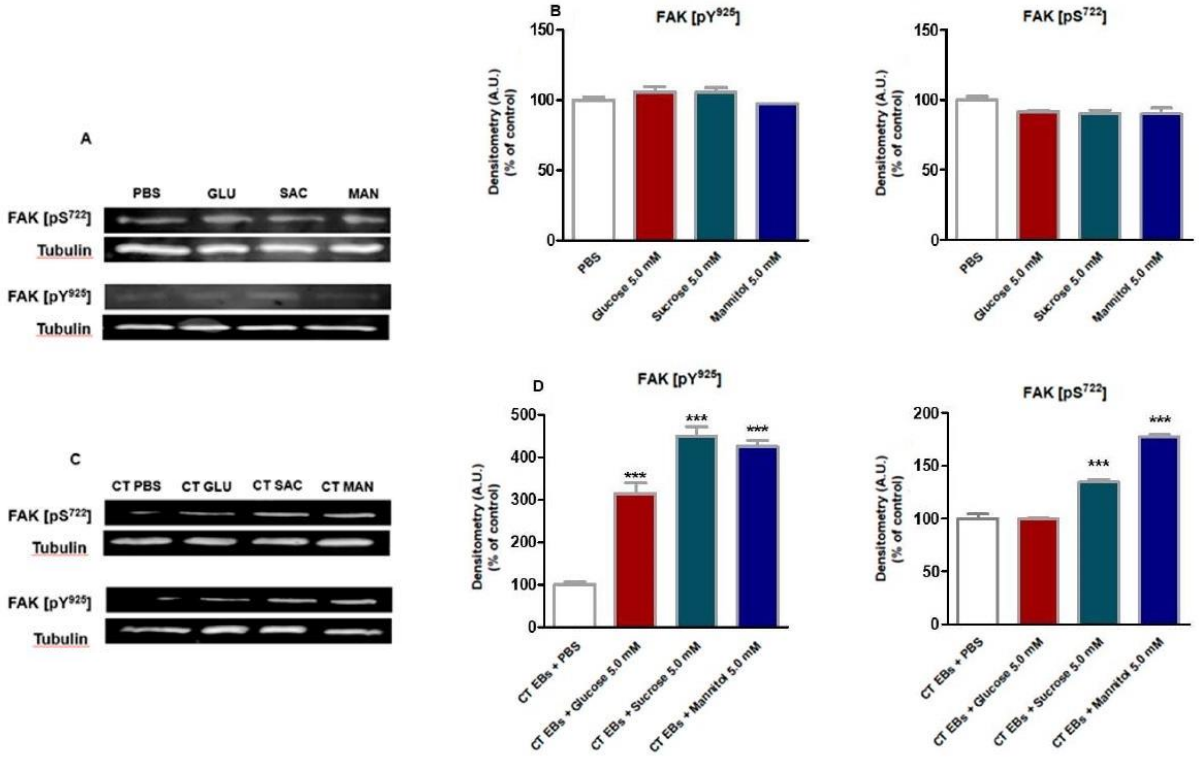

Figure 19: Phosphorylation state of Focal Adhesion Kinase (FAK) in HeLa cells. Two FAK phosphorylation sites were explored (tyrosine 925 -Y925- and serine 722 -S722-), by means of a Western blot analysis. (**A, B**) Hela cells were incubated with PBS or with glucose, sucrose, or mannitol at a concentration of 5 mM for 2 h, and then lysed and analysed by Western blot. (**C, D**) CT EBs were incubated with PBS or with the different sugar solutions (5 mM) for 2 h and then inoculated in Hela cells for 1 h. Subsequently the cells were lysed and analysed by Western blot. Relative quantification of FAK pS722/pY925 was performed using the tubulin signal as control. For each condition, densitometry arbitrary units (A.U.) were normalized by CT infectivity values. Results are given as Means ± SD of three independent experiments and are compared to controls (incubation with PBS), taken as 100%. The asterisks indicate a significant increase in FAK phosphorylation (***, $p < 0.0001$) compared to control. Statistical analysis was performed by ANOVA test, followed by Dunnett's multiple comparison.

Figure 19 (A, B) shows how HeLa incubation with sugar solutions does not lead to any change in the state of phosphorylation of the FAK protein. On the other hand, **Figure 19 (C, D)** shows how EBs incubated with sugar solutions induce a significant increase in the state of phosphorylation of FAK. In particular, phosphorylation on the S722 residue increases after treatment with sucrose or

mannitol, while Y925 phosphorylation was induced by the EBs treated with all three sugar solutions.

5.2 Effect of the two lactic acid isomers produced by two different lactobacilli on conferring HeLa cells protection against *Chlamydia trachomatis* infection

5.2.1 Evaluation of lactobacilli cell pellet and supernatant protective effect against C. trachomatis infection

For this *in vitro* study, two strains of lactobacilli belonging to two species, *L. crispatus* and *L. reuteri* were chosen. Among the differences between the two, lactic acid production represents a crucial characteristic for this study. *L.crispatus* was chosen because it is well known that it is able to produce both the isomers of lactic acids. On the other hand, *L. reuteri,* which is known to be very common in the GI tract, can produce only L (+) lactic acid) and, even if it is less present *in vivo* in the vaginal fluids, nevertheless it is more stable and it is easier to cultivate in comparison with others lactobacilli strains populating the vaginal environment.

The concentration of D(-)- or L(+)-LA was detected and determined in the supernatants of *L. crispatus* and *L. reuteri* after 24 hours of culture, by using HPLC. **Figure 20 (A)** shows how the lactic acid concentration is higher in *L.crispatus* than in *L.reuteri*.

In order to evaluate the role of lactobacilli in the prevention of *C. trachomatis* infection, two different infection evaluations were performed. First, the protective effect of the lactobacillus itself was testes. HeLa cells were incubated with *L. crispatus* or *L. reuteri* at a ratio of 1:100 or 1:200 (HeLa cells: Lactobacillus), for 1 h, and then treated with 5×10^3 CT EBs for 1h (exclusion mechanism).
As shown in Figure 6 (B) *L. crispatus* reduces the infectivity of *Chlamydia trachomatis* by 37% (ratio 1:100) or 73% (ratio 1:200), while *L. reuteri* is less active in protecting against infection.

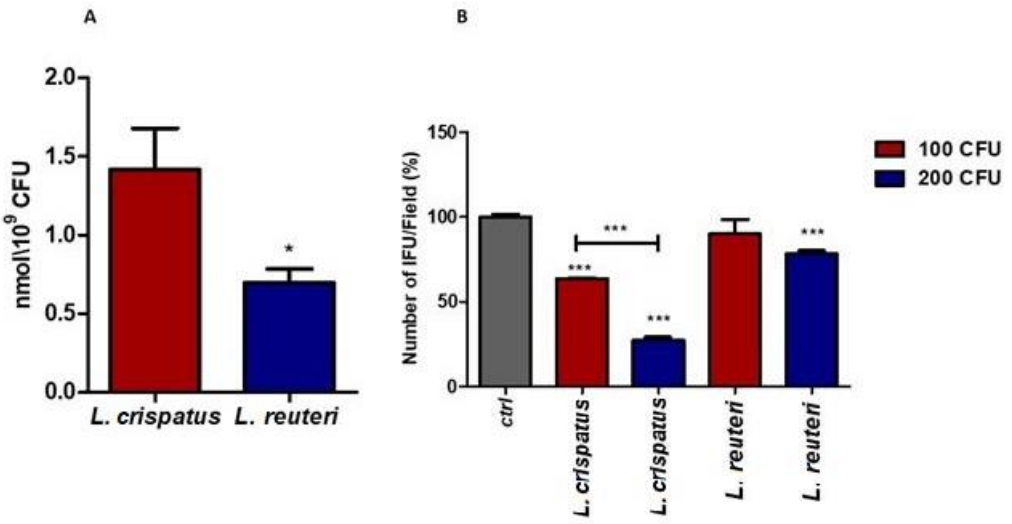

Figure 20: **Determination of the amount of lactic acid in lactobacilli supernatant and effect of lactobacilli cell pellets on *C. trachomatis* infectivity.** A) HPLC analysis of lactic acid content in the supernatant of *L. crispatus* and *L. reuteri.* Results were expressed as nanomoles (nmol) of lactic acid / microliter of supernatant containing 10^9 CFU of lactobacilli. Bars represent mean values and error bars represent standard deviations. Statistical significance was calculated vs. control (* $p \leq 0.01$). 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 ≤ 0.0001)

Secondly, the effect of the lactobacilli colture supernatant on the HeLa cells was tested. As reported in literature [101] lactic acid has directly a Chlamycidial effect on EBs, however lactic acid effect on the host cell, in the view of protecting from infection, is not completely understood. In order to investigate further this aspect, HeLa cells were treated with diluted 1:100 (in PBS) lactobacilli colture supernatants for 1 hour and then infected with CT EBs as reported previously. Control is represented by a treatment of HeLa cells with PBS for the same duration.

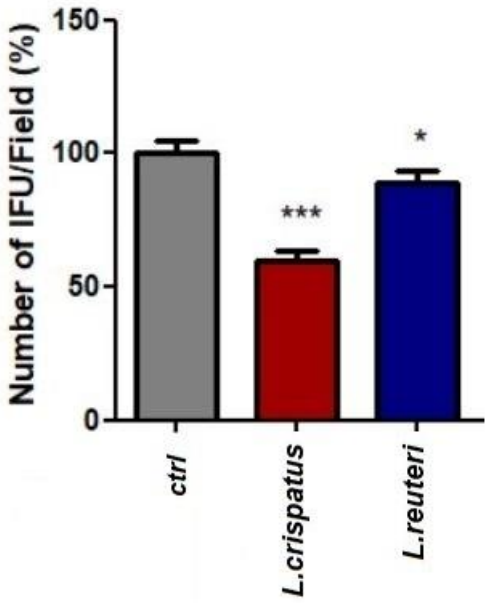

Figure 21: **Effect of lactobacilli diluted supernatants on *C. trachomatis* infectivity**. Quantification of Hela cells positive to CT EBs after treatment with 1:100 diluted supernatants and subsequent infection. Results were expressed in percentage compared to control taken as 100%. Bars represent mean values and error bars represent standard deviations. Statistical significance was calculated vs. control (*P ≤ 0.0001)

The protective properties of lactic acid seem to be correlated to different mechanism: the protonated form of lactic acid can permeate the cell membranes and acidify the cytosol; moreover, lactic acid can also weak the bacterial cell wall or interfere with host cell membranes reducing their susceptibility to infection and finally can induce epigenetic modification that could interfere gene expression of target cells [101] [119] [120]. These properties, independently or together, determine the susceptibility of the host and consequently the host-microbiota relationship.

5.2.2 Assessment of Lactate dehydrogenases activity and presence

In order to evaluate the possible mechanisms underlying the different protective capacity of the two lactic acid isomers against *Chlamydia trachomatis* infection, the activity of both D- and L- lactate dehydrogenase in the two strains of lactobacilli used was evaluated. By this analysis the production of the different isomers of lactic acid by *L.crispatus* and *L.reuteri* can be confirmed.

The enzymes activity was tested on both pellets and bacterial lysates using the resazurin reduction assay.

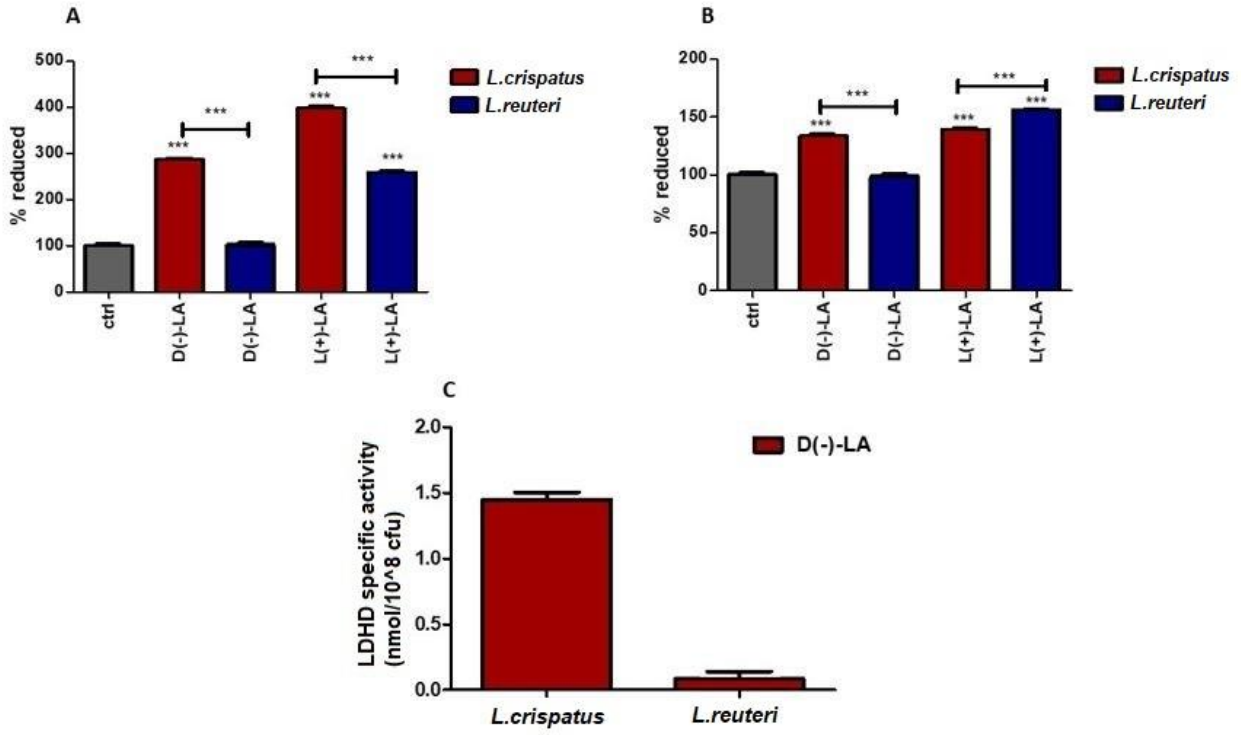

Figure 22: **Lactate dehydrogenase activity assessed by Resazurin assay**. A) bacterial pellets B) bacterial lysates C) specific activity of LDHD in bacterial lysates. Results are given as means ± SD of three independent experiments and are compared to controls, taken as 100%. (* $p < 0.05$; **$p < 0.001$; ***$p < 0.0001$).

The results reported in **Figure 22 (A, B)** confirm the ability of *L.crispatus* to use both D (-) and L (+)- lactic acids as substrate while *L.reuteri* is able to use only the L (+)- isomer. To confirm these results, we have measured the activity of the LDHD in the presence of a saturating concentration of substrate that can be roughly indicative of the amount of enzyme. The results presented in **Figure 22 (C)** confirm the presence of the LDHD in *L.crispatus* lysate, with a maximal specific activity of 1.46 nmol / 10^8 CFU, and the its almost totally absence in the *L.reuteri* lysate in which the maximal specific activity is 0.08 nmol / 10^8 CFU.

These data were to be confirmed by the analysis carried out by LC-MS.

Indeed LC-MS analysis of peptides obtained by the digestion of proteins from *L. Crispatus* led to the identification of 114 proteins among which the D-lactate dehydrogenase was present. The identified peptides along with the other parameters achieved by SwissProt database search are reported in **Table 4**.

Table 4: Results from SwissProt database searching. Identified peptides along with their observed and expected molecular weight, mass error (delta), score, rank, univocity and sequences are reported.

Peptide	Observed	Mr(expt)	Mr(calc)	Delta	Score	Rank	U	Peptide
11-21	717.9360	1433.9360	1433.6827	0.1748	84	1	U	R.KDEEPFLNEWK.E
26-42	969.1105	1936.2065	1936.9677	0.2388	15	1	U	K.DIDVDYTDKLLTPETAK.L
78-88	610.3540	1218.6935	1218.5551	0.1385	11	1	U	R.NVGVDNIDMDK.A
146-163	950.1102	1898.2058	1897.9833	0.2225	21	1	U	R.DQVVGVVGTGHIGQVFMR.I
146-163	633.7457	1898.2154	1897.9833	0.2321	55	1	U	R.DQVVGVVGTGHIGQVFMR.I
164-171	852.5232	851.5160	851.4211	0.0948	14	3	U	R.IMEGFGAK.V
164-171	1426.7667	851.5189	851.4211	0.0978	26	1	U	R.IMEGFGAK.V
227-236	559.8474	1117.6802	1117.5550	0.1252	53	1	U	K.DGVVIVNCSR.G
227236	1118.6879	1117.6806	1117.5550	0.1256	49	1	U	K.DGVVIVNCSR.G
313-319	410.7602	819.5058	819.4239	0.0819	14	3	U	K.AFNNNLK.L

The same analysis performed on proteins from *L. reuteri* led to the identification of 92 proteins, but the D-lactate dehydrogenase was not found.

5.2.3 Supplementation of D/L lactic acid to L.reuteri supernatant

To test whether D-LA acid has a powerful effect on the inhibition of *C. trachomatis* infection, HeLa cells were incubated with *L. reuteri* diluted culture supernatants enriched with D (-)-LA or L (+)-LA at a final concentration of 10 mM for 1 h, and then infected with CT EBs.

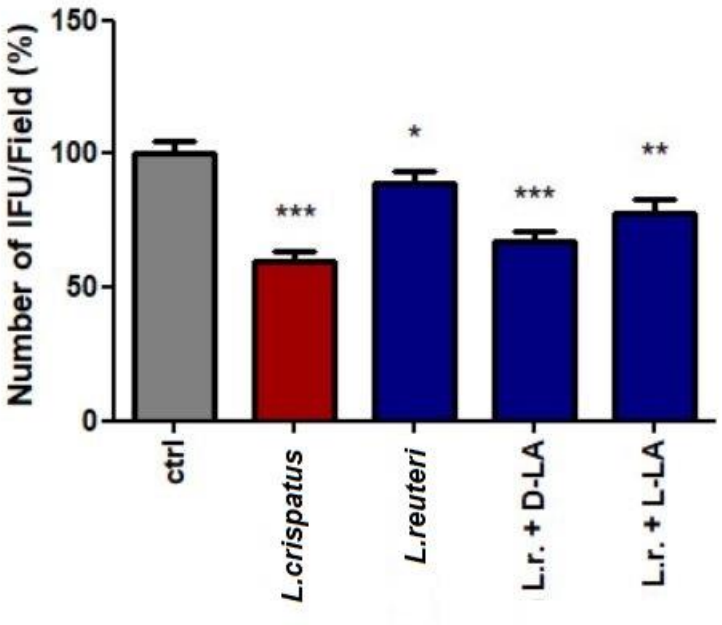

Figure 23: Percentage of *C. trachomatis*-infected HeLa cells pre-exposed to *L.crispatus* culture supernatant (diluted 1:100), *L. reuteri* culture supernatant (diluted 1:100) and *L. reuteri* culture supernatant supplemented with 10mM D (-)-

or L (+)- LA. Results are from three independent experiments. Statistical significance is shown as follows: *P* value < 0.0001.

As expected, supplementation of *L. reuteri* culture supernatant with D (-) Lactic acid has higher inhibitory properties, comparable to those of *L. crispatus* culture supernatant. These results indicate that the D (-) Lactic acid produced can lead to a more potent inhibition of *C. trachomatis* infection than L (+) Lactic acid.

5.2.4 Oxygen consumption rate (OCR) in HeLa cells treated with L.crispatus/ L.reuteri supernatants

Assessed that D (-) Lactic acid has a stronger infection inhibitory effect to HeLa cells, an investigation on the cells metabolic status after the treatment was performed. A switch in the metabolic profile could explain why CT infection and developing are disfavoured, so a Cell Mito Stress Test was carried out. The HeLa OCR was measured in the presence of 10 mM of D (-) Lactic acid in comparison with the effect of *L. crispatus* or *L. reuteri* diluted supernatants (1:100) alone. Control was represented by HeLa in complete medium (RPMI).

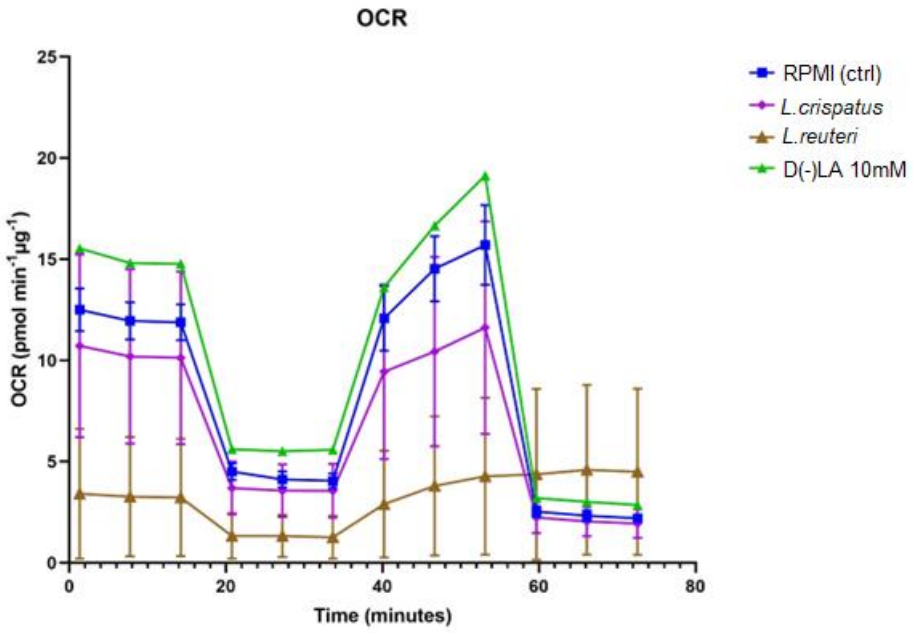

Figure 24: OCR real time measurements of HeLa cells treated with *L.crispatus* or *L.reuteri* diluted supernatants and with D (-) LA 10mM for 1h.

The results reported in Figure 23 show that the supplementation with the D (-) isomer alone stimulates the OCR in HeLa cells (green line) while the addition of the two lactobacilli supernatant decreases both the basal and the uncoupled oxygen consumption rate. This result suggests that in

the presence of D (-) Lactic acid isomer the HeLa cells show a more oxidative metabolic status; while the L (+) isomer, produced by *L.reuteri*, stimulates a more glycolytic metabolism.

5.2.5 Lactobacillus culture supernatants modulate histone modification state and gene expression

Through the inhibition of Histone Deacetylase (HDAC) Lactic acid can promote histone acetylation thus regulating gene expression [121]. Furthermore, inspired by the wide acylation of histones by intracellular metabolites, Zhang et al. recently found that lactic acid can also modify the lysine residues of histones in a new epigenetic modification known as lactylation, that directly stimulates gene transcription from chromatin. [122]. Modification in gene expression caused by epigenetic changes could give some more hints to explain D (-) Lactic acid protective effect.

For this reason, epigenetic modifications induced by supernatants with particular regard to the state of acetylation and lactylation of histone proteins were evaluated.

Hela cells were treated with *L.crispatus* or *L.reuteri* diluted supernatants for 1 h and then histones were extracted 24h after the treatment.

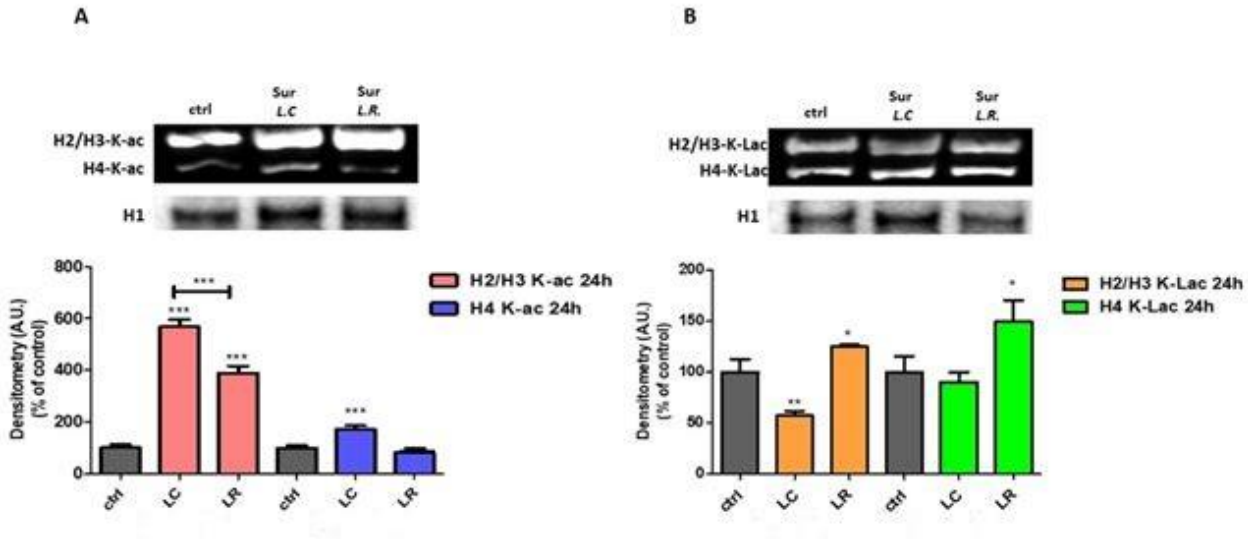

Figure 25: Changes in the state of histone acetylation and lactylation. A) (Above). Western Blot of acetylated histones. (Below). Relative quantification of H2/H3 acetylated histones in HeLa control and exposed for 24 h to culture supernatants of *L. crispatus* or *L. reuteri*. B) (Below). Western Blot of lactylated histones. (Below). Relative quantification of H4 lactylated histones in HeLa control and treated with culture supernatants of *L. crispatus* or *L. reuteri* for 24 h. Densitometry arbitrary units (A.U.) were normalized by H1 histone. Results are given as means ± SD

of three independent experiments and are compared to controls, taken as 100%. (* $p < 0.05$; **$p < 0.001$; ***$p < 0.0001$).

In **Figure 25 (A)** HeLa cells exposed for 24h to culture supernatants of *L. crispatus* or *L. reuteri* significantly increase acetylation of H2/H3 with *L. crispatus* showing a higher increase (5.7 folds vs about 4 folds increase of *L. reuteri*) ($p < 0.0001$). Even in the case of H4 acetylation, the treatment of HeLa cells for 24 h with *L. crispatus* culture supernatant induces a significantly increase (+ 72.6%), while the treatment with *L. reuteri* supernatant has no effect. Interestingly in **figure 25B**, supernatants of both *Lactobacilli* induce changes in the state of histone lactylation but in a different way: - the supernatant of *L. crispatus* significantly decreases the lactylation of H2 / H3 by about 41.7% ± 12.76 and it does not induce modifications of H4 histones; while - the supernatant of *L. Reuteri* significantly increases lactylation of both H2 / H3 and H4 histones by approximately 26.1% ± 15.37 and 52.5% ± 34.59, respectively.

These results indicated that *L. crispatus* and *L. reuteri* induce, via their metabolic products, different epigenetic modifications pattern in HeLa cells, which could lead to a distinct regulation of gene expression.

To verify whether the epigenetic modifications were related to changes in gene expression, we proceeded with the qtRT-PCR analysis and western blot of related host protein products, known to be important for Chlamydia entrance or developing, such as membrane proteins or cellular cycle regulators.

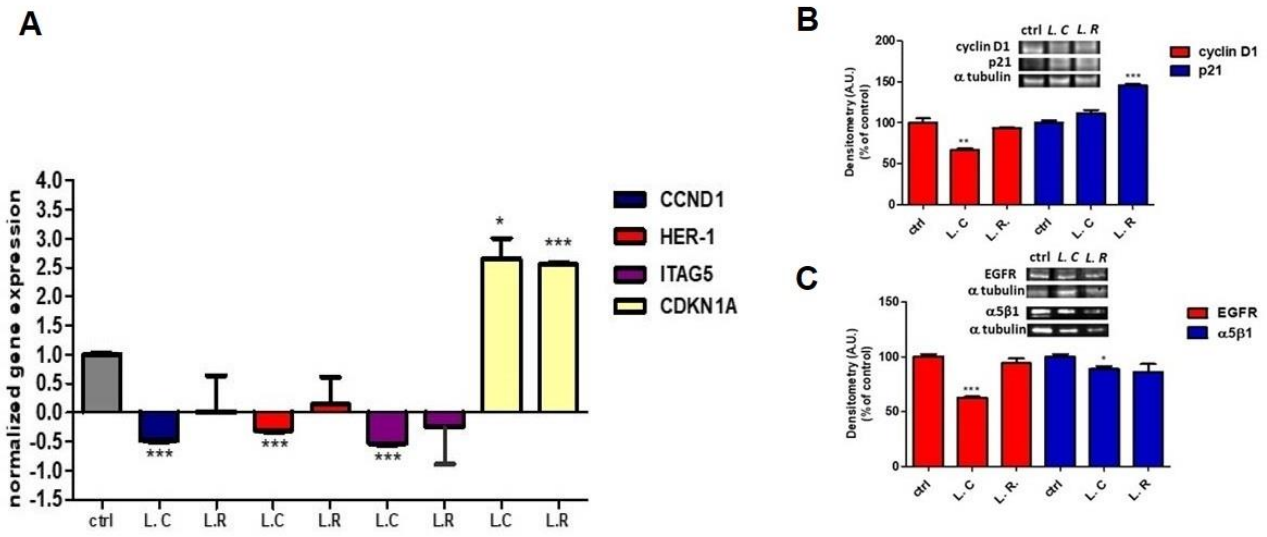

Figure 26: **Diluted supernatant of Lactobacilli modify gene and protein expression. A)** Gene expression for CCND1, HER-1, ITAG5, and CDKN1A analyzed by RT-PCR in HeLa control and treated with Lactobacilli diluted supernatants for 24 hours. **B)** Above: relative quantification of proteins. Below: western blot analysis of cyclin D1 protein and P21 protein. **C)** Above: relative quantification of proteins. Below: western blot of EGFR and α5β1 integrin

1 subunit. Densitometry arbitrary units (A.U.) were normalized by α-tubulin. Results are given as means ± SD of three independent experiments and are compared to controls, taken as 100%.

Transcription levels of CCND1, CDKN1A, HER-1, ITAG5 and were analysed by Real Time PCR performed on cDNA of HeLa control and treated with *L. crispatus* and *L. reuteri* diluted supernatants for 24 hours. Genes transcription was normalized to the reference gene β-actin and the relative variations (fold change) are reported in **Figure 26 (A)**. CCND1, HER-1 and ITAG5 genes expression were significantly downregulated only by *L. crispatus* by a factor of 0.47 ± 0.01 ($p \leq 0.001$ vs control), 0.29 ± 0.01 ($p \leq 0.001$ vs control) and 0.53 ± 0.05 ($p \leq 0.001$ vs control), respectively; on the other hand, CDKN1A transcription was significantly upregulated only by *L. reuteri* by a factor of 1.81 ± 0.19 ($p \leq 0.05$ vs control).

Cyclin D1, p21, EGFR and α5β1 proteins expressions have been analysed by western blot in control HeLa and treated with Lactobacilli supernatants for 48 hours. Supernatant of *L. crispatus* causes a decrease in EGFR, cyclin D1 and α5β1, while both supernatants increase the expression of p21 as reported in **Figure 26 (B, C)**.

6.Discussion and conclusions

The thesis investigates two different aspects of *Chlamydia trachomatis* infection, providing some insight that can be relevant in understanding how sugars can be beneficial for the bacterium, and, on the other hand, how some lactobacilli can be considered an important protective factor against infection.

In the first part of this thesis, the role of three different sugar solutions (glucose, sucrose and mannitol) on the infectivity of CT was studied, elucidating some of the physicochemical mechanisms and molecular factors underlying the interaction of EBs with sugars, in agreement with previous works suggesting that sugars can have a beneficial effect on CT survival, replication, and virulence [60]. First, sugars have been shown to increase the infectivity of CT, acting directly on Chlamydia EBs. In the presence of a dose-response effect, sugar solutions at the highest concentrations (5mM) increase almost by double the number of Chlamydia inclusions (**Figure 15**). In parallel, any effect of sugars on HeLa cells has been excluded (**Figure 16**). Results were later strengthened by the demonstration that sugar solutions significantly increase the fluidity of the EB membrane, without any modifications regarding HeLa cell membranes (**Figure 17**). It is known that environmental factors such as pressure, temperature, pH, nutrients, water activity, ions, and enzyme action can change the structure and physico-chemical properties of microbial membranes [123]

[124]. Indeed, results indicate that mannitol, glucose and sucrose can also be significant factors changing the characteristics of CT membranes. In this context, it has been found that sucrose and mannitol can increase the stability of chlamydial membranes and proteins (i.e., MOMP: major outer membrane proteins), potentially lengthening the viability of CT EBs [125] [126] [127]. Higher levels of sucrose and mannitol can be found in urine in case of peculiar dietary habits (i.e., mannitol: consumption of hard candies, fruits and vegetables, sucrose: sugar-rich diet) [128] [129]. For that reason, during an uro-genital infection, the presence of these sugars in the urethral lumen could favour CT acquisition or delay its clearance. Furthermore, to confirm the hypothesis that sugars can modify the EB membrane, by means of a cytofluorimetric analysis, results shows that the presence of sugars led to a significant decrease in LPS exposure (**Figure 18**). This result is particularly interesting: Chlamydial LPS is involved in bacterial entry into epithelial cells [130] [131], and it is essential for secondary differentiation of RBs to infectious EBs [132]. Moreover, it is characterized by a unique lipid A structure, associated with a significantly less stimulatory activity than enteric LPS in inducing proinflammatory signals by human epithelial cells [133] [134]. Therefore, its reduction could decrease the pro-inflammatory properties of CT, increasing the possibility of asymptomatic infections in vivo. The reduction in LPS exposure due to a new arrangement of EB membranes could be contemporarily accompanied by a higher expression of chlamydial molecules used as ligands for the entry into the epithelial cells; this could potentially explain the increased CT infectivity induced by sugar solutions. A different sugar-induced expression of EB membrane molecules could also be the basis of the significant higher activation of HeLa signalling pathways found after EB attachment. In fact, results have proven that EBs incubated with the sugar solutions, in particular those of sucrose e mannitol, induce phosphorylation of FAK S722 and Y925 residues during early stage of HeLa infection (**Figure 19**). At the same time, experiments excluded that FAK phosphorylation was only due to a higher chlamydial entry into epithelial cells.

FAK is known to be an intracellular protein member of non-receptor tyrosine kinase, activated by an integrin mediated engagement and involved in cellular adhesion and spreading processes. Its autophosphorylation is a prerequisite to trigger its activity as a signalling protein within cytoskeleton associated networks. In particular, it has been shown that the phosphorylation of tyrosine 925 is a prerequisite for anti-apoptotic activity [103], whereas the phosphorylation of serine 722 is involved in the transduction of proliferation signals [104]. Considering that the remodelling of the host cell actin cytoskeleton is usually required for efficient bacterial invasion, there has been increasing interest in the role of FAK in the link between microbial recognition and the initiation of pro-inflammatory responses. As a matter of fact, FAK has been reported to be involved in the

internalization of several intracellular microorganisms including chlamydiae [105] [106] [107]: as reported by literature, integrins have been shown to interact with the pathogen through their extracellular domains and with their intracellular β-tails interact with the FAK, activating it [84] [135] [136]. It's possible that sugars induce a higher exposure of EB ligands able to activate FAK pathways in HeLa cells. In this way, chlamydial EBs can enhance the activation of anti-apoptotic and proliferative signals in the host cells, favouring their infectivity and survival into the host cells. In conclusion, these results indicate that a sugar-rich diet [128] [129] could pose a positive and enhancing factor for *Chlamydia trachomatis* infection, representing therefore an issue, since CT infects mostly young women, which represents a relevant fraction of candy consumers [137].

In the second part of the thesis, two different lactobacilli, *Lactobacillus crispatus* and *Lactobacillus reuteri*, along with their lactic acid production, have been tested to investigate protective effects against CT infection. Since lactic acid is one of the most important antimicrobial compounds produced by vaginal lactobacilli [101], its production was the first evaluation made in lactobacilli free supernatants by HPLC. Results shows that *L.crispatus* is a greater lactic acid producer and exclusion experiments also point that is more protective against CT infection than *L.reuteri* , as expected [109] (**Figure 20**). To further investigate the role of the two different isomers of lactic acid produced by the tested bacteria, properly diluted lactobacilli-free supernatants were also evaluated in CT exclusion experiments. Results confirm that supernatant from *L.crispatus* as the more efficient in prevent CT infection with near 40% reduction of total inclusions number (**Figure 21**). These findings are in agreement with the important *L.crispatus* protective effect described in literature [84] [98] [99] [100] [108]. To understand how the two isomers can have a role in the determination of the protective effect conferred by lactic acid, presence and activity of D-lactate dehydrogenase enzyme were determined for both lactobacilli by resazurin assay, NAD^+ formation and LC-MS measurements. Findings demonstrate that, *L.crispatus* is able to produce both isomers of lactic acid, while *L.reuteri* can produce only L (+)-LA (**Figure 22**). The work of Edwards et al. reports that lactobacilli producing only the L (+)-LA, belong to the low active strain in protection against CT. Since the acid-base properties of the two isomers are quite similar, the high power of the D(-)-LA should be bound to a specific target [138]. To further investigate this hypothesis, exclusion experiments were performed by adding to *L.reuteri* supernatant with 10 mM D(-)- or L(+)-LA. The analysis reveals that D(-)-LA addition results in an increase in protection against CT infection, similar to the *L.crispatus* supernatant treatment (**Figure 23**).

In order to reveal further mechanisms that may explain a greater protective effect of the D (-)-LA, an analysis was performed both on the epigenetic modifications of histone proteins and on the expression of genes involved in the regulation of proliferation.

HeLa cells exposed for 24 hours to *L. crispatus* culture supernatant significantly increased H2/H3 and H4 acetylation, on the contrary treatment with *L. reuteri* supernatant increased H2/H3 acetylation to a lesser extent and has no effect on the H4 acetylation status (**Figure 25 A**). Histone lysine acetylation is well known for being important in the epigenetic regulation of gene expression in eukaryotic cells. Indeed, it is known that reversible acetylation of the N-terminal lysine residues of H4 and of H3 mediates the de-condensation of the nucleosome structure [139] [140] alters histone interactions with the DNA [141] and facilitates access and binding of transcription factors to genes transcribed by RNA polymerase II or III [142] [143].

Interestingly, supernatants of both *Lactobacilli* also induce changes in the state of histone lactylation. The supernatant of *L. crispatus* significantly decreases the lactylation of H2 / H3 and it does not induce modifications of H4 histones, while the supernatant of *L. reuteri* significantly increases lactylation of both H2 / H3 and H4 histones (**Figure 25 B**). This result is difficult to interpret since the study of the epigenetic regulation of genes by histone lactylation is in its infancy. However, it will be interesting to comprehensively define how lactate metabolism influences epigenetic programming under different cellular conditions. The mechanistic set of events that require definition in different cellular contexts includes the mechanisms by which the microenvironment causes fluctuations in metabolites, the effects of metabolite fluctuations on epigenetic modification complexes and epigenetic events, and the mechanisms by which sensitive epigenetic events to the metabolites are translated into specific gene programs of cellular differentiation.

In their work, Zhang and colleagues show that lactate drives the lactylation of histone lysine residues and directly modulates gene transcription. In human HeLa cells and mouse bone marrow–derived macrophages (BMDMs), histone lysine lactylation (Kla) is driven by extracellular lactate or glucose-derived intracellular lactate and is increased during hypoxia, which increases glycolysis and the production of lactate. In transcription assays, histone lactylation promotes gene transcription similar to histone acetylation [122].

The observation that treatment of HeLa cells with the supernatant of the two lactobacilli strains induces an altered pattern of histone acetylation and lactylation suggested that they may activate different epigenetic pathways.

Indeed, the treatment with *L. crispatus* supernatant induces a downregulation of the genes involved in cell cycle regulation (CCND1, CDKN1A and HER-1), and a significant downregulation of the ITGA5 gene encoding the α5β1 integrin. WB analysis of Cyclin D1, p21, EGFR and α5β1 protein expressions confirms the RT-qPCR results (**Figure 26**). These results are in agreement with those

reported by other authors. Edwards et al. [138] has mentioned that the vaginal microbiota can counteract the susceptibility to C. trachomatis infection by controlling the cell cycle. Their finding suggest that lactobacilli producing D(-)-LA downregulate the proliferation of the vaginal epithelial cells protecting them from the susceptibility to *C.trachomatis* infection. Authors showed that culture supernatants of *L. crispatus* and *L. jensenii* modulated the expression of multiple genes related to cell proliferation, including a decrease of CCND1 and HER-1. On the contrary, *L. iners*, which does not have the ability to produce D(-)-LA, was unable to downregulate these genes, not protecting against *C. trachomatis* infection [138]. Furthermore, previous studies have shown that EGFR is required for internalization of chlamydial elementary bodies (EBs) into host cells [144]. Calonghi and colleagues have shown that, when a specific blocking antibody masked the α5 integrin subunit or the corresponding gene expression has been silenced, CT infection was greatly reduced, supporting the hypothesis that the α5 subunit is crucial for CT infection in HeLa cells [84].

Lastly, the additional metabolic evaluation performed by the Seahorse Flux Analyzer shows that HeLa cells treated for 24h with *L.reuteri* supernatant are characterized by a strong decrease in the OCR profile, suggesting a metabolic shift from oxidative to glycolytic metabolism that could favour CT infection, while *L.crispatus* supernatant and 10 mM D(-)-LA treatments do not alter the HeLa metabolic profile (**Figure 24**). CT is an obligate intracellular bacterium with a very small (~1.04 Mb) genome [13] lacking several genes for metabolic enzymes. For this reason, CT is strongly influenced by the host metabolic conditions. Since CT lacks hexokinase, EBs and the RBs require glucose-6-phosphate. Moreover, during the transition from EB to RB, CT is not able to satisfy the increased request for ATP, causing the RB to be entirely dependent on host derived ATP as an energy source [2] [3]. Rother et al. [145] have examined the changes in the host metabolism after CT infection, founding that many metabolites belong the glycolysis and TCA intermediate were upregulated in a fashion that resembles Warburg metabolism.
In conclusion, these findings assess the more powerful effect of D(-)-LA versus the L(+)-LA in conferring protection to the host cell, providing new considerations for the choice of lactobacilli probiotic strains towards prevention of *Chlamydia trachomatis* infection.

7.Bibliography

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