Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN
SCIENZE BIOTECNOLOGICHE E FARMACEUTICHE

XXXII Ciclo

Settore Concorsuale: 03/D2

Settore Scientifico Disciplinare: CHIM 09

DEVELOPMENT OF INNOVATIVE DELIVERY SYSTEMS TO COUNTERACT VAGINAL INFECTIONS

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Esame finale anno 2020

Abstract

The present thesis was focused on the development of innovative delivery systems to counteract vaginal infections and dysbiosis, particularly bacterial vaginosis and vulvovaginal candidiasis. These conditions widely compromise the quality of life of women worldwide and also expose to complications, including higher susceptibility to viral and sexually transmitted infections and increased risks of preterm birth and late termination of pregnancy.

In particular, the project aimed to overcome some drawbacks associated with the currently available therapies, such as the high incidence of recurrent vaginal infections and the growing emergence of drug resistance, and with the use of conventional dosage forms, like the poor retention at the vaginal site that reduces efficacy and patient compliance.

In this contest, both formulations based on well-established drugs (chlorhexidine and econazole nitrate) delivered in innovative systems or formulations containing alternative active agents (probiotics cells and polyphenols) able to counteract vaginal infections have been taken into account. Moreover, in order to improve the vaginal health *status*, local as well as oral administration routes have been investigated.

In the first proposed work (*paper 1*), the strain *Bifidobacterium breve* BC204 was isolated from the vagina of a healthy woman and indagated for its probiotic potential. It showed good ability to adhere to Caco-2 cells, moderate ability to resist to gastrointestinal stress and strong antimicrobial activities towards both urogenital and enteric pathogens. Given these promising features, *B. breve* BC204 was subsequently protect by spray-drying encapsulation and formulated inside orally time-dependent erodible lipid tablets, with the aim to exert beneficial effects both at intestinal and vaginal level. The final dosage forms provided high loading and survival of *B. breve* BC204, as well as a delayed release and mucoadhesive abilities, which are required to guarantee the presence of an adequate amount of beneficial probiotic cells at the treatment site.

In the second work (*paper 2*), the freeze-drying technique was applied to produce lyophilized polymeric matrices for the local delivery of chlorhexidine, with the aim to prolong the vaginal release of this disinfectant, thus reducing the daily dose frequency.

To achieve this objective, chlorhexidine was first complexed by means of ionic interactions with different polyanion polymers, then freeze-dried matrices containing either the isolated complex or the complex along with free drug and polymers were prepared. The selection of suitable polymers and the use of the adequate preparative

method allowed to modulate matrix characteristics. In particular, matrix prepared with hyaluronate showed a flexible structure, the best hydration and mucoadhesive properties and the best profile of drug release, as well as excellent antimicrobial properties.

The last two papers focused on the development of nanosystems based on lipid vesicles for treatment of candidiasis. In this regard, two different strategies were followed.

In a first approach (*paper 3*), econazole nitrate was vaginally delivered in phosphatidylcholine vesicles containing a biosurfactant, which was selected as "green" alternative excipient over chemical surfactants (i.e. Tween 80). The biosurfactant was isolated from a vaginal probiotic strain and characterized. It revealed a peptide-like structure and good surfactant and emulsifying properties. Mixed vesicles were further obtained by film rehydration and extrusion method and presented optimal diameter range for vaginal administration. Compared to vesicles containing Tween 80, those prepared with the biosurfactant showed higher encapsulation efficiency and mucoadhesion ability, as well as a sustained drug release. Notably, they also significantly improved the ability of econazole to eradicate *Candida* biofilm.

The second approach (*paper 4*) aimed to develop new liposomes for the simultaneous vaginal delivery of two polyphenols that could act in combination to eradicate the infection and alleviate candidiasis symptoms, like burning and irritation. Thus, quercetin and gallic acid were proposed as alternative to conventional antifungals to deal with the problem of azole resistance. Film rehydration and sonication method was used to obtain liposomes carrying both polyphenols with the desired size of 200 nm. They showed good entrapment efficiencies and better release profiles with respect to formulations containing only one polyphenol. Moreover, liposomes displayed strong anti-oxidant and anti-inflammatory activities and resulted not cytotoxic to cells. Finally, they were able to counteract the growth of *Candida* owing to the antifungal effect exerted by gallic acid.

Riassunto

Lo scopo del presente lavoro di tesi è lo sviluppo di sistemi di veicolazione innovativi efficaci nel trattamento di infezioni e disbiosi vaginali, con particolare attenzione alla vaginosi batterica e alla candidosi vulvovaginale. Queste condizioni, oltre ad impattare pesantemente sulla qualità di vita di molte donne in tutto il mondo, espongono anche a complicazioni legate a un'aumentata suscettibilità alle infezioni virali e a trasmissione sessuale e a una maggiore probabilità di parto pretermine e aborto tardivo.

In particolare, il progetto di tesi si propone di superare alcune delle criticità associate alle terapie attualmente disponibili, come l'elevata incidenza di infezioni vaginali ricorrenti e il problema crescente della farmaco resistenza, e all'utilizzo delle forme di dosaggio convenzionali, come la scarsa capacità di ritenzione a livello vaginale che riduce l'efficacia del trattamento e la compliance della paziente.

A tal fine, sono stati proposti sia sistemi innovativi per la veicolazione di farmaci impiegati comunemente (clorexidina ed econazolo nitrato), che formulazioni contenenti agenti attivi alternativi (probiotici e polifenoli) in grado di contrastare le infezioni vaginali. Inoltre, sia la via di somministrazione locale che quella orale sono state prese in considerazione per ottenere un miglioramento delle condizioni di salute a livello vaginale.

Nel primo lavoro presentato (*paper 1*), il ceppo vaginale *Bifidobacterium breve* BC204 è stato isolato da donna sana e caratterizzato per il suo potenziale probiotico. Il ceppo ha mostrato una buona capacità di adesione alle cellule Caco-2, una moderata resistenza all'ambiente gastrointestinale e una pronunciata attività antimicrobica nei confronti sia di patogeni urogenitali che enterici. Date queste promettenti proprietà, *B. breve* BC204 è stato successivamente microincapsulazione mediante spray-drying e formulato in compresse orali a base lipidica in grado di cedere il probiotico secondo un fenomeno di erosione tempo-dipendente. Le forme di dosaggio finali presentavano un profilo tecnologico ottimale per garantire la presenza di una quantità adeguata di cellule vitali al sito di trattamento, ovvero un elevato caricamento e sopravvivenza di *B. breve* BC204, un rilascio ritardato del probiotico e capacità mucoadesive.

Nel secondo lavoro (*paper 2*), è stata applicata la tecnica di freeze-drying per produrre matrici polimeriche liofilizzate contenenti clorexidina, allo scopo di prolungare il rilascio vaginale di questo disinfettante e ridurre quindi la frequenza di somministrazioni giornaliere. La clorexidina è stata dapprima complessata con diversi polimeri polianionici tramite interazione ionica e successivamente sono stati preparati due tipi di matrici, uno

contenente solo il complesso isolato e uno contenente anche il farmaco e il polimero non complessati. I risultati hanno mostrato che è possibile modulare le caratteristiche delle matrici tramite la scelta dei polimeri adeguati e del metodo preparativo più idoneo. Nello specifico, le matrici a base di acido ialuronico hanno mostrato una struttura flessibile, le migliori proprietà di idratazione e mucoadesione e il miglior profilo di rilascio del farmaco, oltre che eccellenti proprietà antimicrobiche.

Gli ultimi due lavori sono stati invece incentrati sullo sviluppo di nanosistemi basati su vescicole lipidiche per il trattamento della candidosi e, al riguardo, sono state adottate due strategie.

In un primo approccio (*paper 3*), l'econazolo è stato veicolato per via vaginale in vescicole a base di fosfatidilcolina contenenti un biosurfattante, selezionato come eccipiente "green" alternativo ai surfattanti chimici (ad esempio Tween 80). Il biosurfattante è stato isolato da un ceppo probiotico di origine vaginale e caratterizzato. La molecola ha mostrato una struttura a base peptidica e buone proprietà tensioattive ed emulsificanti. Le vescicole miste, ottenute col metodo dell'idratazione del film sottile seguito da estrusione, presentavano un diametro ottimale per la somministrazione vaginale. Rispetto alle vescicole contenenti Tween 80, quelle preparate con il biosurfattante mostravano una più alta efficienza di incapsulazione, migliori proprietà mucoadesive e un rilascio sostenuto del farmaco. Inoltre, erano in grado di potenziare significativamente la capacità dell'econazolo di eradicare i biofilm di *Candida*.

Il secondo approccio (*paper 4*) ha consistito nello sviluppo di nuovi liposomi per la contemporanea veicolazione di due polifenoli che potessero agire in combinazione per eradicare l'infezione e alleviare allo stesso tempo i sintomi della candidosi, come bruciore e irritazione. La quercetina e l'acido gallico sono stati quindi proposti come alternativa ai convenzionali antifungini per affrontare il problema della resistenza agli azoli. I liposomi formulati con entrambi i polifenoli, ottenuti col metodo del film sottile seguito da sonicazione, avevano le dimensioni volute di 200 nm, buone efficienze di incapsulazione e migliori profili di rilascio rispetto alle formulazioni contenenti un solo polifenolo. I liposomi esplicavano inoltre ottime attività anti-ossidanti e anti-infiammatorie e sono risultati non citotossici per le cellule. Infine, si sono rivelati in grado di contrastare la crescita di *Candida*, grazie all'azione antifungina attribuibile all'acido gallico.

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I. Introduction

I.1 Vaginal environment

I.1.1 Overview of the vaginal cavity

I.1.1.1 Anatomy of the vagina

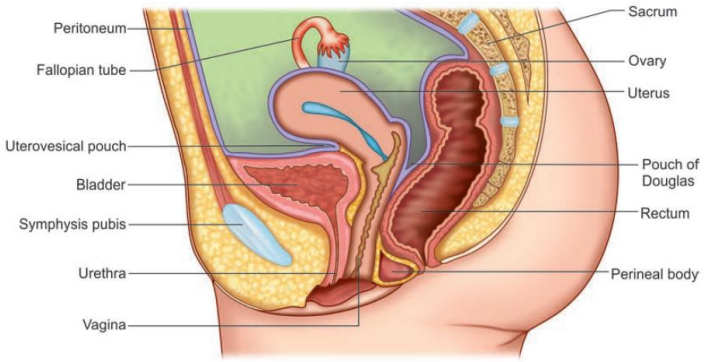

Figure I.1 – Mid-sagittal section of the female pelvis [Konar 2014].

The vagina is one of the most important organs of female genital apparatus and plays a focal role in reproduction. Anatomically, the vagina can be described as a slightly S-shape, fibromuscular and strong tubular organ that extends from the cervix of the uterus to the vestibule of the external genitalia. It is situated between the rectum, bladder and urethra with dimensions ranging from 8-11 cm in length and 2-5 cm in diameter (*figure I.1*) [Baloglu 2009; de Araújo Pereira 2012].

Normally, the vagina appeared as a curved organ, in which two distinct portions can be distinguished: a lower convex portion and a broader upper part that lies in an almost horizontal plane when the woman is in upright position. The two axes form an angle of 130 degrees. The vagina is a collapsed organ; indeed, when viewed in transverse cross-section, the anterior and posterior walls are in contact with each other [Choudhury 2011].

Once the vagina enters to the pelvic region it passes two diaphragms. The bulbocavernosus muscle and the pubococcygeus, from the urogenital and pelvic diaphragms respectively, behave as sphincters to the vaginal introitus [Alexander 2004].

The vaginal wall (*figure I.2*) is composed of four layers and it's estimated that a turnover of about 10-15 layers happens every 7 days [Valenta 2005; Konar 2014]. (i) The most superficial layer consists of non-cornified stratified squamous epithelium, which has no secretory glands. Its thickness varies by approximately 200-300 μm and basically depends

on hormonal fluctuations that occur during different life stages (new born, child, adult and menopause) and periodically during menstrual cycle. In particular, with hormonal activity the vaginal epithelium increases in thickness and is highest in proliferative stage. In women of reproductive age, the vagina is characterized by numerous folds, called "rugae", that offer a high distensibility, support and allow to increase surface area of vaginal wall. (ii) The second layer is the lamina propria, or tunica, made of collagen and elastin, which is highly vascularized and rich in lymphatic channels. (iii) The muscle layer is the third, with smooth muscle fibers running in both circular and longitudinal directions, that confer excellent elasticity to the vagina. (iv) The last layer is formed by areolar connective tissue and a large plexus of blood vessels.

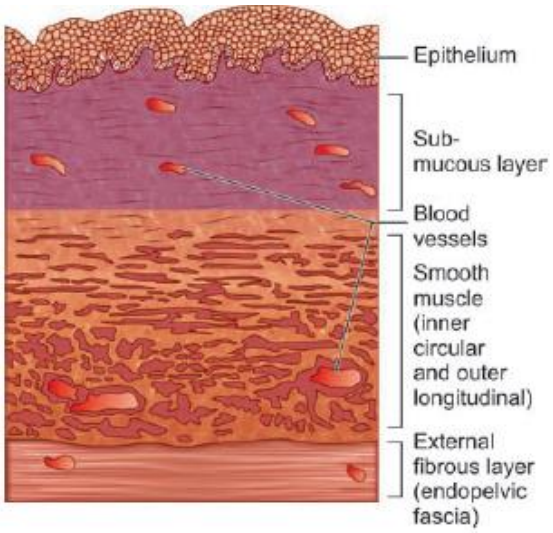

Figure I.2 – Structure of the vaginal wall [Konar 2014].

The vagina's nerve supply arises from two sources [Artico 2012]. The lower quarter of the vaginal is innervate by peripheral nerves, that make it a very sensible area, while autonomic fibers innervate the upper three quarters and respond to stretch but are not very sensitive to pain. For this reason, women rarely feel great discomfort or localized sensations during the use of vaginal products (i.e. suppositories, ovules, vaginal rings and tampons) [Alexander 2005].

The vascular supply involves an extensive network of arteries that surround the vagina from multiple origins, including the uterine artery, the pudendal artery, and the middle and inferior hemorrhoidal arteries. Drugs adsorbed from the vagina avoid the first-pass metabolism because blood leaving the vagina enters the peripheral circulation via a rich venous plexus, which empties primarily into the internal iliac veins. Since vascular circulations of the vagina and uterus are strictly connected, a "first uterine pass effect" has been hypothesized after vaginally administration of hormones [Hussain 2005].

I.1.1.2 Physiology of the vagina

The physiology of the vagina is influenced by age, hormone status, pregnancy and pH changes induced by several factors including semen, menstruation, estrogenic concentration, and bacterial colonization [Alexander 2004].

In healthy women of reproductive age, vaginal pH is generally maintained between 3.8 and 4.2. Lactobacilli that inhabit vaginal niche contribute to the preservation of an acidic environment through the conversion of glycogen from exfoliated epithelium cells into lactic acid and production of hydrogen peroxide [Valenta 2005]. The pH varies according to age, stages of menstrual cycle, pathological states and frequency of coitus. In particular, menstrual, cervical and uterine secretions, and semen cause an increase in pH by acting as alkaline agents.

Despite the absence of glands, a large amount of fluid is secreted in the vaginal cavity. Cervical secretion and transudation from the blood vessels, together with desquamated vaginal cells and leukocytes, mainly form the vaginal fluid. Secretions from the endometrium, fallopian tube and Bartholin's glands, microorganisms and their metabolic products also contribute to formation of vaginal fluid [Hussain 2005]. Thus, the human vaginal fluid is a mixture of a large variety of substances comprising enzymes, enzymatic inhibitors, proteins and amino acids, carbohydrates, alcohols, hydroxylketones and aromatic compounds. This aspect is important because the high enzymatic activity of vaginal epithelium could possibly affect short- and long-term stability of intravaginal dosage forms and devices [Choudhury 2011].

Some factors, like sexual arousal and hormonal cyclical fluctuations, may induce variations in volume and composition of vaginal fluid, thus affecting drug release behaviour from vaginal delivery systems [Choudhury 2011]. In absence of sexual stimulation, the vaginal fluid is principally made up of plasma transudate from the vaginal wall and secretions from the cervical and vestibular glands [Valore 2002]. On arousal, the vaginal lubrification increases as consequence of locally release of vasoactive peptides, which cause arteriolar dilatation and vaginal distention and suppress venous return [Levin 1991]. The rate of discharge produced by reproductive-age women is of 3-4 g every four hours, while the amount of fluid decreases by 50% in postmenopausal women.

For vaginally administered formulations, should be pointed out that the physiologically presence of fluids may cause an overall dilution of active compounds and reduction of drug efficacy, increase leakage and decrease drug residence time at the site of application

[Andrews 2009; Rohan 2009].

Furthermore, vaginal fluid is characterized by the presence of cervical mucus, which in turn can either facilitate or hamper the efficacy of a dosage form [Valenta 2005; de Araújo Pereira 2012].

Mucus is a secretion synthesized by specialized goblet cells and forms an incessantly renewed, semi-permeable, adherent and viscoelastic barrier that lines all female reproductive tract. Within the vaginal cavity, the mucus appeared as a permeable gel layer that assures a proper lubrification and hydration and allows for the exchange of gases and nutrients to and from epithelium. Moreover, it acts as a barrier to protect the underlying layers against pathogens and harmful substances [Carvalho 2010; Lai 2010]. The cervical mucus is composed of mucins and other glycoproteins, inorganic and organic salts, proteins, cholesterol, lipids, carbohydrates, urea, enzymes, amino acids and 95% water by mass, making it a highly hydrated system. Mucin is the most secreted glycoprotein of mucus and is responsible for its structure [de Araújo Pereira 2012].

At physiological pH the mucus network is negatively charged for the presence of sialic acid and sulfate residues, which play an important role in bioadhesion and mucoadhesion processes.

I.1.2 Vaginal microbiota of healthy women

I.1.2.1 Vaginal microbiota of reproductive-age women

The term microbiota (or microbiome) refers to the whole assortment of microorganisms in a particular body niche, including the human gut, oral cavity and vaginal tract [Leyva-Gómez 2019].

The vaginal microbiota of healthy women consists of a plethora of anaerobic and aerobic microorganisms that establish a mutualistic relationship with the host and constitute the first line of defence against the colonization by opportunistic pathogens responsible for urogenital diseases, such as bacterial vaginosis, aerobic vaginitis, fungal infections and sexually transmitted infections [Oakley 2008; Smith and Ravel 2016].

The composition of the vaginal microbiota is not static but dynamic and undergoes modifications in response to hormonal fluctuations throughout the women's reproductive life (i.e. from puberty to menopause and during pregnancy, see section I.1.2.2). Other external factors, such as pathological states, drug intake, sexual intercourses and hygienic behaviours, may also affect the composition of microbiome [Borges 2014].

Despite the proximity of the vagina to the anus, the variety of microorganisms present in the vaginal tract is much lower than in the gut; indeed, about 50 microbial species have been identified in the vaginal microbiome whereas the gut microbiome harbours more than 800 species. The reason for the limited microbial diversity of the vaginal niche is still not completely understood but may be ascribed to reduced competition with indigenous organisms and differences in nutrient availability and immune activity [Cribby 2008].

The vaginal microflora was first described in 1982 by the German gynaecologist Albert Döderlein, who observed the presence of Gram-positive, non-spore-forming rods (referred to as Döderlein's bacilli), sometimes quite long and slim, with square or very tapering ends, occurring single or in chain, producing lactic acid that could inhibit the growth of pathogens [Petrova 2013]. Until 1980 it was believed that *Lactobacillus acidophilus* was the dominant constituent of the vaginal microbiota, as described by culture-dependent and microscope techniques. Knowledge on the composition of the vaginal microbiota has significantly expanded after the development and diffusion of cultivation-independent methods that rely on the analysis of 16-S ribosomal gene sequences [Ma 2012; Borges 2014]. In fact, the culture-dependent methods fail to identify several *Lactobacillus* species, such as *L. iners* that is not able to growth on de Man, Rogosa and Sharpe medium commonly employed for lactobacilli cultivation.

The vaginal ecosystem of healthy adult women is mostly dominated by *Lactobacillus* spp., that generally constitute more than 80% of total microbiome and are present at a concentration of 10^7-10^8 CFU per g of vaginal fluid [Farage 2010; Borges 2014].

Lactobacilli belong to the group of LAB (Lactic Acid Bacteria) and the genus is composed of more than 170 species of facultative, anaerobic or microaerophile, catalase-negative, Gram-positive, non-spore-forming rods. The most recurrent species in vaginal microflora are *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* [Pavlova 2002] but other microbial genera and species may also be present at lower concentrations. These include *Bifidobacterium*, *Staphylococcus*, *Ureaplasma*, *Corynebacterium*, *Streptococcus*, *Peptostreptococcus*, *Gardnerella*, *Bacteroides*, *Mycoplasma*, *Enterococcus*, *Escherichia*, *Veillonella* and *Candida* [Hyman 2005].

Several studies revealed that the *Lactobacillus* spp. composition in vaginal tract varies among women on the basis of geographic locations, races, ethnicities and individual susceptibility. In a study by Ravel et al. the vaginal bacterial communities of 396 asymptomatic North American women representative of four ethnic groups (white, black,

Hispanic and Asian) were investigated through pyrosequencing of barcoded 16S rRNA genes (*figure I.3*) [Ravel 2011]. According to these authors, microbial communities categorized in group I (26.2%), II (6.3%), III (34.1%), and V (5.3%) are dominated by *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, respectively, and are isolated mostly from white and Asian women. The remaining group IV (27%), mainly formed by black and Hispanic women, is a polymicrobial mixture of strict and facultative anaerobes, including species of the genera *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera* and *Peptoniphilus*.

Lactobacillus-dominated vaginal microbial communities have been associated with healthy conditions and are characterized by the production of large amounts of lactic acid, that maintains the vaginal pH below 4.5. This acidic environment is thought to be highly protective against infections or colonization of the vaginal mucosa by opportunistic (i.e. *Escherichia coli*) or non-indigenous pathogens (i.e. *Neisseria gonorroheae* and *Chlamydia trachomatis*). However, additional studies also reported that some women's vaginal ecosystems can be healthy without a *Lactobacillus*-dominant vaginal microbiota. In these cases, other species belonging to the LAB (i.e. *Atopobium vaginae*, *Megasphera* and/or *Leptotrichia* spp.) can be identified as dominant vaginal phylotype [Zhou 2004; Zhou 2007; Srinivasan 2012; Petrova 2013]. The same species were also detected by Ravel et al. in group IV, suggesting that some key functions of vaginal community, such as the production of lactic acid and hydrogen peroxide, must be preserved in order to guarantee an adult healthy vaginal ecosystem [Witkin 2007; Ravel 2011].

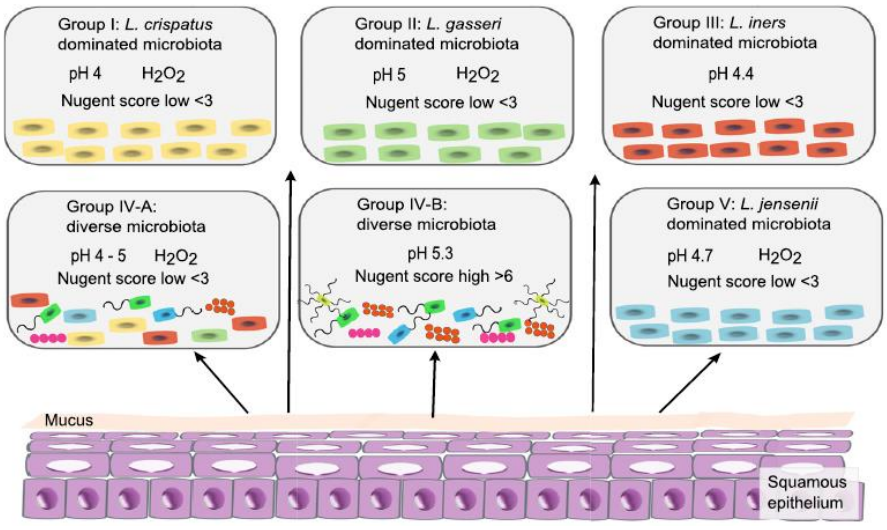

Figure I.3 – Composition of vaginal microbiota in healthy adult women [Petrova 2013]. The figure summarizes the studies conducted by Ravel et al. [Ravel 2011] and Gajer et al. [Gajer 2012].

A similar study conducted by Gajer et al. confirmed the previous observations of Ravel and colleagues. The authors identified five community groups since they split the group IV into two subgroups, IV-A and IV-B (*figure I.3*). Both of them are heterogenous in composition but group IV-A is characterized by a modest proportion of either *L. crispatus* or *L. iners* together with a low number of strict anaerobic bacteria, while IV-B has a higher proportion of members of the genus *Atopobium*, *Prevotella*, *Parvimonas*, *Sneathia*, *Gardnerella* and *Mobiluncus*.

The authors highlight that some of the vaginal bacterial communities noticeably change over time, switching from one to another group, whereas others are relatively stable. For instance, the vaginal communities dominated by *L. crispatus* often transform to group III dominated by *L. iners*, or to IV-A. Group III in turn shifts more often to group IV-B, but in rare cases to IV-A. On the contrary, the community group dominated by *L. gasseri* is quite stable and rarely transits to other types [Gajer 2012].

Furthermore, a strong association between group IV-B (Nugent score > 6) and increased risk to turn into a dysbiosis (i.e. bacterial vaginosis) and to get sexually transmitted infections (i.e. HIV) has been established through epidemiological studies [Smith and Ravel 2016]. Hence, while group IV may be normal (asymptomatic) in some women, in other conditions may lead to adverse sequelae.

I.1.2.2 Vaginal microbiota during different stages of woman's life

The composition of the vaginal microbiota undergoes several changes during the maturation of women as consequence of variations in oestrogen levels (*figure I.4*) [Cribby 2008; Amabebe and Anumba 2018].

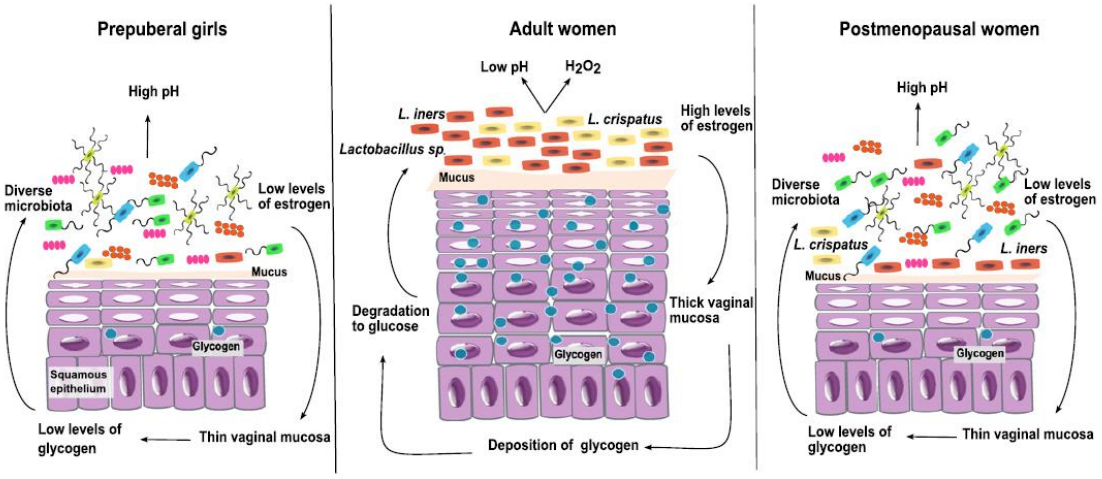

Figure I.4 – Changes in the vaginal mucosae and in microbiome during different stages of the life of a woman [Petrova 2013].

Immediately after birth, the vaginal epithelium is colonized by a large number of microorganisms, that mainly originate from gastrointestinal tract or from the surrounding skin epithelium.

In particular, it seems that vaginally delivered infants acquire bacterial communities that are similar to that of their own mother's vaginal microbiota, usually dominated by lactobacilli. On the contrary, after caesarean delivery infants result more frequently colonized by bacteria present on skin surface, especially *Staphylococcus*, *Corynebacterium* and *Propionibacterium* [Petrova 2013].

During the early stage of infancy, maternal oestrogens induce thickening of the vaginal epithelium and promote the deposition of glycogen in the epithelial cells, which is used by glucose-fermenting microorganisms as carbohydrate source [Boskey 1999].

As soon as the maternal oestrogens are metabolized, a thinning of the mucosa and reduction of glycogen occur. The consequent reduction in glucose-fermenting microorganisms, including lactobacilli, facilitates an increase in the vaginal pH, encouraging the proliferation of a wide range of aerobes and facultative anaerobes. Indeed, during childhood the vaginal microbiome mostly harbours Gram-positive (i.e. *Actinomyces*, *Bifidobacteria*, *Peptococcus*, *Propionibacterium*) and Gram-negative (i.e. *Veillonella*, *Bacteroides*, *Fusobacteria*) anaerobe bacteria, as well as some aerobic bacteria (i.e. *Staphulococcus*, *Enterococcus*, *Corynebacterium* and *Diphteroides*) [Randelovic 2012].

With the beginning of puberty, the vaginal epithelium, under estrogenic control, once again thickens and the glycogen-rich environment selects for glucose-fermenting microorganisms. The microbiota presents in this stage of life is predominantly colonized by *Lactobacillus* spp. (see section I.1.2.1).

During pregnancy the vaginal microbiota is predominated by lactobacilli and is more stable than in non-pregnant state. This is due to the high levels of oestrogens during pregnancy, resulting in increased vaginal glycogen deposition [Amabebe and Anumba 2018].

In postmenopausal period the level of oestrogens decreases, with consequent reduction or completely absence of glycogen and atrophy of vaginal mucosa. In women who don't take hormone replacement therapy, the postmenopausal microbiota is dominated by *L. iners* and *G. vaginalis* while the presence of lactobacilli is strongly reduced, and therefore growth of potential pathogenic bacteria is increased [Petrova 2013].

I.1.2.3 Protective factors of healthy vaginal microbiota

The vaginal ecosystem plays a pivotal role in maintaining a healthy equilibrium, that acts to supply a barrier to new colonization by pathogenic microorganisms and to overgrowth of species that are otherwise commensal.

The switch from homeostasis to a perturbed state can occur through several means, including physical injuries to the mucosa, impairment of the indigenous microbiota by the use of antimicrobials, expression of specific virulence factors and access to the site by excessive quantity of pathogens [Reid 2011].

Restoration of a healthy microbiota is driven by multiple species, especially *Lactobacillus* spp., and requires considerable and prompt reorganization after insult.

The mechanisms adopted by lactobacilli, as well as by exogenous probiotic strains, to stabilize and rapidly re-establish the normal vaginal microbiota are diverse and are listed below (*figure I.5*) [Reid 2011; Borges 2014; Zhang 2018].

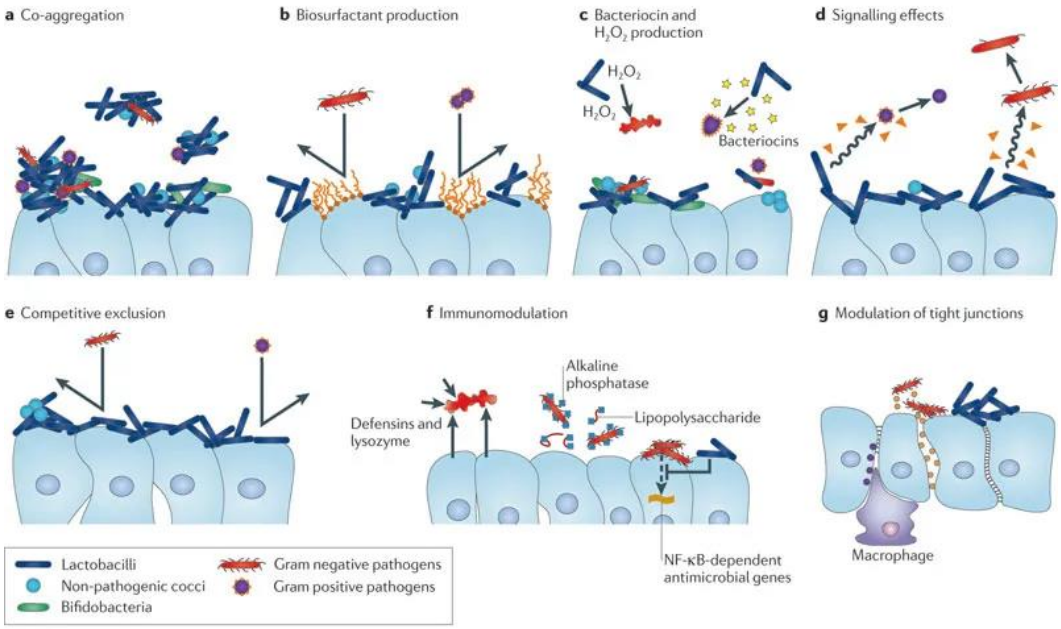

Figure I.5 – Protective mechanisms exerted by vaginal microbiota [Reid 2011].

Co-aggregation – The phenomenon of co-aggregation consists in the assembly of microbial communities into distinct and interlinked structures. Lactobacilli can bind pathogenic microorganisms and form co-aggregates. This mechanism allows to restore the homeostasis of the vaginal tract because it creates a microenvironment biochemically hostile for the growth of the harmful strain and prevents its access to the target epithelial tissues, thus hindering its proliferation in the vaginal niche.

It has been demonstrated that some *Lactobacillus* species, including *L. acidophilus*, *L.*

gasseri and *L. jensenii*, possess the ability to co-aggregate with *E. coli*, vaginal staphylococci and *C. albicans* [Younes 2012].

Biosurfactant production – Biosurfactants are surface-active compounds synthetized as secondary metabolites by a variety of microorganisms. Biosurfactants produced by vaginal lactobacilli are composed of a mixture of proteins, lipids and carbohydrates and show an activity in displacing dense polymicrobial cultures of uropathogenic *E. coli*, *Enterococcus faecalis* and *Gardnerella vaginalis* [Saunders 2007; Reid 2011]. These effects occur even with only few lactobacilli, suggesting that the secreted biosurfactants spread out over the surface of the vaginal mucosa and the resulting modification in surface tension repels hydrophobic pathogens [Banat 2010; Reid 2011].

Production of antimicrobial compounds – Lactobacilli synthetize several metabolites (like lactic acid, hydrogen peroxide and phenyllactic acid) able to exert direct antimicrobial activity against bacteria, viruses and fungi [Zhang 2018].

Lactobacilli metabolize glycogen, deposited in the vaginal epithelium by hormonal activation of oestrogens, with the production of organic acids, especially lactic acid.

Lactobacilli are acid tolerant while most vaginal pathogens are sensitive to low pH values. Lactic acid acts as antimicrobial agent by inhibiting the growth and production of virulence factors of Gram-negative bacteria. Furthermore, the acidic environment induced by accumulation of lactic acid can permeabilize the outer membrane of Gram-negative bacteria and can inhibit urease activity [Alakomi 2000; Zhang 2018].

Hydrogen peroxide is produced approximately by 80% of vaginal strains, in particular by *L. crispatus* and *L. jensenii*, and may also prevent invasions of pathogens [Aroutcheva 2001; Borges 2014]. Indeed, H_2O_2 and its metabolites (hydroxyl radical and superoxide anion) are powerful oxidizing agents, toxic to catalase-negative bacteria like most anaerobe microorganisms. On the contrary, lactobacilli seem to protect themselves from the killing action of free radicals thorough the production of Fe^{3+}-activated extracellular peroxidase [Reid 2011].

Bacteriocins are protein molecules secreted by lactobacilli that have a narrow killing spectrum, being able to control closely related bacteria residing in the same ecological niche via mechanisms that include cytoplasmatic membrane pore formation and permeabilization, interference with cellular enzymatic reaction (i.e. cell wall synthesis) and nuclease activity [Mokoena 2017; Zhang 2018].

Signaling effects – Signaling between bacteria can lead to downregulation of toxin produced in harmful bacteria. Anti-virulence signaling molecules have been identified in

some strains of lactobacilli that are known to be present in the vagina and gut, as well as in some probiotics. For example, *L. reuteri* RC-14 produces a signaling molecule that inhibits the expression of toxic shock toxin 1 in several strains of *S. aureus* and interferes with the P2 and P3 promoters of the staphylococcal global regulatory system *agr* [Laughton 2006].

Competitive exclusion – Lactobacilli can inhibit undesired microbial colonization by adhering to the vaginal epithelium and thus occupying or masking (by steric hindrance) the potential binding sites of pathogens in the mucosa.

The blockage of vaginal pathogens adherence by lactobacilli may occur through three different mechanisms, namely exclusion, competition for receptor sites and displacement of adherent pathogens [Borges 2013].

Immunomodulation – The regulation of immune response by the microbiota can lead to: the production of host factors such as antimicrobial peptides (like defensins), lactoferrin and lysozyme, which can kill pathogens; the production of alkaline phosphatases, which bind to lipopolysaccharide and abolish its toxicity; the deregulation of nuclear factor-κB (NF-κB) signalling in host epithelia [Reid 2011].

Enhancement of epithelial barrier function – The integrity of the vaginal epithelial is crucial to maintaining health. Lactobacilli play important roles in preserving epithelial barrier integrity by acting through diverse mechanisms, such as modulation of the cytoskeleton, induction of mucus production and phosphorylation of tight junction protein, which results in the improvement of tight junction function and the immune response [Kozakova 2016; Zhang 2018].

When this lining is destroyed or breached, the microorganisms on the outer surface gain access to the tissue and induce infection.

For example, HIV entry into the host is facilitate by the capability of the virus to decrease trans-epithelial resistance through disruption of tight junction proteins, the transmembrane protein occludin and the scaffold protein zonula occludens 1 (ZO1). Lactobacilli could potentially counteract this effect, as they upregulate ZO1 and occludin [Reid 2011].

I.1.3 Cervical-vaginal infections and dysbiosis

As mentioned above, several factors (summarized in *figure I.6*) may lead to transition from a health condition (also referred to as eubiosis) to an imbalance in vaginal homeostasis. This can promote colonization by potential pathogenic species, usually

causing a state of "abnormal vaginal microflora", called dysbiosis.

The most prevalent dysbiosis include bacterial vaginosis (BV), aerobic vaginitis (AV) and vulvovaginal candidiasis (VVC). An alteration of vaginal ecosystem can also expose to cervical-vaginal infections, mainly sexually transmitted infections (STIs).

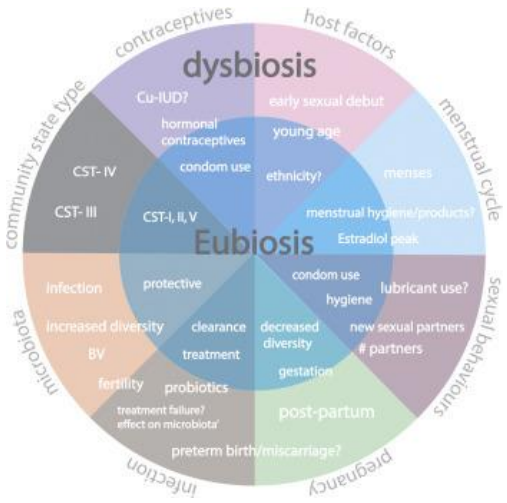

Figure I.6 – Factors associated with dysbiosis or eubiosis of the vaginal microbiota in reproductive age women [Kroon 2018].

I.1.3.1 Bacterial vaginosis

Bacterial vaginosis (BV) is the most prevalent vaginal disease in women of reproductive ages, with a prevalence rate of 12-40% [Kenyon 2013]. Although the exact aetiology of BV is still unclear, the unset of this dysbiosis has been associated with the depletion of lactobacilli, especially of those producing H_2O_2, that in turn favours the proliferation of anaerobes commonly present in the vagina, such as *Gardnerella*, *Atopobium*, *Mobilincus*, *Prevotella*, *Streptococcus*, *Mycoplasma*, *Ureoplasma*, *Dialister* and *Bacteroidetes* [Kaambo 2018]. Thus, BV can be considered a polymicrobial state that leads to a more heterogeneous vaginal environment, with low lactic acid levels and pH above 4.5.

These anaerobic bacteria engage in synergistic interactions and may form a mixed biofilm, which is considered one of the factors responsible for the chronicity and recurrence of the disease [Leyva-Gómez 2019].

BV is asymptomatic in 50% of BV-positive women and no inflammatory processes are detected at the vaginal epithelium. However, symptoms could appear in the form of non-itchy but irritating, white-grey, creamy vaginal discharges, containing exfoliated epithelial cells and, attached to their membranes, Gram-variable polymorphic bacteria. They are also characterized by a fishy odour due to the production and accumulation of

amines (such as putrescine, cadaverine and triethylamine) subsequent to the overgrowth of anaerobic bacteria [Amabebe and Anumba 2018].

Clinically, BV is diagnosed by using Amsel criteria, that include the evaluation of vaginal acidity, the presence of discharges, the appearance of clue cells (desquamated vaginal epithelial cells studded with anaerobes) and a positive "whiff test" (a fishy odour perceived when KOH 10% is added to vaginal discharges).

Interestingly, it seems that BV can increase the risk of acquisition of STIs (i.e. *N. gonorrhoea*, *C. trachomatis*, *T. vaginalis* HSV, HPV and HIV) and other infections (i.e. pelvic inflammatory disease and endometritis). Moreover, women who develop BV during pregnancy are more prone to spontaneous abortions, post abortion sepsis and preterm labour [Donders 2009; Combs 2014].

I.1.3.2 Aerobic vaginitis

Vaginal dysbiosis also manifest as aerobic vaginitis (AV), first described in 2002 by Donders and colleagues as a need to distinguish it from BV [Donders 2002; Leyva-Gómez 2019]. In AV, *Lactobacillus* microflora is dramatically destroyed. In particular, *L. crispatus* and *L. jensenii* in healthy conditions are able to prevent the growth of urogenital pathogens through the action of the surface proteins, and thus their depletion can predispose to the onset of a pathological state [Donders 2017]. The alteration of vaginal microflora triggers an increase in the pH, that is always above 6 in AV, and the proliferation of enteric aerobic microorganisms. In particular, the most isolated bacteria in AV-positive women are *Escherichia coli*, *Staphylococcus aureus*, coagulase-negative staphylococci (such as *S. epidermidis*), group B *Streptococcus* (such as *S. agalactiae*) and *Enterococcus faecalis* [Donders 2002; Donders 2007].

The lack of oestrogens, which induces the redness and atrophy of vaginal mucosa, is also believed to play a role in AV origin. In addition, the numbers of intermediate and parabasal cells increase due to the enhanced turnover and desquamation of superficial epithelial-cell layers, that elicit vaginal inflammation. Indeed, unlike BV, AV is characterized by the presence of a noticeable inflammation response, accompanied with a strong recruitment of leukocytes and neutrophils as well as pro-inflammatory cytokines, specifically IL-6 and IL-1β [Smith and Ravel 2016]. As consequence, AV clinically manifests through redness, itching, burning, pruritus, dyspareunia and purulent yellowish sticky discharges devoid of fishy odour.

It is postulated that AV is the aerobic counterpart of BV and sometimes mixed conditions

(AV and BV) can be found, representing either a transient form or prolonged co-infection. In fact, the vaginal *milieu* should not be considered as a static system, but rather as a complex dynamic one. AV is still not widely known and remains underdiagnosed by many clinicians, or may even be mistaken as BV [Vieira-Baptista 2016].

AV affects 2-25% of women and has been associated with severe gynecological and obstetric outcomes (i.e. ascending genital infection, preterm premature rupture of membranes, preterm labour and preterm birth) and with STIs (i.e. *N. gonorrhoea*, *C. trachomatis*, *T. vaginalis*) [Amabebe and Anumba 2018].

I.1.3.3 Vulvovaginal candidiasis

After BV, vulvovaginal candidiasis (VVC) is the second most common cause of vaginitis and the most prevalent vaginal fungal infection. In fact, it's estimated that VVC affects approximately 70-75% of women, especially those of reproductive ages, at least once during their life. Moreover, 40-50% of patients will experience a recurrence, while 5-8% of adult women manifests recurrent VVC, defined as four or more episodes per year [Sobel 2007]. In the United States alone, 13 million of cases of VVC are observed annually, which further results in 10 million of gynaecologic visits and estimated cost of 1 billion dollars.

The causative agents of VVC are *Candida* species and, among them, *C. albicans* accounts for 85-95% of total vaginal fungal infections. *C. albicans* is a dimorphic fungus, being able to grow both in the yeast and in the filamentous form, and is a resident of the normal vaginal microbiota, colonizing about 20% of women without eliciting any explicit symptoms. VVC may also be related to non-*albicans* species. In particular, *C. glabrata* is the one most commonly associated with candidiasis and can reach a prevalence of 10-20% in many parts of the world [Corsello 2003]. Other non-*albicans* spp., such as *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. dubliniensis* and *C. guillermondii*, are less frequently isolated from VVC-positive women [Nyirjesy 2005]. Vaginitis induced by such species are clinically indistinguishable from that caused by *C. albicans* but are often more resistant to treatment, for example to fluconazole [Holland 2003].

Furthermore, non-*albicans* spp., in particular *C. glabrata*, are frequently responsible for recurrent VVC [Sobel 2007].

The pathogenesis of VVC is still not completely clear. However, it seems that when the balance of vaginal ecosystem gets disturbed, the overgrowth of *Candida* spp. and a simultaneous depletion of lactobacilli occur. Some factors, such as individual

susceptibility, pregnancy, prolonged treatment with wide-spectrum antibiotics, use of contraceptives and spermicide, frequent sexual intercourse, diabetes and immunosuppression, can increase the risk for development of VVC. This happens after initial vaginal epithelial colonization by *Candida* and switch between yeast asymptomatic form and symptomatic pathogenic hyphal form [Johal 2016].

Although VVC is not a life-threatening disease, it can seriously impair the quality of life of patients, leading to physical, psychological and even sexual complications.

Clinical manifestations of candidiasis include vaginal itchiness, dyspareunia (pain during sexual intercourse), external dysuria (pain during urination), vulvar burning, irritation and vaginal soreness. The most well-known symptom, cottage cheese-like discharge, is often minimal and sometimes absent.

Examination reveals erythema and swelling of the labia and vulva, often accompanied with fissures and pustulopapular peripheral lesions. Indeed, one of the hallmarks of vaginal candidiasis is the robust recruitment of neutrophils to the site on infection that, along with cytokine response, actually exacerbates disease symptoms yet fails to properly contain and clear the fungus [Fidel 2004].

The vaginal pH is normal (4-4.5) in VVC, and pH above 4.7 usually indicates the presence of bacterial vaginosis, trichomoniasis or a mixed infection [Sobel 2007].

Nowadays, the major factors that hinder the effective treatment of candidiasis are the difficulty of quick and accurate diagnosis, since VVC is often mistaken for bacterial infection, and the limited number of therapeutic options [Ksiezopolska 2018].

VVC therapy is mainly based on azole drugs (i.e. fluconazole, econazole, ketoconazole, itraconazole, clotrimazole, miconazole, terconazole) that can be administered orally (in the form of tablets or capsules) or topically (in the form of creams, tablets or suppositories) [Johal 2016]. Azoles act as fungistatic agents, blocking ergosterol synthesis, targeting the enzyme lanosterol 14α-demethylase and leading to an accumulation of toxic sterol pathway intermediates.

Unfortunately, the emergence of resistance to multiple antifungal drugs has been increasingly reported in the last years, making the treatment of VVC very challenging.

On one hand, the up-regulation of gene coding for efflux pump is likely the major cause for the acquired planktonic cell resistance. On the other hand, the capability of *Candida* spp. to rapidly form biofilm on host mucosal tissues is an important virulence factor, associated with both resistance and recurrent outcomes (*figure I.7*) [Tsui 2016; Silva 2017].

Generally, a biofilm consists in a community of microorganisms that are irreversibly attached to a surface, behaving very differently from planktonic cells [Cavalheiro 2018]. Biofilm formation of *C. albicans* is a multifaced process that starts with adhesion of yeast cells to a surface, followed by the formation of a discrete colony. Subsequently, cells become organize and begin to produce and secrete extracellular polymeric matrix, that allows the maturation of a three-dimensional structure. Once the formation of mature biofilm, daughter cells can be released from the biofilm and migrate to other niches, thus propagating the fungus infections and colonization.

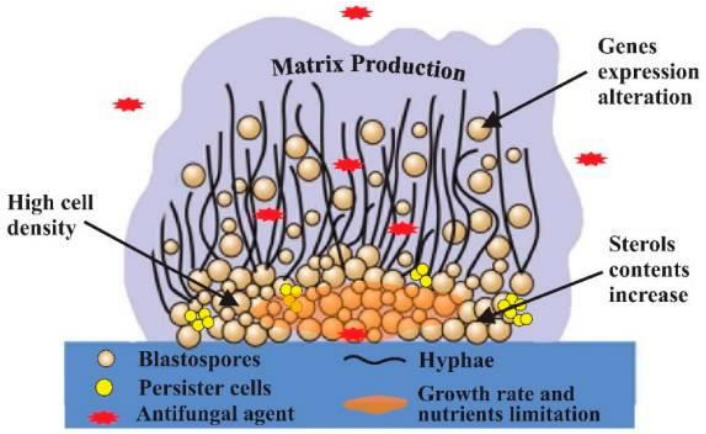

Figure I.7 – Summary of mechanisms involved on *Candida* spp. biofilm resistance [Silva 2017].

Biofilms of *C. albicans* exhibit five- to eightfold higher resistance to all azole drugs compared to planktonic cells due to several factors, such as high concentration of *Candida* cells inside biofilm communities and the presence of extracellular matrix, that provides protection from host immunity and limits the diffusion of antifungal agents. In particular, the major carbohydrate polymer of the matrix, β-1,3 glucans, is responsible for sequestering azoles, acting as a sponge and conferring resistance to *C. albicans* biofilm. The presence of persister cells – a subset of dormant, non-dividing cells located in the deeper layers of biofilm – is also related to a high tolerance to multiple antifungal classes. Moreover, cells from mature biofilms rely less on ergosterol for maintaining their membrane fluidity, and this aspect limits the efficacy of drugs targeting the sterol, including all azoles as stated above [Silva 2017; Cavalheiro 2018].

I.1.3.4 Sexually transmitted infections

Various catastrophic microorganisms can produce severe sequelae when they infect the cervical-vaginal tract. In particular, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*,

Trichomonas vaginalis, *Treponema pallidum*, *Mycobacterium tuberculosis*, human papillomavirus (HPV), human immunodeficiency virus (HIV) and herpes virus (HSV-2) should be mentioned as potential sources of STIs. Among them, *C. trachomatis*, *N. gonorrhoeae*, and less frequently *T. vaginalis*, are responsible for the onset of cervicitis, defined as an inflammation of the cervix that can manifest in acute or chronic form.

Chlamydia trachomatis – *C. trachomatis* (in particular serovars D-K) is one of the most common STIs worldwide, infecting more than 130 million of people every year with an incidence rate of 38 per 1000 females [Chi Wai Wong 2019]. *C. trachomatis* is a Gram-negative obligate intracellular bacterium whose only natural host is humans.

Chlamydia has a unique biphasic developmental cycle of 30-72 h, alternating two distinct bacterial forms. The elementary bodies (EBs) are spore-like, infectious but non-dividing. After the binding with sensitive cells, EBs are internalized through endocytosis to form a phagosome termed inclusion. Within the inclusion EBs differentiate into the reticulate bodies (RBs), the metabolically active and replicative form of the pathogen. Midway through the infectious cycle RBs begin to differentiate back into EBs, which are released to initiate new rounds of infection.

Most women with urogenital *Chlamydia* experience a subclinical and often asymptomatic infection and, therefore, do not pursue treatment. Unfortunately, protracted exposure of the fallopian tube epithelium to *C. trachomatis*, or to antigens released by this bacterium, may compromise tubal integrity, thus leading to infertility and, if conception occurs, increased susceptibility to ectopic pregnancy and pre-term birth.

Other consequences of an upper genital tract infection in women include pelvic inflammatory disease, endometritis, and perihepatitis [Witkin 2017].

Neisseria gonorrhoeae – The host-adapted human pathogen *N. gonorrhoeae* is a Gram-negative coccoid bacterium, accountable for gonorrhoea. In women, urogenital gonococcal infections are often asymptomatic, thus remaining ignored and untreated, and providing an important reservoir for further transmission. Since *N. gonorrhoeae* does not express potent exotoxins, the pathogenesis mainly results from genital mucosa damage caused by the activation of innate immune responses. Symptoms of gonorrhoeal infection in women are usually nonspecific and the vaginal discharges originated from neutrophil influx may be mistaken for BV or yeast infections [Quillin 2018].

If left untreated, gonorrhoea can result in severe complications like pelvic inflammatory disease, infertility, ectopic pregnancy, first trimester abortion, and less frequently, disseminated infections [Foschi 2017].

Trichomonas vaginalis – Trichomoniasis is caused by a single-cell parasite also known as *trichomonad*. This parasite affects vagina, urinary bladder and urethra in females and penis in males. The infected women present stinking foamy yellow-grey-green vaginal discharges, together with other symptoms as painful urination and sexual intercourse, vaginal soreness, burning, redness and irritation [Gupta 2019].

I.1.4 The vagina as a route for drug delivery

Until 1920s it was believed that the vagina was incapable of absorbing drugs systematically. However, the mucus permeability, the dense network of blood vessels and the large surface area make the vagina a valid route to achieve both local and systemic effects. In 1918, Macht observed for the first time that some drugs, specifically morphine, atropine and potassium iodide, were actually absorbed after vaginal administration [Macht 1918].

Traditionally, the vaginal cavity has been used for the delivery of locally acting drugs, such as antibacterial, antifungal, antiprotozoal, antiviral, anti-inflammatory and spermicidal substances, prostaglandins and steroids [Valenta 2005]. Even if most of the drugs approved for vaginal administration are intended to treat local conditions, a part of them reaches blood circulation at sufficiently high levels to elicit systemic effects.

Moreover, the vaginal route can be potentially used for the uterine targeting of active agents like progesterone and danazol [Cicinelli 2000; Einer-Jensen 2002]. Indeed, after vaginal administration, the plasma concentration of progesterone results higher in the uterine artery than in the radial one, suggesting a preferential distribution of this drug to the uterus. Such evidence underlines the existence of direct local transport from the vagina to the uterus, called “first uterine pass effect” [De Ziegler 1997].

I.1.4.1 Advantages of vaginal delivery

Despite the fact that vaginal delivery is only exploitable for females, it offers severe advantages. One of the major benefits of vaginal administration over oral intake is the avoidance of gastrointestinal absorption and hepatic first-passage [de Araújo Pereira 2012]. Absorption from the gastrointestinal tract may be unpredictable and influenced by several issues, such as vomiting, drug-drug interference, presence of food or limited intestinal absorption capacity due to physical-chemical features of drugs (i.e. water solubility) [Valenta 2005].

Furthermore, enzymatic degradation can occur both in the gastrointestinal tract and in the

liver. The possibility of by-passing hepatic first-passage is particularly desirable for drugs that undergo a high degree of hepatic metabolism, as in the case for oestrogens that are 95% metabolized by the liver when are taken orally [Alexander 2004]. Another example is propranolol, whose bioavailability is greater after vaginal delivery compared with oral administration [Patel 1984]. The vaginal drug delivery could therefore permit to lower the dose and systemic exposure, thus reducing incidence of side effects while achieving the same pharmacodynamic effect.

The decrease in plasma fluctuations, characteristic of the daily oral intake, may also contribute to limit side effects. Considering that the onset of side effects is the most important aspect associated with discontinuation of oral contraception, their reduction will increase the acceptability of a dosage form and consequently patient compliance [Rosenberg 1995].

Some compounds have been shown to be more effective when administered vaginally with respect to other routes. For example, indomethacin employed in case of preterm labour appears to have greater effects when used intravaginally as compared with an intrarectal plus oral regimen [Abramov 2000]. Vaginal administration allows also to reduce gastrointestinal mucosal irritation – as observed during the vaginal delivery of bromocriptine [Vermesh 1988] – and hepatic side effects of steroids used in hormone replacement therapy or contraception [Dezarnaulds 2003]. In addition, the vaginal application overcomes the inconveniences caused by parenteral routes such as pain, tissue damage and possible infections [Valenta 2005]. Ease of self-insertion and removal of the final formulations, as well as the possibility of maintaining them for extended periods of time (i.e. daytime and night time) thereby lowering dosing frequencies, are other advantages of this route [Wang 2018]. Indeed, a prolonged contact of a delivery system with the vaginal mucosa can be obtained more easily than at other absorption sites, like rectum and gut mucosa. Several studies also report that a wide range of compounds, including peptides and proteins, show a good permeability trough vaginal mucosa.

It’s also worth to note that vaginal drug delivery permits a selective local exposure where needed, producing little or no chance in exposure throughout the rest of the body [Alexander 2004]. This is particularly important for steroids employed for vaginal treatment of urogenital atrophic diseases.

However, some drawbacks, including cultural sensitivity, personal hygiene, gender specificity, local irritation and influence of sexual intercourse should be considered during the design of a vaginal formulation [Hussain 2005].

I.1.4.2 Factors affecting the vaginal delivery of drugs

Several factors and physicochemical properties of delivered substances may influence the release, and eventually the absorption, of drugs in the vaginal cavity.

As mentioned in section I.1.1.2, cyclic changes in thickness of vaginal epithelium, fluid volume and composition, pH and sexual arousal could potentially affect drug release at the administration site and drug bioavailability. For instance, the thickness of the vaginal epithelium influences the absorption of steroids like oestrogen, which is higher absorbed in postmenopausal women compared to premenopausal ones [Pschera 1989].

Moreover, cervical mucus of the vagina, made of a glycoprotein gel, could be exploited for bioadhesive drug delivery but, on the other side, can represent a permeability barrier for several drug candidates.

Even volume, viscosity and pH of vaginal fluid can positively or negatively influence drug release and absorption. In general, poorly water-soluble drugs are better absorbed when the fluid volume is higher; however, huge volumes of fluid may wash the drug out of the vaginal cavity and subsequently decrease absorption. Variations in vaginal pH can also alter degree of ionization of weak electrolytic molecules and hence impact on the release profile of pH sensitive drugs [Choudhury 2011].

Vaginal absorption can also be influenced by physicochemical properties of drugs, such as molecular weight, lipophilicity, ionization, surface charge and chemical nature. Generally, low molecular weight lipophilic substances have a better chance to be absorbed with respect to large molecular weight lipophilic and hydrophilic drugs [Hussain 2005]. However, considering that vaginal fluid contains a large percentage of water, any compound intended for vaginal delivery requires a certain degree of solubility in water.

I.1.4.3 Vaginal drug delivery systems

Dosage forms traditionally employed for vaginal delivery comprise solutions, suppositories, gels foams and tablets. More recently, vaginal rings have been developed for hormone replacement and contraceptive therapy.

Vaginal drug delivery systems intended for local effect (i.e. spermicidal or antibacterial effect) should be able to distribute uniformly throughout the vaginal cavity and, for this purpose, semi-solid or fast dissolving solid systems are preferred.

Contrariwise, systems able to promote sustained release of drugs, such as vaginal rings, are more useful to obtain a systemic effect (i.e. contraceptives).

Creams and gels – Creams and gels are used for topical delivery of contraceptives and antibacterial drugs. For example, metronidazole and clindamycin creams prescribed for the treatment of BV are found to be as effective as orally administered drugs [Lamont 2003].

Vaginally applied creams and gels are supposed to be easy to use, non-toxic and non-irritating to vaginal mucosal. These dosage forms can be based on the principle of emulsion or hydrogel drug delivery system. In particular, hydrogels are hydrophilic polymers that, when are placed in contact with an aqueous environment, swell and retain huge amount of water in their swollen structure, thus allowing to control the release of drug. For instance, miconazole has been successfully delivered by using a similar system [Mandal 2000].

Vaginal gel has also been employed for intravaginal delivery of cholera vaccine, which displayed a greater mucosal response in female genital tract compared to oral administration of the vaccine [Hussain 2005].

Antibacterial agents and drugs for cervical ripening and induction of labour (i.e. oxytocin, dinoprostone and misoprostol) are also formulated as vaginal gel forms.

Suppositories and vaginal tablets – Several vaginal medications are available as tablets and suppositories. These dosage forms are designed to melt in the vaginal cavity and release the drugs for several hours.

Drugs commonly administered as suppositories comprise dehydroepiandrosterone sulphate for ripening effect on the uterine cervix, miconazole for treatment of VVC and progesterone for hormonal replacement therapy.

Vaginal tablets are instead used for delivery of itraconazole, clotrimazole and prostaglandins. Vaginal tablets are usually easy to produce and insert in position. Moreover, the incorporation of hydrophobic and release retarding materials may prolong the release of drug from formulation, while the presence of penetration enhancers, such as surfactants and bile salts, can significantly improve absorption [Hussain 2005].

Vaginal rings – Vaginal rings (ca. 5.5 cm diameter) are circular ring type drug delivery devices, usually made of polymer or silicone, and thought to guarantee a controlled release of drug after insertion in the vagina. They are used for contraceptive and hormone replacement therapy [Dezarnaulds 2003]. Advantages of this device are that it is user controlled, does not interfere with coition, does not require daily intake of pills and permits continuous delivery of low doses of steroids.

In simple vaginal rings, drug is homogenously dispersed within the polymeric ring.

Therefore, drug at the surface of the ring is released faster, sometimes providing an initial burst release, than drug in the inner layer of the ring. Sandwich or reservoir type rings have instead been developed to obtain a constant release of drug over time. Rate of drug release can be modified by varying the core diameter or thickness of the nonmedicated coating [Alexander 2004]. Furthermore, a single ring can hold several cores of diverse drugs and thereby allowing administration of multiple therapy from the same device.

I.1.4.4 Limitations of conventional vaginal formulations

These conventional vaginal delivery systems are often associated with some disadvantages such as poor distribution and low retention to the vaginal epithelium, mostly owing to the self-cleaning action of the vagina [Johal 2016]. Leakages and messy following the application of creams and gels can also cause embarrassing inconveniences to the user. Moreover, the application can be uncomfortable and the efficacy is quite low as gels may not provide an exact dose because of non-uniformity and leakages.

The requirement of frequent administrations and a prolonged duration of therapy, as in the case of VVC therapy, may also lead to a poor patient compliance.

In addition, conventional dosage forms possess a poor ability to modulate the fate of active compound once they are released into the vaginal cavity and the drug release patter is often inappropriate [Parnami 2013; Singh 2014].

To overcome these limitations, novel approaches for vaginal drug delivery can be adopted to promote both an extended and intimate contact with the vaginal mucosa and a sustained release of active compounds from formulations.

Novel strategies to achieve these goals may include the development of mucoadhesive polymer-based dosage forms and nanocarriers (see sections I.3.2 and I.3.3).

I.2 Active agents selected for the project

I.2.1 Probiotics

Antibiotics and antimycotics commonly used to treat urogenital infections display some relevant drawbacks: they cause a decrease in the number of lactobacilli, do not restore the urinary tract natural barrier to infections and give rise to frequent recurrences and chronic infections, especially in the case of candidiasis, as well as resistance to drug [Iannitti 2010; Al-Ghazzewi and Tester 2016]. Indeed, since the introduction of antibiotics around 1940 and their widespread, several pathogenic microorganisms have developed different mechanisms of drug resistance, like alteration of substance targets, inactivation of the drugs and their limited access to the intracellular target (mainly due to the presence of active drug efflux systems).

Side effects are also related to the disruption of the protective vaginal microbiota. As previously described, healthy vaginal microbiota harbours mainly *Lactobacillus* spp., whose depletion facilitates the overgrowth of species responsible for vaginal dysbiosis (i.e. *G. vaginalis*, *A. vaginae* and *C. albicans*) and urogenital infections (i.e. *E. coli* and *E. faecalis*) [Borges 2014].

In this contest, probiotics could be an effective alternative to antibiotic therapy to face up to vaginal diseases *in virtue* of their health-promoting activities, such as maintenance of an acidic environment, secretion of antimicrobial metabolites (acids, bacteriocins and H_2O_2) and anti-adhesive polysaccharides and competition with pathogens for specific adhesion sites on the urogenital epithelium (see section I.1.2.3).

I.2.1.1 Probiotic bacteria

"Probiotic" derives from the Greek word meaning "for life" [Sarao and Aora 2017].

The term was first introduced in 1965 by Lily and Stillwell to describe products secreted by one organism that stimulate the growth of another [Gupta and Garg 2009].

According to FAO/WHO, probiotics are nowadays defined as "live microorganisms, which, when administered in adequate amounts, confer a health benefit to the host" [FAO/WHO 2006].

Viable probiotics are known to exert several established health effects on the host organism. The beneficial activities attributed to probiotic bacteria include improvement of gut microbial balance by antimicrobial therapy, decrease in gastrointestinal infections, prevention and reduction of diarrhoea of different origin, alleviation of intolerance to

lactose, prevention of food allergies, mitigation of symptoms associated with irritable bowel syndrome and atopic dermatitis, reduction in serum cholesterol, antimicrobial properties (an example is the suppression of infections caused by *Helicobacter pylori*), stimulation of the immune system, and antimutagenic and anticarcinogenic effects. However, functional properties are closely related to species and strains [Lee 2005; Azizpour 2009; Saad 2013; Palacios 2014; Coghetto 2016].

The microbial genera more frequently used as probiotics belong to the group of LAB (Lactic Acid Bacteria) and related microorganisms. In particular, two genera of Gram-positive bacteria, *Lactobacillus* (i.e. *L. rhamnosus, L. acidophilus, L. casei, L. plantarum, L. reuteri, L. delbrueckii*) and *Bifidobacterium* (i.e. *B. breve*, *B. bifidum*, *B. lactis* and *B. longum*) are mainly employed as probiotics [Parvez 2006]. Other bacteria such as *Streptococcus* (*S. thermophilus*), *Enterococcus, Saccharomyces* and *Lactococcus* could also be marked as probiotic genera.

These microorganisms are generally categorized as GRAS (Generally Recognized as Safe) and many of them are also identified as Food Grade Microorganisms (FGM).

In particular, probiotic bacteria have been widely exploited to produce so-called functional foods. Some examples are well-established dairy products – such as yoghurt, ice creams and cheese – as well as novel products – like fruit juice, cereals and chocolate – which turned out to be optimal carriers for probiotic delivery [Sarao and Aora 2017].

Lactobacillus – Lactobacilli are Gram-positive, non-sporing, non-respiring rods, producing lactic acid as the major end-product during the fermentation of carbohydrates. Some species, such as *L. acidophilus*, have been recognized as probiotic bacteria because of their ability to provide advantages for the host and to adhere to human intestinal cells. Moreover, they are intrinsically resistant to very low pH and can survive in harsh environment like high concentrations of bile [Dianawati 2016].

Bifidobacterium – Bifidobacteria are Gram-positive, anaerobic, non-motile, non-sporing rod-shape variably branched bacteria (*figure I.8*). They are known to colonize the oral cavity, the human vagina and, more abundantly, the gastrointestinal tract [Arboleya 2011]. Bifidobacteria are one of the primarily colonizer of the intestine in new-borns and represent 60-90% of gut microbiome in breast-fed infants, while their abundance decreases with age reaching 10% in adult gut microbiome [Turroni 2008].

Although lactobacilli are generally predominant in healthy vaginal microbiota, bifidobacteria may also be present in a subset of women, and contribute to the maintenance of vaginal homeostasis through the proven ability to produce lactic acid and

hydrogen peroxide [Schellenberg 2012]. Freitas et al. also demonstrated that *Bifidobacterium*, particularly the species *B. breve* and *B. longum*, is the dominant genera of vaginal microbiome of some healthy women and that *Bifidobacterium*-dominated communities are able to exert a protective role in the same way as microbiomes dominated by lactobacilli [Freitas 2017].

Since bifidobacteria are capable to provide protection against harmful microorganisms through different mechanisms (including production of bacteriocins, inhibition of pathogen adhesion and modulation of immune system), they have been extensively studied as probiotics [Martinez 2015].

On the other hand, the fact that they are 'fastidious' and anaerobic bacteria poses some technological issues for the development of probiotic formulations. Several factors, such as acidity, pH, temperature and oxygen content, have been claimed to affect *Bifidobacterium* viability [Fritzen-Freire 2012]. In this regard, microencapsulation may be a valid strategy to improve the survival of these microorganisms during manufacturing procedures and the stability during storage and during passage through gastrointestinal tract (see section I.2.1.3).

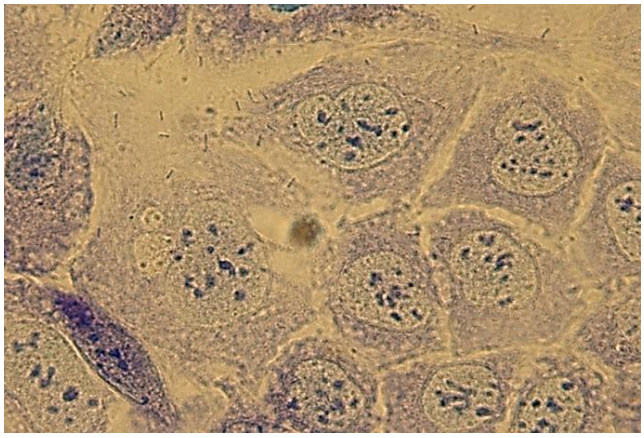

Figure I.8 – *Bifidobacterium breve* BC204 employed in *paper1* attached to Caco-2 cells. Microscope image (1000×) was obtained after May-Grünwald Giemsa staining.

I.2.1.2 The requirements for probiotics

Despite the very widespread use of probiotics, they pose technological challenges. The bacteria used are in fact usually anaerobic and do not survive the extremes of temperature. To be considered as probiotic, a microorganism should satisfy some criteria [Sarao and Aora 2017]. Firstly, it should be isolated from the same species as its intended host and it must be non-pathogenic. Both the microorganism itself and its fermentation products

or cell components must be harmless to the host, not immunogenic and must not trigger allergic, toxic, mutagenic or carcinogenic reactions.

For the maintenance of its favorable properties the strain must also be genetically stable, and plasmid transfer events should not occur. Moreover, the selected strain should have a demonstrable beneficial effect on the host. For example, bifidobacteria can provide protection from pathogens through the production of bacteriocins, inhibition of pathogen adhesion and modulation of the immune system.

It's important to highlight that orally administrated probiotics can exert their action only after colonization and growth in the distal ileum and colon. Thereby, the probiotics should survive passage through the oesophagus and acidic stomach and colonize the intestine, at least temporarily. In this regard, the adhesion to the intestinal epithelium is essential to guarantee adequate permanence of the strain at the site of action; furthermore, the adhesiveness seems to enhance probiotic long-term survival [Morelli 2000].

Similarly, the microorganisms must be able to survive in the bile concentrations encountered in the gut. Lankaputhra and Shaha showed that *B. longum* survives better in acidic conditions and is able to tolerate a bile concentration as high as 4% [Lankaputhra and Shaha 1995]. However, the acid and bile tolerance, as well as the ability of microorganism to adhere to epithelial cells, are to a large extend species and stain dependent. To be beneficial for the host, probiotic cells must reach the colon at a concentration of 10^7 living bacteria per gram of intestinal content. In this regard, to provide functional benefits, the minimum suggested therapeutic dose is 10^8-10^9 viable cells per day/dose [FAO/WHO 2006]. From an industrial perspective, the production of probiotics should be easy and reproducible, and large number of viable bacteria must be able to survive prolonged periods during storage. Indeed, microorganisms able to multiply rapidly on relative cheap nutrients and to remain viable during manufacturing procedure and storage are preferred [Harish and Varghese 2006].

I.2.1.3 Microencapsulation of probiotics

Microencapsulation can be a powerful approach to overcome some limitations related to the administration of probiotics, such as the poor stability exhibited by many strains in acidic environment, as well as during storage conditions. Microencapsulation can be defined as "entrapment of a compound or a system inside a dispersed material for its immobilization, protection, controlled release, structuration, and functionalization" [Poncelet 2006].

According to this definition, microencapsulation can limit contact between probiotic bacteria and environment, thus protecting cells during oral delivery and during exposure to harsh digestive conditions [Islam 2010]. Moreover, it allows to obtain solid and dried dosage forms that facilitate their transportation and storage, as well as the preservation of cell viability due to the reduced moisture content.

The effect of drying on the cell viability varies with the characteristics of strain, type of drying and the composition of excipients (i.e. prebiotics) used for microencapsulation.

Freeze-drying and spray-drying are two techniques largely employed for this purpose [Dianawati 2016]. Since bacteria are typically sensible toward extremely low- or high-drying temperatures, the use of cryoprotectant or thermoprotectant agents is highly recommended to protect cells during the overall process.

Freeze-drying – Freeze-drying is a relatively new concept to microencapsulate probiotic bacteria [Coghetto 2016]. According to this technique, cell suspension is first frozen and then dried by sublimation of the solvent, normally water, under high vacuum [Solanki 2013]. This method, besides having high production cost, can cause damage to cell membrane due to crystal formation and stress condition of high osmolarity. Several cell protectants can be used to preserve cell viability during dehydration, including glucose, trehalose, maltodextrine, skimmed milk powder and whey proteins. These agents accumulate inside cells and act as cryoprotectants by reducing the osmotic difference between the external and internal environments. For example, cellobiose, lactose, sucrose and trehalose were shown to be effective in protecting *B. infantis* UV16PR during freeze-drying, since the survival rate of the strain in presence of these cryoprotectants resulted improved with respect to free cell preparation [Basholli-Salihu 2014].

Spray-drying – Currently, spray-drying has been developed as an alternative to freeze-drying and it offers some advantages, such as low cost and high production rate.

This technique consists in the atomization of a viable cell suspension, containing encapsulating and eventually protecting agents, into hot drying air and fast evaporation; the encapsulated product is then separated as a dry powder from the conveying air in a cyclone [Dianawati 2016]. Various operational conditions (i.e. inlet air temperature, feed temperature and feed rate) need to be optimized to produce small and uniform microparticles and to guarantee at the same time high survival rate of probiotic cells [Coghetto 2016]. The appropriate adjustment of the inlet air temperature is crucial because low air temperatures reduce the rate of water evaporation, resulting in microparticles with high-density membranes and poor flow properties, whereas

excessively high air temperatures can adversely affect cell viability [Rathore 2013].
Some experiments displayed that the use of proteins and milk fat as encapsulating agents increases viabilities of spray-dried probiotic bacteria (*B. breve* R070, *B. longum* R023 and *L.rhamnosus* GG) when exposed to simulated gastric fluid, compared to free cells [Picot and Lacroix 2004]. Also, the employment of skimmed milk as encapsulating agent showed favorable effects on the improvement of cell survival during spray-drying process of a *Bifidobacterium* strain (*Bifidobacterium* BB-12) [Fritzen-Freire 2012].
To provide a better protection of microencapsulated probiotics, they can be further formulated inside solid dosage forms able to pass undamaged through the stomach following oral intake, and release bacterial cells only in the intestine (see section I.3.1).

I.2.1.4 Prebiotics

Another approach to increase probiotic viability during both manufacturing procedures and storage is the employment of prebiotics. A prebiotic is defined as "non-absorbable food component that beneficially stimulate one or more of the gut-beneficial microbial groups and thus has a positive effect on human health" [Gibson and Roberfroid 1995].
In the large intestine, prebiotics act selectively on bifidobacteria and lactobacilli, stimulating their metabolisms [Dianawati 2016]. The two most studied prebiotics are inulin and fructooligosaccharides (FOS). They have specific chemical structures that human digestive enzymes cannot hydrolyse. Inulin is mainly extracted from chicory roots. It is a mixture of poly and oligosaccharides sharing a similar chemical structure GFn (G: glucose; F: fructose; n: number of fructose units linked to one another), with a degree of polymerization ranging between 10 and 60 [Biedrzycka and Bielecka 2004]. FOS are obtained through partial hydrolysis of inulin and therefore have lower molecular weight and lower degree of polymerization, which ranges from 2 to 8 [Franck 2002]. It has been demonstrated that both these prebiotics are able to greatly stimulate the growth of bifidobacteria since most *Bifidobacterium* spp. are adept to the utilization of inulin-type-fructans [Sarao and Aora 2017].

I.2.1.5 Probiotics for treatment of urogenital infections

Usually, the target for probiotic formulations is the gut, but recently other organs and tissues have been taken into account, including skin, hair, the oral cavity and the vagina [Mezzasalma 2017]. Different probiotics were reported to restore the normal vaginal homeostasis after topical application, allowing the lactobacilli colonization [Falagas

2007; Stapleton 2011; Palmeira-de-Oliveira 2015]. Interestingly, other authors suggest that oral administration of lactobacilli and bifidobacteria would allow colonization by probiotics of both the intestinal and vaginal mucosal surfaces. For example, Reid et al. conducted a randomized trial on 64 women, who received once a day for 60 days either a capsule containing two freeze-dried probiotic strains, namely *L. rhamnosus* GR-1 and *L. fermentum* RC-14, or a placebo [Reid 2003]. The study showed that the combination of probiotics was safe to be taken orally on a daily basis for 2 months, since no adverse effects were observed. Notably, the therapy resulted in a significant improvement in the vaginal microbiota, in terms of increased lactobacilli presence and decreased yeast and coliforms associated with BV dysbiosis. In particular, microscopy analysis revealed restoration from asymptomatic BV microflora to a normal *Lactobacillus*-dominated microbiota in 37% subjects during probiotic treatment compared to 13% on placebo; culture findings confirmed a significant increase in vaginal lactobacilli at days 28 and 60, a significant depletion in yeast at day 28 and an important reduction in coliforms at days 28, 60 and 90 for lactobacilli-treated women *versus* control. Hence, this result suggests that intestinal passage of probiotic strains leads to a beneficial impact also at vaginal level. This may occur due to the ascension of the strains themselves from the rectal site to the vagina, that create an inhospitable environment for pathogen colonization [Reid 2001]. Otherwise, the capability of pathogens, particularly *Candida*, to transfer to the vagina could be compromised, thus reducing total vaginal yeast counts. Mezzasalma et al. reported that also a *Bifidobacterium* strain (*B. animalis* subsp. *lactis*) possesses the ability to reach vaginal niche after oral administration [Mezzasalma 2017]. Furthermore, some authors observed that consumed oral probiotics are capable to colonize the vaginal epithelium, though transiently. For instance, Strus et al. documented that, after oral intake of a mixture of lactobacilli (*L. fermentum* 57A, *L. plantarum* 57B and *L. gasseri* 57C), the vagina resulted colonized by these strains for some weeks and this effect was correlated with significant improvement of vaginal health [Strus 2012].

Probiotics can be used as complementary to traditional therapy to improve the treatment of vaginal infections and reduce recurrences. Anukam et al. showed that the association of one probiotic dose (*L. rhamnosus* GR-1 and *L. reuteri* RC-14) with metronidazole is more effective than metronidazole alone [Anukam 2006]. The same probiotic strains have been shown to enhance the activity of tinidazole too [Hummelen 2010]. Obviously, this approach is feasible only if the probiotics are resistant to the antimicrobial agents administered.

I.2.2 Chlorhexidine

Chlorhexidine is a broad-spectrum antiseptic, largely employed for skin and mucous membrane disinfection. It's widely used especially in dentistry as mouth rinses, oral irrigations and slow release devices [Karpiński and Szkaradkiewicz 2015]. Chlorhexidine results very helpful in treating gingivitis, periodontitis and periimplantitis, for prevention of dental caries, in oropharyngeal decontamination and in endodontic interventions [Gomes 2013].

Furthermore, it is used in hospital settings as a topical skin antiseptic to reduce infections, in hand cleanser, in catheter site preparation and in bladder irrigation [Traoré 2000, Tanner 2008]. Chlorhexidine can also be applied in vaginal cavity as a way to reduce vaginal infections caused by both bacteria and fungi.

I.2.2.1 General characteristics of chlorhexidine

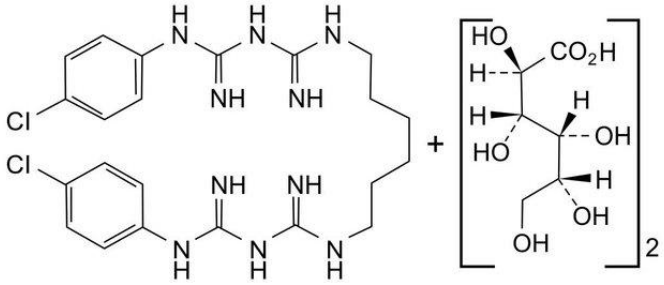

Figure I.9 – Chlorhexidine digluconate structure [Fiorentino 2013].

Chlorhexidine is made of two symmetrical structure with four chlorophenyl rings and two biguanide groups linked by a central hexamethylene bridge (*figure I.9*). The chlorine atoms, one on each phenolic ring, are responsible for its biological activity [Mohammadi and Abbott 2009]. The compound is strongly base and cationic at pH values above 3.5 and is practically insoluble in water. The salts of chlorhexidine – chlorhexidine diacetate, chlorhexidine digluconate and chlorhexidine dihydrochloride – are instead water-soluble. In particular, the most widely used form in disinfectant formulations is chlorhexidine digluconate. Chlorhexidine digluconate is almost colourless or pale-yellow liquid, is highly soluble in aqueous solvents and is usually commercialized as 20% chlorhexidine water solution.

Aqueous solutions of chlorhexidine are relatively resistant to elevated temperatures, indeed heating to 100°C does not cause decay. It has a shelf life of 20 to 24 months and

is stable at room temperature if protected from light and air, otherwise it gradually darkness. The activity of chlorhexidine is influenced by the environmental pH and the optimal range is between 5.5 and 7. Furthermore, the activity is reduced in the presence of serum, blood, pus and other organic material, as well as in presence of soaps and other anionic substances [Karpiński and Szkaradkiewicz 2015].

Chlorhexidine is generally safe to use. Despite its extensive use in medical settings since the 1950s, only isolated side effects – like contact sensitivity, dermatitis, urticaria and photosensitivity – have been reported [Goon 2004]. In obstetric use, few cases of vaginal mucosa desquamation have been observed [McClure 2007].

I.2.2.2 Antimicrobial activity of chlorhexidine

Chlorhexidine has a wide spectrum activity and its antimicrobial effect is dose-dependent; indeed, at low concentrations (0.02-0.06%) it acts as bacteriostatic, while at higher concentrations (> 0.12%) exerts a bactericidal action [Jenkins 1988]. *In virtue* of its cationic nature, chlorhexidine binds non-specifically to negatively-charged membrane phospholipids of bacteria, inducing a perturbation in the osmotic balance, that in turn leads to the release of potassium, phosphorus and other low weight molecules from microbial cells [Hidalgo and Dominguez 2001]. At sublethal concentrations, the loss of potassium ions does not exceed 50% and the alteration is reversable. Contrariwise, at high concentrations other events occur – such as the loss of main cellular components, including nucleotides, changes in protein structures and precipitation/coagulation of cytoplasmatic proteins – that lead to cell death by cytolysis [Karpiński and Szkaradkiewicz 2015].

The bactericidal action of chlorhexidine is very effective against Gram-positive bacteria and weaker against Gram-negative ones [Milstone 2008]. It is also active against virus and fungi, including *C. albicans*, *C. tropicalis* and *C. krusei.* The activity of chlorhexidine against *Candida* spp. is probably due to the inhibition of enzymes necessary for filamentation [Sautour 1999].

I.2.2.3 Vaginal application of chlorhexidine

Topical imidazole and metronidazole or clindamycin are considered standard treatment for VVC and BV, respectively. Unfortunately, concomitant bacterial vaginal infections are detected in up to half of women with candidiasis, and in the case of mixed vaginitis the application of monotherapy with an azole drug or with an antibiotic is ineffective

[Ventolini and Baggish 2003; Molteni 2004].

In obstetrics, vaginal douches containing chlorhexidine 0.25-0.5% are commonly used as an antiseptic treatment [McClure 2007]. It has been shown that the employment every 4-6 hours of chlorhexidine douches during labour reduces peripartum infections and mother-to-child risk of transmission of group B *Streptococcus* [Rouse 2003].

A study conducted by Monteni et al. highlighted that a vaginal chlorhexidine-based gel is able to effectively counteract both bacterial and vaginal infections, with a tolerability profile comparable with the standard therapies commonly applied in these conditions [Molteni 2004]. Moreover, unlike vaginal delivered antibiotics, topical application of chlorhexidine does not affect the normal *Lactobacillus*-dominant microbiota.

The currently available vaginal chlorhexidine-based formulations are douches, gels and ovules (i.e. Neoxene® and Ninfagin®), whose use is impaired mostly because of the washing action of vaginal fluid that rapidly remove dosage forms after application, thus requiring multiple daily doses and consequently reducing patient compliance [Bigucci 2015]. This limitation could be solved through the development of mucoadhesive formulations, able to extend the contact time with the vaginal mucosa (see section I.3.2).

I.2.3 Econazole nitrate

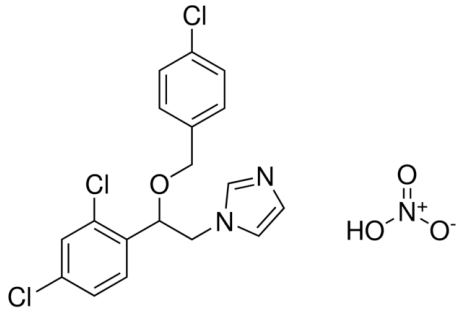

Figure I.10 – Econazole nitrate structure.

Econazole (1-[2-(2,4-dichlorophenyl)-2-(4-chlorobenzyloxyl)-ethyl]-imidazole) is an antifungal agent structurally related to another imidazole derivate, miconazole [Firooz 2015]. It is usually commercialized as nitrate salt (*figure I.10*) and is broadly employed for the treatment of many mycotic infections of skin, hair and mucus membranes. For instance, tinea corporis, tinea pedis, tinea cruris and pityriasis versicolor are common dermatophytic infections treated with topical application of econazole. *Candida* infections are another example of widespread superficial mycosis, that include intertrigo, oropharyngeal thrush, diaper dermatitis and vaginitis [Ambrogi 2010].

I.2.3.1 Antifungal activity of econazole

Econazole displays antimicrobial activities against a variety of dermatophytes, yeasts, actinomycetes, molds and other fungi. It is also active against some Gram-positive bacteria, while it is ineffective against Gram-negative ones [Heel 1978].

Similarly to other azole drugs, econazole exerts its antifungal action by interacting with 14-α demethylase, a cytochrome P-450 enzyme necessary to convert lanosterol to ergosterol (see section I.1.3.3). As ergosterol is an essential component of the fungal cell membrane, inhibition of its synthesis leads to leakage of cellular contents due to increased cell surface permeability.

It has been hypothesized that econazole could also act through alternative mechanisms, such as interaction with membrane phospholipids, inhibition of the endogenous respiration, transformation of yeasts to hyphal form and purine uptake, and impairment of triglyceride and/or phospholipid biosynthesis [Preissner 2010; Warrilow 2010].

I.2.3.2 Vaginal application of econazole

Econazole nitrate is one of the azole drugs normally employed for treatment of vaginal candidiasis. In women affected by VVC, the topical application of econazole follows two different dosage regimens: econazole can be administered either as a pessary of 150 mg for three consecutive days or as a 1% econazole-based cream in a regimen of at least 15 days [Baloglu 2011].

Symptomatic relief of vaginal burning and pruritus seems to occur rapidly in most patients, but vaginal discharge is relieved in a lesser proportion and is negatively influenced by the short-term treatment regimen [Heel 1978].

Topical or intravaginal econazole nitrate is generally well tolerate in most women; side effects have been recorded in about 1-4% of patients and comprise irritation, redness, burning or itching. Econazole action does not appear to be hampered by the concomitant intake of oral contraceptives.

Traditionally dosage forms include solutions, emulsions, suspensions, vaginal tablets, suppositories, creams, ointments and gels [Baloglu 2011].

Examples of commercially available products are: Ecoza® (topical foam 1%), Gyno-Pevaryl® (ovules 150 mg) and Pevaryl® (topical foam 1%).

However, the efficacy of econazole is limited by its poor water solubility and permeation rate; indeed, approximately 90% of drug remains on the skin surface after topical cream application, and thus it is not absorbed [Firooz 2015].

Consequently, it should be applied for several weeks, twice daily, to achieve a complete remission of symptoms.

In this regard, the employment of colloidal drug carrier, such as lipidic vesicular systems, can represent a strategy to improve the bioavailability, and thus the efficacy, of this antifungal (see section I.3.3).

I.2.4 Polyphenols

Polyphenols are phytochemicals, widely present in fruits, vegetable foods, legumes, cereal grains, chocolate, cocoa and beverages like tea and coffee.

More than 8000 polyphenolic compounds have been identified in nature, sharing a chemical structure characterized by the presence of at least one phenolic ring holding hydroxyl groups. Food usually contains complex polyphenols, primarily found in the outer layers of the plants [Ganesan and Xu 2017].

In nature, polyphenols play defensive roles against UV radiations, predators, pathogenic agents, oxidative stress and adverse environmental conditions. Furthermore, they are essential for plant growth and reproduction.

In the last two decades, the dietary intake of polyphenols has gained an increasing interest for the potential benefits on human health as anti-oxidant. The average 100 grams fresh weight of fruits (i.e. grapes, apple, pear, cherries and berries) contain up to 300 mg of polyphenols. Typically, in a cup of tea or coffee or a glass of red wine are present more than 100 mg of polyphenols. Cereals, vegetables, dry legumes and chocolate also contribute to the beneficial daily polyphenolic intake [Pandey 2009].

Numerous epidemiological studies and related meta-analyses strongly suggest that the consumption of these polyphenols positively impact on human health, providing protection against several chronic diseases (*figure I.11*).

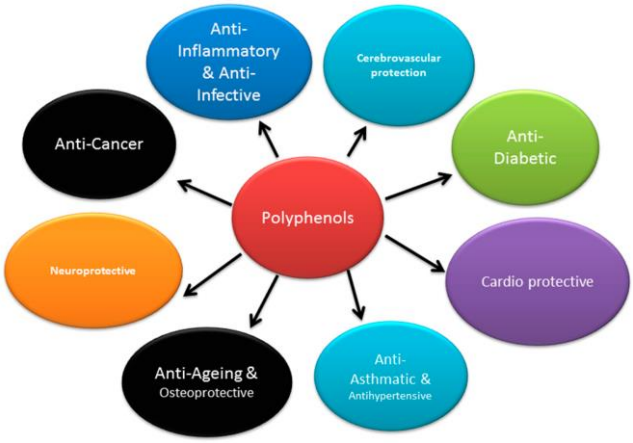

Figure I.11 – Positive effects of polyphenols in humans [Ganesan and Xu 2017].

In fact, besides being anti-oxidant, in human body polyphenols display a plethora of biological activities, since they are able to act as anti-inflammatory [Franceschelli 2017], anti-diabetic [Omodanisi 2017], anti-cholesterol [Tenore 2017], anti-cancer [Luo 2017], anti-asthmatic [Shaw 2016], anti-hypertensive [Gómez 2016], anti-ageing [Nobile 2016], antiseptic [Le Sage 2017], antifungal [Ayub 2017], antibacterial [Miyamoto 2017] and antiviral [Alam 2017] agents. Moreover, they provide protection to cerebrovascular [Forte 2016] and nervous systems [Ben 2017] and to heart [Rodríguez 2017], bones [Léotoing 2016] and liver [Jia 2017].

Despite the many favorable properties of phenolic compounds, their incorporation into food matrices or pharmaceutical delivery systems is very tricky due to poor stability when exposed to common manufacturing procedures; indeed, polyphenols are usually very sensitive to temperature, pH, oxygen, light and enzymes [Jones 2019]. Moreover, most polyphenols have poor water solubility that limits their bioaccessibility and bioavailability, and thus their potential use as active compounds. As in the case of lipophilic drug (like econazole nitrate, see section I.2.3), the incorporation of polyphenols inside nanocarrier could facilitate their delivery (see section I.3.3).

I.2.4.1 Polyphenol classification

Polyphenols can be categorized into four main groups based on the presence of number of phenolic groups and structural element: flavonoids, stilbenes, lignans and phenolic acids.

Flavonoids – Flavonoids share a common structure, consisting of two aromatic rings that are bound together by three carbon atoms that form an oxygenated heterocycle. Flavonoids have a potential effect on radical scavenging activity and inflammatory reaction [Ganesan and Xu 2017]. They are further divided into six subgroups, namely flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins). Flavonols are the most ubiquitous flavonoids in foods, and the main representatives are quercetin and kaempferol [Manach 2004]. Flavonols are mostly found in glycosylated forms in onions, curly kale, leeks, broccoli, and blueberries and, less abundantly, in red wine and tea.

Stilbenes – Stilbenes are found in only low quantities in the human diet. Resveratrol, the most representative compound of this group, is present in wine and it has been associated with anti-cancer, anti-arteriosclerosis and cardioprotective effects [Sessa 2014].

Lignans – Lignans are formed of two phenylpropane units and are metabolized to

enterodiol and enterolactone by the intestinal microflora. The richest dietary source is linseed, which contains secoisolariciresinol and low quantities of matairesinol. Algae, leguminous plants (lentils), cereals (triticale and wheat), vegetables (garlic, asparagus, carrots), and fruit (pears, prunes) are minor sources of lignans [Manach 2004].

Phenolic acids – Phenolic acids can be divided into two classes: derivatives of benzoic acid and derivatives of cinnamic acid [Manach 2004].

The hydroxybenzoic acid content of edible plants is generally very low, with the exception of certain red fruits, black radish, and onions. Gallic acid is the most important compound of this class and it is mainly consumed with tea beverage [Tomas-Barberan and Clifford 2000]. The hydroxycinnamic acids are more common with respect to hydroxybenzoic acids and they essentially comprise p-coumaric, caffeic, ferulic, and sinapic acids.

I.2.4.2 Quercetin

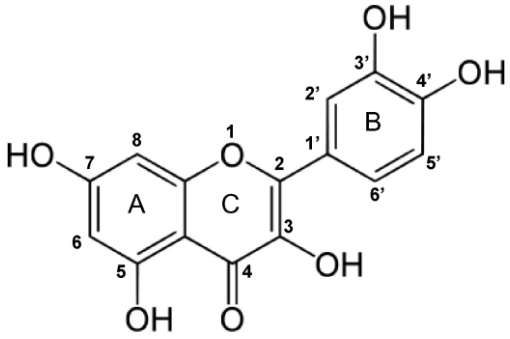

Figure I.12 – Quercetin structure [D'Andrea 2015].

Quercetin (3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-4Hchromen-4-one) belongs to the flavonols family and its chemical structure, typical of that of flavonoids, contains five hydroxyl groups (*figure I.12*). The name quercetin comes from *quercetum,* meaning oak forest. Naturally, quercetin is a polar auxin transporter inhibitor [D'Andrea 2015].

In plants, it is usually in a bound form with sugars, ethers or phenolic acids. In particular, the glycoside derivates – in which one or more hydroxyl groups is replaced by diverse kinds of sugar residues – are the most frequently found [Wang 2016].

Although the associated monosaccharides may be glucose, galactose or xylose, the 3-O-glucoside form is the most widespread. Quercetin derivatives in the form of disaccharides also largely occur in plants and vegetables, and among them the most abundant is rutin (quercetin 3-O-rhamnosylglucoside).

All these lipophilic compounds usually exist in the form of yellow coloured powder or

crystal, very poorly soluble in water (quercetin solubility in water is about 0.01 mg/mL at 25°C) [Gao 2011].

Quercetin is one of the most studied dietary flavonoids ubiquitously present in various vegetables (i.e. onion, tomato, lettuce) and fruits (i.e. black chokeberry) as well as in tea, red wine and caper [Bischoff 2008]. In some countries, this polyphenol is also available as a dietary supplement with daily doses between 200 and 1200 mg.

In the last years, quercetin has gained increasing attentions because of its claimed beneficial properties, such as anti-oxidant, anti-obesity, anti-carcinogenic, antiviral and antibacterial effects [D'Andrea 2015; Wang 2016].

In particular, quercetin is considered to be a strong anti-oxidant *in virtue* of its capability to scavenge free radicals and bind transition metal ions [Souza and Giovani 2004].

Its effect as anti-oxidant is mainly due to two pharmacophores within the molecules – the catechol group in the B ring and the hydroxyl group at position 3 of A ring (*figure I.12*) – which are present in the optimal configuration to exert scavenging activity against free radicals [Heijnen 2002]. Within flavonoid family, quercetin is claimed to be the most potent scavenger of reactive oxygen species (ROS), such as $O_2^{\bullet-}$, $NO^{\bullet}$ and $ONOO^-$.

These properties allow quercetin to excellently inhibit lipid peroxidation, which is responsible for deleterious effects on cells and tissues throughout the body. For instance, the oxidation of low-density lipoproteins can lead to the formation of atherosclerotic plaques responsible for cardiovascular diseases. Furthermore, brain lipid membrane damages owing to lipid peroxidation have been correlated with neurodegenerative conditions, such as Alzheimer's and Parkinson's disease.

Quercetin does not only terminate the propagation of lipid peroxidation by reacting with the radicals formed, but also increases glutathione levels thus preventing free radical formation [D'Andrea 2015; Wang 2016].

Moreover, the capability of quercetin to bind Ca^{2+} ion can protect cells from oxidative stress by preventing Ca^{2+}-dependent cell death [D'Andrea 2015].

Considering that ROS are also involved in inflammatory response, the strong anti-inflammatory activity displayed by quercetin is likely due to its free radical scavenging properties [Comalada 2005].

The anti-inflammatory activity of quercetin is also ascribed to inhibition of enzymes usually induced by inflammation (i.e. cyclooxygenase and lipoxygenase) and inhibition of pro-inflammatory cytokines (i.e. TNFα and IL-8), as well as inhibition of nitric oxide synthase in LPS-induced macrophages [Sah 2011].

I.2.4.3 Gallic acid

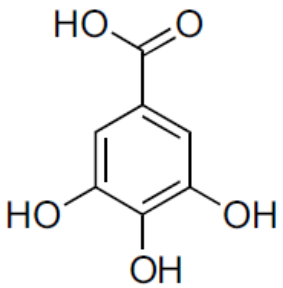

Figure I.13– Gallic acid structure [Choubey 2018].

Gallic acid (3, 4, 5-trihydroxybenzoic acid) is a low molecular colourless or slightly yellowish crystalline triphenolic compound (*figure I.13*), widely distributed across plant kingdom, such as leaves (beriberry), roots and bark (pomegranates and gall nuts) [Mohapatra 2006]. It is also present in some hard wood plant species such as oak (*Quercus robur*) and chestnut (*Castanea sativa*), as well as in processed beverages (i.e. green tea and red wine) [Prince 2009]. Commercially, gallic acid can be produced either by chemical or enzymatic hydrolysis of tannins materials (i.e. pentagalloylglucose) found in plants. Otherwise, it can be obtained through microbial fermentation exploiting microbial enzyme tannase, expressed by some bacteria and fungi, to cleave ester bond and release gallic acid molecule [Kahkeshani 2019].

Gallic acid and its derivatives such as lauryl gallate, propyl gallate, octyl gallate, tetradecyl gallate, and hexadecyl gallate find a lot of applications in the food and pharmaceutical fields, mainly owing to their oxidant activities. In fact, it has been observed that gallic acid is a strong anti-oxidant in emulsion or lipid systems largely employed in food packaging, cosmetics, pharmaceutics and food preservation. The radical scavenging activity is mainly associated with the hydroxyl group at the *para* position to the carboxylic group [Choubey 2018].

Gallic acid is widely used as food additive because of its ability to prevent the rancidity and spoilage of fats and oil. Moreover, it protects the cells from UV-B or ionizing irradiations and thus can be employed in cosmetics. It exhibits also many important biological activities that can be useful in pharmaceutical applications, such as antiallergic, antimutagenic and anti-carcinogenic [Choubey 2015]. It can also be used as an anti-oxidant to protect human cells against oxidative damage, to treat albuminuria and diabetes, and as a remote astringent in case of internal haemorrhage [Abbasi 2011].

Gallic acid and its derivates are also reported to be excellent anti-inflammatory agents,

probably due to their ability to inhibit endothelial nitric oxide, that plays a crucial role in allergic responses [Sanae 2003]. In addition, gallic acid can block mast cell-derived inflammatory response by impeding histamine release and consequently also the histamine-induced release of IgE [Kim 2006]. It has been observed that gallic acid inhibits the IκB kinase complex activity, leading to the decreased activity of the transcription factor NF-κB, which in turn causes the reduction in TNF-α production induced by LPS in macrophages [Yang 2001]. Besides TNF-α, gallic acid can also impair the release of other pro-inflammatory cytokines, such as IL-6, IL-10 and IL-1β [Kim 2006; Chen 2014]. Furthermore, gallic acid possess a broad range of antimicrobial activity. It hampers motility, adherence and biofilm formation of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus mutans*, *Chromobacterium violaceum* and *Listeria monocytogenes* [Shao 2015]. Gallic acid also affects the charge, hydrophobicity and permeability of the membrane surface in Gram-positive and Gram-negative bacteria, and thus leads to microbial cell disruption [Teodoro 2015]. Additionally, it can disintegrate the outer membrane of Gram-negative bacteria by chelating divalent cations. Antimicrobial effects of gallic acid may also be ascribed to inhibitory activity towards bacterial dihydrofolate reductase and excitatory activity on topoisomerase IV-mediated DNA cleavage [Godstime 2014]. Gallic acid affects the replication of some viruses too, including HIV-1, HCV and HSV, as well as the viability of fungi (i.e. *Saccharomyces cerevisiae* and *C. albicans*) and protozoa (i.e. *Caenorhabditis elegans*) [Choubey 2018].

Finally, gallic acid is reported to potentiate the antimicrobial activity of some antibiotics, including erythromycin, gentamicin, norfloxacin, ciprofloxacin, ampicillin, penicillin, and oxacillin via synergism [Kahkeshani 2019].

I.2.4.4 Anti-*Candida* activity of phenolic acids

Despite the availability of a wide spectrum of azole drugs, the incidence of invasive VVC poses the problem to find alternative sources to treat *Candida* infections [Seleem 2017].

Several natural extracts containing phenolic acids show antifungal activity against *Candida* spp. Phenolic acids isolated from these extracts display antifungal activity too. These compounds include gallic, caffeic, cinnamic, benzoic, protocatechuic and phenylacetic acids [Teodoro 2015].

To date, the mechanisms of action through which phenolic compounds exert antifungal activities are not completely elucidated. As stated in section I.1.3.3, the main factors of *Candida* virulence are exoenzymes production, adherence and biofilm formation and

transition from yeast to hyphal form.

Some phenolic acids, including gallic and caffeic acids, are reported to have anti-adhesive and anti-biofilm activities against *Candida* spp. [Alves 2014; De Vita 2014]. Anti-hyphae effects in *C. albicans* were observed following the treatment with epigallocatechin-gallate (a derivative of gallic acid) [Han 2007], licochalcone A, gladribin [Messier and Grenier 2011] and thymol [Braga 2007].

Additionally, bisbibenzyl seems to reduce hyphal and biofilm formations through the upregulation of *Dpp3* gene, that in turn stimulates the synthesis or farnesol, an inhibitor of hyphae formation [Zhang 2011].

It has been proposed that gallic, ferulic and caffeic acids may affect *Candida* cytoplasmatic membranes and cause leakage of cell contents, similarly to what happen for bacterial cells [Borges 2013]. Furthermore, caffeic acid and derivatives have been shown to interfere with 1,3-β-glucan synthase, essential to preserve cell wall integrity [Ma 2010]. Some phenolic acids, including gallic acid and epigallocatechin-gallate, can also considerably reduce biosynthesis of ergosterol, a fundamental component of fungal membrane [Navarro-Martinez 2006; Li 2017].

Other compounds may trigger apoptotic mechanisms in *Candida*, thereby contributing to the overall antifungal activity. For example, eugenol induces apoptosis by inhibiting the cell cycle at G1, S and G2-M phases. Curcumin also causes apoptosis in *C. albicans* by increasing the ROS and inducing *CaMCA1* gene expression [Cao 2009].

Several studies have also underlined a synergistic action between phenolic acids and azole drugs. For instance, epigallocatechin-gallate display a synergistic antifungal effect on *Candida* when combined with fluconazole, intraconazole or ketoconazole [Hirasawa and Takada 2004; Navarro-Martinez 2006]. Azoles directly inhibit the ergosterol biosynthesis, while the effect of epigallocatechin-gallate is indirect. Indeed, epigallocatechin-gallate causes a depletion of the enzyme S-adenosylmethionine and consequently a reduction in production of C24 methyltransferase, which in turn negatively affects the ergosterol biosynthesis. Direct and indirect effects on ergosterol biosynthesis can explain the synergism between epigallocatechin-gallate and azoles [Navarro-Martinez 2006].

I.3 Dosage forms and carries selected for the project

I.3.1 Solid lipid dosage forms

The conventional dosage forms usually require multiple daily administration to maintain the drug concentration within the therapeutic range. The growing interest in controlled drug release delivery is due to its benefits like fewer side effects and the possibility to reduce the frequency of administration, which allow to increase the patient compliance.

In this regard, waxes and fats are non-toxic and biocompatible materials that can be used as barrier coatings to modulate the release of incorporated active ingredients and to protect them from harsh surrounding conditions, such as during the transit through gastrointestinal tract [Gowda 2009].

Lipid dosage forms permit to embed one or more drugs within lipid matrix, and thus offer a great potential to increase apparent solubility of poorly water-soluble drugs. Moreover, they allow to modulate the release of active compounds in a time-controlled manner [Kreye 2008; Schulze and Winter 2009]. A time-controlled release of drug can overcome frequent administrations (i.e. daily injections), required in the case of active ingredients with short half-lives, that are rapidly eliminated from the body.

Moreover, the capability of lipid formulation to preserve its integrity for a few hours after intake is particularly useful in the case of orally administered active agents that exert their function in the intestinal tract, and thereby need to be protected during the transit through the acidic environment of the stomach.

Microparticles, spherical beads and cylindrical implants are the most described lipid dosage forms for controlled drug release [Siepmann and Siepmann 2011]. Various preparation methods have been proposed for the formulation of lipid systems, including compression, extrusion, emulsification methods and melting and casting techniques. Furthermore, a wide range of lipids, lipid mixtures and the addition of other excipients, like water-soluble “pore formers” (i.e. carboxymethylcellulose, see section I.3.2.4), have been suggested [Herrmann 2007].

Lipid microspheres and lipid implants are considered promising strategies for the delivery of proteins, since they can offer a better control of drug release and a more stable environment with respect to polymeric matrices. In fact, the continuous variations of the physical-chemical features inside polymeric matrices, owing to phenomena of polymeric degradation and erosion, may be detrimental for protein and peptide drugs [Maschke 2004]. Lipid implants based on tristearin were developed for the continuous release of

IFN-α over one month [Mohl 2004]. Tripalmitin-based implants were also developed for the sustained release of insulin [Appel 2006], interleukin-18 [Koennings 2006], lysozyme [Guse 2006], and brain-derived neurotropic factor [Koennings 2007].

In the present project, a lipid dosage form is proposed for the oral delivery of the probiotic strain *B. breve* BC204 (see section III). In particular, we selected beeswax as excipient for the formulation of tablets because, besides being safe for human intake, it also resulted harmless to beneficial bacteria.

I.3.1.1 Beeswax

Beeswax can be considered a GRAS and biocompatible excipient. It is an authorized food additive in the European Union, permitted as a glazing agent on confectionary, for the surface treatment of certain fruits, in food supplements and as carrier for colours [FAO 2005].

Naturally, beeswax is widespread produced by the domestic worker bee *Apis mellifera* L. and in some parts of the word may also be produced by *A. cerana, A. florae* and other honeybee species. Production takes place at wax gland complexes consisting of three cell types (epithelial cells, oenocytes and adipocytes), which act in combination to secrete wax. The secretion is a constant process and starts in one-week old honey bee workers, peaks after two weeks of age and hereafter decreases [Cassier and Lensky 1995].

Industrially, beeswax is obtained by melting the honeycombs of bees after removal of the honey by draining and filtering or by centrifuging.

Beeswax is a complex mixture of saturated and unsaturated linear and complex monoesters, hydrocarbons, free fatty acids, free fatty alcohols and other minor exogenous substances [Aichholz and Lorbeer 1999]. More than 300 individual components have been identified in beeswax from various species of honeybees. Its composition depends on some factors, including the bee subspecies, the age of the wax and the climatic circumstances of the production. However, the variation in composition is mainly due to the relative amounts of the diverse components present, rather than in their chemical identity [Tulloch 1980].

Beeswax is commercially available as yellow and white products. In particular, white beeswax is a white or yellowish white solid having a characteristic, but faint, odour of honey. The waxes are insoluble in water, sparingly soluble in alcohol, and very soluble in chloroform, ether, and in fixed and volatile oils [Puleo 1991].

According to the ESFA (European Safety Food Authority), the NOAELs (No Observed

Adverse Effect Level) identified in the toxicological studies on the main constituents of beeswax and chemically related plant waxes were 10 to 50 times higher than the very conservative exposure estimate of 22 mg/Kg bw/day.

These margins of safety can be considered adequate for the assessment of beeswax, which consists of components that generally do not appear to be absorbed from the gastrointestinal tract to any significant extent. However, the ester constituents in beeswax are usually metabolized in the intestinal lumen to their corresponding alcohols and acids that, even if absorbed, are incorporated into normal cellular metabolic pathways and thus result safe [ESFA Journal 2007].

In the last decades, the antimicrobial activities of natural products have gained increasing interest but beeswax has only recently been investigated.

A study conducted by Ghanem et al. revealed that beeswax exerts antimicrobial activities against both pathogenic Gram-positive (*S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228 and *S. pyogenes* ATCC 19615) and Gram-negative bacteria (*B. subtilis* ATCC 27853, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922), as well as against fungus like *C. albicans* [Ghanem 2011]. Notably, our preliminary studies revealed that beeswax does not affect the viability of probiotic strain *B. breve* BC204 formulated in *paper 1* (see section III). A mixture of honey, olive oil, propolis extract and beeswax (called HOPE) was also used successfully for the treatment of mucositis induced by chemotherapy [Al-Waili 2006]. Moreover, a high molecular weight extract called D-002 was purified from beeswax. Interestingly, besides possessing good anti-ulcerative and anti-inflammatory activities, this compound provides a protective action for the gastrointestinal mucosa [Illnait 2013].

I.3.2 Mucoadhesive polymeric matrices

As stated in section I.1.4.4, conventional vaginal formulations are often associated with low retention to the mucosa, mainly due to the cleaning action of the vagina.

Novel vaginal drug delivery systems based on mucoadhesive polymers may be used as promising alternatives over conventional dosage forms in order to assure prolonged contact time within the vaginal cavity, thus improving drug local availability and efficacy.

In 1986, Longer and Robinson defined “bioadhesion” as the “attachment of a synthetic or natural macromolecule to a biological surface” [Longer and Robinson 1986]. “Mucoadhesion” can be considered a kind of bioadhesion in which the biological surface is represented by a mucosal tissue [Salamat-Miller 2005].

The mucosal surfaces, including the vaginal one, are covered with a mucous layer, containing mucins as major component. Mucins are highly glucosylated glycoproteins with a molecular weight of 1-40 million Da and can exist in two forms, soluble secretory mucin and membrane bound mucin. The core of the mucins is a large peptide backbone – made of repeated sequences rich in serine, threonine and proline residues – and O-linked oligosaccharides as side chains, often terminated in either sialic acid, sulfonic acid of L-fructose. Therefore, mucins result negatively charged at physiological pH. The oligosaccharides form about 50-80% of the dry weight of the molecules and confer an extended conformation to the mucins [Edsman and Hägerström 2005].

In this thesis, different polymers were taken into account as potential mucoadhesive excipients for the development of chlorhexidine-based preparations (see section IV). Once placed in aqueous environment, these polymers undergo swelling and thereby allow to efficiently control the release of drug [Valenta 2005].

Moreover, lyophilization technique was used to produce highly porous matrices capable to rapidly hydrate, thus assuring an intimate contact with mucosal surfaces.

I.3.2.1 Mucoadhesion process

Although the exact mechanisms occurring at the interface between a polymeric dosage form and mucosal surface are not yet fully elucidated, certain elements of the process of mucoadhesion are clear. The mucoadhesion process is generally divided into two steps, the contact and the consolidation stages. In the first step the dosage form spreads over the substrate and swells to initiate its deep connection with the mucus layer and increases the surface contact. In some cases, such as for ocular and vaginal formulations, the dosage form is mechanically attached over the mucous membrane. When the direct attachment of the formulation over the membrane is not feasible, as in the case of orally dosage forms, other events (i.e. peristaltic motions) may occur and favour the initial contact with the gastrointestinal mucosa [Carvalho 2010]. In this stage, both attractive (van del Waals forces and electrostatic attraction) and repulsive (osmotic pressure, electrostatic repulsion) forces may arise but, for a successful mucoadhesiveness, the attraction forces must dominate [Lee 2000].

Secondly, in the consolidation step, the mucoadhesive polymers are activated by the presence of moisture. Indeed, when a formulation adheres to the mucosa, three layers can be identified: the dosage form, the mucosa and the interfacial region in which the dosage form results highly hydrated. This aspect is important because the presence of a hydration

layer allows the relaxation of polymeric chains and their interdiffusion with the glycoproteins of the mucus across the interface (*figure I.14*). The interpenetrated chains can then interact, resulting in entanglement and week chemical bonds, deriving from electrostatic attractions, hydrophobic interactions, van der Waals forces and hydrogen bonds [Edsman and Hägerström 2005].

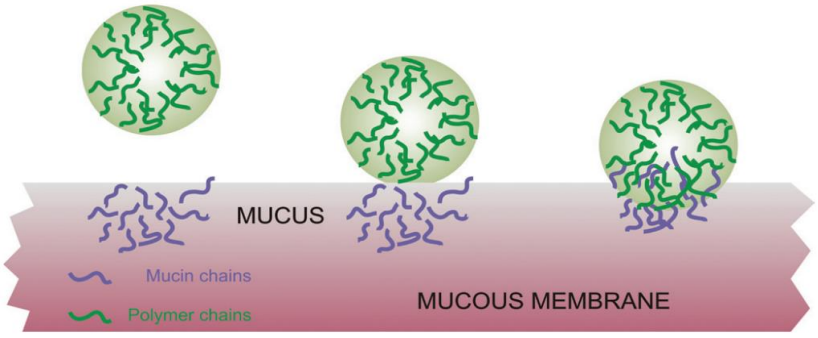

Figure I.14 – Entanglement between polymeric chains of the dosage form and glycoproteins of the mucus [Carvalho 2010].

Five theories have been suggested to play a prominent role in mucoadhesion, namely absorption, diffusion, electronic, fracture and wetting theory. None of these theories can explain mucoadhesion on its own for all the different existing formulations, but they can be combined to obtain an overview of the mucoadhesive process.

Absorption theory – According to this theory, the dosage form adheres to the mucous surface by secondary chemical interactions, as van der Waals and hydrogen bonds, electrostatic attraction or hydrophobic interactions. For polymers containing carboxyl groups, hydrogen bonds are considered the prevalent force at the interface [Smart 2005]. Otherwise, hydrophobic interactions can explain the fact that mucoadhesion to hydrophobic surfaces is often facilitate with respect to hydrophilic substrates [Lee 2000].

Diffusion theory – In the diffusion theory, the chains of the polymeric dosage form and mucin interpenetrate one another to a sufficient depth to allow the entanglement of the molecules and thereby create a semi-permanent adhesive bond. This penetration rate depends on some factors, including the diffusion coefficients of both interacting molecules, flexibility and nature of the mucoadhesive chains, mobility and contact time [Carvalho 2010].

Electronic theory – Considering the different electronic properties of the mucoadhesive polymer and the mucus glycoprotein, the electronic theory states that an electron transfer may occur between the two counterparts. This leads to the formation of an electrical double layer at the interface, where the resulting attractive forces determine the

mucoadhesive strength [Salamat-Miller 2005].

Fracture theory – This theory assumes that the force required for the separation of polymeric dosage form from the mucosal surface after adhesion is equal to polymer adhesive strength. It also assumes that the fractur occurs exactly at the interface, a condition that is rarely observed and only for rigid formulations [Edsman and Hägerström 2005].

Wetting theory – It was initially developed for liquid preparations and describes the ability of a mucoadhesive polymer to spread on biological surfaces. This theory exploits measured surface and interfacial tension to calculate the adhesive bond [Salamat-Miller 2005].

I.3.2.2 Mucoadhesive polymers

Mucoadhesive polymers can be divided into synthetic or natural polymers. Most of the current synthetic mucoadhesive polymers are either polyacrylic acid or cellulose derivates. Others can be considered semi-natural mucoadhesive polymers, such as chitosan and various gum (i.e. guar, xanthan, gellan, carrageenan, pectin and alginate).

In a more functional type of classification, mucoadhesive polymers can be categorized in water-soluble and water-insoluble polymers. The first group mainly consists of linear or random polymers and includes poly(acrylic acid), hydroxypropylmethylcellulose, sodium carboxymethylcellulose (CMC) and sodium alginate. In the second group are comprised the swellable polymers, like polycarbophil and ethylcellulose.

Moreover, polymers can be uncharged (i.e. hydroxypropyllcellulose, PVA and PVP) or charged. The latter can be further divided into cationic (i.e. chitosan) and anionic (sodium alginate, sodium CMC and xanthan gum) polymers [Lee 2000; Salamat-Miller 2005].

Besides environmental factors – such as pH, amount of vaginal fluid and mucus turnover – a variety of polymer-related features can affect the mucoadhesiveness.

In general, it has been shown that the mucoadhesive strength of a polymer increases with molecular weight above 100 kDa [Chen 1970]. This is especially true for linear polymers, because the high molecular weight favours a better entanglement with mucin chains. Moreover, the interdiffusion of polymer inside the mucus layer is promoted when polymeric chains possess a high degree of flexibility. As water-soluble polymers become cross-linked, the mobility of their chains decreases and thereby also the effective length of the molecule that can penetrate into the mucus is reduced, with consequent reduction of mucoadhesion strength. The mucoadhesion is also favoured for polymers that possess

functional groups capable to form several hydrogen bonds.

There is also an optimum concentration of a mucoadhesive polymer to produce maximum mucoadhesion. Indeed, if the concentration of the system is too high, the adhesive strength dramatically drops because the coiled polymeric molecules become separated from the medium and thus the chains available for the entanglement are limited.

Finally, the swelling characteristics may affect the mucoadhesion too. Swelling depends on the polymer concentration, ionic strength, as well as the presence of water. The maximum of mucoadhesion occurs with an optimum water content. Conversely, overhydration results in the formation of a wet slippery mucilage that impairs mucoadhesion process [Lee 2000; Edsman and Hägerström 2005; Salamat-Miller 2005]. The polymers employed in the present thesis (sodium alginate, sodium carboxymethylcellulose, xanthan gum and hyaluronic acid) are briefly described below.

I.3.2.3 Sodium alginate

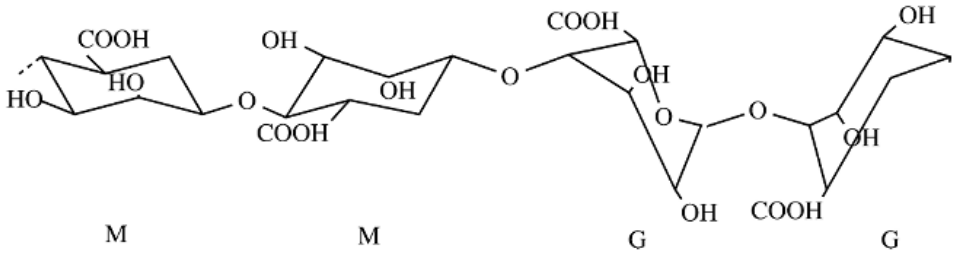

Figure I.15 – Alginate structure [Aravamudhan 2014].

Alginate is a naturally occurring anionic polysaccharides typically extracted from brown seaweeds (*Phaeophyceae*), including *Laminaria hyperborean, L. digitate, L. japonica*, *Ascophyllum nodosum* and *Macrocystis pyrifera* [Lee 2012]. Some bacteria, such as *Azotobacter* and *Pseudomonsas*, also produce alginate. Bacterial biosynthesis can be used as tool to tailor alginate production by genetically engineering selected strains, and thereby to obtain alginate with defined chemical structures and physical properties [Mørch 2007]. Chemically, alginate is a linear copolymer containing blocks of (1,4)-linked β-D mannuronate (M) and α-L- guluronate (G) residues (*figure I.15*).

Alginates extracted from different sources differ in M and G contents, as well as in the length of each block. More than 200 different alginates are currently commercially available with molecular weights that vary widely between 32 and 400 kDa.

The mechanical properties of alginate gels typically are improved by increasing the length of G-block and molecular weight [George 2006]. A high G-block content is also

associated with better biomedical performances because of the ease of processability and low immunogenicity in the body [Christensena 2011].

Alginate has been extensively investigated and used for many biomedical applications, due to its relatively low cost and mild gelation by adding of divalent cations such as Ca^{2+} [Gombotz 1998]. Moreover, alginate extracted from natural sources and highly purified does not induce any significant foreign body reaction and any inflammatory response and thus can be considered non-toxic and biocompatible [Orive 2002; Lee 2009].

It is widely used in wound healing field because of the capability of alginate dressing to maintain a physiologically moist microenvironment, minimize pathogen growth at the wound site and facilitate wound regeneration [Thomas 2000; Queen 2004].

Moreover, alginate gels can be orally and vaginally administered or injected in a minimally invasive manner, and are also promising for cell transplantation in tissue engineering [Valenta 2005; Lee 2012].

I.3.2.4 Sodium carboxymethylcellulose

Over the years, cellulose has been largely exploited by pharmaceutical industry because of its favourable properties, including the stability, versatility and non-toxicity. Cellulose is composed of glucose monomers linked by glycosidic bonds. Cellulose ring structures are attached in a flip-flop orientation, that causes the formation of long and rigid fibers, which result indigestible by humans [O'Sullivan 1997].

Simple chemical modifications of cellulose permit to obtain derivatives, usually referred to as cellulosics, with desired water solubility and swellability properties. These products are mainly the results of esterification, etherification and crosslinking of cellulose derivatives, and are routinely used in many pharmaceutical dosage forms as excipients [Mastropietro and Omidian 2013].

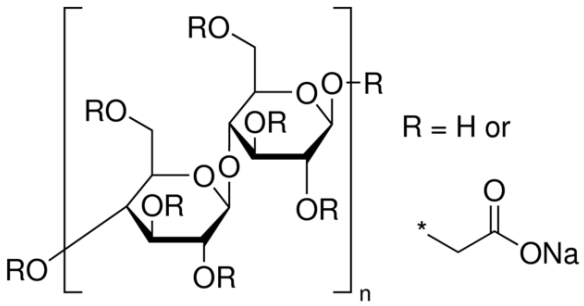

Figure I.16 – Sodium carboxymethylcellulose structure.

The sodium carboxymethylcellulose (CMC) is the major cellulose ether (*figure I.16*) and is produced by reacting alkali cellulose with sodium monochloroacetate. CMC is commonly employed in many dosage forms in order to increase viscosity and as a binder, disintegrant, coating and suspending agent.

CMC has been used satisfactorily as excipient to obtain controlled drug releases in oral suspensions for gastrointestinal drug delivery [Chen 2010], in powder formulations for nasal delivery [Ugwoke 2000] and in injectable products [Mastropietro and Omidian 2013]. CMC is also useful as a scaffold in tissue engineering [Aravamudhan 2014] and, together with chitosan and hydroxyapatite, is employed for bone and dental regeneration purpose too [Chen 2008; Liuyun 2009]. Furthermore, in a work conducted by Bigucci et al., CMC was combined with chitosan in order to obtain polyelectrolyte complexes able to modulate the release of chlorhexidine inside the vaginal cavity [Bigucci 2014].

I.3.2.5 Xanthan gum

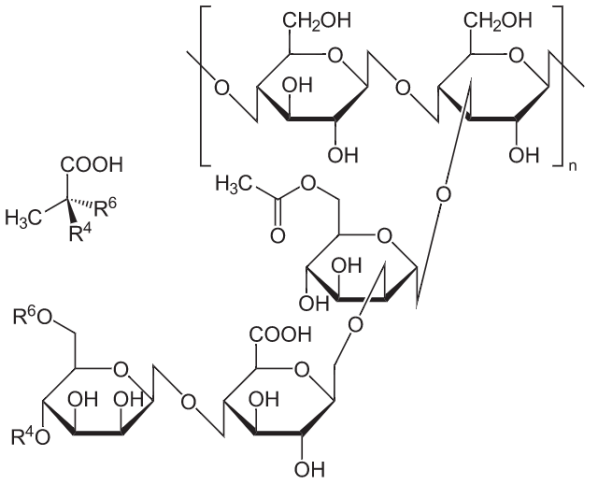

Figure I.17 – Xanthan gum structure.

Xanthan gum is the second microbial polysaccharide, after dextran, which was industrially commercialized [Kumar 2018]. Naturally, it is biosynthetized by different species of Gram-negative *Xanthomonas* bacteria, while the industrial production is usually achieved through the fermentation of glucose by the species *X. camprestris*, a plant-associated bacterium [Petri 2015].

The primary structure of xanthan gum is composed of a backbone of linear (1,4)-linked D-glucose, with branched side chains holding pentasaccharide subunits (*figure I.17*). These comprise D-glucosyl, D-mannosyl and D-glucuronyl acid residues in a 2:2:1 molar ratio with varying proportions of O-acetyl and pyruvyl residues [Faria 2011].

The secondary structure of xanthan gum is shown as five-fold right-hand helical structure with a pitch of 4.7 nm and a diameter of 1.9 nm and undergoes *via* an order-disorder conformational transition that may be thermally-induced at high temperatures or may occurs at low salt concentrations. Indeed, the ordered conformation is stabilized by the presence of salts, which confer extraordinary stability to gum [Kumar 2018].

Xanthan gum is a high molecular weight polymer whose dimension varies from 2000 kDa to 20000 kDa, depending on the association of chains and the variations in fermentation conditions. This gum has a good water solubility and its solutions behave as non-Newtonian fluids. Moreover, it acts as a polyanion at pH above 4.5 due to the protonation of O-acetyl and pyruvyl residues [Petri 2015].

Xanthan gum solutions display excellent resistance to mechanical degradation, high viscosity even at low concentrations and stability over a broad range of temperatures (up to 90°C), salt concentrations and pH (2-11) [Rosalam and England 2006]. In addition, xanthan gum shows excellent biocompatibility, it is non-toxic, non-sensitizing and environmentally friendly [Faria 2011]. Given these superior properties, xanthan gum has extensively been used in food industry and it has been considered also for biomedical applications. For example, it has been demonstrated that xanthan gum can effectively replace hyaluronic acid in intra-articular injection treatment to reduce osteoarthritis progression [Han 2012].

I.3.2.6 Hyaluronic acid

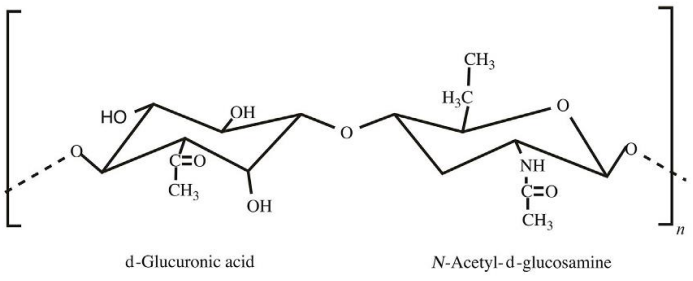

Figure I.18 – Hyaluronic acid structure [Aravamudhan 2014].

Hyaluronic acid is a linear high molecular mass natural polysaccharide made of alternating (1,4)-linked β-D-glucuronic and (1,3)-linked β-N-acetil-D-glucosamine residues (*figure I.18*). The molecular weight of hyaluronic acid can reach as high as 10000 kDa. The disaccharide units of hyaluronic acid form an extended and rigid structure, whose many anionic groups bind water molecules, which make hyaluronate solution

occupy a volume 1000 times higher than in its dry state [Aravamudhan 2014].

The unique viscoelastic and rheological properties predispose hyaluronic acid to play important biological roles in living organisms and make it an excellent biomaterial for a broad range of medical applications [Kogan 2007].

Biologically, hyaluronic acid is an important glycosaminoglycan that primarily occurs in the extracellular matrix of soft tissue, in the synovial fluid and in the vitreous humor of the eye in mammals [Drury 2003]. Besides serving as a matrix in the connective tissue, hyaluronic acid is also involved in other biological pathways, like cell proliferation, differentiation and tissue repair. The length of chains is an essential factor to elicit the biological functions. For example, while the low molecular weight hyaluronic acid (< 35 kDa) is known to be involved in cytokine activity implicated in inflammatory responses [Hodge-Dufour 1997], the bigger molecules (> 200 kDa) are known to inhibit cell proliferation [West and Kumar 1989]. Moreover, smaller fragments (1-4 kDa) have positive effects in promoting vascularization during injuries.

Hyaluronic acid has had a profound impact on the field of tissue engineering. In fact, its incorporation into biomaterials and scaffolds has yielded a new class of biocompatible, controllable and readily degradable materials able to promote beneficial remodelling of engineered tissues [Shu 2004; Aravamudhan 2014].

Hyaluronic acid is largely employed because of its viscoelastic properties in a number of ophthalmological surgeries and in intra-articular applications in joints affected by arthritic disorders (i.e. osteoarthritis and rheumatoid arthritis) [Maltese 2006; Kogan 2007]. Preparations of slightly cross-linked hyaluronic acid are currently commonly used in dermatology and plastic surgery to fill facial wrinkles and depressed scars. In cosmetics, hyaluronic preparations appear to be an excellent alternative to collagen-based fillers because are longer lasting and extremely elastic [Narins 2003]. Wound dressings based on hyaluronic acid are also found to promote healing of fresh skin wounds, as well as the healing of venous leg ulcers associated with chronic wounds [Brown and Jones 2005].

I.3.2.7 Freeze-drying technique

Freeze-drying or lyophilization is a drying method that allows the removal of moisture from the product within a container system by a process of sublimation and desorption.

A typical freeze-drying cycle consists of three main steps, as summarized in *figure I.19*. In the initial freezing stage, the temperature is lowered below the freezing point of water. The crystallization of water causes the remaining solution to become more concentrated,

thus forming a "freeze-concentrate". Some of the drugs and excipients contained in the preparation may crystallize or become amorphous in the freeze-concentrate [Patel and Pikal 2011].

Once the material is completely frozen the primary drying step, in which most of the water is removed through sublimation, can occur. This is achieved by applying the vacuum in order to maintain the chamber pressure well below the vapour pressure of ice (usually between 50 and 200 millitorr). In this phase, it is mandatory that the drying temperature does not exceed the critical eutectic or glass transition temperature of the crystalline or amorphous materials, respectively, as this would result in a "melt-back" or "collapse" phenomenon [Baheti 2010]. Toward the end of the primary drying step, the moisture content of the resulting porous solid cake is used to be 7-8%.

The last step, referred to as secondary drying, is required to remove the remaining bound water by desorption and evaporation still under vacuum. The residual water content at the completion of entire cycle is about 1-2% [Siow 2016].

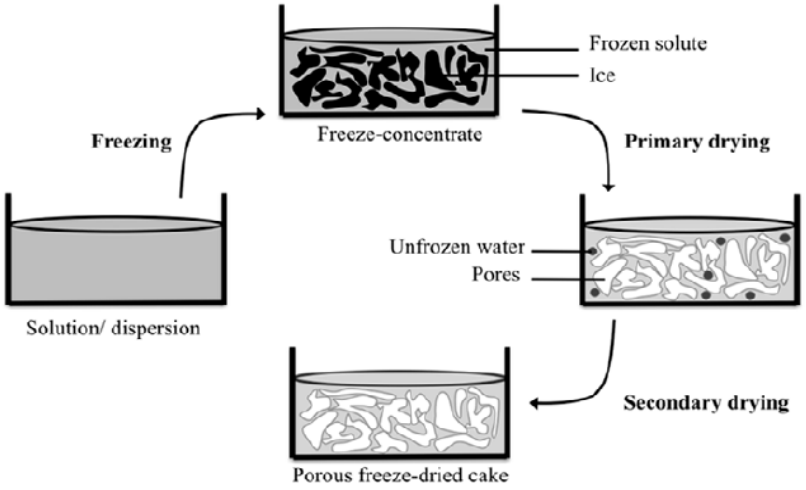

Figure I.19 – Main phases of a freeze-drying cycle [Siow 2016].

The expansion of freeze-drying application into the pharmaceutical field is mainly due to the understanding that viable shelf-life and stability may be improved by significantly reducing moisture content [Siow 2016]. Freeze-dried orodispersible tablets are a good example of freeze-drying pharmaceutical application.

Lyophilization is particularly suitable for the production of biologics (i.e. proteins) and small molecule parenteral formulations, which are sensitive to high temperatures and thus are not compatible with conventional oven drying techniques.

However, phase separation of the drug from the liquid vehicle (especially in the case of lipophilic compounds) and re-crystallization of amorphous molecules are problems

associated with this drying method. In this regard, some excipients (i.e. hydrophilic polymers or sugars) can be added to overcome these detrimental effects, in addition to being used as bulk agents [Elgindy 2011]. Several mechanisms by which polymers may prevent drug precipitation have been proposed: shielding of drugs by the polymer molecules, formation of hydrogen bonds between drug and polymer and increase in viscosity of dissolution medium [Moes 2011].

The mechanical properties of drug after lyophilization result also ameliorate compared to unprocessed drug, probably owing to the high porosity, compressibility and tensile strength of freeze-dried products.

I.3.3 Liposomes

Nanopharmaceutical comprises an emerging multidisciplinary branch in the field of nanotechnology, which is currently experiencing exponential growth since it is regarded as a potent tool to revolutionize medical treatment [Vanić and Škalko-Basnet 2013].

The general advantages of nanocarriers can be ascribed to their tiny particle size (< 1 μm) and can be summarized as follows: (i) decrease patient-to-patient variability; (ii) enhance drug solubility; (iii) increase surface area, thus improving absorption and bioavailability; (iv) allow to reduce the dose required; (v) increase rate of dissolution; (vi) improve pharmacokinetic and biodistribution of therapeutic agents (vii) elicit a more rapid onset of therapeutic effect; (viii) facilitate targeted drug delivery to the diseased site; (ix) increase retention in biological systems thus prolonging the drug efficacy (x) increase patient compliance [Leyva-Gómez 2018; Chen 2019].

Thus, nanocarriers – including lipid-based carriers, polymeric nanoparticles, inclusion complexes, micelles and nanofibers – can be considered more potent and less toxic therapeutics able to improve distribution, retention and ultimately pharmaceutical efficiency of a broad range of active molecules.

In the present work, liposomal vesicles were chosen as nanocarriers for the vaginal delivery of active agents able to counteract and reduce symptoms of vulvovaginal candidiasis. In particular, a well-known azole drug (econazole nitrate) was incorporated inside lipid vesicles enriched with surfactants in order to improve its topical delivery (see section V).

Subsequently, two polyphenols (quercetin and gallic acid) were formulated inside conventional liposomes and proposed as alternative to common antifungal products (see section VI).

I.3.3.1 Conventional liposomes

Liposomes are widely appreciated nanocarriers owing to their biocompatible and biodegradable properties [Immordino 2006].

The term liposome comes from two Greek words, namely “lipos” (meaning fat) and “soma” (meaning body), which describe the chemical composition and physical structure of these vesicles [Xu and Burgess 2012]. Liposomes are mainly composed of phospholipids, a class of amphiphilic molecules holding both hydrophilic (water-soluble) and hydrophobic (water-insoluble) groups. A typical liposome-forming phospholipid, such as lecithin, is characterized by two hydrocarbon chains (hydrophobic tails) and one hydrophilic group (hydrophilic head) linked to three hydroxyl groups on a glycerol backbone at positions 1, 2 and 3, respectively (*figure I.20*).

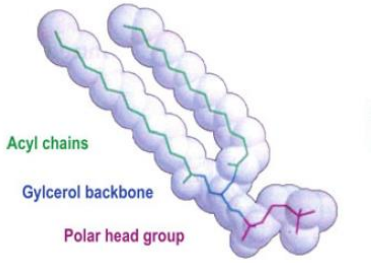

Figure I.20 – General structure of a phosphatidylcholine [Xu and Burgess 2012].

When dispersed in aqueous solution, phospholipids have a strong tendency to spontaneously form spherical, self-closed structures composed of one or more concentric curved lipid bilayers (called lamella), separated from each other by aqueous spaces (*figure I.21*). Indeed, due to the amphiphilic nature of phospholipids, their polar heads establish contacts with the outer aqueous environment, while the apolar chains tend to interact each other, thus favouring the formation of inner lipid bilayers which act as permeability barriers, both inward and outward. If drugs or other molecules are present during the vesicle formation, they can be entrapped inside the inner liposomal compartments.

The formation of lipid bilayers is mainly driven by hydrophobic interactions and van der Waals forces that keep the long hydrocarbon tails together, thus strengthening this architecture. Lastly, hydrogen bonds and polar interactions between the water molecules of the aqueous environment and the polar heads of lipids stabilize this organization [Bozzuto and Molinari 2015].

However, the final organization of lipids depends on their nature, concentration, temperature, and geometric form [Frolov 2013].

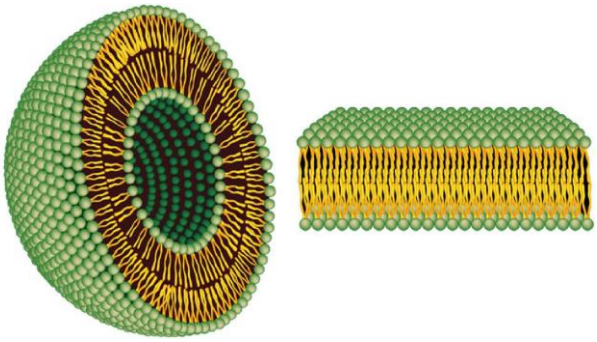

Figure I.21 – Schematic representation of liposome (left) and lipid bilayer (right) [Bozzuto and Molinari 2015].

Liposomal size can vary from 20 nm to several dozen micrometers, while the thickness of the bilayer is about 4-5 nm. They can be classified on the basis of their size (small, intermediate or large) and lamellarity (uni-, oligo- and multilamellar vesicles).

Multilamellar vesicles (MLVs) are made of several concentric lipid bilayers and have diameters of 1-5 μm. When small vesicles are trapped within the large ones the resulting structure is known as multivesicular vesicles (MVVs). Finally, when only one lipid bilayer is present, liposomes are named unilamellar vesicles (ULVs). Depending on their particle size, ULVs can be further divided into small unilamellar vesicles (SUVs), with a diameter of 20-100 nm, and large unilamellar vesicles (LUVs), with a diameter of 100-1000 nm. Liposomes above 1000 nm can also be formed and are called giant vesicles (GV) [Xu and Burgess 2012].

I.3.3.2 Liposome preparation

For the formulation of liposomes, a variety of lipids can be used, including phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols and sphyngomyelines. Lipids employed can be cationic, anionic or zwitterionic, as well as modified phospholipids. An example are long-circulating stealth liposomes capped with polyethylene glycol, which reduces mononuclear phagocyte system uptake [Cattel 2004]. Furthermore, the stability of liposomes may be improved by adding cholesterol in the bilayer or replacing unsaturated phospholipids with the high-melting distearolylphosphatidylcholine or with sphyngomyelines [Sarnad 2007].

The formation of ULVs or MLVs basically depends on the preparative method adopted and whether reduction size approaches are applied.

Thin film hydration method – It is one of the most widely utilized method for liposome preparation. According to this procedure, lipids are dissolved in an organic solvent, typically chloroform or methanol, which is further evaporated in order to obtain a lipid

film deposited on the walls of a round bottom flask. The addition of aqueous medium – always performed at temperatures above the phase-transition temperature of lipids – leads to the swell of the dried film and spontaneous formation of liposomes. The drug to be entrapped can be added to the aqueous phase, for hydrophilic molecules, or to the organic phase, for lipophilic compounds. Liposomes obtained with this technique are predominantly large MLVs of very heterogenous size distribution [Xu and Burgess 2012; Bozzuto and Molinari 2015].

Organic solvent injection method – It implies the injection of a lipid solution – usually prepared in ethanol or ether – into the aqueous phase under vigorous agitation, which causes the precipitation of ULVs. Subsequently, the solvent must be removed by dialysis [Xu and Burgess 2012; Bozzuto and Molinari 2015].

Reverse-phase evaporation method – Similar to the previous method, lipids are introduced into an aqueous solution from an organic solvent. In this case, the two-phase resulting system is sonicated in order to form a temporary stable water-in-oil emulsion, which is then stabilized by the accumulation of amphiphilic lipids at the organic solvent/water interface. Liposomes formation arises when the organic solvent is carefully evaporated, giving rise first to a viscous gel and subsequently to an aqueous suspension. Liposomes obtained by this method are predominantly poly-dispersed LUVs or MLVs [Xu and Burgess 2012; Bozzuto and Molinari 2015].

Detergent depletion method – This technique involves the hydration of a lipid film with a detergent, forming mixed micelles. The detergent is then removed leading to the disruption of mixed micelles and the precipitation of phospholipids in the form of polydispersed ULVs [Garidel 2006].

Considering that most of the aforementioned techniques typically generate heterogenous polydispersed vesicles, either ULVs or MLVs, post-formation treatments are required to obtain vesicles with the desired size, lamellarity and homogeneity. These methods include sonication, extrusion and high-pressure homogenization. Sonication is carried out by applying ultrasonic waves (through a probe or bath sonicator) to liposome suspension, which cause the rupture and rearrangement of lipid bilayers and ultimately the formation of small ULVs. The membrane-extrusion method is based on forcing MLVs through a membrane filter with a defined pore size [Xu and Burgess 2012; Bozzuto and Molinari 2015]. The high-pressure homogenization is instead a fluid mechanical process that involves the subdivision of vesicles into smaller size and occurs in special homogenizing equipment at extremely high pressures [Barnadas-Rodríguez and Sabés 2001].

I.3.3.3 Liposomes as drug carrier

The advantages of liposomes as drug carriers rely on their optimal biological and physicochemical properties. From a physicochemical point of view, liposomes are kinetically stabilized rather than thermodynamically stabilized systems. This is an advantage because, as opposed to systems in thermodynamic equilibrium (i.e. micelles and microemulsions), kinetically stabilized systems are not affected by rapid changes that occur in the surrounding environment [Lasic 1998; Xu and Burgess 2012].

Since liposomes are usually composed of natural phospholipids and possess a structure similar to that of cellular membranes, they result biologically inert, weakly immunogenic and safe to human administration [Immordino 2006]. Moreover, liposomes are suitable carriers to entrap drugs with different lipophilicity. In particular, strongly lipophilic drugs are located almost completely in the lipid bilayer and are more easily incorporated in MLVs *in virtue* of the high lipid content of these vesicles. On the contrary, very hydrophilic drugs are entrapped exclusively in the aqueous compartments, and in this case ULVs are preferred as carrier because of the presence of a large aqueous core that can accommodate water-soluble molecules. Finally, drugs with intermediate logP usually distribute between the lipid bilayers and aqueous moieties [Gulati 1998].

Molecules are entrapped in liposomes by passive loading, following their equilibrium with the external medium. As consequence, lipophilic compounds can be loaded with far higher efficiency than hydrophilic ones, since their affinity for the lipid membrane is much higher than that of the external aqueous phase. In particular, the entrapment efficiency results very high for hydrophobic drugs with logP above 3 [Xu and Burgess 2012]. Contrariwise, entrapment efficiency of hydrophilic compounds is usually lower because the drug concentrations outside and inside the vesicles are the same, but the environmental volume is greater than that of the liposome core [Frieze 2006].

Liposomes are reported to possibly protect encapsulated compounds against naturally occurring environmental stimuli, such as enzymatic and chemical degradations, and immunologic inactivation [Wang 2016]. Thus, liposomes prevent the active agent from being metabolized before reaching target sites, and simultaneously they minimize the exposure of healthy tissues to encapsulated drug during its circulation. These effects allow to achieve a double objective, namely to increase the therapeutic index and reduce undesirable adverse effects and thereby the toxicity of delivered drugs [Bozzuto and Molinari 2015]. Moreover, they provide protection also from light, extreme temperatures and pH fluctuations; this aspect is important especially for molecules which are very

sensitive to external conditions, such as polyphenols [Xia 2012].

Liposomes also permit to enhance solubility of very lipophilic molecules, thus improving their bioavailability. However, it should be pointed out that drug loaded inside liposomes become available only when they are released. Different approaches can be pursued in order to obtain a controlled release of the drug from liposomes. For instance, the incorporation of cholesterol or sphingomyelin into liposomes causes a switch from a fluid-phase phospholipid bilayer to a solid-phase bilayer, which in turn increases the retaining of cargo inside vesicles [Bozzuto and Molinari 2015]. In this regard, liposomes made of phosphatidylcholine and cholesterol have been reported to promote sustained release of clotrimazole for vaginal therapy [Ning 2005].

Several clinical studies have demonstrated that liposomal delivery of drugs may lead to a chance in toxicity profile. For examples, liposomal amphotericin B shows better tolerance and higher efficiency than the free antibiotic amphotericin B deoxycholate [Moen 2009]. Moreover, when used in clinical settings, liposomal treatments are proved to reduce some of the side effects associated with unloaded chemotherapist drug, such as cardiotoxicity, nausea and vomiting [Zamboni 2008].

Regarding vaginal delivery, liposomes are particularly appropriate as nanocarriers because they have been reported to not interfere with healthy vaginal microbiota and pH and to minimize irritation to the vaginal mucosa. Finally, as stated above, they effectively protect drugs from sudden changes in the vaginal microenvironment (i.e. variations in the vaginal fluid due to arousal and release of semen) [Vanić and Škalko-Basnet 2013; Brako 2017].

I.3.3.4 Surfactant lipid vesicles

The structure of conventional liposomes can be modified by incorporating surfactants together with lipid component (*figure I.22*) [Bnyan 2018]. For example, niosomes were developed as an alternative delivery system to liposomes and are characterized by a structure containing non-ionic surfactants in combination with cholesterol [Chen 2019]. Niosomes share with liposomes several general features and can be prepared by using the same methods describe in section I.3.3.2. However, compared to conventional liposomes, niosomes are reported to have better permeability and stability properties [Geusens 2011].

Transferosomes are another example of a next liposome generation containing surfactants, and are defined as ultra-deformable and elastic vesicles, able to easily squeeze themselves through skin pores and biological barriers [Maghraby 2000].

The presence of surfactants in the composition of lipidic vesicles has been stated as the reason for the improvement in several properties, such as entrapment efficiency, stability and permeability [Bnyan 2018]. In particular, surfactant lipid vesicles have been widely studied for the transdermal delivery to enhance the penetration rate through the stratum corneum, which represents the first barrier for drug absorption across the skin [Kassem 2017; Singh 2017]. Moreover, these vesicles have been shown to favour the transmucosal delivery of various compounds through the oral, nasal and vaginal mucosa. For instance, cationic niosomes containing the surfactant Span 80 were successfully developed for the transmucosal vaginal delivery of metformin for the treatment of polycystic ovary syndrome [Saini 2016].

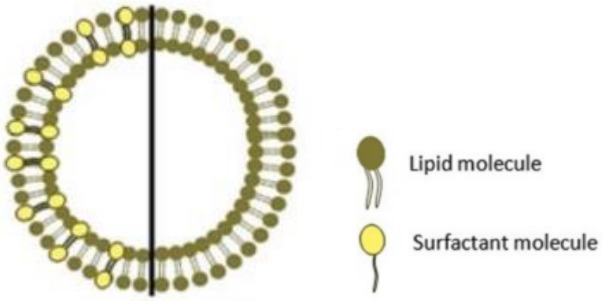

Figure I.22 – Comparison between surfactant vesicles (left side) and conventional liposomes (right side) [Bnyan 2018].

In our work (section V) a biosurfactant (microbial-derived surfactant) was characterized and studied as alternative to synthetic surfactants (chemically-synthetized surfactant) for the formulation of lipidic vesicles (referred to as mixed vesicles).

I.3.3.5 Synthetic surfactants

Surfactants, also known as surface-active agents, are amphiphilic molecules composed of two principal moieties: the polar hydrophilic head linked to the non-polar lipophilic tail. The lipophilic part is usually a straight or branched hydrocarbon chain, which consists of 8-18 carbon atoms [Bnyan 2018]. When present at low concentrations in aqueous medium, surfactants exist as monomer and have the tendency to adsorb on the interfacial surfaces (solution-air interface); consequently, they displace some surface molecules and reduce the intermolecular forces, thereby lowering the surface tension [Summerton 2017]. For each surfactant a CMC (Critical Micelle Concentration) value can be identified and defined as the concentration above which the molecule exists in the form of micelle aggregations. The formation of micelles is owing to the hydrophobic effect that, in aqueous medium, leads the hydrophilic heads face the surrounding water and the

hydrophobic tails orientate towards the inner part of micelle [Maibaum 2004].

Surfactants are usually classified in cationic, anionic, amphoteric and non-ionic, according to the charges on their hydrophilic head groups [Chen 2019].

Non-ionic surfactants are the most employed for lipid vesicles formulation due to their low toxicity and biocompatibility. Non-ionic surfactants comprise wide range of classes, including alcohol ethoxylate, sorbitan esters ethoxylate and fatty acids ethoxylates. In addition, there are multihydroxy products like glycol esters, glycerol esters, glucosides and sucrose esters [Bnyan 2018]. Among these surfactants, sorbitan fatty acid esters (Span) and polyoxyethylene sorbitan fatty acid esters (Tween) are the most commonly employed because are non-toxic and non-irritating.

I.3.3.6 Biosurfactants

It is currently accepted that the widespread use of synthetic surfactants in many area – including pharmaceutical and medical manufacturing, the food and feed industry, agriculture, environmental remediation and petroleum industry – have a negative impact on the environment [Naughton 2019]. In the recent years, biosurfactants have been extensively studied as sustainable alternative to synthetic surfactants.

Biosurfactants are surface-active biomolecules produced as secondary metabolites by a variety of microorganisms, namely bacteria, yeast and microscopic filamentous fungi, and can exist either in extracellularly secreted form or associated with the cell membrane [Hajfarajollah 2018; Paraszkiewicz 2019].

Similarly to other surfactants, biosurfactants are amphiphilic biochemical compounds containing both a hydrophilic moiety (usually a carbohydrate, amino acid, linear or cyclic peptide, phosphate, carboxylic acid or an alcohol) and a hydrophobic group, which may be a long fatty acid, hydroxyl fatty acid or α-alkyl β-hydroxy fatty acid.

Biosurfactants are complex molecules, usually anionic or neutral, comprising different structures. According to their molecular weight, they can be classified into two groups: (i) low-molecular weight surface active agents named biosurfactants, which efficiently lower the surface and interfacial tension; and (ii) high-molecular weight compounds, also called bioemulsifier, more effective as emulsion-stabilizing agents [Paraszkiewicz 2019]. The first group mainly includes glycolipids, lipopeptides and phospholipids, whereas the second one comprises lipoproteins, lipopolysaccharides, polymeric and particulate biosurfactants [Rivera 2019]. Among biosurfactants, the most investigated are rhamnolipids (a glycolipid) and surfactin (a lipopeptide), produced by *Pseudomonas*

aeruginosa and *Bacillus subtilis*, respectively [Anvari 2016].

Biosurfactants offer numerous advantages over their synthetic counterparts, such as high biodegradability, low ecotoxicity, high selectivity, high foaming capacity and high activity at extreme conditions of temperature, pH and salinity [Hajfarajollah 2018]. The high biocompatibility and digestibility make them good candidates also for applications in cosmetics, pharmaceuticals and as functional food additives. Furthermore, they can be easily obtained from relatively cheap raw materials which are available in large amounts and are renewal energy resources [Makkar and Cameotra 2002; Paraszkiewicz 2019].

Despite the favourable properties of biosurfactants, the major concern about their use for human applications is due to the fact that the principal microbial surfactant producers are pathogenic microorganisms, specifically *Pseudomonas* spp., *Acinetobacter* spp., *Bacillus* spp. and *Arthrobacter* spp. [Sharafi 2015]. For this reason, the use of probiotic biosurfactants, mainly produced by LAB microorganisms (i.e. *Lactobacillus rhamnosus*, *L. casei*, *L. acidophilus* and *L. fermentum*), offers new dimensions to overcome health issues, as probiotics constitute healthy indigenous microflora and therefore are safe to use (see section I.2.1.1) [Fariq 2016].

Biosurfactants have wide possible applications in many fields, such as oil, cosmetic, pharmaceutical and agricultural industries. Indeed, they display industrially valuable properties of detergency, emulsification and foaming [Naughton 2019].

Biosurfactants, in particular surfactin and rhamnolipid, have been employed in a new technique called MEOR (Microbial Enhanced Oil Recovery), developed to recover residual oil by using microorganisms or microbial surfactant [Thavasi 2011]. Biosurfactants properties can also be exploited for bioremediation, to solve the problems involved in ocean oil spills and enhance the biodegradation of polycyclic aromatic hydrocarbon compounds [Makkar and Rockne 2003]. Furthermore, they can be used in agriculture applications to improve plant growth by removing phytopathogens [Sachdev and Cameotra 2013], and in food industry as emulsifier in foods containing fats and oils (i.e. for stabilization of mayonnaise) [Ranasalva 2014].

Probiotic biosurfactants are also emerging biotherapeutics and have been investigated in terms of antimicrobial and anti-adhesive properties.

Finally, microbial surfactants, *in virtue* of their versatile self-assembling and biochemical properties, have been suggested as valuable molecules for the design of drug formulations, in particular for the development of stable nanoparticles, microemulsions and liposomes [Rodrigues 2015].

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Experimental part

II. Overview of the project

II.1 Aim of the thesis

My PhD project was focused on the development of innovative delivery systems for the treatment of vaginal infections and dysbiosis characterized by alteration of local microbiota.

The homeostasis of vaginal ecosystem depends on complex interactions and synergies between the host and diverse microorganisms that inhabit the vaginal mucosa. The vaginal microbiota of healthy woman is commonly dominated by the genus *Lactobacillus* (*L. crispatus, L. gasseri, L. vaginalis, L. jensenii* and *L. iners*) but other microbial species, such as *Bifidobacterium* spp. (*B. breve, B. longum* and *B. bifidum*), may also be present at lower concentration and can contribute to the maintenance of vaginal health. These bacteria form a critical line of defense, preventing the overgrowth of potential pathogens thorough different mechanisms, i.e. the preservation of an acidic environment, the modulation of the immune system, the competition for nutritional substances and adhesion sites on the vaginal epithelium, the production of bacteriostatic and bactericidal compounds, signaling molecules and biosurfactants [Ravel 2011; Freitas and Hill 2017]. Unfortunately, under certain circumstances this ecological balance may be disrupted and turn into a pathological state.

Vaginal infections, especially bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC), are highly prevalent; indeed, it is estimated that more than 70% of adult women have suffered from vaginal problems that have required therapeutic intervention [Palmeira-de-Oliveira 2015]. These infections not only compromise the quality of life of patients, but can also expose to complications, including a higher susceptibility to other pathogens, such as HIV, *Herpes simplex* virus, *Neisseria gonorrohoeae* and *Chlamydia trachomatis*, and an increased risk of preterm birth and late termination of pregnancy [Van Der Pol 2010]. However, the high incidence of recurrent vaginal infections and the poor patient compliance, together with the growing emergence of drug resistance, underline the inadequacy of currently available therapeutic strategies and the need for the development of more effective approaches.

In this contest, both formulations based on well-established drugs (such as chlorhexidine and econazole nitrate) delivered in innovative ways or formulations containing alternative active ingredients able to counteract vaginal infections (such as probiotics cells and

polyphenols) have been taken into account.

Moreover, in order to improve the vaginal health *status*, local as well as oral administration routes have been investigated.

In particular, the project has been divided into two main themes:

(i) Oral administration of living probiotics (*paper 1*).

(ii) Vaginal delivery of antimicrobial drugs, even in association with metabolites produced by vaginal lactobacilli, or natural substances that can provide a benefit in case of vaginal alterations (*paper 2-4*).

II.2 List of the papers

The thesis is based on the following papers:

- *Paper I*: "Vaginal *Bifidobacterium breve* for preventing urogenital infections: development of delayed release mucoadhesive oral tablets" [published: doi:10.1016/j.ijpharm.2018.09.003].
- *Paper 2*: "Freeze-dried matrices based on polyanion polymers for chlorhexidine local release in the buccal and vaginal cavities" [published: doi:10.1016/j.xphs.2019.02.026].
- *Paper 3*: "Novel mixed vesicles containing lactobacilli biosurfactant for vaginal delivery of an anti-*Candida* agent" [published: doi:10.1016/j.ejps.2017.11.012].
- *Paper 4*: "Utilizing liposomal quercetin and gallic acid in localized treatment of vaginal *Candida* infections" [published: doi:10.3390/pharmaceutics12010009].

In the next section brief descriptions of each paper are provided.

II.3 Summary of the papers

In the first proposed work (***paper 1*, section III**), a strain belonging to *Bifidobacterium breve* species was selected as probiotic bacterium and formulated inside orally tablets, with the aim to provide beneficial effects both at intestinal and vaginal level. According to FAO/WHO definition, probiotics are "living microorganisms which, when ingested in adequate amounts, confer a positive effect on the host" [FAO/WHO 2006].

B. breve BC204 was first isolated from the vagina of a healthy woman, taxonomically characterized and indagated for its health-promoting potential. In particular, the capability of *B. breve* BC204 to exert antimicrobial activities was tested against different pathogenic strains responsible for urogenital (*Candida* spp., *C. trachomatis*, *E. coli*, *E. faecium*, *E. faecalis*, *E. hirae*, *S. aureus* and *P. mirabilis*) and gastrointestinal (enterotoxigenic *E. coli*, *S. typhimurium* and *Y. enterocolitica*) diseases. To provide functional benefits to the gut and urogenital tracts, oral probiotics must survive passage through the acidic stomach and colonize colon [Sarao 2017]. Thus, tolerance to low pH and bile salts, as well as adhesion to Caco-2 and HeLa cells (chosen as model of gut and vaginal epithelium, respectively), were investigated.

Since *B. breve* BC204 successfully satisfied the probiotic requisites, the second part of the study was focused on the development of an oral enteric and mucoadhesive formulation which could favour cell survival during storage and after administration and to promote intestinal colonization. This goal was achieved by applying a two-step procedure consisting of microencapsulation followed by tablet production.

In a first step *B. breve* BC204 was suspended in a solution of skimmed milk, used as microencapsulating and protective agent, and subjected to spray-drying in order to obtain microparticles able to preserve bacterial viability [Fritzen-Freire 2011]. Subsequently, spray-dried microparticles were melted together with beeswax and spread inside the dies of the Optima Tablet device (*figure II.1*). Beeswax was exploited as natural excipient able to guarantee a time-dependent degradation behavior, useful to prevent the undesired release of cells in the stomach [Del Curto 2014]. Moreover, carboxymethylcellulose was added at different concentrations to the melted mixture in order to modulate the characteristics of the final formulations.

This procedure allowed the production of tablets in a simple and reliable way, avoiding at the same time the application of compressing forces which could impair probiotic viability. The homogeneity of the final dosage forms was evaluated in terms of size,

weight and probiotic loading. Moreover, tablets were tested for their ability to adhere to intestinal mucosa, to hydrate in gut environment and to assure the proper release of *B. breve* BC204 during the gastrointestinal transit. Finally, tablets were stored at 4°C and the survival of formulated probiotic was monitored for up to 12 months.

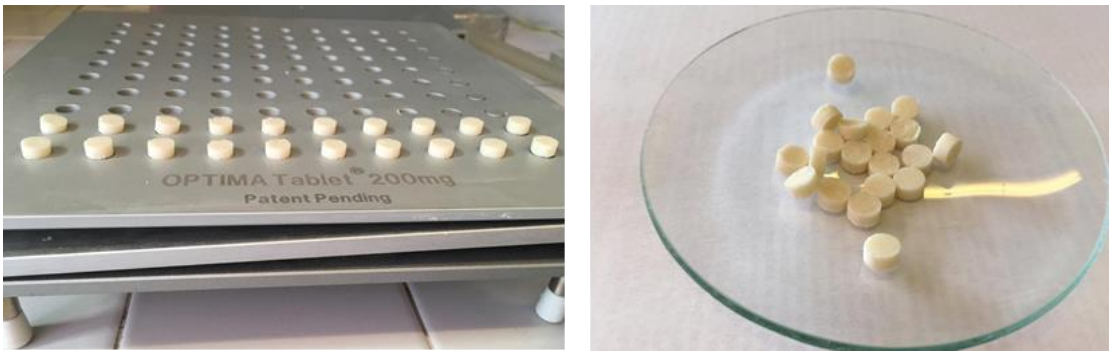

Figure II.1 – Beeswax-based tablets for the delivery of *B. breve* BC204, obtained by using Optima Tablet device.

In the second work (***paper 2*, section IV**) the freeze-drying technique was applied to produce lyophilized polymeric matrices containing chlorhexidine (CLX), a well-known cationic disinfectant commonly employed to prevent and treat infections of both vaginal tract (including BV and VVC) and oral cavity [Milstone 2008]. The aim of this study was to overcame some drawbacks of commercially available dosage forms for CLX delivery (i.e. vaginal ovules and mouthwashes), like the short-term permanence at the application site and the immediate release of drug, that require multiple daily administration [Pereira and Bruschi 2012]. In this regard, mucoadhesive polymers were selected as excipients and freeze-drying was used to obtain highly porous matrices that rapidly hydrate *in situ* after application, leading to gel formation [Cavallari 2015]. On the one hand, this behaviour favours the polymer's relaxation, which in turn allows the interpenetration of polymeric chains into the mucus layer and thus an intimate contact of the formulation with the mucosal surface. On the other hand, drug diffusion through gelled polymeric network ensures the sustained release of drug, thus reducing the application frequency [Akiyode and Boateng 2018]. For the first time in literature, the same dosage form containing CLX was investigated for the possible application not only in the vaginal environment but also in the buccal one.

All polymers employed in this study – sodium alginate, carboxymethylcellulose, xanthan gum and sodium hyaluronate – were of natural origin, biocompatible, biodegradable and presented several negatively charged functional groups able to interact with positive

charges of CLX. The drug was first complexed with polymers to slow down its release. In particular, the ionic interactions between CLX and polymeric chains provided the precipitation of solid complexes, while free (non-complexed) polymers and drug remained in solution. Besides the employment of diverse polymers, two different preparative methods were adopted to control functional properties of matrices. In the method "b" the CLX-polyanion complexes were isolated, placed inside cavities of a blister pack and freeze-dried (*figure II.2*). In the method "a" the complexes were not isolated and the final freeze-dried matrices contained both the complex and free polyanion and drug. All obtained matrices were characterized in terms of drug content, solid state, morphology of inner porous network and resistance to compression stress. Moreover, phosphate buffers at pH 4.5 and 6.8 were used for hydration ability and release studies while vaginal and esophageal mucosa were employed for mucoadhesion studies in order to simulate the vaginal and buccal environments, respectively. Finally, the antimicrobial activity was assessed against common pathogens responsible for vaginal (i.e. *C. albicans* and *E. coli*) and buccal (i.e. *S. mutans*) infections.

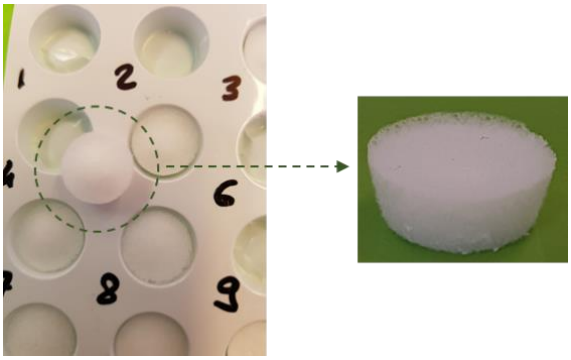

Figure II.2 – Appearance of freeze-dried matrices based on sodium alginate (produced with method "a") loaded with chlorhexidine.

The last two papers focused on the development of nanosystems based on lipidic vesicles for treatment of VVC. In particular, two different approaches were followed: (i) the delivery of econazole nitrate (EN) inside vesicles containing a natural surfactant as excipient and (ii) the delivery of two phytochemicals as alternative to conventional antifungal drugs. Nanocarriers were selected as delivery systems because they allow to solve some limitations of conventional formulations (gels, creams, capsules, foams, vaginal ovules), such as reduced efficacy, poor retention, mostly due to the self-cleaning action of the vagina, and poor ability to modulate the fate and the release behavior of active compounds after application in the vaginal mucosa [Leyva-Gómez 2018].

In the third work (***paper 3*, section V**) we prepared and characterized an innovative formulation for the vaginal delivery of EN, a broad-spectrum lipophilic azole drug commonly prescribed for the treatment of candidiasis [Calvo 2019] and characterized by poor water solubility that limits its bioavailability [Abd El-Gawad 2017]. In this regard, EN incorporation in lipidic vesicles may improve and extend its availability at the application site, thus reducing the necessary daily-dose and consequently increasing patient compliance.

Classical phospholipid vesicles are often enriched with chemical surfactants, thus modifying their elasticity and fluidity, with consequent improvement of their performance in terms of drug delivery. In the recent years, the growing awareness towards the use of "green products" has stimulated the research about no-chemical surfactants, such as biosurfactants (BS). BS are amphiphilic compounds produced by diverse microorganisms on their cell surface or secreted extracellularly and show several advantages, such as low toxicity and high biodegradability [Jimoh 2019].

In the present work we proposed a novel BS produced by *Lactobacillus gasseri* BC9 – a probiotic strain previously isolated from the vagina of a healthy premenopausal women as well as *B. breve* BC204 (see *paper 1*) [Parolin 2015] – as alternative to Tween 80, a commonly employed non-ionic surfactant.

In the first part of the study BS was extracted from *L. gasseri* BC9 culture, lyophilized and indagated for its chemical structure, by means of Fourier-transformed infrared spectroscopy and mass spectrometry, and its technological properties, including determination of critical micelle concentration and emulsification capacity.

Since BS revealed a peptide-like structure and excellent properties as surfactant, in the second part of the study it was used to prepare phosphatidylcholine based mixed vesicles, loaded or unloaded with EN. Vesicles containing Tween 80 were also formulated as comparison (*figure II.3*).

All formulations were obtained by using the lipid film rehydration method followed by extrusion. Mixed vesicles were characterized in terms of size, zeta potential, encapsulation efficiency and stability in phosphate buffer at pH 4.5. The interaction with mucin, the major component of mucus that covers vaginal epithelium, was also investigated. Moreover, the loaded mixed vesicles prepared with BS were compared with those prepared with Tween 80 for their ability to control the release of drug.

The antifungal effect of proposed mixed vesicles was then assessed against planktonic culture of *Candida albicans*, the most frequent etiological agent of VVC.

Finally, since the tendency of *Candida* to rapidly form biofilm is strongly associated with recurrent infections and development of azole resistance, we sought for capability of mixed vesicles to improve biological activity of EN in eradicating preformed *C. albicans* biofilms.

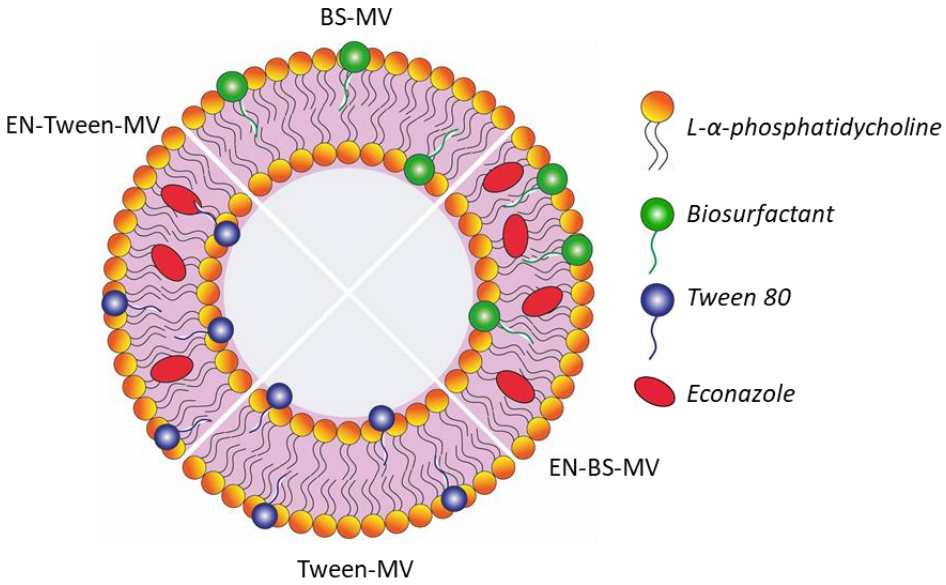

Figure II.3 – Mixed vesicles (MV) containing biosurfactant or Tween 80, unloaded or loaded with econazole.

In the fourth work (***paper 4*, section VI**) we moved the attention to natural compounds as potential active molecules to counteract VVC.

VVC therapy is mainly based on azole drugs, such as fluconoazole and econazole (see *paper 3*). Although in a large part of patients most of conventional therapeutic approaches are effective in reducing symptoms of an initial *Candida* infection in a large part of patients, cells repetitively exposed to azole drugs may adapt to antifungal pressure and became resistant [Ksiezopolska 2019]. For this reason, new strategies to counteract this gynecological infection are highly required.

Polyphenolic compounds are phytochemicals able to exert several positive effects in human body, like anti-oxidant, anti-inflammatory, anti-diabetic, cardioprotective and antimicrobial activities. Moreover, they have gained an increasing interest in the last few years as potential anti-*Candida* candidates. For the first time, in the present study quercetin (Q) and gallic acid (GA) have been taken into account for the local delivery to vaginal mucosa. Candidiasis is often associated with strong anti-inflammatory response and increased levels of free radicals that can exacerbate tissue damage and symptoms, including irritation, burning and pain at vulvar and vaginal level. In this regard, this work

aimed to develop new liposomes for the simultaneous delivery of Q and GA, that could act in combination when released in the vaginal cavity to eradicate the infection and alleviate symptoms of VVC at the same time. In particular, Q was chosen because of its anti-itching properties and because is one of the most potent anti-oxidant *in vitro* [Maramaldi 2016], while GA was selected *in virtue* of its reported activity towards *Candida* spp., especially *C. albicans* [Teodoro 2015]. For our purpose liposomes are particularly suitable as nanocarriers because they can carry both lipophilic (such as Q) and hydrophilic (such as GA) molecules in the lipid bilayer and in the aqueous core, respectively. They are also biodegradable, non-irritating to vaginal mucosa and provide good protection against rapid perturbations that naturally occur in the vaginal environment [Voltan 2016].

Liposomes containing only Q, only GA or the combination of the two polyphenols were prepared by lipid film rehydration method followed by sonication (*figure II.4*). For the technological characterization size, zeta potential, entrapment efficiency and storage stability of vesicles were measured. The interaction with mucin and the capability of liposomal formulations to guarantee sustained release of both polyphenols were also verified. Furthermore, liposomes were biologically characterized in terms of anti-oxidant and anti-inflammatory activities and a possible synergistic effect between Q and GA was indagated. Finally, we tested the ability of proposed liposomal formulations to inhibit *Candida* growth.

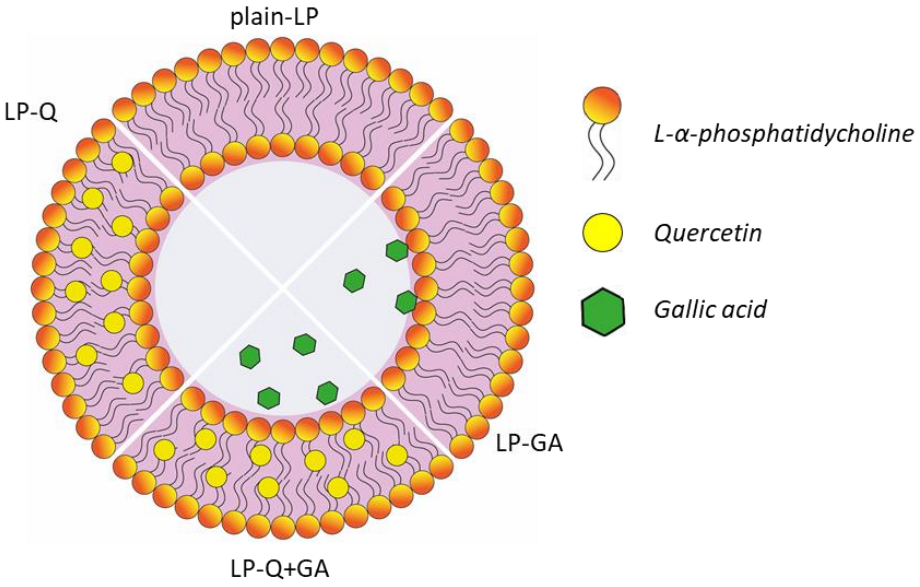

Figure II.4 – Liposomes (LP) carrying only quercetin (LP-Q), only gallic acid (LP-GA) or both polyphenols (LP-Q+GA).

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III. Vaginal *Bifidobacterium breve* for preventing urogenital infections: development of delayed release mucoadhesive oral tablets (*paper 1*)

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Published on International Journal of Pharmaceutics (doi:10.1016/j.ijpharm.2018.09.003) [Giordani 2018].

III.1 Abstract

Bifidobacteria are predominant microorganisms in the intestinal flora, but at the same time represent a subdominant group of the vaginal microbiota. For this reason, oral administration of these probiotic bacteria can provide beneficial effects for both intestinal and urogenital ecosystems.

The first aim of this study was to test the strain *Bifidobacterium breve* BC204, isolated from a vaginal swab of a healthy woman, for its capability to adhere to human cells, to survive to gastric acids and bile salts and to exert antimicrobial activities. The second aim of the work was to develop an oral formulation able to guarantee bacterial survival during storage and administration, thus favouring intestinal and vaginal colonization. *B. breve* BC204 was encapsulated by spray-drying and subsequently formulated in time-dependent erodible tablets.

B. breve BC204 showed good ability to adhere to Caco-2 cells and moderate ability to resist to gastrointestinal stress. Moreover, it exerted a strong antimicrobial activity against urogenital and enteric pathogens. Microencapsulation followed by tablet production allowed high loading and survival of *B. breve* BC204, associated to a delayed release and mucoadhesive ability. These characteristics are required to achieve appropriate amount and persistence of viable microbial cells in the treatment site.

Keywords

Bifidobacterium breve BC204; urogenital infections; microencapsulation; delayed release; mucoadhesion.

List of abbreviations

AA: ascorbic acid; BW: beeswax; CMC: sodium carboxymethylcellulose; CFS: cell free supernatant; CP: cell pellet; CT: *C. trachomatis*; FOS: fructooligosaccharides; IN: inuline; SDM: spray-dried microparticles; SM: skimmed milk; VitE: vitamin E.

III.2 Introduction

Probiotics represent a healthy support to antibiotics for the management of urogenital infections as they can interfere with various pathogens without the risk of appearance of adverse effects and drug resistance [Borges 2014; Gupta 2017; Vitali 2017]. The vaginal microbiota of healthy, reproductive aged women is generally dominated by strains of *Lactobacillus*. However, *Bifidobacterium* strains can also be found in human vagina, contributing to contrast local infections, due to their ability of producing lactic acid and hydrogen peroxide [Schellenberg 2012; Freitas and Hill 2017]. In this work, the strain *Bifidobacterium breve* BC204 was isolated from a vaginal swab of a healthy woman, characterized for its antimicrobial activity and formulated in an oral dosage form, with the aim to provide beneficial effect towards urogenital environment. In fact, considering the evidence that the oral intake of probiotics permits the colonization of the vaginal mucosa [Reid 2001; Mezzasalma 2017], an appropriate oral formulation containing adequate dose of probiotic bacteria and suitable excipients can be a convenient treatment choice. An effective oral dosage form for probiotic delivery should be developed taking into account that a minimum dose of 10^8-10^9 viable cells per day is strongly recommended [FAO/WHO 2006] and that probiotic bacteria should be able to survive the passage through the acidic stomach to colonize the intestinal and the vaginal mucosa.

Thus, *B. breve* BC204 was firstly encapsulated by applying two distinct techniques (spray-drying or freeze-drying) in the presence of favourable excipients such as skimmed milk, inulin, fructooligosaccharides and ascorbic acid and subsequently formulated in tablets containing a time-dependent excipient, a mucoadhesive polymer and an antioxidant agent, such as beeswax, sodium carboxymethylcellulose, and vitamin E acetate, respectively. Survival of probiotics during drying process can be assured by controlling the process conditions and selecting suitable encapsulating agents [Broeckx 2016]. Improvement of cell survival can be greatly achieved with the use of skimmed milk [Coghetto 2016]. However, Fritzen-Freire and coauthors provided the evidence that skimmed milk can be partially replaced by prebiotic agents such as fructooligosaccharides and inulin [Fritzen-Freire 2012]. In a previous study, we reported the addition of the antioxidant ascorbic acid in order to achieve a multiple goal: to improve the survival of beneficial bacterial cells during production and storage of the formulation, to stimulate probiotic growth and to protect epithelial cells from the oxidative stress at the treatment site [Vitali 2016].

Protection of the probiotic cells can be further achieved by the use of excipients that assure the bacterial release in the intestinal environment. Time-dependent erodible dosage forms have been successfully employed for site specific drug release [Del Curto 2014], including wax-based formulations [Maestrelli 2015]. The use of waxes to deliver probiotics has the advantage not only of delaying their release over the gastric tract, but also of providing barriers to oxygen, acid, and moisture that can lead to the reduction of viable cells.

After the transit through the stomach, the persistence of the probiotic cells in the intestine can favour the colonization of this tract, and consequently the colonization of the vaginal environment. Sodium carboxymethylcellulose is a safe excipient previously used for probiotic local delivery due to its ability to hydrate and adhere to mucosal epithelia as well as to modulate the release [Saha 2013]. In this study, microencapsulated *B. breve* BC204 was delivered in time-dependant erodible and mucoadhesive tablets containing beeswax and sodium carboxymethylcellulose. The final dosage form was easily produced by Optima Tablet device avoiding the use of a compressing machine and thus possible reduction of bacterial viability [Muller 2014].

III.3 Materials and methods

III.3.1 Materials

Fructooligosaccharides (FOS) and sodium carboxymethylcellulose (CMC; MW 250 kDa, substitution degree 0.78) were purchased from Farmalabor (Barletta-Andria-Trani, Italy); inulin (IN), vitamin E acetate (VitE) and beeswax (BW) from A.C.E.F. (Piacenza, Italy); ascorbic acid (AA) from Fluka (Milan, Italy); skimmed milk (SM), de Man, Rogosa and Sharpe medium (MRS) and Brain-Heart Infusion medium (BHI) from Difco (Michigan, USA).

Dulbecco's minimal essential medium (DMEM) and RPMI 1640 medium were supplied by EuroClone (Pero, Italy); Sabouraud Dextrose medium (SD) by Oxoid (Basingstoke, UK).

Tolerance of *B. breve* BC204, water-uptake, mucoadhesion and release studies were carried out in simulated gastric fluid (SGF: 125 mmol/L NaCl, 7 mmol/L KCl, 45 mmol/L $NaHCO_3$, 3 g/L pepsin, pH 2) and in simulated intestinal fluid (SIF: 0.1% w/v pancreatin, 0.15% w/v Oxgall bile salt, pH 7).

Candida strains and *Chlamydia trachomatis* strain GO/86 serotype D were isolated, respectively, from vaginal swabs and a urethral swab submitted to the Microbiology Laboratory of Sant'Orsola-Malpighi University Hospital of Bologna for routine diagnostic procedures and belong to the laboratory collection. HeLa cells ATCC® CCL-2™, Caco-2 cells ATCC® HTB-37™, *Escherichia coli* ATCC® 11105™, *Staphylococcus aureus* ATCC® 29213™ and *Proteus mirabilis* ATCC® 29906™ were purchased from the American Tissue and Cell Culture Corp. (Virginia, USA). *Enterococcus faecalis* BC101, *Enterococcus faecium* BC104, *Enterococcus faecium* BC105, *Enterococcus hirae*, *Salmonella choleraesuis* serovar *typhimurium* and *Yersinia enterocolitica* belong to Department of Pharmacy and Biotechnology of University of Bologna.

III.3.2 Isolation, cultivation and taxonomic characterization of *B. breve* BC204

Bifidobacterium breve BC204 was isolated from a pre-menopausal Caucasian woman following the procedure described by Parolin et al., in accordance with the Ethics Committee of the University of Bologna (52/2014/U/Tess) [Parolin 2015]. *B. breve* BC204 was cultured in MRS medium supplemented with 0.05% L-cysteine and incubated anaerobically at 37°C with the addition of Anaerocult C (Merck, Milan, Italy). To prepare *Bifidobacterium* fractions, 48-h culture (OD600 = 1, corresponding to 5×10^8 CFU/mL)

was centrifuged (5000 *g* for 10 min); supernatant was filtered through a 0.22 μm membrane filter to obtain cell free supernatants (CFS) while cell pellet (CP) was washed in sterile saline.

Genomic DNA was extracted from *B. breve* BC204 culture using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), the 16S-23S rRNA intergenic transcribed spacer (ITS) was amplified with the universal primers L and R [Brigidi 2000] and sequenced. The obtained sequence was searched with nucleotide BLAST web service (blast.ncbi.nlm.nih.gov) for the taxonomic identification. The sequence is available in DDBJ nucleotide sequence database under accession number LC381965.

III.3.3 Adhesion of *B. breve* BC204 to HeLa and Caco-2 cells

The capability of *B. breve* BC204 to adhere to HeLa cells and to Caco-2 cells was evaluated following the methods previously reported by Parolin et al. [Parolin 2015]. Briefly, HeLa and Caco-2 cells were grown on glass coverslip to 70% confluent monolayers in 5% CO_2 at 37°C in DMEM supplemented with 10% foetal bovine serum, 1% L-glutamine, 100 IU/mL penicillin G and 100 μg/mL streptomycin. Both HeLa and Caco-2 cells were washed twice with PBS and treated with 100 μL of *B. breve* BC204 suspension (5×10^8 CFU/mL) for 1 and 3 h, in order to evaluate its adhesiveness after different contact time to cells. The adhesion of *B. breve* BC204 was assessed by light-microscopy (1000×) after May-Grünwald Giemsa staining by counting the number of *Bifidobacterium* attached to 200 randomly chosen HeLa or Caco-2 cells.

III.3.4 Tolerance of *B. breve* BC204 to gastric acids and bile salts

The resistance of *B. breve* BC204 to gastric and intestinal environments was investigated according to Fernández et al. [Fernández 2003].

Briefly, *B. breve* CP (48-h bacterial culture, ca. 10^{10} CFU) was first suspended in 20 mL of SGF (pH 2). In order to evaluate the effect of the enzymatic component of the SGF, the final pH was also adjusted to 7. The suspension was incubated for 3 h at 37°C under anaerobic conditions and agitation to simulate peristalsis. Subsequently, cells were harvested by centrifugation (5000 *g* for 10 min), suspended in 20 mL of SIF (pH 7) and incubated as above for further 3 h.

Aliquots were taken every 1.5 h, serially diluted in sterile saline and seeded on MRS agar plates in order to evaluate cell viability over time.

III.3.5 Antimicrobial activity of *B. breve* BC204

Antimicrobial activities of *B. breve* BC204 were assessed both against bacteria and fungi responsible for urogenital disease and against gastrointestinal pathogens.

III.3.5.1 Anti-*Candida* activity

Vulvovaginal candidiasis is a common yeast infection compromising the quality of life of many women, mainly caused by *C. albicans* and to a lesser extent by other *Candida* species [Rathod and Buffler 2014]. The fungistatic activity of *B. breve* BC204 CFS was evaluated against 4 clinical isolates of *C. albicans* (named *C. albicans* 1-4), and against one clinical isolate of each of the species *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* and *C. lusitaniae*, according to the EUCAST guidelines [EUCAST 2014] and as previously described [Parolin 2015]. Briefly, *Candida* suspensions (OD530 = 0.5, corresponding to 1-5 × 10^5 CFU/mL) were prepared in RPMI 1640 medium from 24-h culture on SD agar plates. 100 µL of yeast suspensions were inoculated together with 100 µL of *B. breve* CFS (corresponding to 5 × 10^7 CFU) in each well of a flat-bottom microdilutions tray. A growth control well, containing 100 µL of sterile MRS medium and 100 µL of *Candida* suspension, was set up. The growth was observed after 24 and 48 h of incubation at 35°C and the results were read by measuring the absorbance at 450 nm with Multiskan FC Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, USA). Fungistatic activity was defined as a reduction of at least 50% of yeast growth compared with control. 20 µL of samples exhibiting less than 50% of growth were then spotted onto SD agar plates and incubated at 35°C for 24/48 h, in order to determine a fungicidal effect of CFS. Fungicidal activity was defined as a 3 $\log_{10}$ reduction from the starting inoculum.

III.3.5.2 Anti-*Chlamydia* activity

C. trachomatis (CT) is an obligate intracellular bacterium that causes the most prevalent bacterial sexually transmitted infection worldwide [Senior 2012].

The capability of *B. breve* BC204 fractions (CP and CSF) to reduce infectivity of *C. trachomatis* strain GO/86 serotype D in HeLa cells was studied by considering three different mechanisms of inhibition: competition, exclusion and displacement. In these assays, HeLa cells, grown on coverslips in individual tubes at a confluence of 95-100%, were infected with 5 × 10^3 CT elementary bodies (EBs) and treated with *B. breve* BC204 fractions corresponding to 5 × 10^7 cells, following different timelines. In the competition

assay, *Bifidobacterium* fractions and CT EBs were inoculated simultaneously onto HeLa cells and incubated for 1 h at 37°C with 5% CO_2. In the exclusion assay, HeLa cells were incubated with *Bifidobacterium* fractions for 1 h at 37°C with 5% CO_2; successively, CT EBs were added and further incubated for 1 h. In the displacement assay, CT EBs were inoculated onto HeLa cells for 1 h at 37°C with 5% CO_2; afterwards, *Bifidobacterium* fractions were added and further incubated for 1 h. At the end of the incubation, the medium was replaced with 1 mL of DMEM supplemented with 1 μg/mL cycloheximide, HeLa cells were centrifuged (640 *g* for 2 h) to facilitate cell penetration and then incubated 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, Ohio, USA), as previously reported [Nardini 2016]. Slides were observed under epi-fluorescence microscope (Eclipse E600, Nikon, Japan) equipped with a super high pressure mercury lamp and Plan Fluor DLL 20×, 40×, 100× lenses. The number of IFUs was counted in 30 randomly chosen 200× microscopic fields. Results were expressed as the percentage of CT infectivity inhibition, comparing the number of IFUs of the single experiments with the EBs infectivity of the control tubes without *Bifidobacterium* fractions.

III.3.5.3 Antibacterial activity against extracellular urogenital and gastrointestinal pathogens

The antimicrobial activity of *B. breve* BC204 was evaluated also against 11 bacteria, responsible for urogenital (*E. coli* ATCC11105, *E. coli* K12, *E. faecalis* BC101¸ *E. faecium* BC104, *E. faecium* BC105, *E. hirae, S. aureus* ATCC29213 and *P. mirabilis* ATCC29906) or gastrointestinal (*E. coli* ECET H10407, *S. choleraesuis* serovar *typhimurium* and *Y. enterocolitica*) disorders. The antibacterial activity of *B. breve* BC204 was tested by using agar spot test, as previously reported by Siroli et al. [Siroli 2017]. Briefly, 5 μL of 48-h *B. breve* BC204 culture (5×10^8 CFU/mL) were spotted over the surface of MRS agar plates and allowed to grow under anaerobic conditions at 37°C for 24 h. Afterwards, 0.1 mL of an overnight culture (10^8 CFU/mL) of each target microorganism was inoculated into 10 mL of BHI soft agar (containing 0.7% of agar) and poured on the plates were *B. breve* BC204 had grown. Plates were aerobically incubated at 37°C for 24 h. The antimicrobial activity of *B. breve* BC204 was evaluated by measuring the diameter of the zone of growth inhibition.

III.3.6 Microencapsulation of *B. breve* BC204

Given the probiotic activities of BC204, the second aim of the work was to develop an appropriate oral formulation. The final dosage form was obtained with a two-step procedure consisting of microencapsulation followed by tablet production.

III.3.6.1 Preparation of microparticles by spray-drying

B. breve BC204 was cultured in 300 mL of MRS broth supplemented with L-cysteine (0.05% w/v) at 37°C in anaerobic conditions for 48 h. Cells were harvested by centrifugation (5000 *g* for 10 min) and suspended in 100 mL of drying medium (ca. 5×10^{10} CFU/mL), containing SM (10% w/v), IN (5% w/v), FOS (5% w/v) and AA (2.5% w/v). The spray-drying process was performed with a Büchi Mini Spray Dryer (Model 191, Büchi Laboratoriums-Technik, Switzerland) at constant air inlet temperature of 120 ± 2°C and outlet temperature of 65 ± 3°C. The suspension was kept under magnetic stirring at room temperature and pumped with feed flow of 7.5 mL/min and with drying air flow rate of 35 m^3/h. Microparticles were collected and stored at 4°C.

III.3.6.2 Preparation of microparticles by freeze-drying

B. breve BC204 was microencapsulated also using another method, namely freeze-drying, with the aim to compare the effectiveness and feasibility of the two techniques. Cells were suspended in drying medium ad described in section III.3.6.1, frozen overnight at -20°C and freeze-dried at 0.01 atm and -45°C (Christ Freeze Dryer ALPHA 1-2, Milan, Italy). Freeze-dried powder was then stored at 4°C.

III.3.6.3 Size and morphology of microparticles

Particle size and morphology of the microparticles obtained with spray-drying and freeze-drying were observed by scanning electron microscopy (SEM). Microparticles were fixed on supports and coated with gold-palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. Samples were then observed with LEO 420 (LEO Electron Microscopy Ltd., Cambridge, UK) using secondary electron imaging at 15 kV.

III.3.6.4 Survival of *B. breve* BC204 after microencapsulation and during storage

The impact of spray-drying and freeze-drying process on cell viability was assessed immediately after particle production and during storage at 4°C for up to 12 months.

Microparticles (0.1 g) were suspended in MRS (1 mL), incubated at 37°C for 1 h, serially diluted in sterile saline and seeded on MRS plates. Plates were anaerobically incubated at 37°C and *B. breve* BC204 was allowed to growth for 24 h before counting viable cells.

III.3.7 Formulation of microencapsulated *B. breve* BC204 in oral tablets

Spray-dried microparticles (SDM) were then formulated in time-dependent erodible tablets.

III.3.7.1 Preparation of oral tablets by spreading and cooling

Three formulations containing fixed amounts of BW, SDM, VitE and variable amounts of CMC and/or FOS were obtained as reported in *table III.1*.

For the preparation, BW was melted at 80°C together with VitE. CMC and/or FOS were then incorporated and the temperature was lowered to 55°C before adding microencapsulated viable probiotics. Tablets were finally obtained by using Optima Tablet device (Farmalabor Tech., Barletta-Andria-Trani, Italy). The warm paste was spread in the dies of the device and the cooling process was achieved at room temperature.

***Table III.1* –** Composition of mixtures used for the preparation of oral tables (% w/w).

% (w/w)	Tab A	Tab B	Tab C
BW	45	45	45
CMC	15	10	0
VitE	5	5	5
FOS	0	5	15
SDM	35	35	35

The same procedure described above (section III.3.7.1) was also applied to incorporate freeze-dried microparticles inside final oral formulation. Unfortunately, the freeze-dried powder did not give rise to compact and homogenous tablets, that were consequently excluded from the study and were not further characterized.

III.3.7.2 Size, weight and friability of the tablets

After preparation, tablets were evaluated in terms of height, diameter and friability. Friability test was conducted at 25°C as reported by Abruzzo et al. [Abruzzo 2015].

Ten tablets were weighted, then subjected to repeated revolutions (25 rpm for 4 min) using a friability tester and weighted again.

The friability was measured as a percentage (% friability) of weight lost during a standardized abrasion.

III.3.7.3 Survival of *B. breve* BC204 after tablet production and during storage

The impact of spreading and cooling process on bacteria viability was assessed immediately after tablet production and during storage of final formulations at 4°C.
Accurately weighted tablets were manually grinded, dispersed in MRS (1 mL) and incubated at 37°C for 1 h before viable count on MRS plates as reported in section III.3.6.4.

III.3.7.4 Water-uptake ability

Water-uptake study was performed according to the method described by Bigucci et al. [Bigucci 2015]. Briefly, accurately weighted tablets were placed on a filter paper (3 cm × 3 cm) soaked in SIF and placed on the top of a sponge (7 cm × 5 cm × 2 cm), previously soaked in the hydration medium. The sponge was positioned in a Petri dish filled with the same medium to a height of 0.5 cm. Water-uptake (WU) was calculated as weight increase of the tablet for 5 h, according to the following equation:

$$WU(\%) = (W_{HTP} - W_{HT} - W_{DT}) \times 100 / W_{DT}$$

where W_{HTP} is the weight of hydrated tablet and wet paper filter, W_{HT} is the weight of wet paper filter and W_{DT} is the weight of the dry tablet.

III.3.7.5 *In vitro* mucoadhesion

The ability to establish a prolonged contact with mucosa epithelia was measured in terms of the force needed to pull out a freshly isolated intestinal mucosa from tablets with an adapted tensiometer (Krüss 132869; Hamburg, Germany). Rabbit intestinal mucosa was fixed to a support (surface area 1 mm^2) with cyanoacrylate glue and attached to the tensiometer spring. The mucosa was lowered until it just contacted the surface of the tablet, previously hydrated with SIF for 5 min. Then the mucosa was raised until it was separated from the tablet surface. This point represents the adhesive strength between the mucosa and tablet and is expressed as a positive force in dyne.

III.3.7.6 *In vitro B. breve* BC204 release

The release of *B. breve* from the tablets was investigated in simulated media at 37°C under stirring. Each tablet was incubated in 20 mL of SGF for 3 h, recovered by filtration

(0.22 µm cellulose nitrate filter, Sartorius, Goettingen, Germany) and subsequently incubated in 20 mL of SIF for 4.5 h. Aliquots were taken at predetermined intervals of time to detect viable cells released by counting on MRS plates.

III.3.8 Statistical Analysis

All the results are reported as mean ± standard deviation (SD), calculated from 3 independent experiments, each with 3 replicates, except for mucoadhesion and friability results that were calculated from 5 and 10 independent experiments, respectively. Statistical analysis was performed using ANOVA test (GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Differences were deemed significant for $p < 0.05$.

III.4 Results and discussion

III.4.1 Adhesion of *B. breve* BC204 to HeLa and Caco-2 cells

The ability of a probiotic strain to adhere to mucosal surfaces of the intestine and the vagina represents the first step for the formation of a barrier to prevent undesirable pathogens colonization [Reid 2011].

B. breve BC204 was examined for its ability to adhere to HeLa cells, a cell line originated from human cervix adenocarcinoma, and to Caco-2 cells, epithelial cells derived from a colorectal adenocarcinoma (*figure III.1*). *B. breve* BC204 adhered at low levels to HeLa cells (< 2 bacteria/cell after a contact time of 3 h) while it strongly adhered to Caco-2 cells (>10 bacteria/cell). The higher adhesion to the gut epithelium supports our hypothesis to include a *Bifidobacterium* strain of vaginal origin in an oral formulation able to guarantee a high intestinal colonization, as a reservoir for a subsequent colonization of the probiotic strain at the vaginal level. These results are in accordance with the fact that bifidobacteria are a minor component of the vaginal ecosystem, while they are dominant in the gastrointestinal habitat [Freitas and Hill 2017].

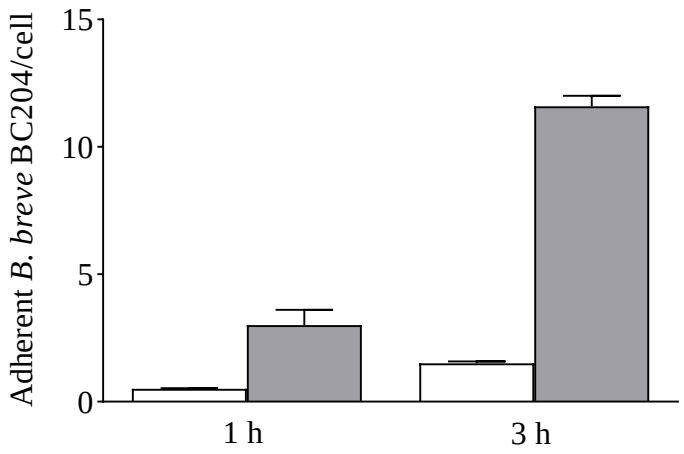

Figure III.1 **–** Adhesion of *B. breve* BC204 to HeLa (white bars) and Caco-2 (grey bars) cells after a contact time of 1 or 3 h. The results are expressed as average number of adherent bacteria per one HeLa/Caco-2 cell (mean ± SD, $n = 3$).

III.4.2 Tolerance of *B. breve* BC204 to gastric acids and bile salts

To provide functional benefits to the gut and urogenital tract, oral probiotics must survive the passage through the acidic environment of the stomach [Albertini 2010].

First, *B. breve* BC204 was incubated under SGF at pH 2 or 7 for 3 h (*figure III.2*).

B. breve BC204 was able to survive under acidic conditions during the first 90 min.

After 180 min of incubation at pH 2 the cell viability decreased from 8.73 ± 0.15 to 6.83

± 0.19 log $CFUmL^{-1}$. At pH 7 no significant reduction in viability was observed, suggesting that cell survival depends on the environment pH. After incubation under SGF, cells were exposed to SIF at pH 7 for subsequent 3 h. No significant reduction in cell viability was found when bifidobacteria were previously incubated in SGF at pH 7.

On the contrary, when previously subjected to simulated gastric fluid at pH 2, *B. breve* BC204 viability decreased (from 6.83 ± 0.19 to 4.95 ± 0.25 log $CFUmL^{-1}$), but it was not completely abolished, showing a moderate intrinsic ability of the strain to resist to gastric acidity.

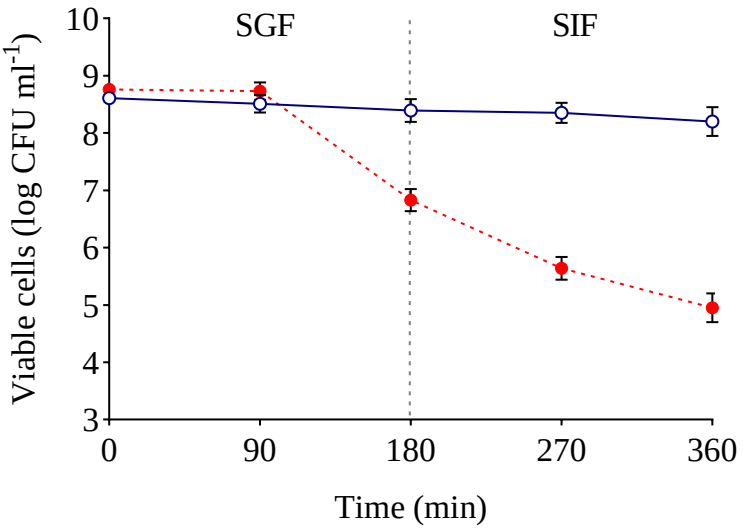

Figure III.2 – Survival of *B. breve* BC204 in simulated gastric fluid (0-180 min) at pH 7 (○) or pH 2 (●), and in simulated intestinal fluid (180-360 min) at pH 7 (mean ± SD, *n* = 3).

III.4.3 Antimicrobial activity of *B. breve* BC204

The fungistatic and fungicidal activities of *B. breve* BC204 CFS were evaluated against 4 clinical isolates of *C. albicans* and 5 clinical isolates of *Candida* non-*albicans*. *B. breve* BC204 showed a broad spectrum of anti-*Candida* activity since it exerted a fungistatic activity against all *C. albicans* strains, *C. tropicalis*, *C. parapsilosis* and *C. lusitaniae*. Moreover, it was fungicidal towards all *C. albicans* strains, *C. tropicalis* and *C. lusitaniae*. We also investigated the ability of *B. breve* BC204 CP and respective CFS to counteract the infection process of *C. trachomatis* in HeLa cells. *B. breve* BC204 CP significantly reduced *C. trachomatis* invasion to HeLa cells through all 3 mechanisms of inhibition examined (competition, exclusion and displacement). In particular, CP completely abolished *Chlamydia* infectivity throughout the competition mechanism and reduced the capability of *C. trachomatis* to infect HeLa cells of 77.61 ± 7.39 % and 82.95 ± 8.52 %

in exclusion and displacement assays, respectively.

On the contrary, CFS did not exercise an inhibitory effect, suggesting that the probiotic cells, rather than their metabolites, are the major responsible for the reduction of *Chlamydia* infectivity.

The antagonistic activity of *B. breve* BC204 was also evaluated against 11 representative bacterial strains, responsible for urogenital and gastrointestinal disorders (*table III.2*).

B. breve BC204 strongly inhibited the growth of commensal species responsible for opportunistic infections, such as *E. coli* and *S. aureus*, and extremely virulent species, like *Y. enterocolitica* (zone of inhibition ranging between 6.2 mm and 14.5 mm).

It showed a good antagonistic activity, with a diameter of the inhibition zone ranging between 3.4 and 4.6 nm, also against *E. coli* ECET H10407, *S. choleraesuis* serovar *typhimurium*, *E. faecalis* BC101 and *E. faecium* BC104.

Table III.2 – Evaluation of the antagonistic activity of *B. breve* BC204 against 11 urogenital and gastrointestinal extracellular pathogens. The results are expressed as diameter of the zone inhibition (mean ± SD, $n = 3$).

Target microorganism	Zone of inhibition (mm)
Escherichia coli ATCC11105	14.5 ± 2.8
Escherichia coli K12 BC107	6.2 ± 0.9
Escherichia coli ECET H10407	4.1 ± 1.1
Enterococcus faecalis BC101	3.4 ± 0.9
Enterococcus faecium BC104	4.6 ± 0.4
Enterococcus faecium BC105	2.7 ± 0.3
Enterococcus hirae	1.8 ± 0.4
Staphylococcus aureus ATCC29213	6.7 ± 1.0
Proteus mirabilis ATCC29906	2.1 ± 0.3
Salmonella choleraesuis serovar *typhimurium*	3.7 ± 0.4
Yersninia enterocolitica	9.9 ± 0.8

III.4.4 Microencapsulation of *B. breve* BC204

III.4.4.1 Size and morphology of microparticles

Microparticles containing *B. breve* BC204 were prepared by spray-drying and freeze-drying.

Microparticles obtained by spray-drying presented spherical shape, as revealed by SEM micrograph (*figure III.3A*). The concavities observed on the surface were typical of spray-dried microparticles and were the result of particle shrinkage due to the fast solvent

evaporation of water from the atomized droplets.

Despite the microparticles showed smaller particle size (ranging between 2.5 μm and 7.5 μm) with respect to the majority of the microparticles obtained by spray-drying (10-100 μm) [Fang and Bhandari 2010], they appeared suitable for encapsulating *B. breve* BC204 cells (about 2 μm length) that were not visible on the particles surface [Muthukumarasamy 2006]. On the contrary, microparticles obtained by freeze-drying appeared as irregular and jagged fragments (*figure III.3B*), having non homogenous dimensions (the length of fragments varied from 3 μm to 200 μm).

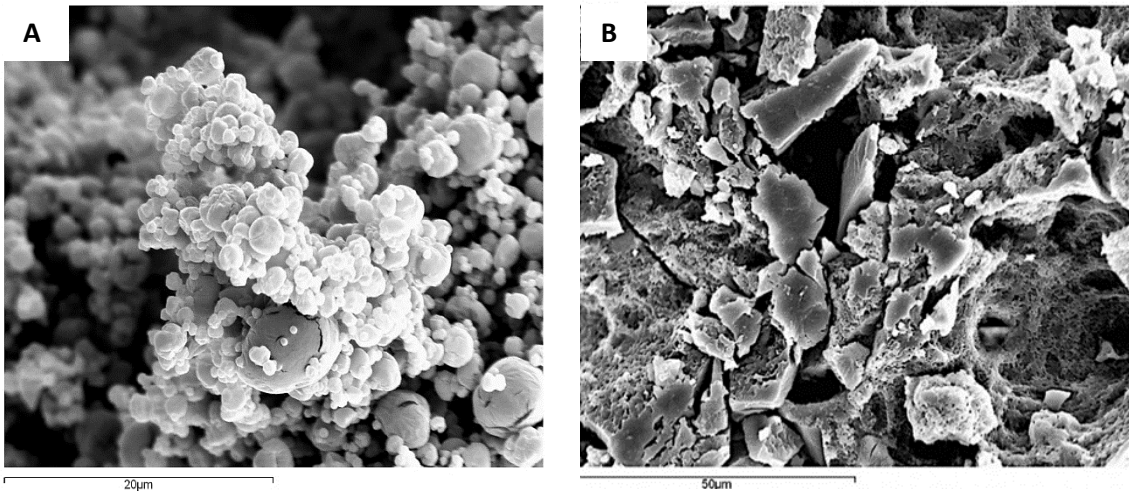

Figure III.3 - Scanning electron micrographs of *B. breve* BC204 microencapsulated by using (A) spray-drying (2000×) or (B) freeze-drying (1000×).

III.4.4.2 Viability of *B. breve* BC204 after microencapsulation

Spray-drying and freeze-drying are two widely employed techniques for the microencapsulation of probiotics. Since probiotic bacteria are highly sensitive to high and low temperatures, thermoprotective agents or cryoprotectants are necessary to preserve cell viability during drying process [Dianawati 2017].

In general, spray-drying of probiotic suspensions, performed under adequate conditions and in presence of suitable excipients, guarantees sufficient survival of the bacterial cells [Cook 2012; Broeckx 2016]. As expected, viability of *B. breve* was subjected to very low decrease (0.4 log units) after spray-drying procedure. This is in accordance with the protecting effect of IN, FOS and AA and the set drying conditions (inlet temperature of 120 ± 2°C and outlet temperature of 65 ± 3°C) [Coghetto 2016].

On the other hand, after freeze-drying the loss in viability was higher (1 log unit), suggesting that *B. breve* BC204, if resuspended in the same drying medium (see section III.3.6.1), was more sensitive to the lyophilization procedure rather than to spray-drying process.

Spray-dried microparticles contained a high concentration of viable cells (10.60 ± 0.17 log $CFUg^{-1}$), that was greatly maintained up to 12 months. Indeed, *figure III.4* shows that the viability of *B. breve* BC204 inside spray-dried microparticles was excellent both after 3 months (10.10 ± 0.07 log $CFUg^{-1}$) and after 6 months (9.73 ± 0.18 log $CFUg^{-1}$) of storage at 4°C. After 12 months the viability was reduced by about 2 log unit but the amount of viable cells contained in spray-dried microparticles was still very high (> 8.60 log $CFUg^{-1}$).The high survival of *B. breve* BC204 during this storage period suggested the possibility of storing microencapsulated probiotics for months before approaching the second step of the production.

Conversely, the viability of *B. breve* BC204 microencapsulated through freeze-drying was considerably compromised during storage at 4°C. In this case, the initial probiotic concentration in freeze-dried powder was lower (9.86 ± 0.05 log $CFUg^{-1}$) because, as discussed above, the lyophilization process greatly affected the survival of *B. breve* BC204. Moreover, the viability was further reduced by 1.3 log units after 3 months (8.55 ± 0.24 log $CFUg^{-1}$), by 2.3 log units after 6 months (7.56 ± 0.20 log $CFUg^{-1}$) and by 5.8 log units after 12 months (4.10 ± 0.15 log $CFUg^{-1}$) of storage at 4°C (*figure III.4*).

Thus, to improve the survival of *B. breve* 204 after freeze-drying it would be necessary to optimize the composition of the lyophilization medium and the choice of production parameters.

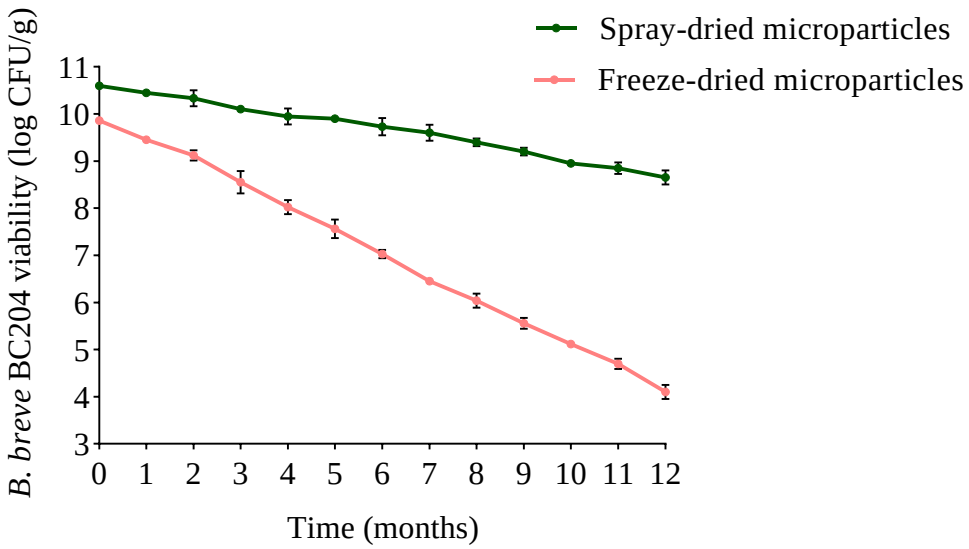

Figure III.4 – Survival of *B. breve* BC204 microencapsulated by spray-drying and freeze-drying during storage at 4°C (mean ± SD, *n* =3).

III.4.5 Formulation of *B. breve* BC204 in oral tablets

III.4.5.1 Preparation of oral tablets by spreading and cooling

The Optima Tablet device was used for the production of the final dosage form containing *B. breve* BC204 spray-dried microparticles. The addition of VitE decreased the melting point of BW from 65°C to 53°C (melting point system SMP1, Bibby Scientific™ Stuart™, UK) favouring the incorporation of the dried microparticles at lower temperature and consequently the viability of probiotic cells. Moreover, after the mixing of the solid components (CMC, FOS and SDM), a warm and uniform paste was obtained that could be easily and completely spread into the dies of the device. This assured the formation of monolithic matrices with regular geometry (height and diameter), weight and homogeneous content of *B. breve* BC204.

All the prepared tablets showed a diameter of 7.21 mm and no significant difference in height (Tab A: 3.71 ± 0.08 mm, Tab B: 3.67 ± 0.12 mm, Tab C: 3.75 ± 0.05 mm) and weight (Tab A: 0.158 ± 0.005 g, Tab B: 0.157 ± 0.003 g, Tab C: 0.159 ± 0.003 g).

Tablets contained an average amount of viable cells equal to $1.58 \pm 0.53 \times 10^9$ CFU/tablet, with no significant differences between different formulations (Tab A: $2.16 \pm 0.68 \times 10^9$ CFU/tablet, Tab B: $1.46 \pm 0.87 \times 10^9$ CFU/tablet, Tab C: $1.12 \pm 0.96 \times 10^9$ CFU/tablet, $p > 0.05$). This content of probiotic was very close to the theoretical one, corresponding to the experimental amount determined in the spray-dried microparticles ($2.21 \pm 0.05 \times 10^9$ CFU/tablet). Along with microparticle protection ability, the spreading step at low temperature, the rapid cooling and the absence of compression forces and subsequently the increase of temperature inside the compression die [Allouche 2018], guaranteed complete survival of the probiotic during tablet formation.

Finally, the manufacturing method associated with the excipients used for tablet production determined optimal resistance of the final dosage form to mechanical stress. In fact, considering that friability values lower than 1% are suitable for oral tablets, all the formulations prepared can be considered hard enough (% friability < 0.003) to be easily removed from their packaging and swallowed without damage.

The same procedure was also used in an attempt to produce tablets containing *B. breve* BC204 freeze-dried microparticles. In this case, the molten mass containing freeze-dried microparticles resulted lumpy and not homogenous, difficult to distribute inside the dies of the Optima Tablet. Therefore, it was not possible to obtain compact and uniform tablets.

This result was probably due to the irregular morphology and dimensions of freeze-dried microparticles with respect to spray-dried ones, as well as the more hygroscopic nature of the lyophilized product.

III.4.5.2 Water-uptake ability

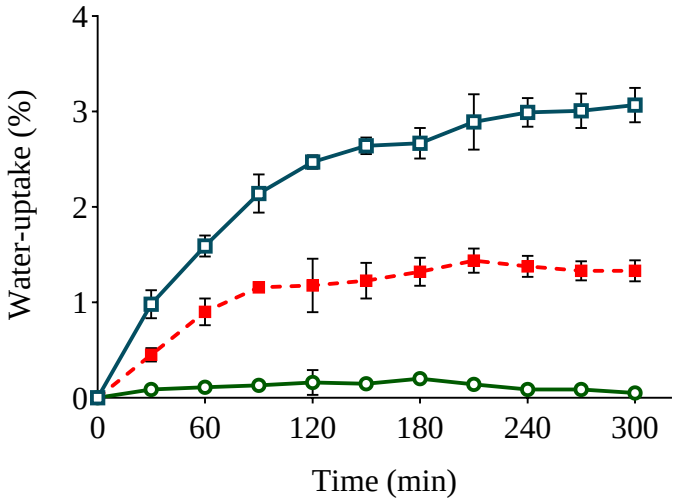

Figure III.5 – Water-uptake ability of Tab A (□), Tab B (■) and Tab C (○) (mean ± SD, *n* = 3).

The ability of the tablets to hydrate can be influenced by their structure and composition. All the formulations were homogeneous matrices containing high percentage of BW, a lipophilic excipient that limits tablet wettability. However, the presence of hydrophilic excipients could favour water entry in the matrices, thus increasing their weight.

Figure III.5 shows that, after 180 min in presence of SIF, Tab A (15 % w/v CMC) allowed to reach higher water-uptake than Tab B (10% w/v CMC) and Tab C (0% w/v CMC), accordingly to the amount of CMC in the formulations. Considering that Tab C was not able to hydrate, the increase in water-uptake ability of the tablets cannot be ascribed to the presence of FOS.

III.4.5.3 *In vitro* mucoadhesion

Anionic polymers such as CMC can interact with the mucous network via hydrogen bonds and chain entanglement [Owens 2005; Debotton and Dahan 2017]. Thus, it is not surprising that the mucoadhesive strength increased with the increase of CMC content in the tablet. Tab A showed a significantly ($p < 0.01$) higher detachment force with respect to Tab B and C (*figure III.6*).

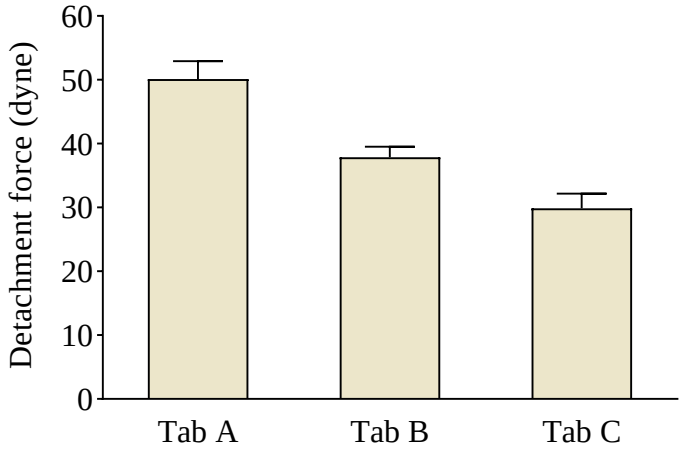

Figure III.6 **–** Mucoadhesion capacity of Tab A, Tab B and Tab C (mean ± SD, *n* = 5).

III.4.5.4 *In vitro B. breve* BC204 release

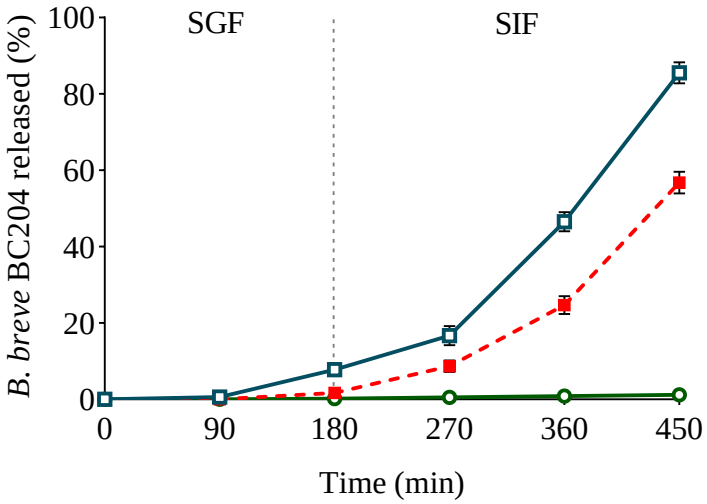

Figure III.7 – *B. breve* BC204 release in simulated gastric fluid (0-180 min) and in simulated intestinal fluid (180-450 min) from Tab A (□), Tab B (■) and Tab C (○) (mean ± SD, *n* = 3).

In *figure III.7* the percentage of *Bifidobacterium* released in simulated media was plotted over time. After 3 h in SGF, corresponding to the passage through the stomach, all tablets released less than the 10% of bacteria content. After further 4.5 h in SIF, the total release, compared to initial quantity in the tablets, varied according to the formulation and reached 85.57 ± 2.80 % and 56.75 ± 2.88 % for Tab A and Tab B, respectively. That means that Tab A and Tab B could release about 10^9 living cells in 7 h. This is in accordance with the suggested therapeutic daily dose of 10^8-10^9 viable cells [FAO/WHO 2006]. On the contrary, Tab C released only a small amount of probiotic. This behaviour is in accordance with the composition of the tablets and their different water-uptake ability.

The CMC incorporated in Tab A and B favoured the uptake of water from the gastrointestinal medium, thus enhancing tablet hydration and erosion.

III.4.5.5 Survival of *B. breve* BC204 during tablet storage

The final dosage form should ensure probiotic cells survival at high levels for a sufficient storage period. *Figure III.8* depicts the survival of *B. breve* BC204 during tablets storage up to 6 months at 4°C. All formulations were able to preserve high viability of *B. breve* BC204 up to 3 months (> 8.50 log CFU/tablet). After 6 months the count decreased at 7.15 ± 0.17 log CFU/tablet for Tab A while Tab B and Tab C maintained a viability of 8.45 ± 0.17 log CFU/tablet and 8.55 ± 0.08 log CFU/tablet, respectively. This data indicated that the presence of FOS was important to improve the survival of *B. breve* BC204. Considering all the results obtained, Tab B appeared to be the best formulation because it combined the ability to adequately protect the bacterium during storage with good mucoadhesive properties and high release of probiotic cells.

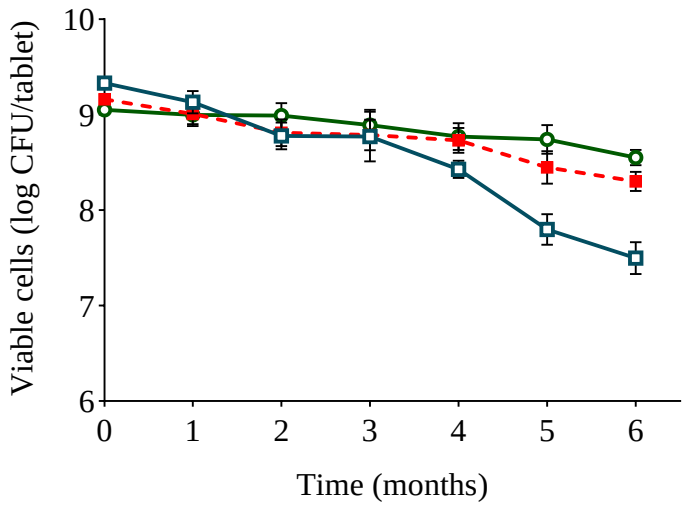

Figure III.8 **-** *B. breve* BC204 survival in Tab A (□), Tab B (■) and Tab C (○) during a storage period of 6 months at +2-8 °C (mean ± SD, *n* = 3).

III.5 Conclusions

The potential benefits of oral probiotics on the modulation of the vaginal microbiota strongly deserve to implement the research concerning their clinical application as preventive agents for urogenital infections. Considering that the international guidelines correlate the efficacy of probiotics to the number of viable cells reaching the target site, the selection of strains with effective probiotic capacity and the design of an appropriate delivery system are essential prerequisites. This work highlighted the possibility of combining the favourable characteristics of *B. breve* BC204 strain and the release of adequate amount ($>10^9$ CFU/dose) of living cells at the treatment site, by taking into account all the critical aspects of probiotic administration. The formulation design and the manufacturing method used in this work allowed to obtain a reliable product that can be elaborated at large scale.

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IV. Freeze-dried matrices based on polyanion polymers for chlorhexidine local release in the buccal and vaginal cavities (*paper 2*)

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Published on Journal of Pharmaceutical Sciences (doi:10.1016/j.xphs.2019.02.026) [Giordani 2019].

IV.1 Abstract

Chlorhexidine (CLX) is a wide spectrum cationic antimicrobial used for prevention and treatment of infections of buccal and vaginal cavities. In order to increase the residence time of CLX based formulations at the application site, and consequently reduce the daily dose frequency, new formulations composed of mucoadhesive polymers should be designed. The objective of this work was the development of matrices based on polyanionic polymers, such as sodium alginate, carboxymethylcellulose, xanthan gum and sodium hyaluronate, aimed to prolong the local release of CLX into the buccal or vaginal cavity. Matrices were prepared by freeze-drying comply with two different preparative methods and characterized in terms of resistance to compression, water-uptake ability, mucoadhesion, *in vitro* drug release behavior and antimicrobial activity toward representative pathogens of buccal and vaginal cavities. Results showed that the selection of suitable polymers associated to the adequate preparative method allowed to modulate matrix ability to hydrate, adhere to the mucosa and release the drug as well as to exert antimicrobial activity. In particular, matrix based on sodium hyaluronate was found to be the best performing formulation and could represent a versatile system for local release of CLX with potential application in both buccal and vaginal cavities.

Keywords

Drug-excipient interaction(s); hydrogel(s); lyophilization; mucoadhesive; polymeric drug delivery system(s); solid dosage form(s); mucosal drug delivery.

List of abbreviations

ALG: sodium alginate; CLX: chlorhexidine digluconate; CMC: sodium carboxymethylcellulose; HYA: sodium hyalorunate; XG: xantan gum.

IV.2 Introduction

Chlorhexidine digluconate (CLX) is a cationic bisguanide with a wide spectrum activity against Gram-positive and Gram-negative bacteria. At low concentrations, chlorhexidine alters membrane integrity, while at high concentrations, cytoplasmic contents precipitate, resulting in cell death. It is also effective against some fungi and yeasts, including *Candida*, and some viruses like HIV and HBV [Denton 1991; Milstone 2008]. Chlorhexidine has been widely used for prevention and treatment of infections in the oral cavity (caries, periodontal disease, oral candidiasis, reduction of bacteremia after implant surgery and intracanal irrigation) [Pupe 2011] and in the vaginal tract (bacterial vaginosis and vulvovaginal candidiasis) [Molteni 2004]. A number of chlorhexidine-based preparations are commercially available, although these preparations usually provide short-term efficiency, requiring repeated applications (multiple daily doses) to maintain antimicrobial activity. This behavior could be mainly attributed to the short contact time of the formulation with mucosa, due to the rapid dilution by saliva and the swallowing reflex in the oral cavity as well as the self-cleaning action of the vaginal tract. The need to enhance the retention time in the administration site can be satisfied using mucoadhesive polymers which enhance adhesion of the dosage form to mucosa. This adhesion process involves a contact stage between the mucoadhesive formulation and the mucus membrane, with hydration, wetting and spreading as a main step. Subsequently, the strengthening of polymer-mucin junction occurs thanks to the interpenetration of the polymer chains into the mucus layer and the establishment of polymer-mucin bonding (mainly weak van der Waals and hydrogen bonds or electrostatic interactions).

Freeze-drying of polymeric solutions or gels allows to obtain dosage forms that, *in virtue* of their high porosity and specific surface area, offer the advantage of rapid hydration associated with gel development. Drug diffusion through gelled polymeric network could ensure drug release for extended period and so reduce the application frequency of dosage forms. At the best of our knowledge freeze-dried matrices containing chlorhexidine were widely studied as drug delivery system for wound infections [Ng 2014], but only few papers deal on formulations for the local drug release in the buccal or vaginal cavities.

Recently, Cavallari and colleagues reported that buccal films containing the gel-forming polysaccharide psyllium and the film-forming sodium carboxymethylcellulose showed good mucoadhesive properties and a prolonged zero-order release of chlorhexidine [Cavallari 2015].

Likewise, Onnainty and colleagues developed bionanocomposites based on chitosan and montmorillonite characterized by long-term sustained release of chlorhexidine in the buccal cavity and good mucoadhesion properties, maintaining the drug antimicrobial activity [Onnainty 2016].

As regard vaginal administration, freeze-dried inserts based on chitosan complexes with alginate [Abruzzo 2013] or carboxymethylcellulose [Bigucci 2015a] showed that the selection of suitable chitosan/polyanion molar ratio allowed to modulate insert ability to hydrate, adhere to the mucosa, and release chlorhexidine, maintaining the drug antimicrobial activity toward common pathogens responsible for vaginal infections.

In the present study we developed new mucoadhesive matrices based on polyanionic polymers such as sodium alginate, carboxymethylcellulose, xanthan gum and sodium hyaluronate aimed to the local release of chlorhexidine into the buccal and vaginal cavities. Briefly, the main steps were: (i) ionic complexation between polymer and chlorhexidine and its characterization in terms of yield; (ii) preparation of freeze-dried matrices comply with two different preparative methods and evaluation of drug content, solid state, morphology and mechanical properties; (iii) investigation of water-uptake ability, mucoadhesion potential and *in vitro* drug release behavior; (iv) evaluation of antimicrobial activity against common pathogens responsible for buccal and vaginal infections.

IV.3 Materials and methods

IV.3.1 Materials

Sodium alginate (ALG; MW 140 kDa, pKa = 3.14) [Shi 2006] was obtained from Farmalabor (CanoALG di Puglia, Italy). Sodium carboxymethylcellulose (CMC; MW 250 kDa, pKa 4.3, substitution degree 0.78) [Bigucci 2015a], xanthan gum (XG; MW 10000 kDa, pKa = 2.65) [Darwesh 2018] and sodium hyaluronate (HYA; MW 1800-2300 kDa, D-glucuronic acid > 42 %, pKa = 2.9) [Bigucci 2015b] were purchased from A.C.E.F. (Piacenza, Italy). Chlorhexidine digluconate (CLX; MW 897.8 Da, pKa = 10.52) aqueous solution (20% w/v) and mucin from porcine stomach (type II, bound sialic acid ~1%) were purchased from Sigma Aldrich (Milano, Italy). Sabouraud Dextrose (SD) medium, Brain Heart Infusion (BHI) medium and GasPak EZ used for microbiological assays were supplied by Becton Dickinson and Company (Sparks, MD, USA). All other chemicals were of analytical grade and were purchased from Carlo Erba (Milan, Italy).

Buffer solution at pH 6.8, simulating human saliva pH (healthy saliva pH = 6.7-7.4) [Marques 2011], was prepared as follow: 8.38 mM $Na_2HPO_4 \cdot 12H_2O$, 7.35 mM KH_2PO_4 and 94.11 mM NaCl. Buffer solution at pH 4.5, simulating vaginal pH (vagina pH 3.5-5.5) [Boskey 2001], was prepared as follow: 0.1 M KH_2PO_4.

IV.3.2 Freeze-dried matrix preparation

The weighed polymer powder was added gradually into distilled water and mixed for at least 24 h using a magnetic stirrer at room temperature. Then, CLX solution was added to the polymeric solution under stirring in order to obtain a final drug and polymer concentration of 0.25% and 0.5% w/v, respectively. The addition of drug to the polymer provided a dispersion of polyanion/CLX complex in a solution of free drug and polyanion. After homogenization of dispersion (50 mL) at 6.500 rev/min for 2 min (Ultra-Turrax, T 25 basic homogenizer, IKA, Dresden, Germany), two different preparative methods were adopted.

Method “a”: about 0.9 g of the dispersion were placed into each cavity (diameter 13 mm) of a blister pack (Farmalabor, Canosa di Puglia, Italy), frozen overnight at -20°C, freeze-dried at 0.01 atm and -45°C (Christ Freeze Dryer ALPHA 1-2, Milan, Italy) and stored in a desiccator until use.

Method “b”: the polyanion/CLX complex was isolated by ultracentrifugation (ALC 4239R centrifuge; Milan Italy) at 12000 rpm/min for 60 min, washed with deionized

water and dispersed again in 20 mL of water (with exception for HYA/CLX complex for which it was not possible the dispersion in water); finally, about 0.9 g of the dispersion were placed into each cavity (diameter 13 mm) of a blister pack and freeze-dried.

For the solid complex weight measurement, the precipitate based on polyanion/CLX complex was isolated as previously described and weighed after freeze-drying.

Each formulation was coded based on the polymer followed by the subscript "a" and "b" for the matrix prepared using the method "a" and "b", respectively.

IV.3.3 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) analysis were conducted in order to investigate the interactions between polyanions and CLX. Infrared spectra of raw material polymers, CLX powder (obtained after freeze-drying of the commercial solution at the same conditions described in the section IV.3.2) and freeze-dried polyanion/CLX complexes (obtained accordingly to method b reported in section IV.3.2) were recorded with a Jasco FT-IR 4100 spectrophotometer (Jasco, Lecco, Italy). The samples were prepared as compressed KBr disks.

IV.3.4 Matrix dimensions, weight and drug content

Matrices obtained from freeze-drying process were weighed and measured for diameter and thickness through an electronic digital caliper (art. 1367 E 2900, Shanghai ShangErBo Import & Export Co., Shanghai, China).

For the determination of drug content, each matrix was dissolved in 40 mL of phosphate buffer at pH 4.5 or 6.8 and the solutions obtained were analyzed by HPLC method. The chromatographic system was composed of a Shimadzu (Milan, Italy) LC-10ATVP chromatographic pump and a Shimadzu SPD-10AVP UV-Vis detector set at 254 nm. Separation was obtained on a Phenomenex (Torrance, CA, USA) Synergi Fusion-RP 80A (150 mm × 4.6 mm I.D., 5 μm) coupled to a Phenomenex (Torrance, CA, USA) SecurityGuard C18 guard cartridge (4 mm × 3.0 mm I.D., 5 μm).

The mobile phase was a mixture of acetate buffer (CH_3COONa 30 mM adjusted with glacial acetic acid to pH 3.3) and acetonitrile (40:60, v/v) [Padois 2012]. The flow rate was 0.4 mL/min and manual injections were made using a Rheodyne 7125 injector with a 20 μL sample loop. Data processing was handled by means of a CromatoPlus computerized integration system (Shimadzu Italia, Milan, Italy).

Calibration curve was plotted at concentration range of 0.5-50.0 μg/mL and a good

linearity was found ($R^2 = 0.9946$).

Accordingly to matrix weight and drug content, the weight ratio (WR) between polyanion and drug was calculated as follow:

$$WR_{polyanion/drug} = \frac{\text{weight of the matrix - amount of drug inside the matrix}}{\text{amount of drug inside the matrix}}$$

IV.3.5 Scanning electron microscopy

Scanning electron microscopy (SEM) analysis were performed to evaluate the internal morphology of the polymeric matrices that could affect their functional properties, such as hydration ability, drug release and mucoadhesion characteristics. Matrices were cut with a razor blade, fixed on supports and coated with gold-palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. Samples were then observed with LEO 420 (LEO Electron Microscopy Ltd., Cambridge, UK) using secondary electron imaging at 15 kV.

IV.3.6 Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments were performed on freeze-dried matrices to evaluate their solid state (DSC analysis of CLX was not performed since drug was purchased as aqueous solution). Calorimetric measurements were done using a Netzsch DSC200 PC differential scanning calorimeter (Metzsch, Germany). The samples were placed in aluminum pans and then hermetically sealed with aluminum lids. Thermal analyses were recorded from 25°C to 350°C under a dry nitrogen atmosphere and a heating rate of 10°C/min.

IV.3.7 Moisture content

The moisture content was measured to investigate the efficiency of freeze-drying process to eliminate water from formulations [Hazzah 2015; Freag 2018]. The matrices were weighed after the freeze-drying process (W_i) and then heated to 100°C until reaching a constant weight (W_f). The moisture content was estimated as percent weight loss following the equation below:

$$\% \text{ weight loss} = \frac{(W_i - W_f) \times 100}{W_i}$$

IV.3.8 Mechanical characterization

The assay was performed at 25°C using a dynamometer equipped with a 50 N load transducer (2712-041 Instron machine, Norwood, USA). Before compression measurements, the thickness of the matrix was measured by calliper. A flat cylindrical stainless-steel probe (23 mm diameter) was used to compress the samples (around 13.05 mm diameter, *table IV.1*) to a defined depth. The contact area is assumed to be equal to sample diameter through all the test. The run depth of the probe was set at 33% the sample height to minimize irreversible damage of the inner structure of the matrices. Other test parameters were setup as follow: pre-test speed (2 mm/min); load speed (6 mm/min); displacement speed (6 mm/min). Load and displacement forces were recorded, and the following parameters were calculated: compression stress at 33% strain (σ_{33}; kPa) and the compressive work (W; mJ).

Hysteresis loop (H) calculated as the difference between the loading and unloading works was calculated and expressed in mJ and as percentage. The results were calculated by using Bluehill Software Version 3.31 (Instron, Norwood, USA). The results are the mean of 5 determinations.

IV.3.9 Water-uptake ability

Water-uptake ability was studied to investigate the hydration properties of polymeric matrices. Accurately weighed matrix was placed on cellulose nitrate membrane filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany; diameter = 4.7 cm, pore size = 0.45 mm) soaked in phosphate buffer at pH 4.5 or 6.8 and positioned on top of a sponge (7 cm × 5 cm × 2 cm), previously soaked in the hydration medium. The sponge was placed in a petri dish filled with the same solution to a height of 0.5 cm [Bertram 2012].

Water-uptake (WU) was determined as weight increase of the matrix for 180 min, according to the following equation:

$$WU\ (\%) = (W_{HMF} - W_F - W_{DM}) \times 100 / W_{DM}$$

where W_{HMF} is the weight of hydrated matrix and wet cellulose filter, W_F is the weight of wet cellulose filter and W_{DM} is the weight of the dry matrix.

IV.3.10 Mucoadhesion ability

Mucoadhesion ability was measured in terms of the force needed to pull out a freshly porcine esophageal or vaginal mucosa from polymeric matrices with an adapted tensiometer (Krüss 132869; Hamburg, Germany).

With respect to buccal application, porcine buccal mucosa is frequently used as its structure and permeability characteristics are close to those of human tissue. Despite the availability and low cost, buccal porcine mucosa is characterized by some limitations, such as the restricted surface, the damage due to the mastication and the firm attachment to the underlying muscular tissue that make the excision fastidious and time-consuming. Pig esophageal mucosa has been proposed as an alternative to the buccal one due to the high histology resemblance. Moreover, it is easier to separate from the underlying tissue, its surface area is greater and undamaged by mastication [Diaz 2005].

Regarding vaginal application, porcine vaginal mucosa was used because the tissue exhibits striking similarities to the human one in cellular structure, intercellular barrier lipid composition and organization [D'Cruz 2005; Squier 2008].

Pig esophageal and vaginal tissues were obtained from a local slaughterhouse (CLAI, Faenza, Italy), immediately transported to the laboratory and used within 2 h. Esophagus and vagina were cut longitudinally and rinsed with isotonic saline. The mucosa was separated from the muscular layer by cutting the connective fibers with a scalpel. Subsequently, it was fixed to a support (surface area 1 mm^2) with cyanoacrylate adhesive, suspended from the tensiometer spring and hydrated for 5 min with 200 µL of mucin suspension (0.5% w/v) in phosphate buffer at pH 4.5 or 6.8 for vaginal and buccal mucosa, respectively.

The mucosa was lowered until it just contacted the surface of the matrix and a 10-dyne force, measured by the torsion balance of the instrument as a negative force, was applied to the matrix for 30 sec. Then, the mucosa was raised until it was separated from the matrix. This point represents the adhesive bond strength between mucosa and matrix and is expressed as a positive force in dyne.

IV.3.11 *In vitro* release studies

In vitro release studies were performed in order to evaluate the drug amount released from matrices over the time. For this study, each matrix was attached on a glass slide using cyanoacrylate adhesive. This configuration ensures that the matrix is prevented from floating during the entire testing period. The glass slide was immersed inside a beaker containing 40 mL of phosphate buffer at pH 4.5 or 6.8, simulating vaginal and buccal pH, respectively, and maintained under agitation with a magnetic bar. Aliquots of 1 mL were withdrawn at different time intervals and replaced with fresh medium. All experiments were performed under sink conditions (C_{max} in medium < 10% $C_{saturation}$).

The studies were carried on for 360 min and samples were analyzed by the same HPLC method previously described. The results of release studies are shown as cumulative drug amount released (expressed as fractional amount) plotted as a function of time.

IV.3.12 Antimicrobial studies

The antimicrobial activity was evaluated against three pathogenic strains responsible for buccal (*Streptococcus mutans*) and vaginal (*Candida albicans* and *Escherichia coli*) infections.

S. mutans was grown in BHI medium at 37°C for 24 h in anaerobic jars containing GasPak EZ, *E. coli* was grown aerobically in BHI medium at 37°C for 24 h, *C. albicans* was grown aerobically in SD medium at 30°C for 24 h. A microbial suspension, prepared from a broth culture in log phase of each target microorganism, was used to inoculate Erlenmeyer flasks containing 40 mL of phosphate buffer at pH 6.8 (*S. mutans*) or 4.5 (*E. coli* and *C. albicans*) (T0 concentration: about 6 log CFU/mL, corresponding to the physiological amounts in case of infection).

Viability of *S. mutans*, *E. coli* and *C. albicans* was compared with viability of the same microorganisms cultured in the presence of CLX and polymeric matrices (HYA_a, CMC_a and CMC_b). HYA_a, CMC_a and CMC_b were selected for the antimicrobial studies as a function of their mucoadhesion and drug release behavior.

Counts of viable microorganisms were carried on BHI agar (*S. mutans* and *E. coli*) or on SD agar (*C. albicans*) plates at the inoculum time (T0) and after 6 h (T6), 24 h (T24) and 48 h (T48) of incubation at 37°C (physiological temperature). BHI plates were incubated at 37°C for 24 h aerobically for *E. coli* and anaerobically for *S. mutans*. SD plates were incubated aerobically at 30°C for 48 h.

IV.3.13 Statistical analysis

All experiments were done at least in triplicate. Results are expressed as mean ± standard deviation (SD). Student's t-test were used to determine statistical significance of studies. The criterion for statistical significance was $p < 0.05$.

IV.4 Results and discussion

Conventional liquid and semisolids preparations containing CLX and intended for buccal or vaginal application usually provide a short-term efficiency, due to the low contact time of the formulation with mucosa, requiring multiple daily doses to maintain antimicrobial activity. For this reason, the employment of mucoadhesive polymers could be useful in order to develop new formulations, able to be retained at the administration site for a long time. Literature revealed that there is no single mucoadhesive formulation that can be administered either through buccal and vaginal routes for the local release of CLX.

In this study, we reported the preparation and characterization of new mucoadhesive matrices containing CLX and different polyanionic polymers and proposed them as formulations with a dual potential application for the treatment of infections of both buccal and vaginal tracts.

IV.4.1 Solid complex weight measurement

The addition of CLX to polymeric solutions provided the precipitation of a solid complex due to the ionic interaction between the large negatively charged polyanion with the positively charged drug.

Yields of solid complexes were 54.27 ± 3.24 %, 26.05 ± 1.32 %, 25.57 ± 1.25 % and 19.62 ± 1.01 % for XG, CMC, ALG and HYA, respectively.

The different yield values could be attributed to the peculiar affinity of polyanionic polymers for the dicationic drug. In fact, factors such as the medium pH, drug pKa, charge density, molecular weight and pKa of the polymer influenced the interaction between polymer and drug [Berger 2004].

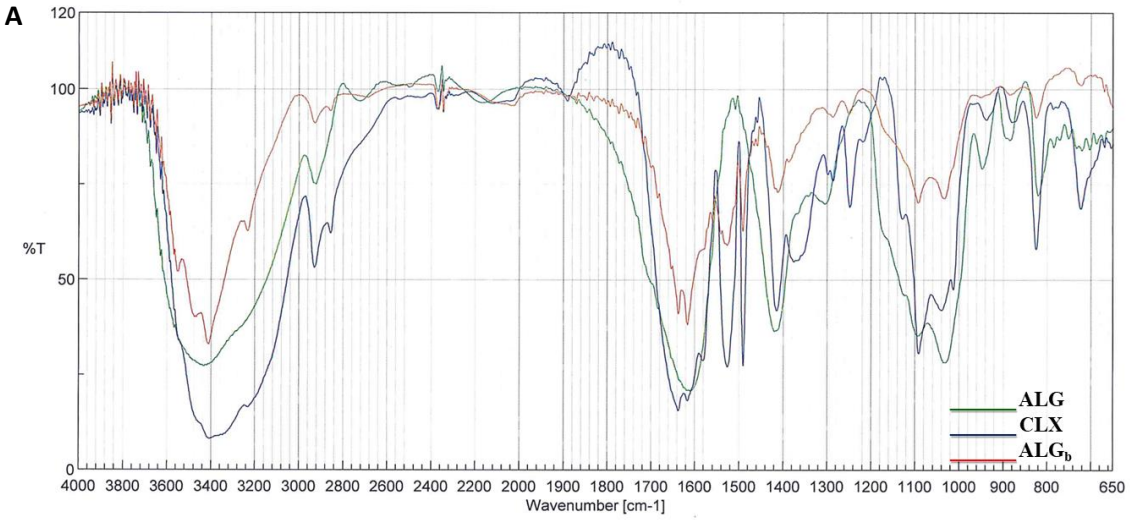
IV.4.2 Fourier transform infrared spectroscopy

Figure IV.1 reports the FT-IR spectra of CLX, polyanions and freeze-dried polyanion/CLX complexes. Spectrum of CLX (blu trace in *figure IV.1A-D*) reported at 3400 cm^{-1} the stretching bands for the aromatic of the groups Alkyl-NH-Aryl and $(Alkyl)_2NH$ and the group =NH, and the characteristic peaks at 1639 cm^{-1} and 1617 cm^{-1} assigned to the O-H bending vibration and to carbon-nitrogen double bond stretching vibration (C=N), respectively. Bands at 2938 and 2860 cm^{-1} were assigned to asymmetric and symmetric C-H stretching bands. Moreover, the peak at 1582 cm^{-1} can be attributed to the NH bending vibration of secondary amine and imine groups. Absorption at 1414

cm^{-1} belonged to the C=C stretching vibrations of aromatic ring and at 825 cm^{-1} to the C-H out-of-plane deformation vibrations of a 1,4-disubstituted aromatic ring [Holešováa 2015].

Regarding FT-IR spectra of polyanions (green trace in *figure IV.1A-D*), the large peak within the range of 3100-3600 cm^{-1} was the axial deformation of OH, while at around 2900 cm^{-1} appeared the axial deformation of C-H (symmetrical and asymmetrical stretching of CH_3 or even groups of CH_2). ALG (*figure IV.1A*) showed the typical band at 1610 cm^{-1} relative to the vibration of C=O group [Abruzzo 2013]. In the FT-IR spectrum of CMC (*figure IV.1B*), two strong peaks at 1617 cm^{-1} and 1421 cm^{-1} were observed due to the asymmetrical and symmetrical stretching of COO groups [Bigucci 2015a]. FT-IR spectra of XG (*figure IV.1C*) and HYA (*figure IV.1D*) presented an intense group of overlapped bands in the region of the carbonyl stretching vibration (1500-1700 cm^{-1}) derived from the vibration of acetamide and carboxylate/pyruvate groups present in the d-N-acetylglucosamine (for HYA) and d-glucuronic acid (for HYA and XG) units. Moreover, between 1410 and 1420 cm^{-1} the spectra displayed a band derived from the symmetric stretching vibration in the carboxylate/pyruvate group. Finally, XG presented the absorption band at 1720 cm^{-1} which can be attributed to carboxyl [Faria 2011; Bigucci 2015b; Wang 2017].

FT-IR spectra of freeze-dried polyanion/CLX complexes displayed the presence of characteristic peaks of the drug, with the exception of peak at 1582 cm^{-1} that shifted to around 1561 cm^{-1}, as a proof of the interaction between drug and polyanion [Abruzzo 2013; Bigucci 2015a]. Moreover, in the spectrum of XG_b the peak at 1720 cm^{-1} related to carboxyl group disappeared as a consequence of its interaction with drug [Freag 2018].



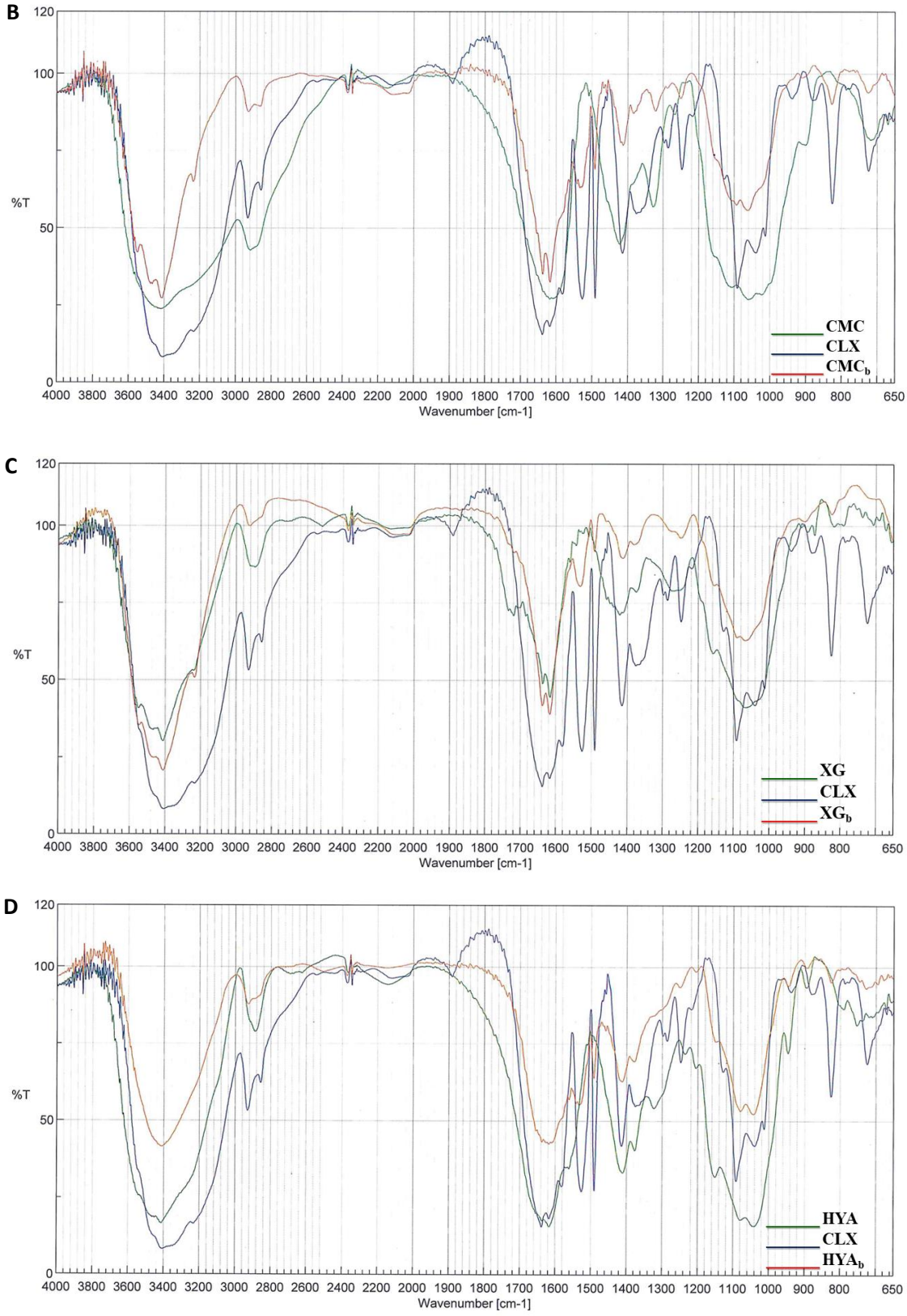


Figure IV.1 – FT-IR spectrum of CLX (blu trace) superimposed with polyanion (green trace) and freeze-dried polyanion/CLX complexes (red trace): (A) CLX, ALG and ALG_b; (B) CLX, CMC and CMC_b; (C) CLX, XG and XG_b; (D) CLX, HYA and HYA_b.

IV.4.3 Matrix dimensions, weight and drug content

Matrices showed cylindrical shape and presented a smooth surface with spongy texture. Moreover, they were easy to remove from blisters, with no or minimum residue and with good handling characteristics. Matrix dimensions and weight are listed in *table IV.1*. Matrices showed a mean thickness of 5.77 ± 0.26 mm and a mean diameter of 13.05 ± 0.33 mm. As described in literature, thickness affects the rate of hydration and the drug diffusion path through the swollen gel with significant effects on release profiles [Kianfar 2014]. The preparative method "a" produced matrices characterized by the same weight ($p > 0.05$) since it was based on the freeze-drying of a dispersion containing the same total amount of polyanion and CLX (free and complexed).

On the other hand, matrices produced with method "b" showed different weights accordingly with the yield results. In particular, XG_b presented a higher weight with respect to ALG_b and CMC_b ($p < 0.05$), as a consequence of the dispersion in water of a higher amount of recovered XG_b/CLX complex.

Table IV.1 – Characterization of freeze-dried matrices: thickness, diameter, weight and moisture content %. 5 (mean ± SD, $n = 5$).

Matrix	Thickness (mm)	Diameter (mm)	Weight (mg)	Moisture content %
ALG_a	5.70 ± 0.31	13.01 ± 0.58	6.6 ± 0.6	4.7 ± 0.4
CMC_a	5.81 ± 0.39	12.92 ± 0.37	7.6 ± 0.7	1.5 ± 0.1
XG_a	5.66 ± 0.38	13.55 ± 0.36	5.5 ± 1.7	1.1 ± 0.1
HYA_a	6.18 ± 0.52	12.94 ± 0.23	7.5 ± 0.5	1.7 ± 0.2
ALG_b	5.41 ± 0.33	12.73 ± 0.38	4.8 ± 1.1	1.7 ± 0.1
CMC_b	5.61 ± 0.47	12.73 ± 0.41	3.8 ± 0.7	2.3 ± 0.3
XG_b	6.03 ± 0.46	13.47 ± 0.33	9.4 ± 1.4	1.0 ± 0.1

In the *table IV.2* drug contents measured in phosphate buffer at pH 4.5 are reported (no significant differences were observed with respect to drug contents measured in phosphate buffer at pH 6.8). As concerned matrices obtained with method "a", the experimental drug content was close to the theoretical one (33.33 %). In the case of matrices obtained with the method "b", drug content of XG_b was lower with respect to matrices ALG_b and CMC_b ($p < 0.05$), probably due to a different affinity of XG chains for the drug with respect to other polymers.

The $WR_{polyanion/drug}$ into the matrix was calculated as it reflected the different degree of interaction between drug and polyanion and allowed to evaluate the amount of polyanion

able to affect the hydration ability and consequently the drug release [Puttipipatkhachorn 2001]. The calculated $WR_{polyanion/drug}$ between XG and drug ($WR_{XG/drug}$ = 2.04) was higher than weight ratio of ALG ($WR_{ALG/drug}$ = 0.72) and CMC ($WR_{CMC/drug}$ = 0.72).

Table IV.2 – Drug content % measured in phosphate buffer at pH 4.5 and drug fractional amount (Mt/M0) released from matrices after 360 min in phosphate buffers at pH 6.8 and 4.5 (mean ± SD, *n* = 3).

		Mt/M0 after 360 min	
Matrix	Drug content %	pH 6.8	pH 4.5
ALG_a	26 ± 9	0.50 ± 0.02	0.32 ± 0.03
CMC_a	27 ± 7	0.75 ± 0.10	0.53 ± 0.07
XG_a	34 ± 5	0.74 ± 0.01	0.47 ± 0.06
HYA_a	32 ± 8	0.96 ± 0.05	0.66 ± 0.02
ALG_b	58 ± 11	0.31 ± 0.07	0.24 ± 0.03
CMC_b	58 ± 13	0.38 ± 0.03	0.35 ± 0.09
XG_b	33 ± 3	0.40 ± 0.03	0.32 ± 0.07

IV.4.4 Scanning electron microscopy

SEM analysis were employed to assess the morphology of the freeze-dried matrices and determine its relation to water-uptake ability, *in vitro* drug release and mucoadhesion characteristics. As previously reported, a great porosity generally allows a greater ingress of water, which is expected to influence drug release and mucoadhesion ability [Kianfar 2013; Kianfar 2014]. *Figure IV.2* shows the morphology of the matrices observed by SEM. As a consequence of the water sublimation during the freeze-drying process, matrices obtained with both method "a" and "b" displayed a different interconnecting, porous network, reflecting the different polymeric composition and $WR_{polyanion/drug}$.

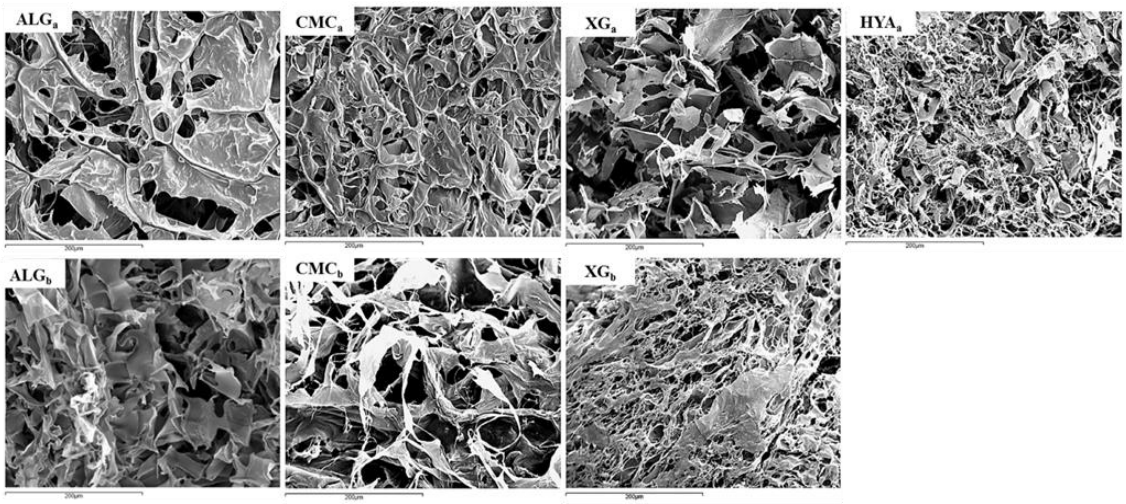


Figure IV.2 – SEM images (200×) for the cross-section of the freeze-dried matrices obtained with method "a" and "b".

IV.4.5 Differential scanning calorimetry

In order to evaluate solid state of matrices DSC was used. As reported in *figure IV.3*, the DSC patterns of matrices obtained with method "a" (*figure IV.3A*) and "b" (*figure IV.3B*) were characterized by a large endothermic peak around 60-70°C, related to the water loss and exothermic peaks at high temperatures between 230°C and 280°C, corresponding to polymeric decomposition process. No peaks related to CLX were observed indicating the presence of drug into the matrices in the amorphous state. This state should produce big advantages in terms of solubility and bioavailability [Murdande 2010].

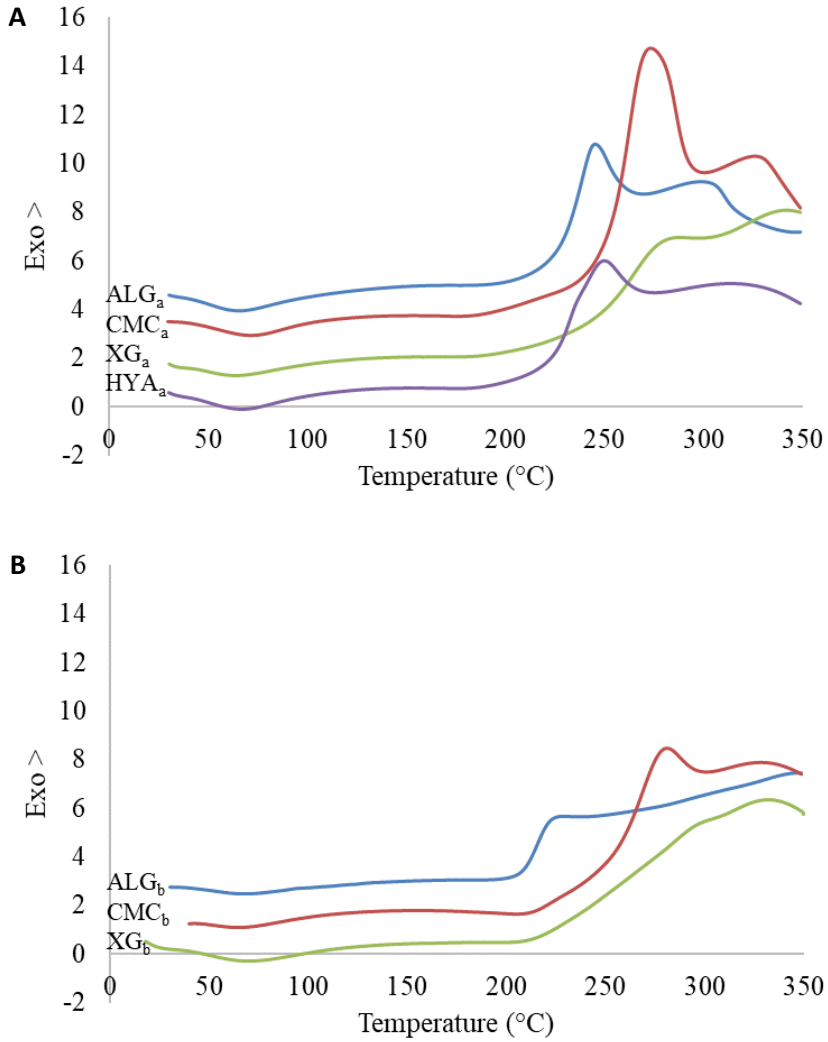


Figure IV.3 – DSC thermograms of freeze-dried matrices obtained with (A) method "a" and (B) method "b".

IV.4.6 Moisture content

Following freeze-drying process, all the formulations showed an acceptable moisture traces ranging from 1.0 to 4.7% (*table IV.1*) which confirmed the efficiency of drying step and its ability to remove water from the formulations [Hazzah 2015].

IV.4.7 Mechanical characterization

The data concerning resistance to compression stress of the matrices are reported in *table IV.3*.

Table IV.3 – Resistance to compression of freeze-dried matrices: σ33, compression stress at 33% strain; W, compressive work; H, hysteresis loop (mean ± SD, $n = 5$).

Matrix	σ_{33} (kPa)	W (mJ)	H	
			(mJ)	(%)
ALG_a	5.439 ± 0.454	0.495 ± 0.014	0.327 ± 0.022	65.951 ± 4.216
CMC_a	6.711 ± 1.048	0.481 ± 0.110	0.355 ± 0.088	73.703 ± 2.263
XG_a	0.550 ± 0.039	0.044 ± 0.004	0.025 ± 0.003	57.991 ± 0.436
HYA_a	3.071 ± 0.468	0.282 ± 0.056	0.158 ± 0.039	55.535 ± 0.856
ALG_b	1.097 ± 0.180	0.106 ± 0.042	0.072 ± 0.027	68.158 ± 1.601
CMC_b	1.074 ± 0.082	0.100 ± 0.003	0.063 ± 0.003	62.437 ± 1.130
XG_b	2.263 ± 0.964	0.155 ± 0.094	0.111 ± 0.067	72.692 ± 3.745

The σ_{33} values appeared more significantly influenced by the preparative method than the material used for the preparation of the matrix. Indeed, the rank order found in the case of formulations prepared by method "a" ($CMC_a \geq ALG_a > HYA_a > XG_a$, $p < 0.0001$) was not detectable in the case of the matrices prepared by the method "b", where the differences were not significant ($p > 0.05$). The difference noticeable in the case of CMC and ALG pairs could be justified considering that the isolation and resuspension of the polyanion/CLX complex led to a different organization of the polymeric freeze-dried network influencing the mechanical resistance. In other words, the presence of polymeric material non-complexed with CLX probably allowed the formation of a network with a higher interconnectivity. Moreover, the presence of free polymeric material caused a slight modification of the networks leading to a partial modification from a sponge-like structure towards the leaf-like structure as can be observed in the SEM images (*figure IV.2*). These features were not noticed in the case of XG formulations and the σ_{33} values obtained with this material appeared more related to the weight of the matrix (*table IV.1*). Moving the attention to the comparison of the forces registered during the load and unload step, the following considerations can be withdrawn. During the load step of the test, the matrices were forced to reorganize their inner structure in compacted and dense matter. If the physical organization of a matrix was too weak, their inner structure was stimulated to break. During the displacement step, the matrix was free to regain the lost structure and, presumably, the lost inner porosity. A hysteresis area, represented by the H-values,

is inevitable in the loading-displacement cycle owing to the dissipated energy for plastic deformation of the structure. On this basis, it is noteworthy that the H-values of HYA_a resulted significantly lower with respect to CMC_a ($p = 0.024$) and ALG_a ($p = 0.001$). Furthermore, the calculated hysteresis area of HYA_a expressed as a percentage was the lowest among all formulations ($p < 0.05$) suggesting that, even if the HYA_a was softer than ALG_a and CMC_a, it displayed a more flexible structure, which could be advantageous considering the patient handling and the possible movement of the mouth occurring over the application time.

IV.4.8 Water-uptake ability

Water-uptake studies were performed in order to evaluate matrix hydration ability that could influence drug diffusion and release. *Figure IV.4* shows WU % of matrices obtained with method "a" (*figure IV.4A*) and "b" (*figure IV.4B*) after 180 min at pH 6.8 and 4.5, simulating buccal and vaginal pH, respectively. Despite matrix morphology can generally affect water-uptake ability, in our study no close correlation between SEM results and hydration properties was observed and the water-uptake of the different formulations was mainly influenced by other factors, like the different $WR_{polyanion/drug}$ and the physical-chemical properties of polyanions.

ALG_a, CMC_a, XG_a and HYA_a (based on polyanion/CLX solid complex and free polyanion and free drug) were characterized by different hydration ability in relation to the yield of solid polyanion/drug complex: the higher yield of the solid complex, the lower amount of free polyanion in solution, the lower WU % of the freeze-dried matrix. In addition, HYA_a was characterized by the greatest water-uptake *in virtue* of its physicochemical properties, such as the molecular weight and the presence of a large number of hydroxyl and carboxyl groups, which provide hydrogen bond donors/acceptors [Schantéa 2011; Trastullo 2016]. Moreover, water-uptake values for all matrices at pH 6.8 were higher than at pH 4.5, accordingly with the pKa values of the polymers. This result suggests that matrix hydration could be favoured into the buccal environment characterized by higher pH with respect to the vaginal one.

In the case of ALG_b, CMC_b and XG_b (based only on polyanion/CLX solid complex) the hydration ability depended probably on the $WR_{polyanion/drug}$ in the complex: the higher $WR_{polyanion/drug}$, the higher amount of polyanion in the complex, the higher WU % of the freeze-dried matrix. In fact, XG_b presented a higher WU % with respect to ALG_b and CMC_b ($p < 0.05$) *in virtue* of the higher polyanion content (see section IV.4.3). Moreover,

no significant differences were observed between pH 6.8 and pH 4.5 ($p > 0.05$).

At both pH, water-uptake values of ALG_a and CMC_a were higher than of ALG_b and CMC_b, respectively ($p < 0.05$), due to the presence of free polyanion in the matrices. On the contrary, water-uptake of XG_b matrix was higher with respect to XG_a ($p < 0.05$) in relation to its higher weight (see *table IV.1*).

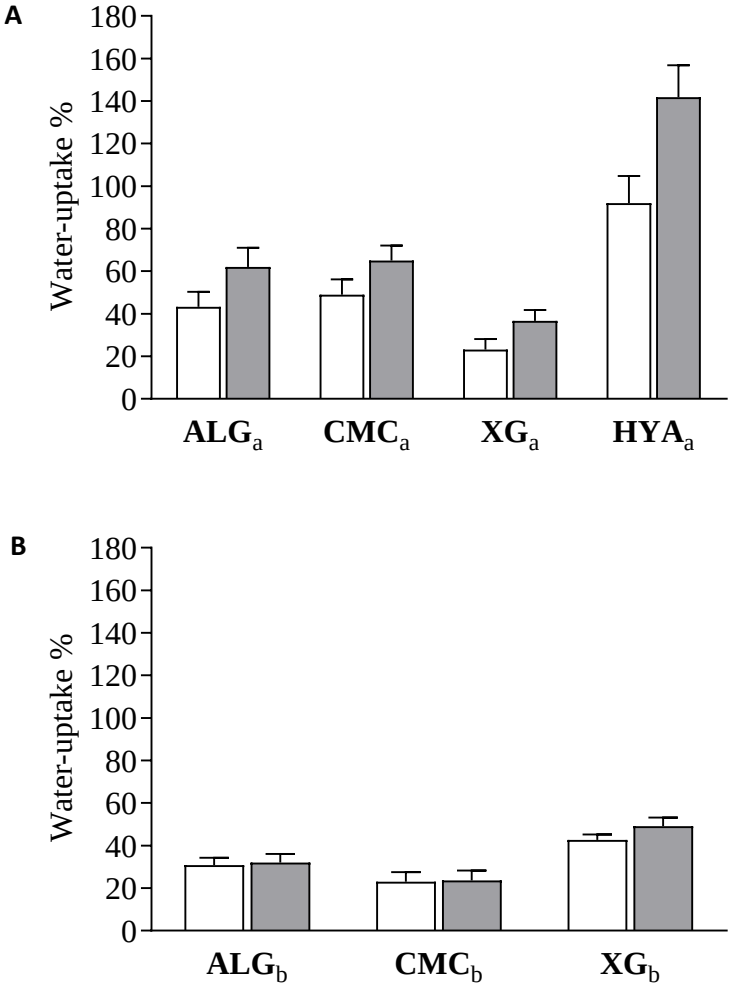


Figure IV.4 – Water-uptake % of matrices obtained with (A) method "a" and (B) method "b" after 180 min at pH 4.5 (white bars) and 6.8 (grey bars) (mean ± SD, $n = 3$).

IV.4.9 Mucoadhesion ability

In order to overcome the rapid removal from the buccal and vaginal cavities, mucoadhesive formulations could represent a promising approach. In fact, these formulations can be retained in the application site for a longer period, guarantying the drug effective dose over the time and consequently reducing the application number and improving the patient compliance. A prerequisite for the adhesion of the formulations on the mucosal membranes is their hydration that promote the formation of mucoadhesive

joints. Once administered into the oral or vaginal cavity, matrices must spread over the mucosa to initiate intimate contact. Then the chains of the mucoadhesive polymer have to interdiffuse into the mucus substrate to create a greater area of contact and have to establish attractive forces. A variety of factors affects the mucoadhesive properties of polymers, such as molecular weight, chain flexibility, charge, hydrogen bonding capacity, cross-linking density, and hydration ability. In particular, hydration ability is an important property that favours the interpenetration process between polymer and mucus [Salamat-Miller 2005].

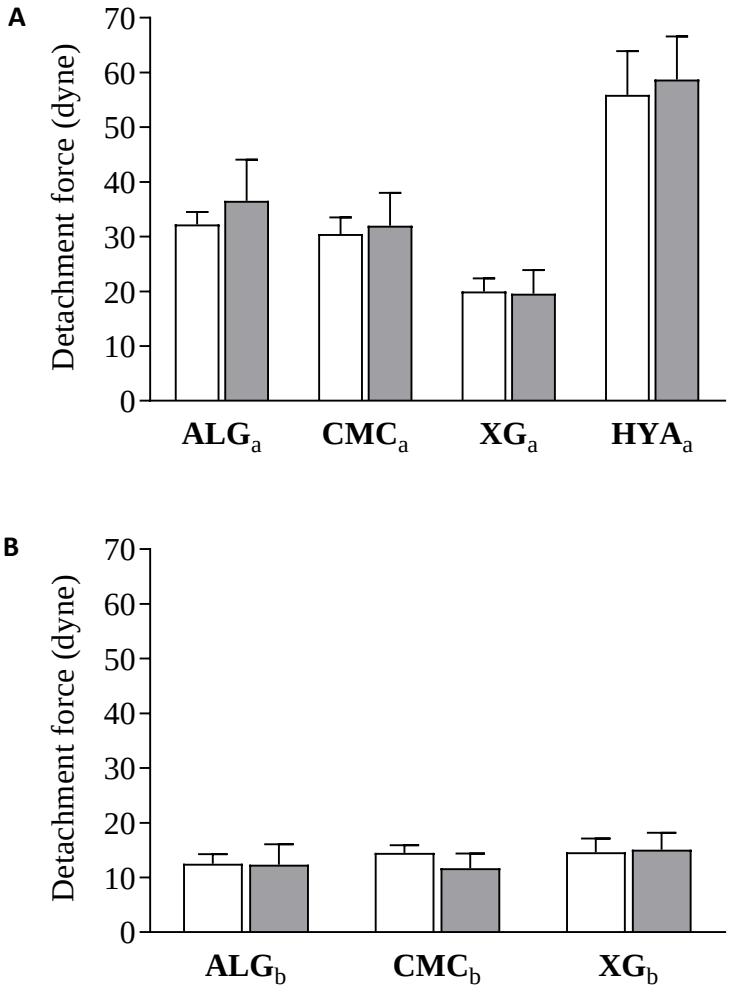


Figure IV.5 – Mucoadhesive capacity (expressed as detachment force) of matrices obtained with (A) method "a" and (B) method "b" at pH 4.5 (white bars) and 6.8 (grey bars) (mean ± SD, n = 3).

Mucoadhesion results are reported in *figure IV.5*. Coherently with the hydration properties, HYA_a, showing the highest hydration ability, was characterized by a higher mucoadhesion with respect the other matrices ($p < 0.05$), while XG_a, containing a lower amount of free polyanion, presented the lowest mucoadhesion (*figure IV.5A*).

On the contrary, matrices obtained with method "b" did not show significant differences

in our experimental conditions ($p > 0.05$), despite the higher WU values of XG_b with respect to ALG_b and CMC_b (*figure IV.5B*). Moreover, for all matrices detachment force at pH 6.8 was not significantly different from that obtained at pH 4.5 ($p > 0.05$).
Finally, at both pH ALG_b and CMC_b presented lower mucoadhesion abilities with respect to matrices "a" with the same polyanions ($p < 0.05$) in agreement with water-uptake results, while no significant difference was observed between XG_a and XG_b ($p > 0.05$).
In accordance with these results, HYA_a possessed the highest mucoadhesive capacity at both pH, and therefore it could be the best candidate to prolong the CLX permanence time in the buccal and vaginal cavities.

IV.4.10 *In vitro* release studies

Figures IV.6 and *IV.7* show *in vitro* release profiles of matrices obtained with method "a" and "b", respectively. Experiments were carried out both in phosphate buffers at pH 4.5 (*figures IV.6A* and *IV.7A*) and at pH 6.8 (*figures IV.6B* and *IV.7B*).
For matrices "a" (containing complexed and free CLX), the release behavior was widely affected by matrix hydration ability and content of free drug. In fact, HYA_a provided the release of a higher drug amount over the time with respect to CMC_a and ALG_a ($p < 0.05$), probably due to its higher water-uptake ability. This behavior was in agreement with the study of Boateng et al. in which it was observed a faster rate of drug release in relation to a faster hydration ability [Boateng 2009]. Despite the water-uptake results, XG_a provided a higher drug release with respect to ALG_a ($p < 0.05$), probably due to the presence of a higher amount of free drug. In fact, as reported in section IV.4.3, the higher $WR_{XG/drug}$ in the solid complex led to the presence of a higher amount of free drug into XG_a.
Table IV.2 reports the fractional amount of drug released from matrices after 360 min. As can be seen, drug fractional amounts released at pH 6.8 were higher than at pH 4.5 in agreement with WU results. In fact, accordingly to the different pKa of the polymers, at pH 6.8 matrices showed a greater ionization leading to a greater WU and consequently to an easier drug diffusion and release. In the case of matrices obtained with the method "b", no significant differences were observed between CMC_b, ALG_b and XG_b at both pH 4.5 and 6.8. Moreover, at both pH matrices obtained with method "a" were characterized by a greater amount of released drug over the time with respect to matrices "b" (*table IV.2*), due to the presence of free drug more available to be released. Considering these results, HYA_a could represent a suitable formulation able to provide the release of a greater amount of CLX in a prolonged manner in both buccal and vaginal cavities.

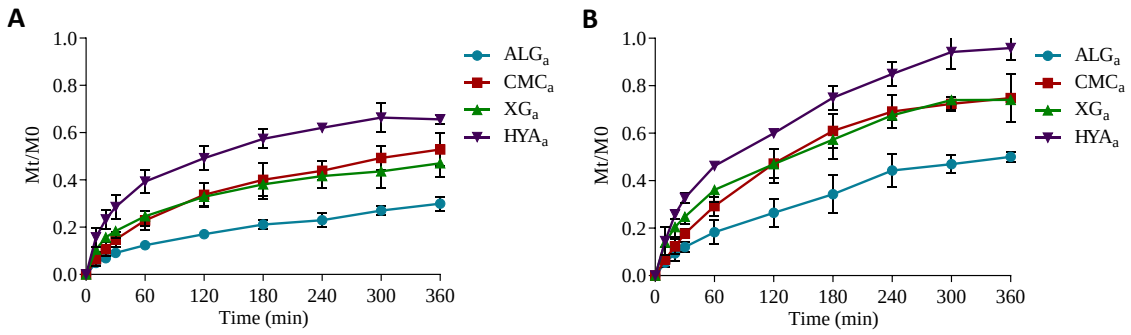


Figure IV.6 – *In vitro* release profiles of CLX from matrices obtained with method "a" in phosphate buffers at (A) pH 4.5 and (B) pH 6.8 (mean ± SD, *n* = 3).

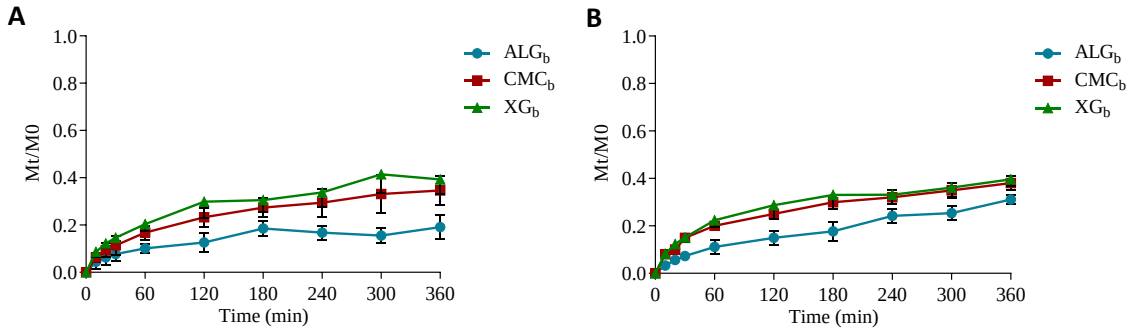


Figure IV.7 – *In vitro* release profiles of CLX from matrices obtained with method "b" in phosphate buffers at (A) pH 4.5 and (B) pH 6.8 (mean ± SD, *n* = 3).

IV.4.11 Antimicrobial studies

Viability of *S. mutans*, *E. coli* and *C. albicans* in phosphate buffer at pH 4.5 or 6.8 was compared with viability of the same bacteria and yeast strains in presence of free CLX and HYA$_a$, CMC$_a$ and CMC$_b$ (*table IV.4*).

For *S. mutans* and *E. coli*, no cell viability was found after 6 h of incubation in presence of free CLX and matrices, thus evidencing a maintenance of antibacterial activity by CLX when formulated in these matrices.

A different behavior was observed for *C. albicans*. CLX exerted a lower anti-*Candida* activity as compared to its antibacterial activity, determining a progressive, but not complete, reduction in the yeast viable count from T0 (5.68 log CFU/mL) to T48 (3.32 log CFU/mL). CMC$_a$ and CMC$_b$ maintained CLX activity showing similar patterns. Comparing the two CMC-based formulations, slight differences can be highlighted: CMC$_b$ elicited a greater reduction of *Candida* viability in the first 6 h (T0: 5.68 log CFU/mL; T6: 4.44 log CFU/mL; T24: 4.69 log CFU/mL; T48: 4.13 log CFU/mL),

whereas CMC_a showed a greater antifungal effect between T24 and T48 (T0: 5.68 log CFU/mL; T6: 5.45 log CFU/mL; T24: 5.61 log CFU/mL; T48: 3.29 log CFU/mL).

Among the selected matrices, HYA_a exerted the highest anti-*Candida* activity, determining a progressive reduction of *C. albicans* load up to the time point T24. After this time point no survival was observed (T0: 5.68 log CFU/mL; T6: 4.43 log CFU/mL; T24: <1 log CFU/mL; T48: <1 log CFU/mL). This result is of interest considering the documented activity of growth inhibition of high molecular weight HYA towards *C. albicans* [Sakai 2007].

The present microbiological data demonstrated that HYA_a exerts the best profile of antimicrobial activity, covering both bacteria and *Candida* strains, responsible for oral and vaginal infections.

Table IV.4 – Viability of *S. mutans* (phosphate buffer at pH 6.8), *E. coli* (phosphate buffer at pH 4.5), and *C. albicans* (phosphate buffer at pH 4.5) in absence of treatment (control) or with CLX, HYA_a, CMC_a and CMC_b (mean ± SD, $n = 3$).

Time	control	CLX	HYA_a	CMC_a	CMC_b
S. mutans					
T0	5.37 ± 0.19	5.37 ± 0.19	5.37 ± 0.19	5.37 ± 0.19	5.37 ± 0.19
T6	5.10 ± 0.17	< 1	< 1	< 1	< 1
T24	3.23 ± 0.47	< 1	< 1	< 1	< 1
T48	1.00 ± 0.10	< 1	< 1	< 1	< 1
E. coli					
T0	6.43 ± 0.10	6.43 ± 0.10	6.43 ± 0.10	6.43 ± 0.10	6.43 ± 0.10
T6	5.60 ± 0.05	< 1	< 1	< 1	< 1
T24	4.93 ± 0.24	< 1	< 1	< 1	< 1
T48	4.89 ± 0.15	< 1	< 1	< 1	< 1
C. albicans					
T0	5.68 ± 0.09	5.68 ± 0.09	5.68 ± 0.09	5.68 ± 0.09	5.68 ± 0.09
T6	5.40 ± 0.05	4.30 ± 0.02	4.43 ± 0.04	5.45 ± 0.04	4.44 ± 0.02
T24	5.79 ± 0.08	3.90 ± 0.17	< 1	5.61 ± 0.18	4.69 ± 0.17
T48	5.52 ± 0.07	3.32 ± 0.15	< 1	3.29 ± 0.11	4.13 ± 0.38

IV.5 Conclusions

Matrices based on HYA, ALG, CMC and XG were prepared by freeze-drying of polymer/CLX dispersions and by using two preparative methods. The selection of the polymer and the preparative method allowed to modulate matrix mechanical properties, ability to hydrate, to adhere to buccal or vaginal mucosa and to release the drug over the time. In particular, HYA_a showed a flexible structure, the best mucoadhesion properties and provided the release of a greater amount of drug over the time with respect to other formulations. Moreover, its mucoadhesion properties and ability to provide a prolonged drug release should guarantee a higher retention time of the drug at the application site, thus improving drug efficiency and consequently reducing administration frequency. Finally, HYA_a exerted the best profile of antimicrobial activity and could represent a versatile system for drug local release in both buccal and vaginal cavities.

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V. Novel mixed vesicles containing lactobacilli biosurfactant for vaginal delivery of an anti-*Candida* agent (*paper 3*)

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Published on European Journal of Pharmaceutical Sciences (doi: 10.1016/j.ejps.2017.11.012)

[Abruzzo & Giordani 2018].

V.1 Abstract

The purpose of this work was to prepare and characterize an innovative formulation for the vaginal delivery of econazole nitrate, commonly used for the treatment of *Candida* infections. A novel biosurfactant isolated from a vaginal *Lactobacillus* strain was used to prepare phosphatidylcholine based mixed vesicles. Biosurfactant was produced by *Lactobacillus gasseri* BC9, a probiotic strain isolated from the vagina of a healthy premenopausal woman, and was chemically characterized by FT-IR and ESI-MS.
Mixed vesicles, obtained through film rehydration and extrusion method, were characterized in terms of size, zeta potential, encapsulation efficiency, mucoadhesion properties and econazole release. Moreover, the antimicrobial activity of the mixed vesicles was tested towards both planktonic cultures and biofilms of *Candida albicans*. Biosurfactant produced by *L. gasseri* BC9 was composed by peptide-like molecules containing hydrocarbon chains and possessed a high surface activity together with a low critical micelle concentration. All the mixed vesicles presented optimal diameter range (226-337 nm) for topical vaginal administration. Econazole-loaded mixed vesicles containing biosurfactant showed higher encapsulation efficiency and mucoadhesion ability with respect to vesicles containing Tween 80. Further, they allowed a sustained release of econazole nitrate, maintaining the antifungal activity against *C. albicans* planktonic culture. Notably, biosurfactant-based vesicles were significantly more active than free econazole in the eradication of *Candida* biofilm. In conclusion, mixed vesicles are promising new vaginal delivery systems for the potential employment in the treatment of chronic infections.

Keywords
Lactobacillus; biosurfactant; mixed vesicles; vaginal delivery; antimicrobial activity; *Candida*.

List of abbreviations
BS: biosurfactant; EN: econazole nitrate; MV: mixed vesicles.

V.2 Introduction

The vulvovaginal candidiasis (VVC) is the most common and highly prevalent gynecological condition among reproductive-aged women. The VVC is generally caused by *Candida albicans* and its treatment involves the use of topical and oral antifungal azole medications [das Neves 2008].

Econazole nitrate is a broad-spectrum antifungal azole used to treat skin and vaginal infections caused by fungi. Chemically, econazole nitrate is 1-[2-[(4-Chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl] imidazole nitrate (MW: 444.7 g/mol); it shows a poor aqueous solubility which is considered a challenge in formulation process [Abd El-Gawad 2017].

In the last years, several delivery systems have been investigated for econazole delivery such as microemulsion, microemulsion based gel, solid lipid nanoparticles, mucoadhesive microspheres, sustained-release suppositories, microparticles, liposomes, vaginal tablets and douches [Kumar 2014]. In particular, nanosystems have been shown to play an important role for the treatment of biofilm-related vaginal infections and the use of lipidic vesicles have been proven to influence biofilm eradication due to their ability to interact with the microorganisms and deliver the entrapped drug to the cell membrane [Rukavina and Vanić 2016]. A biofilm is a thin layer of microorganisms that adhere to an organic or inorganic surface and are enveloped in an extra polymeric substance. Biofilms are considered as a prominent mode of microbial life in nature and disease [Costerton 1995]. *C. albicans* biofilms are intrinsically resistant to conventional antifungal therapeutics, the host immune system, and other environmental perturbations, making biofilm-based infections a significant clinical challenge [Nobile and Johnson 2015]. Considering the very low water solubility of econazole, its incorporation in vesicles of nanometric size may improve its solubility as well as its availability at the site of administration, thus improving the eradication efficacy towards *C. albicans* biofilm.

In a recent work, Vanić et al. investigated the potential use of mixed vesicles based on phospholipids and surfactants for vaginal administration of metronidazole and demonstrated that they were able to enhance drug permeability more effectively than the conventional liposomes, as shown in the *in vitro* model of the epithelial barrier [Vanić 2013]. Traditionally, chemically surfactants such as sodium cholate, sodium deoxycholate, Spans, Tweens and dipotassium glycyrrhizinate, were used for the preparation of mixed vesicles. However, in the recent years the growing awareness

towards the use of renewable-based products and "green products" has stimulated the development of alternatives to these chemical surfactants [Vaz 2012].

Biosurfactants (BS) are an example of such environmentally friendly options and can be obtained either by chemical synthesis from renewable resources, by microbial fermentation processes or by enzymatic syntheses. Comparing with chemical surfactants, these compounds have several advantages such as lower toxicity, higher biodegradability and effectiveness at extreme temperatures or pH values [Van Hamme 2006; Singh 2007].

The first part of this work describes the production of BS by *Lactobacillus gasseri* BC9, a strain isolated from the vagina of a healthy premenopausal woman exhibiting antimicrobial activities [Parolin 2015; Siroli 2017; Ñahui Palomino 2017]. *Lactobacillus* BS was characterized in terms of chemical structure and technological parameters. Subsequently, the study was focused on the preparation of loaded and unloaded mixed vesicles (MV), containing BS (BS-MV) or Tween 80 (Tween-MV) by the lipid film hydration method.

Vesicles were characterized in terms of size, zeta-potential, encapsulation efficiency, mucoadhesion properties and release of EN from MV. Moreover, the ability of MV to exert anti-*Candida* activity against both planktonic culture and biofilm was evaluated.

V.3 Materials and Methods

V.3.1 Materials

L-α-phosphatidylcholine from egg yolk, mucin (Type II: crude, from porcine stomach) and crystal violet and LC-MS grade methanol were purchased from Sigma-Aldrich (Milan, Italy). Tween 80 was provided from Fluka (Milan, Italy).

All other chemicals and solvents were of analytical grade and purchased from Carlo Erba (Milan, Italy). Econazole nitrate (EN) was provided from Erregierre S.p.A., San Paolo D'Argon (BG, Italy). The culture media and GasPak EZ were supplied by Becton Dickinson and Company (Sparks, MD, USA). L-Cysteine hydrochloride monohydrate was purchased from Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA).

Buffer solutions were prepared as follows: 2.38 g/L Na_2HPO_4; 0.19 g/L KH_2PO_4; 8 g/L NaCl for buffer at pH 7.4 (PBS); 13.61 g/L KH_2PO_4 (0.1 M) for buffer at pH 4.5; sodium acetate 0.1 M adjusted to pH 4.0 with acetic acid for acetate buffer used for drug quantification.

V.3.2 Microorganisms and culture conditions

L. gasseri BC9 was cultured in de Man, Rogosa and Sharpe (MRS) broth supplemented with 0.05% L-cysteine, at 37°C for 24 h in anaerobic jars containing GasPak EZ.

Candida strains were used as test microorganisms to evaluate the antimicrobial activity of loaded MV. *Candida* was cultivated in Sabouraud Dextrose (SD), aerobically for 24 h at 35°C. *L. gasseri* BC9 and all *Candida* strains used in this study were previously isolated from vaginal samples [Parolin 2015].

V.3.3 Biosurfactant production and isolation

To isolate cell-bound BS, the following protocol was pursued according to previously published methods for lactobacilli BS purification [Gudiña 2010; Sambanthamoorthy 2014]. Briefly, *L. gasseri* BC9 was cultured in 10 mL of MRS broth for 24 h, and cell pellet was harvested by centrifugation (10000 *g* for 10 min). Cells were washed twice in sterile water, re-suspended in 3 mL of PBS and gently stirred at room temperature for 2 h to release the cell-bound BS. Afterwards, the suspension was centrifuged and the supernatant was filtered through a 0.22 µm pore size filter. Cell-free supernatant was then subjected to dialysis against demineralized water in a Cellu-Sep© membrane (molecular

weight cut-off 6000-8000 Dalton; Spectra/Por 2 dialysis membrane Spectrum Laboratories Inc, USA) for 24 h at room temperature, and freeze-dried.

For bulk production of BS, 40 mL of an overnight culture of *L. gasseri* BC9 were inoculated in 400 mL of MRS broth and allowed to grow for 24 h. Cell-bound BS were recovered as described above, by resuspending cell pellet in 150 mL of PBS.

V.3.4 Chemical characterization of biosurfactant

V.3.4.1 Fourier transformed infrared spectroscopy

Infrared spectrum was recorded with a Jasco FT-IR 4100 spectrophotometer (Jasco, Lecco, Italy) in order to identify different types of chemical bonds (functional groups) of the isolated BS. Freeze-dried BS was gently triturated with KBr powder in a weight ratio of 1:10 and then pressed using a hydraulic press at a pressure of 100 tons for 5 min to prepare KBr discs containing BS. These discs were placed in the sample holder and scanned between 4000 and 450 cm^{-1}.

V.3.4.2 Mass Spectrometry (ESI-MS)

Mass spectrometric (MS) analysis was performed by using a Waters (Milford, MA, USA) Micromass Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ion source (ESI), working in both positive and negative ionisation mode.

Full scan MS spectra were acquired within a mass range of *m/z* 20-2000 (scan duration of 500 ms) using the following parameters: capillary voltage was set at 3.0 kV, while cone voltage was screened between 15 and 100 V; source temperature was 120°C and desolvation one 150°C; cone gas (N_2) flow was set to 50 L/h while desolvation gas (N_2) flow was 200 L/h. Sample were analysed in ESI+ and ESI- modes to provide satisfactory information.

Isolated BS methanolic solutions at the concentration of 10 µg/mL, before and after mild hydrolysis, were analysed by direct infusion into the ESI source by means of a Harvard Apparatus (Holliston, MA) 11 Plus programmable syringe pump set at a flowrate of 10 µL/min. Data processing was performed using Waters MassLynx 4.1 software.

V.3.5 Surface-activity determination and critical micelle concentration

The surface tension of solutions with different BS concentrations (0.3-4.0 mg/mL) was measured using the Ring method, as previously described [Kim 2000; Rodrigues 2006;

Gudiña 2010; Fracchia 2012].

For this study, a tensiometer (K8600E Krüss GmbH, Hamburg, Germany) equipped with a 1.9 cm platinum ring was used. Surface tension values (dyne/cm) represent averages of three independent measurements performed at room temperature. The critical micelle concentration (CMC) was determined by plotting the surface tension as a function of the logarithm of BS concentration and it is represented as the point at which the baseline of minimal surface tension intersects the slope where surface tension shows a linear decline.

V.3.6 Emulsification properties

A mixture of 1 mL of BS solution (1 mg/mL), 4 mL of water and 6 mL of oil (olive oil, sunflower oil and wheat germ oil) was vigorously shaken in graduated glass test tubes for 2 min to obtain maximum emulsification [Cooper and Goldenberg 1987]. After 24 h, the height of the emulsion was measured and emulsification index (EI_{24}) was calculated using the following equation:

EI_{24} = Height of emulsion layer × 100/ Total height

Water and Tween 80 (1 mg/mL) were used as negative and positive controls, respectively.

V.3.7 Mixed vesicles preparation

Mixed vesicles (MV) were prepared by the film rehydration and extrusion method described by Uchino et al. with some modifications [Uchino 2011]. Briefly, L-α-phosphatidylcholine (300 mg) and the surfactant (100 mg of Tween 80 or BS) were dissolved in a mixture of $CHCl_3$-CH_3OH (10 mL, 9:1 v/v) in a round-bottomed flask. The organic solvent was removed by rotary evaporation (Buchi Rotavapor R-200, Flawil, CH) under vacuum at 55°C for 2 h. Subsequently, the dry lipid film was hydrated with 40 mL of PBS for 1 h. The resulting suspension was extruded 12 times through a polycarbonate membrane with a pore size of 200 nm (LiposoFast manual syringe extruder, Avestin Europe GmbH, Mannheim, Germany). For loaded MV preparations, EN was solubilized in the organic phase (1 mg/mL). Unloaded and loaded formulations with Tween 80 (named Tween-MV and EN-Tween-MV, respectively) and unloaded and loaded formulation with BS (named BS-MV and EN-BS-MV, respectively) were obtained.

V.3.8 Determination of encapsulation efficiency

The percentage of EN incorporated into MV was determined by centrifuging the MV suspensions at 14500 rpm (Microspin 12, High-speed Mini-centrifuge, Biosan, Riga,

Latvia) for 15 min. The supernatant was diluted with methanol, stirred for 2 h and analyzed by HPLC. The % encapsulation efficiency (EE %) of drug was calculated as follows:

$$\% \text{ EE} = \frac{Total\ amount\ of\ drug - Amount\ of\ drug\ contained\ into\ the\ supernatant}{Total\ amount\ of\ drug} \times 100$$

EN was analyzed through HPLC-UV method. The chromatographic system was composed of a Shimadzu LC-10ATVP (Milan, Italy) chromatographic pump and a Shimadzu SPD-10AVPUV-Vis detector set at 272 nm. Separation was obtained on a PhenomenexSinergy Fusion-RP 80A (150 × 4.6 mm I.D., 5 μm) (Torrance, CA, USA) coupled to a Phenomenex Security Guard C18 guard cartridge (4 × 3.0 mm I.D., 5 μm). The mobile phase was composed of a mixture of acetonitrile-acetate buffer at pH 4.0 (70:30 V/V). The flow rate was 0.4 mL/min and manual injections were made using a Rheodyne 7125 injector with a 20 μL sample loop. Data processing was handled by a Shimadzu CromatoPlus computerized integration system. A calibration curve was set up in the 0.03-1.00 mg/mL range and a good linearity was found (R^2 = 0.9972).

V.3.9 Mixed vesicle size distribution and zeta potential

MV size distribution was measured by PCS (Photon-Correlation Spectroscopy) using an instrument (Brookhaven 90-PLUS) with He-Ne laser beam at a wavelength of 532 nm (scattering angle of 90°). MV suspensions were diluted (1:1000; v/v) in ultra-filtered water. Zeta potential measurements were performed at 25°C on a Malvern Zetasizer 3000 HS instrument, after the same dilution.

V.3.10 Stability studies

Stability studies of loaded MV were carried out in order to evaluate the aggregation of vesicles in phosphate buffer at pH 4.5, which simulate the pH of vaginal environment. One mL of each MV preparation was added to 4 mL of phosphate buffer at pH 4.5 or distilled water (used as comparison), gently mixed and left in contact for 6 h. Every hour, the resulting vesicle suspensions were diluted and analysed to determine the size distribution as reported in section V.3.9.

V.3.11 Mucoadhesion studies

Mucoadhesion properties were investigated through turbidimetric measurement of a suspension containing mucin and the formulation [Cerchiara 2015]. For this study, a

mucin dispersion (0.08 % w/v) was prepared. After stirring for 6 h, mucin dispersion was centrifuged at 7500 rpm (GS-15R Centrifuge, Beckman Coulter, Milan, Italy) for 20 min to separate excess amount of mucin. Mucin dispersion and MV suspension were then mixed at a 1:4 volume ratio and vortexed for 1 min. The turbidity of the samples was measured at 650 nm (UV-Visible Spectrophotometer, Shimadzu Corporation, Australia). The absorbance (ABS) of mucin dispersion itself and MV suspension without mucin were also measured as references.

V.3.12 *In vitro* drug release studies

For these studies, MV suspensions (containing unentrapped and entrapped drug) were placed in phosphate buffer at pH 4.5, under sink conditions. The system was put in a shaking water bath at 37°C with an agitation speed of 100 rpm. At predetermined time intervals (up to 6 h), aliquots of the sample were taken from the medium, centrifuged at 14500 rpm for 15 min and the supernatant was analyzed through HPLC (as reported in section V.3.8). Drug release over time was determined as Mt/M0 (fractional amount), where Mt represents the amount of EN released at each time and M0 the total EN mass loaded into the MV.

V.3.13 Anti-*Candida* activity

V.3.13.1 Preliminary evaluation of activity against *Candida* spp.

The anti-*Candida* activity of MV formulations was initially evaluated against 2 *Candida albicans* strains (named *C. albicans* 1 and 2) and 2 non-*albicans* strains (*C. krusei* and *C. tropicalis*) [Parolin 2015] by using the disk-diffusion agar method. The following samples were tested: (i) free EN (1 mg/mL, as in loaded MV); (ii) EN-Tween-MV (containing unentrapped and entrapped drug); (iii) EN-BS-MV (containing unentrapped and entrapped drug). Solutions of Tween 80 and BS (2.5 mg/mL, as in MV), as well as unloaded MV, were also tested as controls.

Paper disks (6 mm in diameter) were imbued with 20 µL of the sample and placed on SD agar plates, on which 100 µL of each *Candida* suspensions (10^6 CFU/mL) were previously spread. Plates were aerobically incubated at 35°C and the diameters of inhibition zones were measured after 24 h.

Since *C. albicans* is the most isolated species in women affected by VVC, *C. albicans* 1 (in the following sections simply referred to as *C. albicans*) was chosen to further investigate the antifungal effect of loaded MV.

V.3.13.2 Inhibitory activity against *C. albicans* planktonic culture

The anti-*Candida* activity towards planktonic cultures was evaluated according to the method of Albertini et al., with slight modifications [Albertini 2009]. Briefly, *C. albicans* was grown in culture media alone or together with EN (50 μg/mL) in the following form: (i) free EN; (ii) EN-Tween-MV (containing unentrapped and entrapped drug); (iii) EN-BS-MV (containing unentrapped and entrapped drug).

SD medium and simulated vaginal fluid (SVF) were used as culture media (volume: 10 mL).

SVF was prepared as previously reported [Owen and Katz 1999]: 3.51 g/L NaCl, 1.40 g/L KOH, 0.222 g/L $Ca(OH)_2$, 0.018 g/L bovine serum albumin, 2 g/L lactic acid, 1 g/L CH_3COOH, 0.16 g/L glycerol, 0.4 g/L urea and 5 g/L glucose. The pH was corrected at 4.2 with HCl 0.1N.

A yeast suspension, prepared from a broth culture in log phase of growth, was used to inoculate the Erlenmeyer flasks containing SD (T0 concentration: 10^5 CFU/mL) or SVF (T0 concentration: 10^6 CFU/mL). Counts of viable *C. albicans* cells were carried out on SD agar plates at the inoculum time (T0) and after 6 h (T6), 24 h (T24) and 48 h (T48) of incubation at 35°C. Plates were then aerobically incubated at 35°C for 24 h.

V.3.13.3 Eradication of *C. albicans* biofilm

To evaluate the ability of *Lactobacillus* BS to disperse preformed biofilms of *C. albicans*, the following procedure was used. The wells of a sterile 96-well flat-bottomed plastic plate were filled with 200 μL of a 10^6 CFU/mL yeast suspension in SD medium.

Candida biofilms were established at 35°C under gentle shaking conditions (50 rpm). After 72 h, the medium was removed and the biofilms were gently washed with PBS. Biofilms were subsequently treated for 48 h with EN at three different concentrations in PBS (50 μg/mL, 500 μg/mL and 1000 μg/mL) in the following forms: (i) free EN; (ii) EN-Tween-MV (containing unentrapped and entrapped drug); (iii) EN-BS-MV (containing unentrapped and entrapped drug). As control experiments, BS and Tween 80 alone were also tested for their ability to eradicate *Candida* biofilm.

Adherent biofilms were fixed with 200 μL of 99% ethanol for 15 min prior to staining for

2 min with 200 μL of 2% (w/v) crystal violet in 12% ethanol. Excess stain was rinsed out by washing the multi-well plate with PBS for three times. Subsequently, the plate was air dried, the dye bound to the adherent microorganisms was resolubilized with 200 μL of 33% (v/v) ethanol and the ABS was measured at 595 nm using EnSpire Multimode Plate Reader.

The eradication percentages were calculated as:

% Eradication = $[1- (OD_T / OD_C)] \times 100$

where OD_T represents the optical density of the well subjected to a treatment and OD_C is the optical density of the control well.

V.3.14 Statistical analysis

All the experiments were performed at least in triplicate. Results are expressed as mean ± standard deviation (SD). Student's t-test was used to determine statistical significance of studies. Differences were considered significant for values of $p < 0.05$.

V.4 Results and discussion

V.4.1 Chemical characterization of biosurfactant

V.4.1.1. Fourier transformed infrared spectroscopy

Figure V.1 reports the FT-IR spectrum of BS. The absorbance maxima recorded at wavelengths of 3413 is typical of the stretching of N-H bond. The absorption peaks at 1639 and 1617 cm^{-1} correspond to the stretching mode of the CO-N bond and to the stretching of C=C bond, respectively; the peak at 1544 cm^{-1} is related to the deformation mode of the N-H bond combined with C-N stretching mode. In addition, it is also clear the presence of aliphatic chains ($–CH_3$, $–CH_2–$), represented by the bands at 2926 cm^{-1}, 1460 cm^{-1} and 1384 cm^{-1} [Sharma 2014].

The FT-IR spectrum demonstrated that the chemical composition of BS produced by *L. gasseri* BC9 is non-homogeneous and is characterized by peptide-like molecules containing hydrocarbon chains.

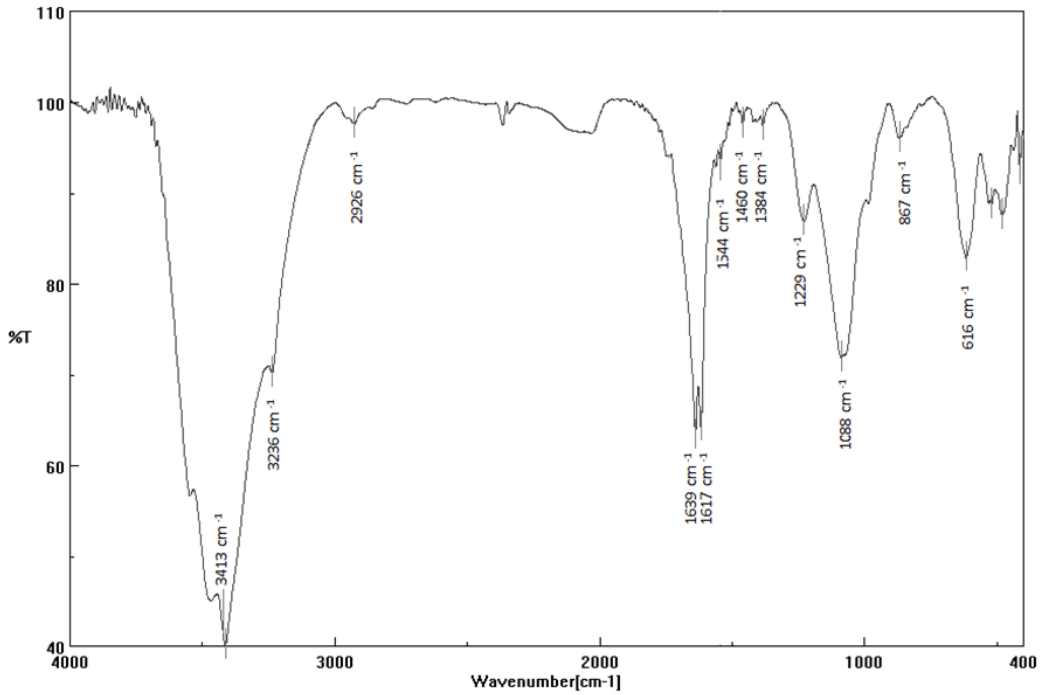

Figure V.1 – FT-IR spectrum of BS produced by *L. gasseri* BC9.

V.4.1.2 ESI-MS analysis

BS produced by *L. gasseri* BC9 strain were analysed through ESI-MS direct infusion in both positive and negative full scan mode.

In ESI- mode, no significant peaks were observed under any of the screened parameters. This would suggest the absence of chemical entities having organic acid functions, such

as glycolipids (mainly rhamnolipids), which show intense negative signals referred to pseudomolecular ions in the 200-700 *m/z* range.

In ESI+ mode, $[M-H]^+$ molecule-ion peaks appeared between *m/z* 988 and 1044 in ESI full MS scan. The respective, $[M-Na]^+$ sodium adduct peaks were observed between *m/z* 1010 and 1066. Each molecule-ion peaks mass differed by 14 (equal to the mass of $[CH_2]$), thus suggesting the components might be homologues depending on different lengths of fatty acids chains.

After mild hydrolysis, MS spectra showed lower mass peaks compatible with the partial loss of aminoacids, namely histidine, valine and threonine.

V.4.2 Surface-activity determination and critical micelle concentration

An important property of BS is the potential to reduce the surface tension of liquids. *Figure V.2* shows that the surface tension decreased with the increase of BS concentration. For BS concentrations higher than 2 mg/mL no significant surface tension modification was observed.

A CMC value of 2 mg/mL was found, which is in agreement with previous studies concerning biosurfactants isolated from other *Lactobacillus* strains [Sharma 2014]. Moreover, the CMC determined in this study is close to the CMC of synthetic sodium dodecyl sulfate (1.8-2.9 mg/mL) that is known for its strong ability in lowering the surface tension (from 72 to 37 dyne/cm) [Mulligan and Gibbs 2004].

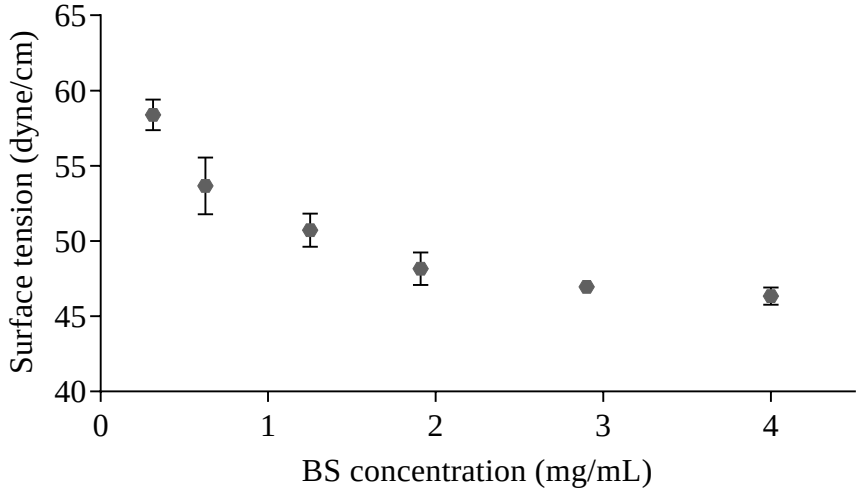

Figure V.2 – Surface tension (dyne/cm) as a function of concentration (mg/mL) of BS produced by *L. gasseri* BC9 (mean ± SD, *n* = 3).

V.4.3 Emulsification properties

The emulsification properties of the produced BS (1 mg/mL) were evaluated against different substrates like olive oil, sunflower oil and wheat germ oil. Tween 80 was used as a reference control. Emulsion stabilizing capacity can be defined as the ability to maintain at least 50% of the original emulsion volume 24 h after its formation [Das 1998]. In all cases, EI_{24} of BS was higher than 50% and only slightly lower than that measured for Tween 80 ($p < 0.05$), thus indicating the good emulsification properties of this BS isolated from a *Lactobacillus* strain (*Figure V.3*).

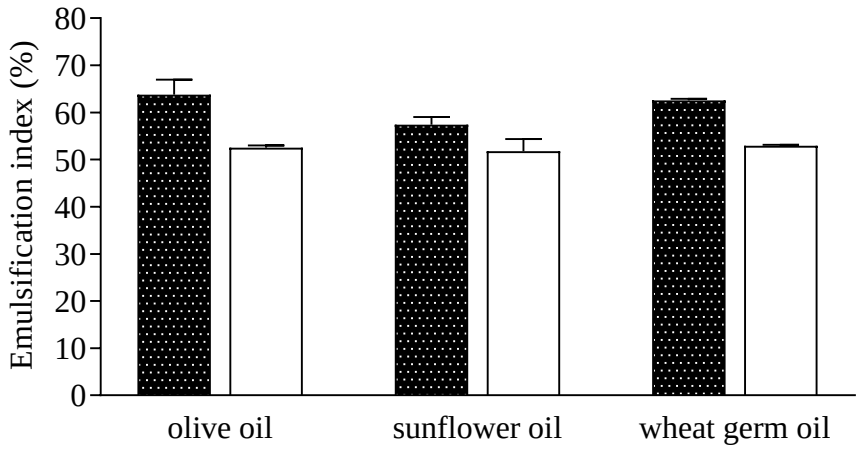

Figure V.3 – Emulsification index at 24 h of Tween 80 (black bars) and BS produced by *L. gasseri* BC9 (white bars), calculated on different substrates (olive oil, sunflower oil and wheat germ oil) (mean ± SD, $n = 3$).

V.4.4 Determination of encapsulation efficiency

The desirable therapeutic effect of MV as drug carriers can be achieved if they are loaded with a sufficient amount of an active compound. Therefore, suitable encapsulation efficiency (EE %) of drugs is required. The EE % of EN inside the vesicles was 73.77 ± 6.57 % and 89.92 ± 4.60 % for EN-Tween-MV and EN-BS-MV, respectively.

The higher EE % obtained in the case of EN-BS-MV with respect to EN-Tween-MV ($p < 0.05$) can be attributed to the presence of BS that provide a more favourable phospholipid bilayer environment or possible interactions with the drug [Cogswell 2006].

V.4.5 Mixed vesicle size distribution and zeta-potential measurements

Table V.1 reports the characteristics of MV, loaded or unloaded with the drug.

No significant difference was observed between size of unloaded and loaded formulations, and all the MV presented optimal diameter range (226-337 nm) for topical

vaginal administration, accordingly to literature where nanocarriers in the size range of 200-500 nm are described as more performant to both much smaller and larger nanosystems [das Neves 2011a; das Neves 2011b; Rukavina and Vanić 2016].

EN-BS-MV showed higher mean diameter with respect to EN-Tween-MV ($p < 0.05$), probably due to the entrapment of a greater amount of drug (see section V.4.4).

Moreover, all the prepared formulations had a good polydispersity index (PDI), thus confirming that the extrusion could be a suitable method to obtain MV with a homogenous distribution. All the MV exhibited a negative zeta potential. Several authors reported the formation of slightly negative vesicles based on L-α-phosphatidylcholine, that is a neutral zwitterion at physiological pH [Perttu 2012]. The presence of the BS in the unloaded MV (BS-MV) did not provide a significant difference in the zeta potential compared to Tween-MV. Notably, EN-BS-MV showed a more negative zeta potential value with respect to EN-Tween-MV ($p < 0.05$). This behaviour can be probably attributed to the higher loading of EN, that presented a negative charge at physiological pH (pKa = 6.6) [Verma and Pathak 2012]. Since all the measured zeta potential values are near or above the theoretically appointed 30 mV limit required for stability, it would expect that the prepared MV have sufficient charge to avoid aggregation due to electrostatic repulsion [Budai 2013].

Table V.1 – Vesicle size, polydispersity index (PDI) and zeta potential of EN loaded and unloaded MV containing Tween 80 or BS (mean ± SD, $n = 3$).

	Vesicle size (nm)	PDI	Zeta potential (mV)
Tween-MV	329.9 ± 82.0	0.303 ± 0.006	-34.9 ± 0.9
EN-Tween-MV	260.2 ± 12.4	0.313 ± 0.054	-29.2 ± 2.3
BS-MV	318.6 ± 55.8	0.352 ± 0.002	-32.7 ± 1.3
EN-BS-MV	337.2 ± 16.0	0.305 ± 0.022	-44.3 ± 0.8

V.4.6 Stability studies

The stability of EN-Tween-MV and EN-BS-MV was evaluated in terms of size and PDI (*figure V.4*) in distilled water and in phosphate buffer at pH 4.5 for a period of 6 h.

As can be noticed in *figure V.4A*, dimension distribution of EN-BS-MV did not varied over time in distilled water, while in the same conditions mean diameter of EN-Tween-MV slightly increased after 2 h ($p < 0.05$). This result can be correlated with the zeta potential values of MV.

Indeed, as reported in section V.4.5, the surface charge of EN-BS-MV in distilled water was more negative than that of EN-Tween-MV, thus limiting the aggregation of the vesicles.

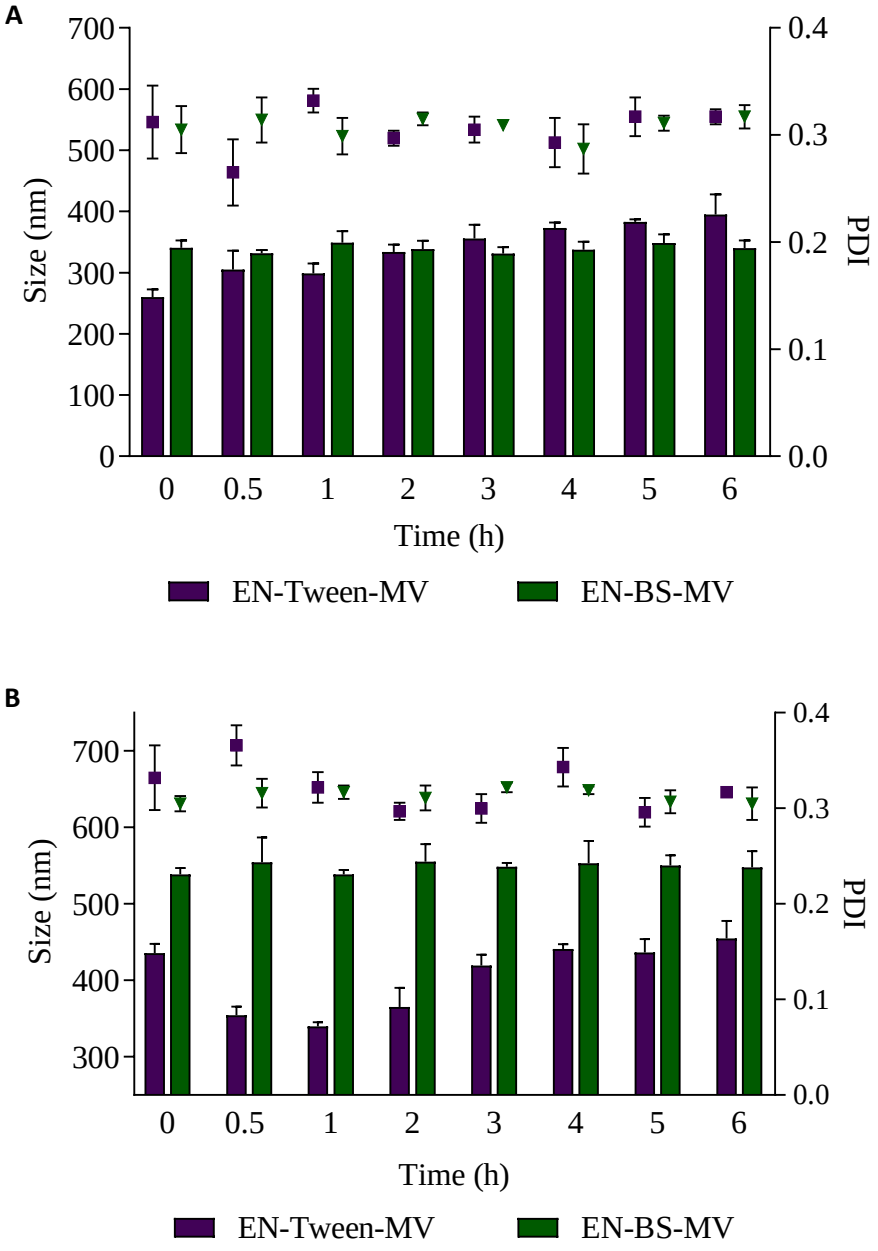

Figure V.4 – Variations of MV size (bars, left Y axis) and PDI (symbols, right Y axis) over a period of 6 h in (A) distilled water and (B) phosphate buffer at pH 4.5 (mean ± SD, $n = 4$).

On the other hand, *figure V.4B* shows that EN-BS-MV were considerably bigger in phosphate buffer at pH 4.5 than in distilled water ($p < 0.05$), even if the dimensions appeared to be stable over time and are still inside the optimal range for vaginal administration. Considering that BS possess a peptide-like structure, the change in pH and ionic strength probably affect the ionization of the molecule and, consequently, the

dimensions of the vesicles. The size of EN-BS-MV was, in fact, equal to 340.8 ± 11.8 nm in distilled water and 538.4 ± 8.3 nm in phosphate buffer at pH 4.5 (immediately after dilution).

A smaller increase in size was instead observed for EN-Tween-MV immediately after dilution in phosphate buffer at pH 4.5 (435.7 ± 12.0 nm).

However, as previously observed for distilled water, size and PDI of EN-Tween-MV fluctuated over time, thus resulting not completely stable.

V.4.7 Mucoadhesion studies

Prolonged residence time in the vaginal cavity is an important requirement in order to maintain drug-loaded formulations in full contact with the vaginal mucosa and to retain them for a long period at the site of application [Vanić 2013]. For this reason, the interaction between vesicles and mucin is desirable in order to improve the potentiality of the prepared formulations.

Mucoadhesion studies were performed by measuring the turbidity at 650 nm of MV in the presence or not of mucin. The ABS measured for aqueous dispersions of MV was due to scattering.

In the presence of mucin, an increase of the ABS % (percentage increase of the sample ABS in the presence of mucin with respect to the same samples without mucin) was observed (*figure V.5*). In particular, EN-BS-MV presented a significant increase of the ABS % with respect to EN-Tween-MV ($p < 0.05$), thus demonstrating a greater interaction with mucus components.

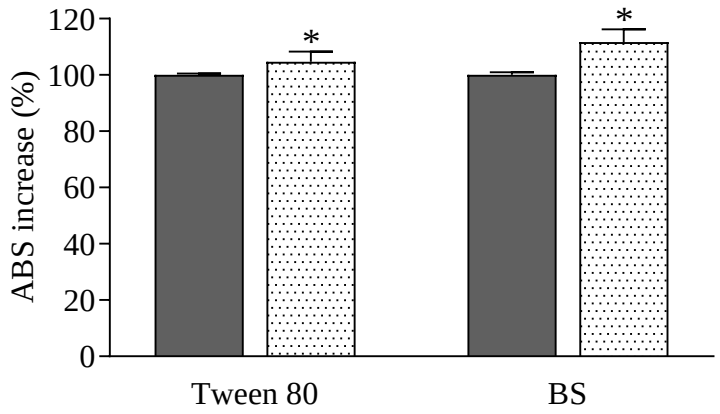

Figure V.5 – Percentage increase of the absorbance (ABS %) at 650 nm of EN-MV containing Tween 80 or BS in absence (grey bars) or presence of mucin (white bars) (mean ± SD, $n = 5$). The statistical significance was calculated with respect to EN-MV in absence of mucin; * $p < 0.05$.

V.4.8 *In vitro* drug release studies

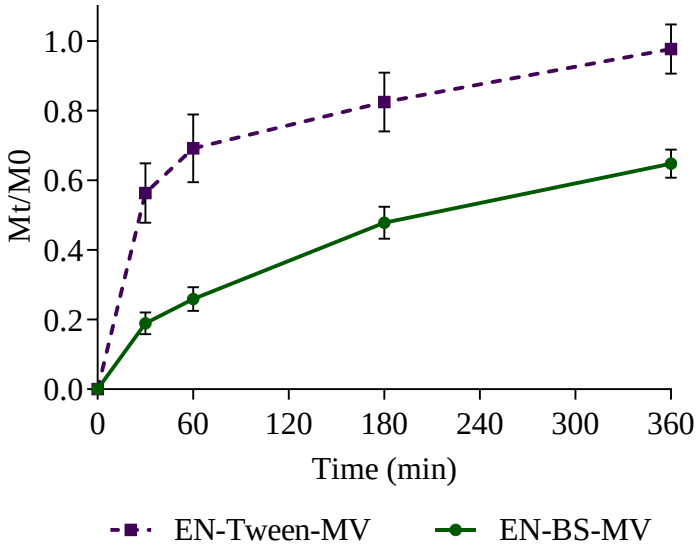

Figure V.6 – Fractional amount (Mt/M0) of EN released over time from EN-BS-MV and EN-Tween-MV in phosphate buffer at pH 4.5 (mean ± SD, *n* =3).

The effectiveness of a drug delivery system at the mucosal site depends on its distribution and retention on the mucosal surface as well as on the release behavior of the drug [Vanić and Škalko-Basnet 2013].

Figure V.6 shows the cumulative percentage of EN released from MV as a function of time. In the case of EN-Tween-MV approximately 69% of drug was released in the first hour. On the contrary, EN-BS-MV provided a sustained release of the encapsulated drug. This may be attributed to a greater affinity of the drug for the bilayer of BS-MV and the possible interaction of EN with the BS.

V.4.9 Anti-*Candida* activities

V.4.9.1 Activity against *Candida* spp.

Table V.2 reports the diameters of inhibition zones obtained for free EN, EN-Tween-MV and EN-BS-MV. EN was able to exert a strong activity against both clinically isolated *C. albicans* and non-*albicans* strains. In all cases, free EN provided a significantly higher reduction of *Candida* growth with respect to loaded MV ($p < 0.05$). This result is probably a consequence of the release behavior of entrapped drug (see section V.4.8). Indeed, since MV slowed the release of EN, not all the drug was immediately available when the disks where applied on plates, thus leading to an apparent reduction in activity. For this reason,

to better clarify the anti-*Candida* effects of MV, other experiments on *Candida* planktonic cultures were performed (see section V.4.9.2).

No significant differences were instead recorded between EN-BS-MV and EN-Tween-MV. Moreover, Tween 80, BS and unloaded MV failed to inhibit *Candida* growth, indicating that the observed antifungal effects were only due to EN.

Table V.2 – Evaluation of the antifungal activity of free EN, loaded and unloaded MV, Tween 80 and BS against 4 *Candida* strains. The results were expressed as diameter of the zone of inhibition (mean ± SD, *n* = 3). na: not active.

	Zone of inhibition (mm)			
	C. albicans 1	*C. albicans* 2	*C. krusei*	*C. tropicalis*
free EN	36.00 ± 1.15	29.50 ± 1.91	24.75 ± 0.96	28.50 ± 0.58
Tween-MV	na	na	na	na
EN-Tween-MV	27.75 ± 2.06	19.00 ± 4.69	13.33 ± 1.83	16.50 ± 2.38
BS-MV	na	na	na	na
EN-BS-MV	29.75 ± 1.50	19.50 ± 5.20	13.25 ± 0.96	17.00 ± 3.16
Tween 80	na	na	na	na
BS	na	na	na	na

V.4.9.2 Inhibition of planktonic cultures

The susceptibility of *C. albicans* planktonic culture to free EN, EN-Tween-MV and EN-BS-MV is depicted in *figure V.7A-B. Figure V.7A* describes the killing curves in SD medium. In the control culture (*C. albicans* without antimycotic), cell concentration increased over time (T0 = 5.68 log CFU/mL; T24 = 8.19 log CFU/mL; T48 = 8.02 log CFU/mL). The fungicidal effect of free EN was already evident after 6 h of treatment as the yeast concentration was reduced by an order of magnitude (T6 = 4.85 log CFU/mL). After 24 h no viable *Candida* cells were detected. The killing curves of *C. albicans* in presence of EN formulated in MV (EN-Tween-MV and EN-BS-MV) were similar to each other. In both cases a lack of killing activity in the first 6 h was observed (EN-Tween-MV: T6 = 5.73 log CFU/mL; EN-BS-MV: T6 = 5.86 log CFU/mL), but a complete depletion of *Candida* cells was registered after 24 h of incubation.

Figure V.7B reports the killing curves in SVF. *C. albicans* was able to grow in SVF as demonstrated by the increase of cell concentration over time, especially in the first 6 h (T0 = 6.12 log CFU/mL; T6 = 6.75 log CFU/mL; T24 = 6.87 log CFU/mL; T48 = 6.90 log CFU/mL). As expected, *Candida* growth in SVF was lower than that observed in SD

medium. Free EN caused a progressive killing of *Candida* up to a complete depletion after 48 h (T6 = 5.44 log CFU/mL; T24 = 3.95 log CFU/mL). Compared to free EN, EN-Tween-MV and EN-BS-MV exhibited a delayed antiproliferative effect similar to that observed in SD medium. In fact, fungicidal activity was observed only after 24 h and was complete after 48 h (EN-Tween-MV: T6 = 6.52 log CFU/mL, T24 = 4.30 log CFU/mL; EN-BS-MV: T6 = 6.45 log CFU/mL, T24 = 4.43 log CFU/mL).

These results demonstrate that the insertion of EN into MV allows maintaining antifungal activity against *C. albicans* planktonic cultures with a delayed effect, consistent with the time requested for the release of the active principle from the formulation. It is worth noting that MV containing *Lactobacillus* BS have the same activity profile as MV containing Tween.

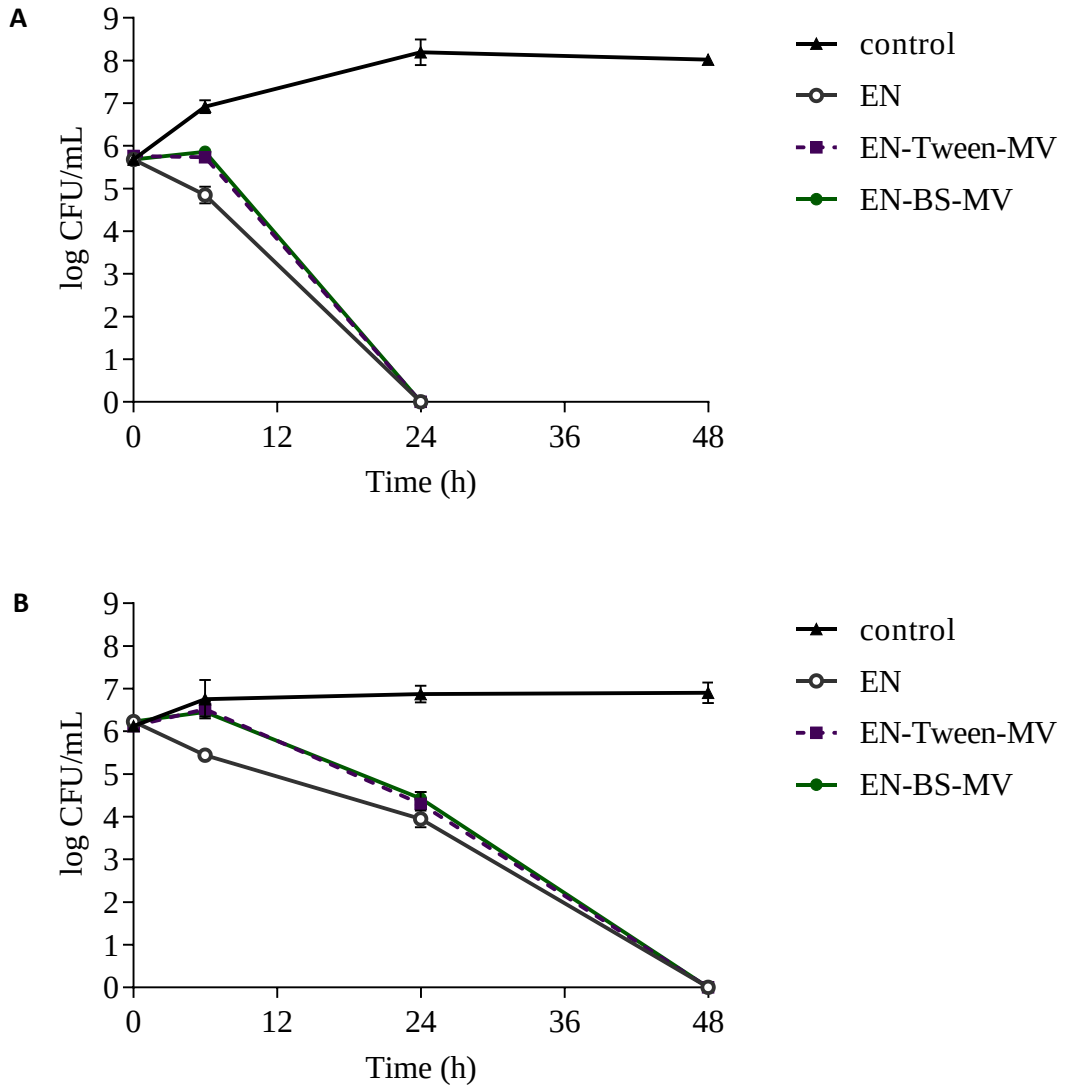

Figure V.7 – Viability (log CFU/mL) of *C. albicans* incubated in (A) SD medium or in (B) SVF in presence of EN in different forms: free EN, EN-Tween-MV and EN-BS-MV (mean ± SD, *n* = 3).

V.4.9.3 Biofilm eradication

The capability of EN (free EN, EN-Tween-MV and EN-BS-MV) to eradicate *C. albicans* biofilm is depicted in *figure V.8*. The concentration of 50 μg/mL of EN was active neither in the free form nor in both types of MV. An increase in EN concentration resulted in an improvement of anti-biofilm activity. Treatments with free EN at concentrations of 500 μg/mL and 1000 μg/mL caused a biofilm eradication of 28.98% and 43.60%, respectively. Interestingly, the formulation of EN in MV significantly increased the eradication rate ($p < 0.005$). Tween-MV loaded with EN 500 μg/mL (EN_{500}-Tween-MV) and EN 1000 μg/mL (EN_{1000}-Tween-MV) resulted in biofilm eradication of 52.48% and 79.09%, respectively. BS-MV loaded with EN 500 μg/mL (EN_{500}-BS-MV) and EN 1000 μg/mL (EN_{1000}-BS-MV) dispersed *Candida* biofilm at percentages of 68.03% and 86.47%, respectively. BS and Tween 80 alone were not active in biofilm dispersion at the concentration used in the preparation of MV (2.5 mg/mL).

The present data clearly indicate the potential of MV to improve the antifungal profile of EN, especially towards *Candida* biofilm. The increased anti-biofilm activity of EN formulated in MV might be attributed to a more efficient penetration of lipophilic MV into the extracellular matrix of the biofilm [Rukavina and Vanić 2016].

Notably, MV containing BS were more active in biofilm eradication than MV containing Tween ($p < 0.005$), opening up the intriguing perspective to use BS-based MV to deliver antibiotics for the treatment of chronic infections sustained by biofilms of pathogens.

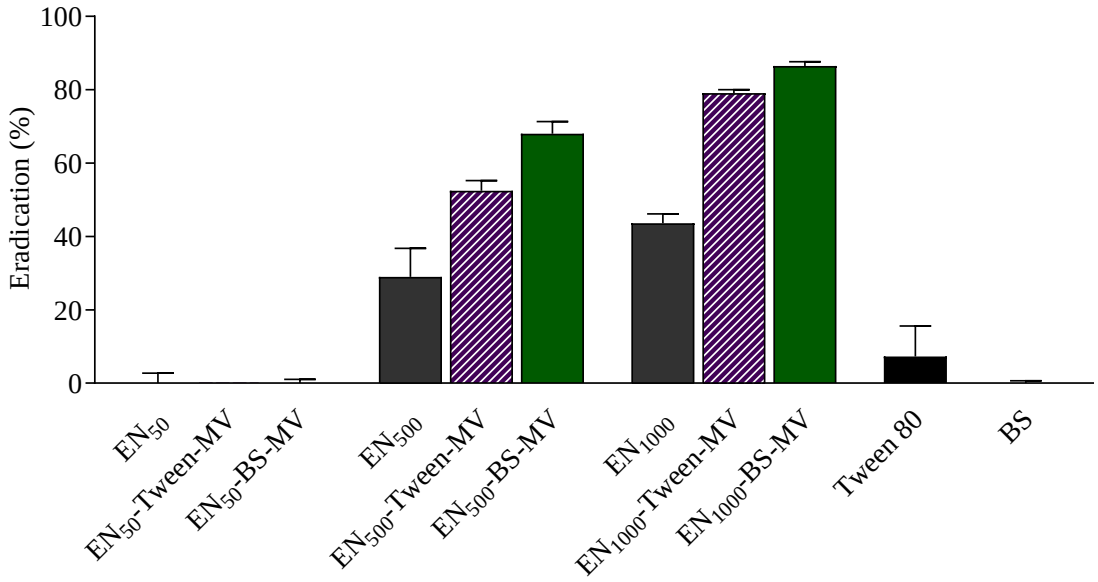

Figure V.8 – Capability of EN, EN-Tween-MV and EN-BS-MV at different concentrations of drug (50 μg/mL, 500 μg/mL, 1000 μg/mL) to eradicate *C. albicans* biofilm. Results are expressed as eradication percentage (mean ± SD, $n = 3$).

V.5 Conclusions

BS produced by *L. gasseri* BC9 was composed by peptide-like molecules containing hydrocarbon chains and possessed a high surface activity together with a low critical micelle concentration. MV presented a diameter in the range of 226-337 nm. Compared to EN-Tween-MV, EN-BS-MV showed higher size and a lower zeta-potential value, greater encapsulation efficiency and mucoadhesion ability along with a sustained release of EN over time. Finally, the antimicrobial tests demonstrated an improvement of the antifungal activity of EN formulated in the MV. In particular, EN-BS-MV could almost completely eradicate *Candida albicans* biofilm.

In conclusion, EN-BS-MV could be considered a promising econazole delivery system since it assured not only the mucoadhesiveness but also the prolonged drug release. This is of great importance considering the vaginal administration. In fact, a reduced frequency of administration can result in better patient compliance. In addition, EN-BS-MV showed the best profile in the dispersion of *Candida* biofilm. This finding opens the perspective to use *Lactobacillus* BS as green ingredients for the formulation of mixed vesicles active against chronic vaginal infections, replacing synthetic surfactants that are less compatible with human health and environmental protection.

Additional investigations on mixed vesicles and their incorporation in a suitable gel vehicle should be considered to further evaluate their applicability in vaginal drug delivery.

V.6 References

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VI. Utilizing liposomal quercetin and gallic acid in localized treatment of vaginal *Candida* infections (*paper 4*)

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Published on Pharmaceutics (doi:10.3390/pharmaceutics12010009)

[Giordani 2020].

VI.1 Abstract

Vulvovaginal candidiasis (VVC) is widely spread fungal infection that causes itching, pain and inflammation at vaginal site. Although common, currently available treatment suffers from limited efficacy and high recurrence. In addition, the growing problem of resistance to azole drugs used in current treatment, emphasises the need for superior treatment options. Antimicrobial polyphenols are an attractive approach offering multitargeting therapy. We aimed to develop novel liposomes for simultaneous delivery of two polyphenols (quercetin, Q and gallic acid, GA) that, when released within vaginal cavity, act in combination to eradicate infection while alleviating the symptoms of VVC. Q was selected for its antiitching and antiinflammatory properties, while GA for its reported activity against *Candida*. Novel liposomes containing only Q (LP-Q), only GA (LP-GA) or both polyphenols (LP-Q+GA) were in size range around 200 nm. Q was efficiently entrapped in both LP-Q and in LP-Q+GA (85%) while the entrapment of GA was higher in LP-Q+GA (30%) than in LP-GA (25%). Liposomes, especially LP-Q+GA, promoted sustained release of both polyphenols. Q and GA acted in synergy increasing the anti-oxidant activities of single polyphenol. Polyphenol-liposomes were not cytotoxic and displayed stronger anti-inflammatory effect than free polyphenols. Finally, LP-GA and LP-Q+GA considerably reduced *C. albicans* growth.

Keywords

Vaginal delivery; liposomes; *Candida*; vaginal infections; polyphenols; quercetin; gallic acid.

List of abbreviations

LP: liposomes; GA: gallic acid; Q: quercetin; SPC: phosphatidylcholine.

VI.2 Introduction

Vulvovaginal candidiasis (VVC) is an ever-living problem affecting 70-75% of women of reproductive age at least once during their life and 40-50% of them will experience a recurrence. *Candida albicans*, and other non-*albicans* related species, is the major causative agent of VVC [Sobel 2007]. Although *C. albicans* may be a commensal microorganism, the perturbation of vaginal homeostasis could facilitate the overgrowth of this opportunistic fungus and the onset of symptomatic candidiasis. Even if VVC is not a life-threatening problem, it can impair the quality of life of patients leading to several physical and sexual impediments, with economic costs estimated for one billion of dollar per year [Ahangari 2018]. Candidiasis clinically manifests mostly through itching, pruritus, irritation, burning, pain, white cheesy discharges, vulvar and vaginal erythema and edema [Richardson 2018].

Fidel et al. proved that the pathogenesis of VVC has a prominent immunological component, involving the recruitment of neutrophil to the vaginal mucosa and the activation of related proinflammatory cytokines and chemokines [Fidel 2004]. This strong inflammatory response, not only resulted ineffective in adequately controlling *Candida* burden, but can also exacerbate the tissue damage and discomfort and even trigger for chronic infections.

Therapeutic approaches for VVC comprise both local and oral administration of different azole drugs, such as fluconazole, ketoconazole and clotrimazole, that are able to reduce symptoms of an initial infection in a large part of patient [Johal 2016]. Unfortunately, since all azoles have a similar fungistatic effect toward *Candida* spp., the cells repetitively exposed to these antifungals may adapt to drug pressure and became resistant [Ksiezopolska 2018]. Taking into consideration the emerging problem of drug resistance, as well as the high incidence of VVC, it is clear that new therapeutic strategies based on both the administration of alternative compounds and the development of non-conventional delivery systems, are extremely desirable.

Among natural molecules, polyphenols have gained an increasing interest in the last few years as potential candidates for *Candida* treatment [Matins 2015; Seleem 2017]. Polyphenolic compounds are phytochemicals that can be principally found in cereals grains, legumes, fruits, vegetables and in beverage, i.e. tea, coffee, fruit juice and cocoa [Ganesan and Xu 2017]. Based on their chemical structure, polyphenols can be categorized into four main groups: flavonoids, stilbenes, lignans and phenolic acids.

In humans, besides the well-established anti-oxidant activity, polyphenols display many others biological effects, being able to act as anti-inflammatory, anti-diabetic, cardioprotective, antiaging [Rasines-Perea and Teissendre 2017] and antimicrobial [Özçelik 2011; Gehrke 2013] agents.

In the present work, we have, for the first time, utilized quercetin and gallic acid as polyphenols of interest in topical therapy of vaginal *Candida*.

Quercetin (3,3′,4′,5,6-pentahydroxyflavone) is the most potent anti-oxidant among polyphenols that has been proposed for the treatment of a wide spectrum of pathologies, including diabetes, circulatory dysfunctions and cancers [D'Andrea 2015]. The therapeutic effects of quercetin mainly depend on its ability to scavenge oxygens radicals, like O_2^- and $ONOO^-$, to protect from lipid peroxidation and to chelate metal ions. In addition, its inhibitory effects on cytokine production (i.e. TNF-α and IL-8) and histamine release lead to a reduction of the inflammation state [Mlcek 2016]. Some authors also stated that quercetin may be an anti-nociceptive in animal models, thus relieving the pain associated to inflammation [Valério 2009; Britti 2017]. Interestingly, Maramaldi et al. demonstrated that phytosomes containing quercetin were able to exert lenitive and anti-itch effects on a group of volunteers with skin damages [Maramaldi 2016].

Gallic acid (3,4,5-trihydroxybenzoic acid) shares with quercetin some important biological features, mostly imparted by the hydroxyl group. It's largely studied for its strong anti-oxidant and anti-inflammatory properties, as well as anti-carcinogenesis and anti-arteriosclerosis effects. Furthermore, gallic acid is reported to inhibit microbial biofilm formation, to possess bactericidal effects towards both Gram-positive and Gram-negative bacteria and antiviral activities against HIV-1 [Choubey 2018]. Several authors proposed that gallic acid is also able to exert an antifungal activity against planktonic cultures and biofilm of *C. albicans* [Özçelik 2011; Gehrke 2013; Teodoro 2015]. In particular, Li et al. demonstrated that gallic acid inhibited the growth of different clinically isolates of *Candida* spp. and suggested that the fungicidal outcome was due to the impairment of biosynthesis of ergosterol, a fundamental component of the fungal membrane [Li 2017].

Despite the favourable pharmacological properties of polyphenols, their applicability remains limited by the low solubility and bioavailability as well as high susceptibility to environmental conditions, including biological environment.

In this regard, the incorporation of polyphenols inside a nanocarrier can be an appropriate approach to avoid the degradation of active molecules and to promote their deposition at

the site of administration, thus assuring enhanced biological effect [Vanić and Škalko-Basnet 2013; Brako 2017].

In this work, a new liposomal system for the simultaneous delivery of quercetin and gallic acid to the vaginal cavity was developed. The combination of quercetin and gallic acid was chosen with the aim of achieving a double effect, namely the immediate and long-lasting alleviation of VVC symptomatology and the eradication of the fungus infection. The purpose was to obtain a formulation able to guarantee a modified release of both active compounds, thus assuring adequate concentration of polyphenols in the vagina mucosa. Liposomes are vesicular nanostructures composed of one or more lipid bilayers, and are particularly suitable for the combined delivery of two molecules with different chemicals properties since they are able to accommodate both the lipophilic compounds, such as quercetin, and more hydrophilic substances, like gallic acid, inside the lipid and aqueous compartments, respectively [Voltan 2016].

Liposomal formulations were characterized for their technological (size, zeta potential, entrapment efficiency, release behaviour and stability over time) and biological properties (anti-oxidant and anti-inflammatory activities and cytotoxicity). Finally, the capability of proposed formulations to play an antifungal effect against *Candida* was also investigated.

VI.3 Materials and Methods

VI.3.1 Materials

Lipoid S 100 from fat free soybean lecithin, containing not less than 94% phosphatidylcholine (SPC), was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Quercetin (Q, MW: 302.2, logP: 2.16, purity ≥ 95%), gallic acid (GA, MW: 188.1, logP: 0.7, purity ≥ 99%), potassium phosphate monobasic, propylene glycol (PG), mucin (Type II: crude, from porcine stomach), 2,20-azino bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), vitamin C (ascorbic acid), vitamin E (α-tocopherol), RPMI 1640 medium, bovine serum albumin (BSA), glutamine and Cell Counting Kit-8 (CCK-8) were purchased from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany). Lipopolysaccharide (LPS; *Escherichia coli*, 055:B5), sulfanilamide, naphthylethylenediamine dihydrochloride, phosphoric acid and sodium nitrite were from Sigma Life Science (Sigma-Aldrich Norway AS, Oslo). Murine macrophage RAW 264.7 cell line and *Candida albicans* strain (ATCC 10231) were supplied by ATCC (Manassas, USA). All solvents were of analytical grade and were provided from VWR International bvba/sprl (Leuven, Belgium).

Candida was grown in potato dextrose broth (Difco, BD, Franklin Lakes, NJ, USA) supplemented with glucose 2% w/v (PDB_{Glu}) or on agar plates of the same medium (PDA_{Glu}).

Phosphate buffer solution at pH 4.5 simulating vaginal pH (3.5-5.5) [Ravel 2011] was prepared as follows: KH_2PO_4 0.1 M. The composition of Griess reagent was: sulphanilamide 1%, naphthylethylenediamine 0.1% dihydrochloride, phosphoric acid 2.5%.

VI.3.2 Preparation of liposomes

The Q and GA co-loaded liposomes (LP-Q+GA) were prepared by film hydration method as previously described, with some modifications [Jøraholmen 2019]. Briefly, Q (10 mg) and SPC (200 mg) were dissolved in 10 mL of methanol in a round-bottom flask. The solvent was removed by evaporation (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland) under vacuum (45 mmHg) for 2 h at 50°C. The resulting dry lipid film was rehydrated with a solution of GA (10 or 20 mg) in distilled water (10 mL), obtaining multilamellar vesicles (MLVs). Liposomes containing only Q (LP-Q) or GA (LP-GA) were formulated in the same way,

by adding only Q (10, 15 or 20 mg) in the organic phase or GA (10 or 20 mg) in the aqueous phase, respectively. Plain liposomes (plain-LP) were prepared using only SPC (200 mg).

All liposomal suspensions were allowed to stabilize at 4-8°C overnight prior to vesicles size reduction. Sonication was used to obtain liposomes with the desired size [Andersen 2017]. The sonicator (Ultrasonic processor 500 W, Sigma-Aldrich, St. Louis, MO, USA) was set to 40% amplitude and the liposomes were exposed to ultrasonic irradiation for 4 cycles (30 sec on/60 sec off). An ice bath was used to prevent heating of the dispertions. All sonicated liposomal formulations were stored at 4-8°C overnight before further use.

VI.3.3 Determination of vesicle size distribution

The particle size and polydispersity index (PDI) of liposomal preparations were determined by photon correlation spectroscopy (Submicron particle sizer model 370, Nicomp, Santa Barbara, CA, USA). In order to avoid interference from dust particles, the preparative procedure was carried out in a laminar airflow bench, as previously described [Jøraholmen 2014]. Small aliquots of the liposomes were diluted with filtered distilled water (0.22 μm syringe filter, VWR International, Leuven, Belgium) to obtain suitable particle intensity (250-350 kHz). All measurements were run (run time of 10 min) in the vesicle mode and intensity-weight distribution at room temperature (24-25°C).

VI.3.4 Liposomes zeta potential

Zeta potential analysis were performed using a Malvern Zetasizer Nano-ZS (Malvern, Oxford, UK [Ternullo 2018]. Measurement cells (DTS1060) were rinsed with ethanol and filtered water (0.22 μm syringe filter) before loading the sample. Liposomal suspensions were diluted 1:20 (v/v) with filtered water to achieve an attenuation value of 6-7. Measurements were made at 25°C with an equilibration time of 180 sec and number of run set to automatic.

VI.3.5 Determination of polyphenol entrapment efficiency

Since Q and GA display very different water solubility (the experimentally determined values were 4.1 ± 0.3 μg/mL and 13.7 ± 0.2 mg/mL, respectively), different protocols were applied for the determination of their entrapment efficiency. Therefore, the entrapment efficiency of quercetin (Q EE %) in LP-Q and LP-Q+GA was determined after mild centrifugation (3000 *g* for 15 min, room temperature; Biofuge stratos

centrifuge, Heraeus instruments GmbH, Hanau, Germany) to precipitate not-encapsulated Q. Then, Q was quantified by UV-visible spectroscopy (Spark Multimode Microplate Reader, Tecan® Trading AG, Männedorf, Switzerland) at 372 nm both in supernatants containing quercetin incorporated inside vesicles, and in not-centrifuged liposomes after dilution with methanol. The standard curve of Q in methanol was plotted at concentration range of 2.5-37.5 μg/mL ($R^2 = 0.9997$).

Considering the good solubility of GA in distilled water, to determine its entrapment efficiency (GA EE %) in LP-GA and LP-Q+GA, liposomes were separated from supernatant (containing freely unentrapped polyphenol) by ultracentrifugation (92000 *g* for 1 h, 10°C; Beckman model L8-70M with SW 60 Ti rotor, Beckman Instruments, Brea, CA). Aliquots of pellet and supernatant, as well as not-centrifuged liposomes, were diluted with methanol and GA was quantified in each fraction by UV-visible spectroscopy (Spark Multimode Microplate Reader) at 272 nm. The standard curve of GA in methanol was set up in the 2.5-37.5 μg/mL range ($R^2 = 0.9996$).

VI.3.6 Evaluation of liposomes storage stability

Samples were stored at 4-8°C and kept out of light with aluminium foil. The stability of prepared liposomes was evaluated my measuring their mean size, PDI, zeta potential and entrapment efficiency of Q and GA after 15, 30, 60 and 90 days. Four different batches of each formulation were analysed in triplicate.

VI.3.7 Mucoadhesion studies

The ability of liposomal formulations to interact with mucin was indagated through the method reported by Abruzzo et al. with slightly modifications [Abruzzo 2018].

Briefly, mucin dispersion (0.08%, w/v) and liposomes were mixed at 1:3 volume ratio and kept in agitation for 1 h (200 rpm). Samples containing only mucin dispersion and liposomes without mucin were also set up as references. The turbidity of the samples (ABS) was measured at 650 nm (Spark Multimode Microplate Reader) and the results were expressed as percentage increase of the sample ABS (ABS %) in the presence of mucin with respect to the same sample without mucin.

VI.3.8 *In vitro* polyphenol release studies

The *in vitro* polyphenol release studies were performed by using Franz diffusion cells (1.77 cm^2 diffusion area, 12 mL acceptor volume; PermeGear, Bethlehem, USA)

equipped with a V6A Stirrer (PermeGear, Bethlehem, USA) [Jøraholmen 2017]. The heating circulator (Julabo Laboratechnik, F12-ED, Seelbach, Germany) was set to maintain a temperature of 37°C. In order to mimic the vaginal pH the acceptor chambers were filled with phosphate buffer at pH 4.5 [Abruzzo 2018; Giordani 2019] and ethanol was added at the final concentration of 20% (v/v) [Park 2013] to ensure sink conditions during the study (the solubility of Q and GA in the releasing conditions was found to be 0.226 ± 0.042 mg/mL and 78.7 ± 3.4 mg/mL, respectively).

Dialysis membranes (Mw cut-off: 12000–14000 Daltons; Medicell International Ltd, London, UK) pre-soaked in phosphate buffer pH 4.5 were fixed between donor and acceptor compartments [Bose 2013].

LP-Q, LP-GA and LP-Q+GA were added to the donor chamber (500 μL). Solutions of Q (in PG solution 50%, w/v) and GA (in distilled water) at the same concentrations as in liposomes were used as comparisons. Samples (500 μL) were withdrawn from the receptor chamber every hour for 8 h and immediately replaced by an equal amount of fresh medium. The amounts of Q and GA were quantified spectrophotometrically at 372 nm and 260 nm, respectively. Calibration curves for Q (R^2 = 0.9998) and GA (R^2 = 0.9993) in releasing medium were obtained within the concentration range 2.5-25 μg/mL. Results are expressed as cumulative percentage of polyphenols released over time.

VI.3.9 Anti-oxidative assays

The *in vitro* anti-oxidant activity of liposomal formulations and free polyphenols [Liang 2017a] was assessed by employing two different methods, namely ABTS and DPPH assay. LP-Q, LP-GA and LP-Q+GA and plain-LP were serially diluted in ethanol and tested at various SPC concentrations (10-600 μg/mL), corresponding to Q and GA concentrations of 0.5-30 μg/mL. The anti-oxidant properties of Q and GA (alone or in combination) were evaluated applying the same dilutions.

The activity of vitamin E and vitamin C were also tested under the same experimental conditions and used as comparison.

VI.3.9.1 ABTS $^{\bullet+}$ radical scavenging

ABTS $^{\bullet+}$ radicals were generated by mixing equal volumes (3 mL) of the stock solutions of ABTS (7.4 μM) and potassium peroxodisulphate (2.6 μM) in distilled water at room temperature [Jøraholmen 2019]. The reaction mixture was stabilized for 18 h and then diluted with 100 mL of ethanol. A total of 300 μL of ABTS•+ radicals working solution

were then added to equal volume of samples, shaken vigorously and kept in dark for 30 min at room temperature. Reduction of blue-green coloured radical solution by hydrogen-donating anti-oxidant was measured spectrophotometrically at 751 nm (Spark Multimode Microplate Reader).

VI.3.9.2 DPPH radical scavenging

DPPH scavenging activity was determined as reported by Jøraholmen et al. [Jøraholmen 2019]. A total of 300 µL of ethanolic DPPH solution (60 µM) were thoroughly mixed with equal volume of each sample and kept in dark for 30 min at room temperature. The disappearance of violet colour after incubation time indicated a high free radical scavenging activity, spectrophotometrically quantifiable at 519 nm (Spark Multimode Microplate Reader). The radical scavenging activity (RSA) was calculated according to the following equation:

$$RSA\ (\%) = (1 - A_{sample} / A_{control}) \times 100$$

where A_{sample} is the absorbance of the sample and $A_{control}$ is the absorbance of the control samples prepared by mixing the same amount of ethanol and ABTS•+ or DPPH solution. Ethanol was used as blank sample.

The combined effect of Q and GA was investigated according to Loewe additivity model [Lee 2007] and the interaction index (γ) was calculated as follows:

$$\gamma = d_1 / D_1 + d_2 / D_2$$

where D_1 and D_2 are the doses of Q (alone) and GA (alone) that have the RSA value of 50 (EC_{50}) while d_1 and d_2 are the doses of Q and GA in the mixture that elicit the same effect. Values of $\gamma > 1$, $= 1$ and < 1 mean that the combination effect is antagonism, additive and synergy, respectively.

VI.3.10 Cell culture

The murine macrophage RAW 264.7 cell line was used to investigate possible cytotoxic effects of free and formulated polyphenols and their ability to exert anti-inflammatory activities. Cells were maintained in RPMI 1640 medium supplemented with BSA 10%, streptomycin 100 µg/L, and penicillin 100 IU/mL at 37 °C in a 5% CO_2 atmosphere.

VI.3.11 *In vitro* cell viability study

To assess the *in vitro* toxicity, RAW 264.7 cells, grown as described in section VI.3.10, were seeded (90 µL) in 96-well flat bottom plates at the density of 5×10^4 cells/well. The

plates were pre-incubated for 24 h at 37°C in 5% CO_2 to allow the cells to stabilize.

The adherent cells were then treated with 10 μL of media only (negative control) or liposomal formulations (LP-Q, LP-GA and LP-Q+GA) and subsequently incubated for 24 h.

In particular, liposomes were diluted in the growth medium and tested at three different final SPC concentrations, namely 1, 10 and 50 μg/mL, corresponding to Q and GA concentrations of 0.05, 0.5 and 2.5 μg/mL, respectively [Ternullo 2018].

Solutions of Q and GA (alone or in combination) were prepared in PG, diluted in growth medium and tested at the same concentrations as in liposomal formulations. Plain-LP, at the same SPC concentrations as in loaded liposomes, were also evaluated. Distilled water and PG solution (added at the final concentration of 0.25% w/v, corresponding to the maximum amount applied to cells) served as controls.

After incubation time, living cells were quantified using the Cell Counting Kit-8 (CCK-8), following the manufacturing instructions. Briefly, 10 μL of CCK-8 were added to the cells and the absorbance was measured at 450 nm (Spark Multimode Microplate Reader) after 4 h of incubation at 37°C. Since slight spontaneous absorbance may occur in a culture medium incubated with CCK-8, growth medium without cells was used as blank. Results were expressed as percentage of living cells with respect to control (untreated cells).

VI.3.12 Anti-inflammatory activity determination

The capability of liposomes to inhibit nitric oxide (NO) production in LPS-induced macrophages was evaluated as previously described and expressed as anti-inflammatory activity [Basnet 2012].

Cells were cultured in 24-well plates (5×10^5 cells/mL) for 24 h (section VI.3.10). Old medium was then removed and replaced with fresh RPMI 1640 medium (1 mL) containing LPS (1 μg/mL) to induce NO production. Cells were treated with 10 μL of liposomal formulations or solutions at the same concentrations as applied in toxicity assay (section VI.3.11), and incubated for 24 h. For a negative control, cells were treated with only LPS.

The NO production by macrophages was correlated to nitrite formation in the media which was quantified by Griess method. Briefly, equal volumes of media and Griess reagent (300 μL) were mixed and incubated for 30 min. The absorbance was determined at 550 nm (Agilent Technologies, Santa Clara, CA, USA) and a calibration curve (0.5-20

μM) was constructed by using $NaNO_2$ as standard with Griess reagent. The anti-inflammatory activity was expressed as percentage of NO production's inhibition calculated with respect to control (untreated cells).

VI.3.13 Anti-*Candida* activity testing

The antifungal activity towards *Candida albicans* ATCC 10231 was evaluated by broth microdilution following the method reported by Andersen et al. [Andersen 2017].

C. albicans was grown aerobically in PDB_{Glu} at 37°C. After 24 h, *Candida* suspension was diluted in PDB_{Glu} to reach a concentration of 4×10^5 cells/mL (determined by counting in a Bürker chamber). The yeast suspension (50 μL) was then inoculated in 96-well plates along with 50 μL of liposomal formulations (LP-Q, LP-GA and LP-Q+GA) or solutions of Q (in DMSO) and GA (in distilled water). Samples were diluted in a two-fold sequence in order to test concentrations ranging from 250 to 2 μg/mL.

Plain-LP, DMSO and distilled water were used as negative control, solvent control and growth control, respectively. Blank control, consisting only of growth medium, and sterility controls, containing formulations and sterile medium, were also included.

Plates were incubated aerobically without shacking at 37°C for 24 h. Afterwards, the growth inhibition was established through microscope observation (20×; Axiovert 40 Inverted Microscope, Carl Zeiss, Thornwood, USA) and IC_{50} was defined as the minimal concentration of test substance that inhibits 50% or more of the visible growth, as compared to untreated control.

Aliquots of the samples (20 μL) were spotted onto PDA_{Glu} and the minimal lethal dose (MLD) was defined as the concentration at which no growth was observed after 24 h of incubation at 37°C.

VI.3.14 Statistical analysis

All experiments were performed at least in triplicate and results are expressed as mean ± standard deviation (SD). For the comparison of two means, Student's *t*-test was applied. One-way ANOVA followed by Bonferroni correction was used for multiple comparison. All statistical analysis were performed using GraphPad Prims version 8.1.2 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and differences were deemed significant for $p < 0.05$.

VI.4 Results and discussion

Vaginal drug administration for localized therapy is an interesting therapeutic choice considering treatment of sexually transmitted diseases, fungal and bacterial infections, and cancer. Vaginal route offers different advantages over orally administered drugs, for instance the avoidance of hepatic first-passage effect and degradation during the transit through the gastrointestinal tract [Ensign 2014; Leyva-Gómez 2018]. As a consequence, topical application requires smaller doses, ensuring at the same time higher drug local concentrations and improved efficacy. Vaginal cavity is regarded as one of the highly challenging site for drug action and different approaches have been proposed as superior treatments in topical vaginal therapy. Conventional vaginal pharmaceutical forms are often associated with poor retention, low bioavailability, inability to modulate the release of drug and need for frequent administrations that reduce the patient compliance [Johal 2016]. Among nanocarriers, liposomes are particularly suitable for vaginal delivery because they are reported to not interfere with vaginal microbiome and to protect active substances against external enzymatic degradation and rapid perturbations that can occur in the vaginal cavity [Brako 2017]. Liposomes are also biodegradable, biocompatible, weakly immunogenic and non-irritating to vaginal [Immordino 2006].

Their characteristics, such as composition, size and surface properties will affect their fate at vaginal site mucosa [Vanić and Škalko-Basnet 2013]. This study focused on the feasibility of simple phosphatidylcholine-based liposomes. Indeed, the entrapment of highly lipophilic molecules, such as Q, could be hampered by the presence of additional components in the lipidic bilayer. Considering that antifungal formulations need to act primarily on the vaginal epithelial surface, where *Candida* infection occurs, conventional liposomes could be a valid choice to obtain not-expensive and simple vesicles able to simultaneously accommodate two different active molecules.

VI.4.1 Technological characterization of liposomes

VI.4.1.1 Liposomal size and zeta potential

In order to optimize the loading of polyphenols, liposomal formulations varying in concentrations of Q and/or GA were prepared and characterized in terms of size, PDI and zeta potential. Results are summarized in *table VI.1*.

Firstly, liposomes containing only Q were investigated.

Although extrusion is a widely used vesicle size reduction method [Ong 2016; Jøraholmen 2019], sonication was found more suitable in this work. Indeed, when forced step-wise through polycarbonate membranes (0.8, 0.4 and 0.2 μm pore size filters, respectively) LP-Q precipitated, possibly because Q was expelled out the lipid bilayer during the process, leading to unstable dispersions (mean size: 366.5 ± 9.2; PDI: 0.558 ± 0.004).

Considering the maximum Q loaded in vesicles, we observed that size and PDI increased with the increasing starting concentration of Q. In particular, when Q was added at the concentration of 2 mg/mL, liposomes were significantly bigger and the high PDI (> 0.8) indicated the presence of precipitates. This was probably due to the fact that Q, being very poorly soluble in water, immediately precipitated when not incorporate in the lipid bilayer [Riva 2019]. LP-Q obtained with the intermediate concentration (Q 1.5 mg/mL) were also excluded because of the stability issue; although equal in size to liposomes prepared with Q 1 mg/mL, they were not stable after just one week of storage at 4°C (mean size: 504.6 ± 14.1; PDI: 0.564 ± 0.052).

On the contrary, liposomes formulated with GA in the aqueous phase at 2 mg/mL were stable over time. However, when co-entrapped with Q 1 mg/mL, the resulting LP-Q+GA formulation precipitated.

Table VI.1 – Characteristics of liposomal preparations: size, polydispersity index (PDI) and zeta potential (mean ± SD, n = 4). In brackets are indicated the starting concentrations of Q and/or GA. Formulations selected for further studies are in bold.

	Vesicle Size (nm)	PDI	Zeta Potential (mV)
plain-LP	**166.9 ± 18.0**	**0.347 ± 0.140**	**-1.5 ± 0.2**
LP-Q (Q 1 mg/mL)	**194.4 ± 28.5**	**0.348 ± 0.052**	**-4.9 ± 0.7**
LP-Q (Q 1.5 mg/mL)	194.6 ± 8.8	0.444 ± 0.013	-5.6 ± 0.2
LP-Q (Q 2 mg/mL)	594.3 ± 72.8	0.898 ± 0.006	-6.4 ± 0.3
LP-GA (GA 1 mg/mL)	**180.7 ± 21.1**	**0.378 ± 0.025**	**-3.7 ± 0.5**
LP-GA (GA 2 mg/mL)	289.4 ± 8.3	0.354 ± 0.079	-5.6 ± 0.2
LP-Q+GA (Q 1 mg/mL; GA 1 mg/mL)	**220.4 ± 21.6**	**0.438 ± 0.035**	**-7.1 ± 0.4**
LP-Q+GA (Q 1 mg/mL; GA 2 mg/mL)	366.5 ± 9.2	0.548 ± 0.019	-7.8 ± 0.7

Based on the aforementioned consideration, the liposomal preparations LP-Q (Q 1 mg/mL), LP-GA (GA 1 mg/mL) and LP-Q+GA (Q 1 mg/mL; GA 1 mg/mL) were selected for further studies. They displayed an average diameter of around 200 nm that is estimated to be optimal for delivery to vaginal mucosa [Das Neves 2011] and for targeting microorganisms able to grow as biofilm [Rukavina and Vani 2016], such as *Candida* spp. [Cavalheiro and Teixeira 2018].

In particular, mean size of liposomes containing either only Q or GA did not differ significantly from that of plain-LP, while LP-Q+GA were slightly bigger ($p = 0.0012$) as consequence of the simultaneously incorporation of both polyphenols. Although sonication led to the formation of dispersions less homogeneous than those obtained with extrusion technique [Ong 2016], results were reproducible and the PDI values were all below 0.7 (0.348-0.438) and could be considered acceptable for liposomal formulations [Refai 217].

As previously observed [Jøraholmen 2015; Ternullo 2018; Jøraholmen 2019], plain-LP exhibited a zeta potential close to neutral because phosphatidylcholine is a zwitterionic lipid that can acquire slightly negative values in distilled water, as a result of orientation of lipid headgroup and formation of a hydration layer around vesicles [Garcia-Manyes 2005]. Liposomes incorporating Q and/or GA were more negative than plain-LP ($p < 0.05$), coherently with the fact that both Q and GA were negatively charged in water (-18.5 ± 0.6 mV and -14.3 ± 0.7 mV, respectively).

VI.4.1.2 Polyphenols entrapment efficiency

Despite the wide spectrum of beneficial properties exerted by Q, the low solubility of this molecule limits its wider therapeutic use [D'Andrea 2015]. Moreover, chemical stability of Q is highly compromised in the presence of oxygen [Buchner 2006] and metal ions [Chen 2009], as well as in alkaline conditions [Moon 2008].

Among polyphenolic acids, GA exhibits several biological potential activities [Choubey 2015]. However, its pharmaceutical application is also hampered by low bioavailability [Bhattacharyya 2013] and susceptibility to environmental factors [Acevedo 2018], such as the tendency to easily oxidize at pH above 7 giving rise to metabolites, namely 4-omethylgallic acid and pyrogallol, that possess lower anti-oxidant activity compared to GA [Eslami 2010].

Due to their structure composed of phospholipid bilayers, liposomes can straightforwardly entrap both hydrophilic and lipophilic substances. In this regard,

encapsulation in liposomal vesicles is expected to increase the physicochemical stability, local accumulation of active substances at the site of administration and therefore the therapeutic efficiency of both polyphenols [Manosroi 2011].

Co-encapsulation of active molecules in the same nanocarrier is particularly attractive because it allows the simultaneous delivery of compounds to their target, thus simplifying and improving the therapy. To date, Q was successfully co-encapsulated in liposomes with resveratrol [Caddeo 2016] and epigallocatechin-3-gallate [Chen 2019], whereas GA was co-loaded with resveratrol [Vitonyte 2019]. These formulations were mainly focused on the treatment of skin pathologies related to microbial infections or oxidative stress.

To the best of our knowledge, no formulations comprising Q and GA have been proposed for localized vaginal treatment.

In *table VI.2* are reported the EE % and polyphenol/lipid ratio for Q and GA formulated either as single polyphenol or in combination after 120 sec of sonication (see section VI.3.2).

Table VI.2 – Characteristics of liposomal preparations: entrapment efficiency of Q (Q EE %) and GA (GA EE %) in the final formulations (liposomes sonicated for 120 sec) (mean ± SD, $n = 4$).

	Q EE %	Q/Lipid ratio (μg/mL)	GA EE %	GA/Lipid ratio (μg/mL)
LP-Q	85.1 ± 4.6	42.54 ± 2.31	-	-
LP-GA	-	-	25.4 ± 0.9	12.68 ± 0.47
LP-Q+GA	86.0 ± 7.0	43.00 ± 3.49	30.2 ± 1.7	15.10 ± 0.84

Q was efficiently entrapped both in LP-Q and LP-Q+GA (EE % > 85%), as a consequence of its lipophilic nature that favours its incorporation inside the lipid bilayer. This is in agreement with the high EE % of Q inside liposomes, ranging from 67% to 89%, reported by several authors [Castangia 2014; Caddeo 2016; Jangde and Singh 2016; Zhou 2018]. Recently, Riva et al. also demonstrated that the employment of phytosomes can increase the solubility of this polyphenol, thus promoting the entrapment of Q into the vesicles [Riva 2019].

The EE %, size and PDI of GA inside liposomes before and after different sonication times are reported in *table VI.3*. The EE % of GA inside MLVs was found to be 50.6 ± 0.6 % and 47.5 ± 1.0 % for LP-GA and LP-Q+GA, respectively. After sonication, the EE% decreased with increasing sonication time. Two minutes of sonication were required

to obtain vesicles with the desired size; indeed, mean diameter of LP-GA was 600.1 ± 19.0 nm after 60 sec of sonication and 314.8 ± 4.5 nm after 90 sec, whereas LP-Q+GA had a size of 897.7 ± 5.4 nm after 60 sec of sonication and 369.7 ± 4.7 nm after 90 sec of sonication. Following 2 min of sonication the EE % was 25% for liposomes containing only GA and, interestingly, was higher for LP-Q+GA (30%, $p = 0.0005$). These values were higher than those obtained by Vitonyte et al. for GA and resveratrol co-loaded liposomes [Vitonyte 2019].

Considering its hydrophilic nature, GA was probably located inside aqueous core. During ultrasound treatment, MLVs undergo breakage and rearrangements that induce instability and formation of smaller vesicles. Our results suggest that the presence of Q in the surrounding lipid bilayer partially prevented leaks of GA from liposomes due to sonication process.

Table VI.3 – Characteristics of liposomes containing GA before (multilamellar vesicles, MLV) and after different sonication times (60 sec and 90 sec): size, polydispersity index (PDI) and GA EE % (mean ± SD, $n = 4$).

Liposomes	Vesicle Size (nm)	PDI	GA EE %
LP-GA MLV	> 1 μm	>0.9	50.6 ± 0.6
LP-GA sonicated-60 s	600.1 ± 19.0 nm	0.58 ± 0.09	36.8 ± 0.9
LP-GA sonicated-90 s	314.8 ± 4.5	0.44 ± 0.02	31.0 ± 0.7
LP-Q+GA MLV	> 1 μm	> 0.9	47.5 ± 1.0
LP-Q+GA sonicated-60 s	897.7 ± 5.4	0.64 ± 0.02	39.9 ± 1.5
LP-Q+GA sonicated-90 s	369.7 ± 4.7	0.56 ± 0.01	35.4 ± 0.7

VI.4.1.3 Stability of liposomes during storage

Storage stability of liposomal dispersions is an important aspect to predict the quality of formulations since the leakage of active molecules as well as aggregation should be avoided [Liang 2017b]. We evaluated stability by using particle size, entrapment efficiency and zeta potential as parameters (*figures VI.1, VI.2* and *VI.3*).

Figure VI.1 shows that no relevant variations in terms of size and PDI were recorded for all loaded formulations, that appeared more stable than plain-LP, for which an increase in the dimensions was noticed after 2 months of storage at 4°C.

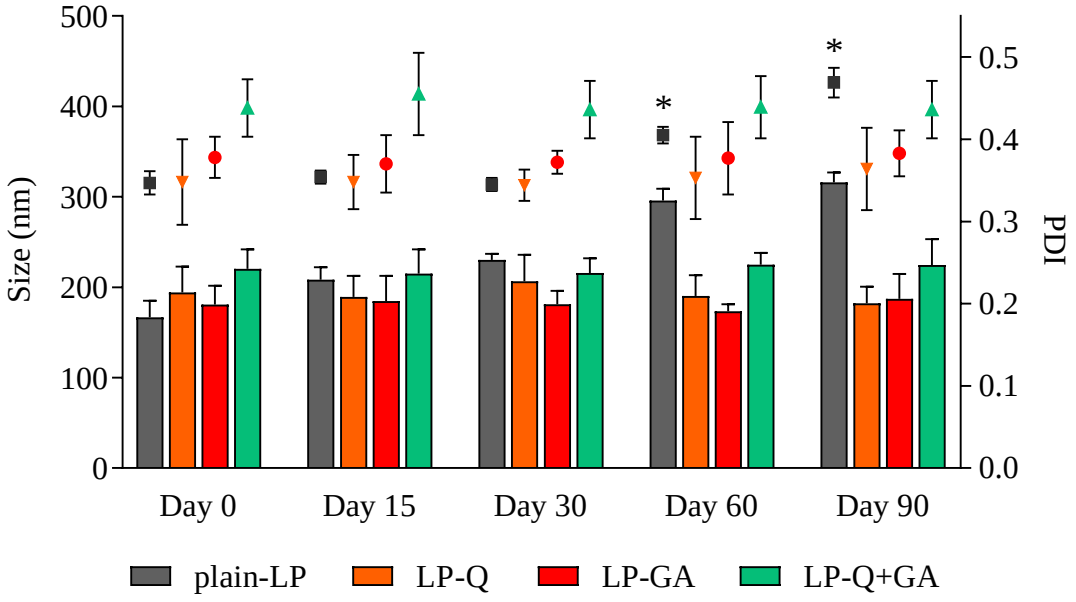

Figure VI.1 – Variations of liposomes size (bars, left Y axis) and PDI (symbols, right Y axis) over a storage period of 90 days at 4°C (mean ± SD, n = 4). The statistical significance was calculated with respect to day 0; * $p < 0.05$.

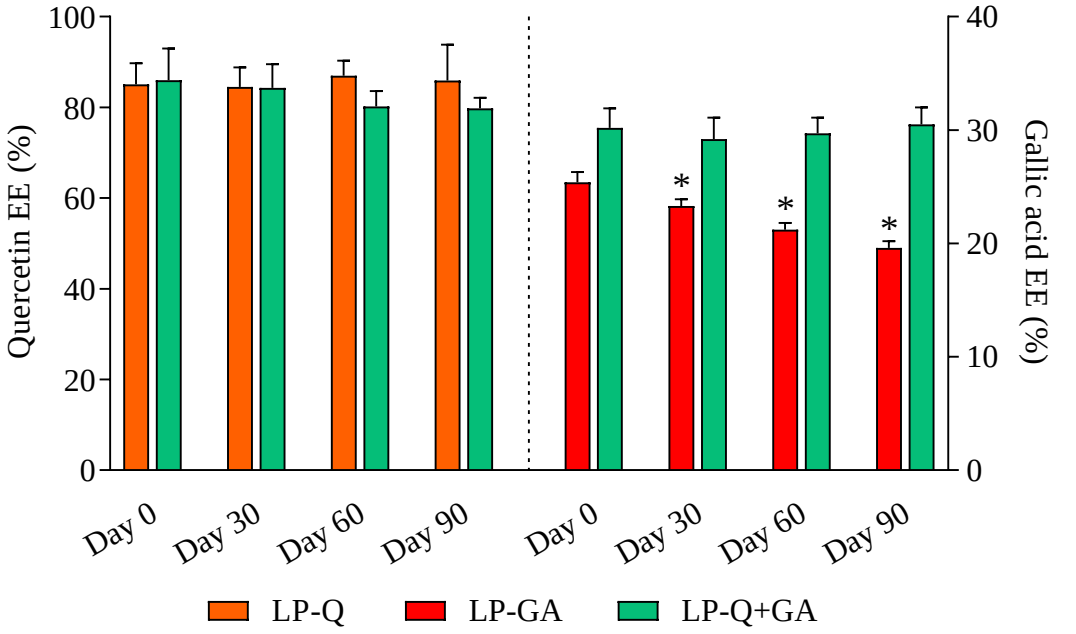

Figure VI.2 – Variations of Q and GA EE % over a storage period of 90 days at 4°C (mean ± SD, n = 4). The statistical significance was calculated with respect to day 0; * $p < 0.05$.

As reported earlier by other authors [Jangde 2016; Zhou 2018], Q was effectively retained inside liposomes over the course of 3 months, with no significant loss and no signs of degradation (*figure VI.2*). On the contrary, the EE % of GA formulated alone tended to slowly decrease after more than one month of storage, indicating leakage from liposomes.

After 90 days the EE % of LP-GA fell to 19.6 ± 0.6 %, which corresponds to a decline of 22.8%, similarly to what was described by Manosroi et al. for GA encapsulated inside noisome formulations [Manosroi 2011]. Notably, this phenomenon was not observed for liposomes comprising both Q and GA, which were able to prevent leakage of both polyphenols, coherently with our previous findings about EE % (section VI.4.1.2).

Zeta potential of LP-GA and LP-Q+GA became more negative during storage while the surface charge of LP-Q was extremely stable (*figure VI.3*). Indeed, significant ($p < 0.0001$) decrease in zeta potential occurred after 30 days for LP-GA and after 60 days for LP-Q+GA. This evidence could be attributed to the tendency of GA to move and accumulate at the lipid bilayer-water interface.

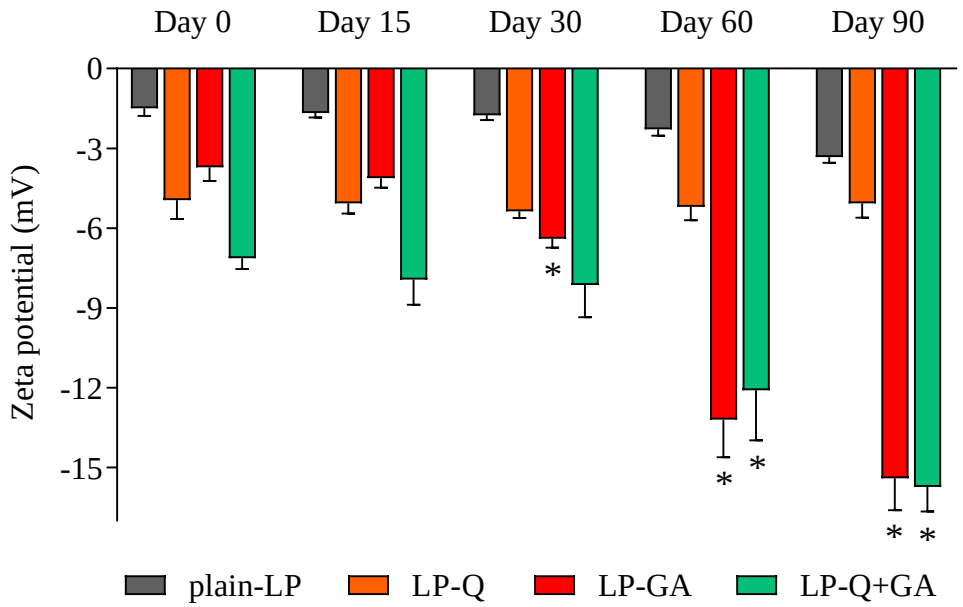

Figure VI.3 – Variations of liposomes zeta potential over a storage period of 90 days at 4°C (mean ± SD, $n = 4$). The statistical significance was calculated with respect to day 0; * $p < 0.05$.

VI.4.1.4 Mucoadhesion studies

The interaction of liposomal vesicles with mucus represents an important step to allow a prolonged contact with the vaginal mucosa and, consequently, the retention of formulations at the application site for a period of time adequate to assure the action of active delivered compounds. In this regard, the interaction with mucin, one of the main components of mucus layer, was indagated and results are summarized in *figure VI.4*.

LP-Q and LP-GA, as well as plain-LP, displayed a significant increase of the ABS % with respect to the corresponding controls without mucin (~ 140%). No significant differences were instead observed between plain-LP, LP-Q and LP-GA.

The mucoadhesiveness was significantly higher for LP-Q+GA (ABS % increased of ~ 195%) with respect to other formulations ($p < 0.01$), probably due to the more pronounced surface charge and the higher size that could favour the interaction with mucin chains.

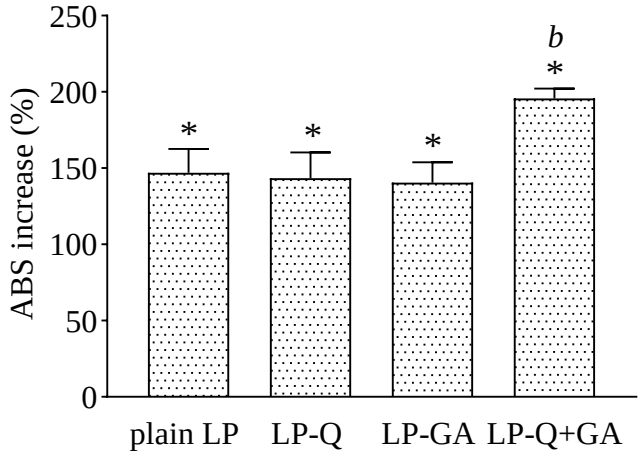

Figure VI.4 – Percentage increase of the absorbance (ABS %) at 650 nm of liposomal formulations in presence of mucin calculated with respect to the same samples without mucin (mean ± SD, $n = 4$). * $p < 0.05$: significantly higher compared to corresponding control without mucin; *b:* $p < 0.01$: LP-Q+GA significantly higher compared to single polyphenol-containing liposomes.

VI.4.1.5 *In vitro* release of polyphenols from liposomes

The *in vitro* polyphenols release studies were performed by using Franz diffusion cells and phosphate buffer at pH 4.5 as receiving phase to mimic the acidity of vaginal environment.

The release behaviors of Q and GA from liposomes are depicted in *figure VI.5*. Free Q and GA in solutions were also tested and used as comparison. For all liposomal formulations, the cumulative release rates of polyphenols from vesicles were much slower compared to the respective controls ($p < 0.001$).

The release profiles of GA (*figure VI.5B*) from both LP-GA and LP-Q+GA were biphasic, with an initial burst release in the first hour followed by a sustained release. Interestingly, significant differences in GA release rates were detected between LP-GA and LP-Q+GA at the earlier time points up to 4 h ($p < 0.002$). In particular, after 1 h, ~ 50% of GA was released from LP-GA and only ~ 34% from LP-Q+GA. After 8 h, both formulations released more than 80% of GA ($p > 0.05$) and the release was complete within 24 h. The lipophilic nature of Q allowed a more evident sustain release of this polyphenol from liposomes (*figure VI.5A*) [Jangde 2016]. About 47% of Q was released after 8 h from LP-Q. Notably, the co-presence of GA inside liposomes favoured the release of Q ($p < 0.03$),

that reached ~ 58% from LP-Q+GA after 8 h. Thus, the combination of two polyphenols not only extended the release of GA, guaranteeing a long-lasting activity at the site of administration, but also improved the release of Q required to elicit a stronger biological effect.

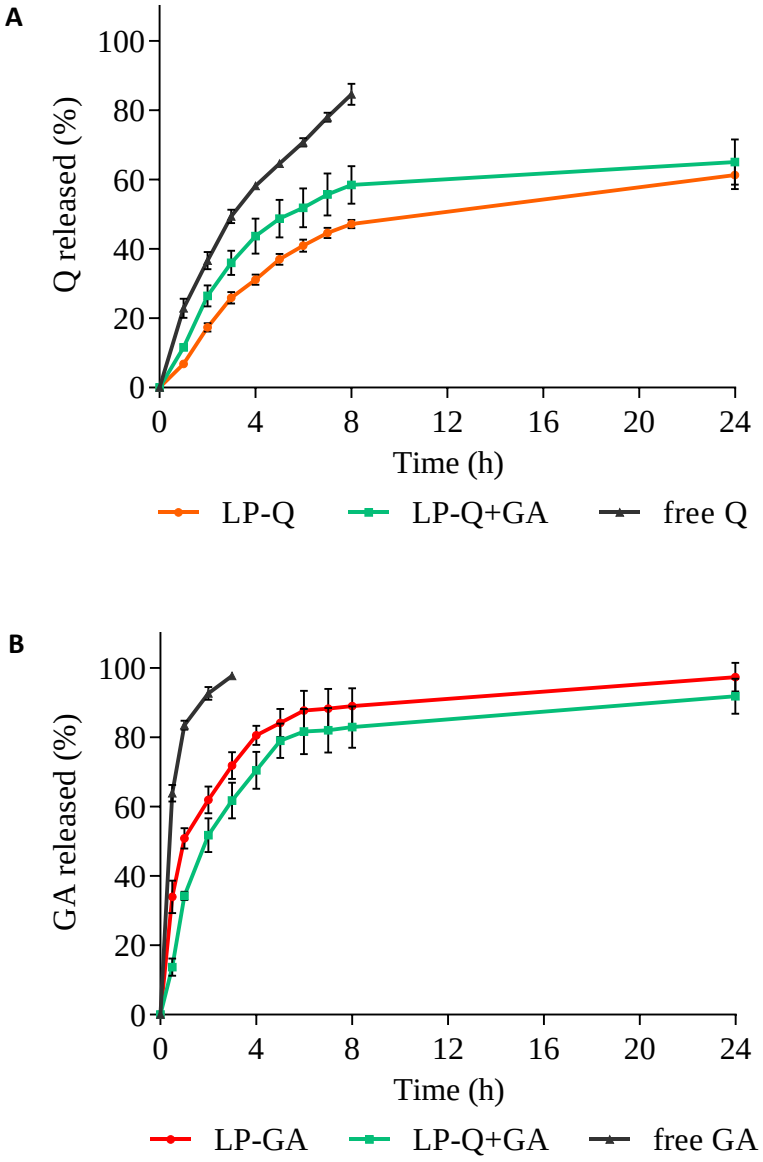

Figure VI.5 – Cumulative percentages of (A) Q and (B) GA released over time from LP-Q, LP-GA and LP-Q+GA compared to free Q and free GA (mean ± SD, *n* = 3).

VI.4.2 Biological characterization of liposomes

VVC is associated with increased levels of nitric oxide which lead to inflammatory response and consequent vulvar pain, that can heavily impair the quality of life of many women worldwide [Alvendal 2017].

Fidel et al. also demonstrated that symptomatic, but not asymptomatic, women displayed high levels of polymorphonuclear leukocytes (PMN) responsible for release of pro-inflammatory mediators and free radicals in the vaginal lumen [Fidel 2004]. The authors highlighted that recruitment of was PMN not only ineffective in protecting from fungus infections, but actually exacerbated VVC symptoms, including itching, burning and redness at the vulva and vaginal mucosa.

In this regard, polyphenols are attractive natural substances able to exert anti-oxidative and anti-inflammatory activities, minimizing in this way tissue damages and preventing the onset of chronic infections [Ganesan and Xu 2017].

VI.4.2.1 Anti-oxidant activity

Anti-oxidant effect of polyphenols was evaluated after complete dissolution of liposomal formulations in ethanol, according to the two colorimetric methods employed.

Figure VI.6 reports the results at three different concentrations of Q and/or GA (1, 2.5 and 10 μg/mL) obtained for ABTS (*Figure VI.6A*) and DPPH assays (*Figure VI.6B*).

With respect to well-known anti-oxidants (vitamin E and vitamin C), the two polyphenols exhibited significantly higher concentration-dependant scavenging activity against both free radicals ($p < 0.0001$), while plain-LP were ineffective, as expected.

It is evident that Q and GA possessed strong anti-oxidant potential, since they were active also at very low concentration (~ 11-31 % at 1 μg/mL) and both ABTS $^{\bullet+}$ and DPPH radicals were almost completely inhibited at 10 μg/mL (~ 90-99 %). This is in agreement with evidences present in literature for free Q [Caddeo 2016] and free GA [de Cristo Soares Alves].

Table VI.4 – Anti-oxidant activity of Q and GA (alone or in association), expressed as EC_{50} (effective concentrations required for the 50% decrease of radicals in ABTS and DPPH assays) (mean ± SD, $n = 3$).

	ABTS assay		DPPH assay	
	EC_{50} (μg/mL)	γ	EC_{50} (μg/mL)	γ
Q	1.61 ± 0.01		2.94 ± 0.06	
GA	2.27 ± 0.07	-	3.41 ± 0.15	-
Q+GA	0.88 ± 0.04	0.81	1.52 ± 0.06	0.85
LP-Q	1.61 ± 0.02	-	2.92 ± 0.17	-
LP-GA	2.49 ± 0.17	-	3.48 ± 0.03	-
LP-Q+GA	1.11 ± 0.06	0.79	1.54 ± 0.02	0.88

Taking into account that anti-oxidant activity plays a crucial role in Q and GA pharmaceutical effects, it's noteworthy that their incorporation inside lipid nanocarriers, and the entire preparative method, did not compromise the capacity of the molecules to quickly and efficiently remove free radicals.

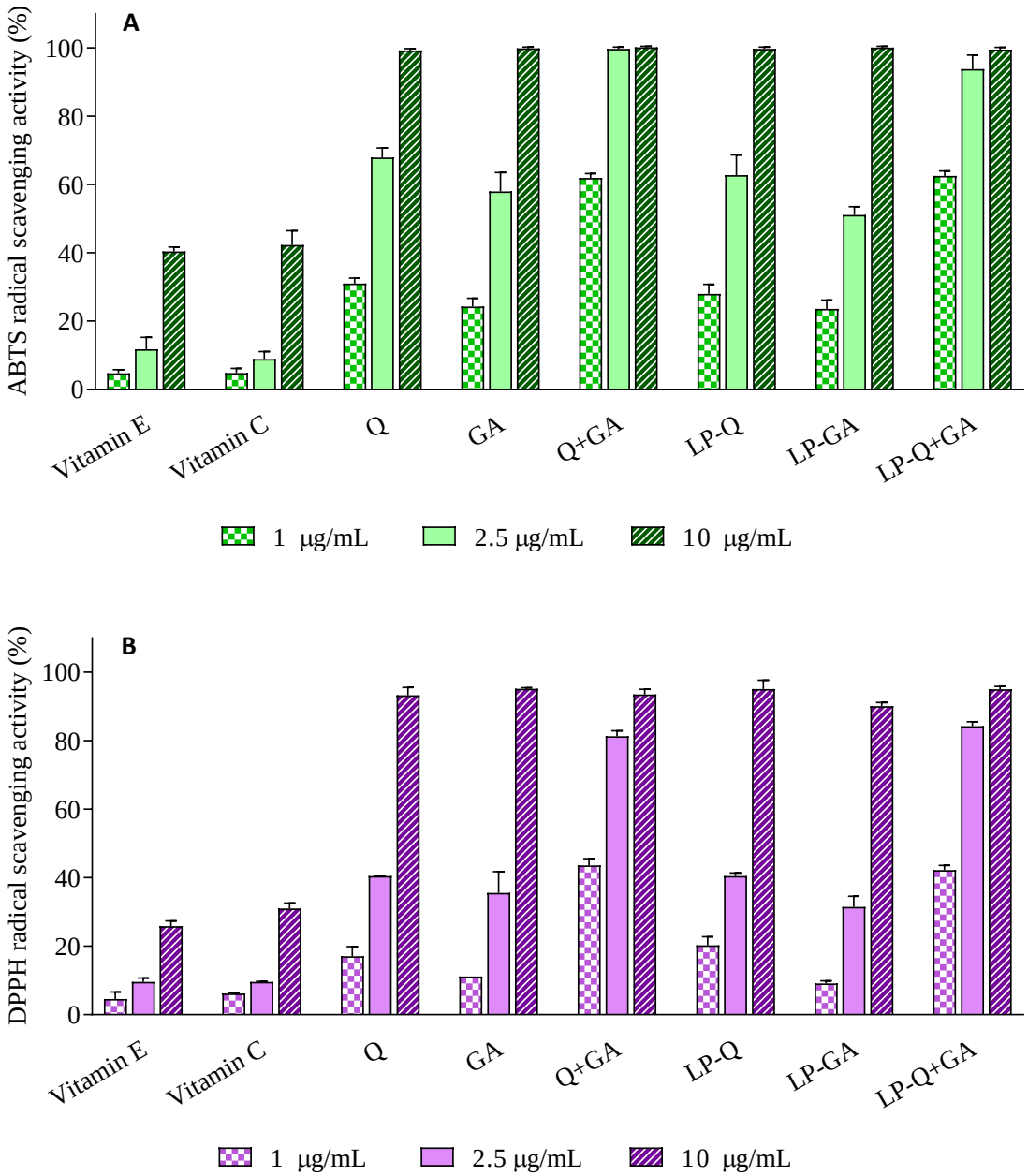

Figure VI.6 – Anti-oxidant activities of free polyphenols (Q, GA and A+GA), liposomes (LP-Q, LP-GA and LP-Q+GA), vitamin E and vitamin C expressed as (A) ABTS and (B) DPPH free radicals scavenging activity (mean ± SD, n = 3).

It seems that the therapeutic activity of many polyphenols could be potentiated when co-administered with other polyphenols or antibiotics. Recently, Q was reported to act in synergism with epigallocatechin-3-gallate [Chen 2019] and with curcumin [Güran 2019], enhancing the anti-oxidant and anti-inflammatory activities, respectively.

In order to investigate the effect of the combination of Q and GA (free or incorporated inside liposomes), EC_{50} values were obtained from linear regression analysis (*table VI.4*). The γ values, calculated according to the equation reported in section VI.3.9 for ABTS and DPPH assays, were all below 1, indicating that co-delivered polyphenols exerted an anti-oxidant activity in a synergistic way.

VI.4.2.2 Anti-inflammatory activity of free and liposomal polyphenols

It's well documented that both Q and GA are able to reduce inflammation response in LPS-induced macrophages by acting at different levels of phlogosis cascade [Li 2016; Choubey 2018].

In the current work, we evaluated the inhibitory effects of liposomal polyphenols on the production of NO, a signaling molecule that plays a pivotal role in the pathogenesis of inflammation and infections. LPS-induced macrophages were treated for 24 h with liposomal (*figure VI.7A*) or free polyphenols at three different Q/GA concentrations (0.05, 0.5 and 2.5 μg/mL) (*figure VI.7B*). Plain-LP were also tested at the same SPC concentration as in liposomes containing polyphenols.

No effect on NO production was observed at the lower concentration, but at 0.5 and 2.5 μg/mL polyphenol-containing liposomes displayed a strong concentration-dependant inhibition of NO production, that was significantly higher compared to plain-LP ($p < 0.0001$). A concentration-dependant behaviour was also confirmed by free polyphenols. Notably, in all cases the inhibitory effect on NO production was higher when Q and/or GA were incorporated inside liposomes ($p < 0.009$), in agreement with previous works on liposomal curcumin [Basnet 2012], resveratrol [Jøraholmen 2015] and epicatechin [Jøraholmen 2019] formulations.

LP-Q seemed to be more active with respect to LP-GA (inhibition of ~ 67% and ~ 43%, respectively, at 2.5 μg/mL) and the anti-inflammatory activity significantly increased ($p < 0.0005$) when the two molecules were delivered together (LP-Q+GA inhibited ~79% of NO production at 2.5 μg/mL), consistently with what observed for the anti-oxidant activity. These results suggest that the choice of appropriate delivery systems improves the beneficial activity of polyphenols. Furthermore, combining Q and GA can be a promising strategy to take advantage of different mechanisms of action, thus heightening the overall anti-inflammatory effect and improving the relief of pain associated with inflammation.

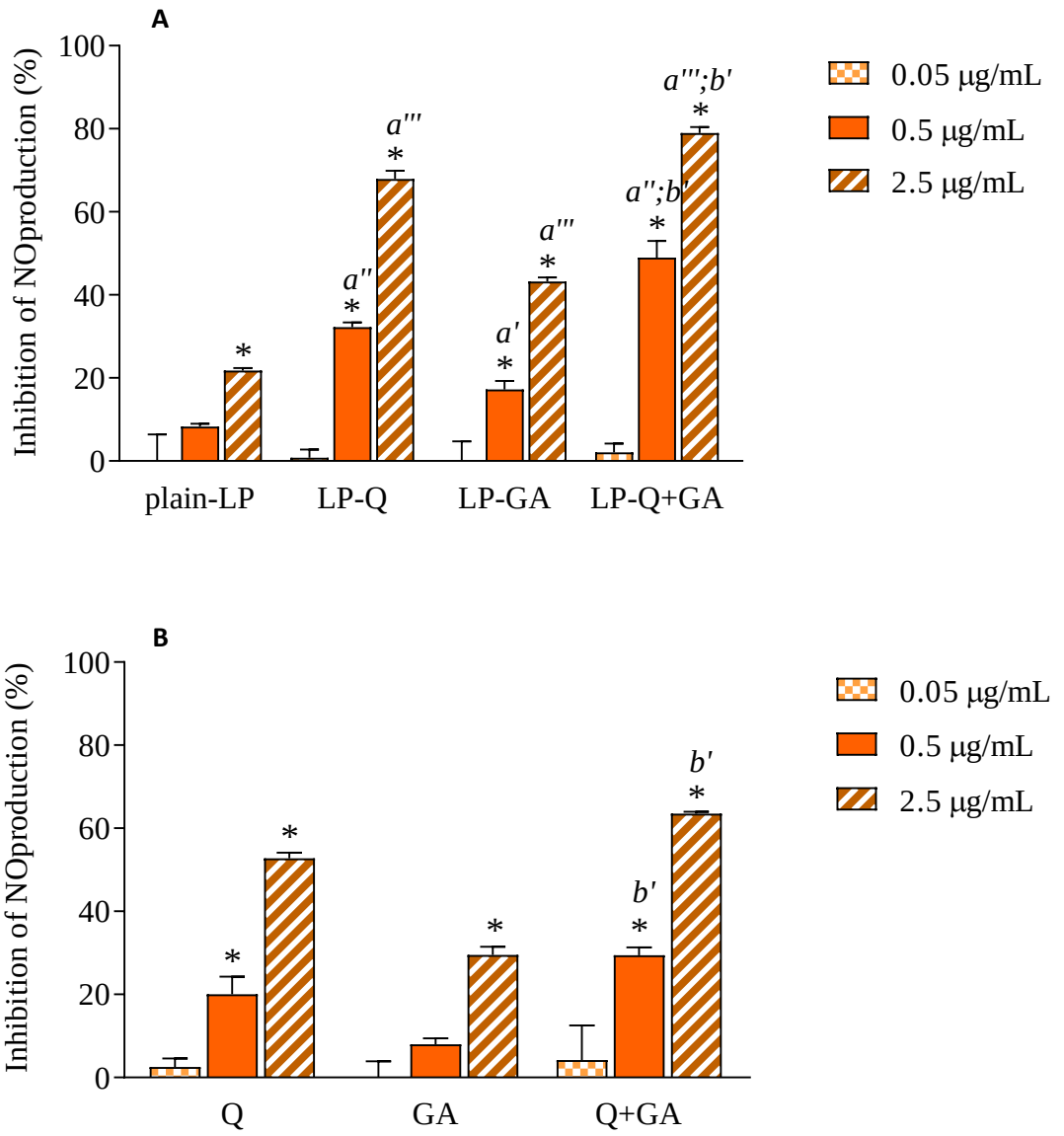

Figure VI.7 **–** Inhibitory effect of (A) liposomal and (B) free Q and/or GA on NO production in LPS-induced macrophages (mean ± SD, $n = 3$). The statistical significance was calculated with respect to control (untreated cells); * $p < 0.0001$. Statistical differences between liposomes and corresponding free polyphenols were reported as follows: *a*': $p < 0.01$; *a*'': $p < 0.001$; *a*''': $p < 0.0001$. Statistical differences between coupled polyphenols (LP-Q+GA or Q+GA) and single polyphenol (LP-Q/LP-GA or Q/GA) were also investigated; *b'* < 0.001.

VI.4.2.3 Effect of free and liposomal polyphenols on cell viability

The effect of liposomal formulations and free Q and/or GA on macrophages viability was also indagated and results are depicted in *figure VI.8*. Cells were exposed to free (*figure VI.8A*) and liposomal polyphenols (*figure VI.8B*) for 24 h at the same concentrations tested for the anti-inflammatory activity. No cytotoxic effects were found.

LP-Q and LP-GA expressed weak mitogenic effects at the higher concentration tested (2.5 µg/mL), that was also observed for LP-Q+GA at all concentrations.

These preliminary results indicated that both formulated and free polyphenols were not toxic to living cells at the polyphenol concentration up to 2.5 µg/mL, corresponding to lipid concentration of 50 µg/mL. Moreover, the results suggest that the decreased NO production (section VI.4.2.2) was actually due to inhibition of inflammation and not to the inhibition of cell proliferation.

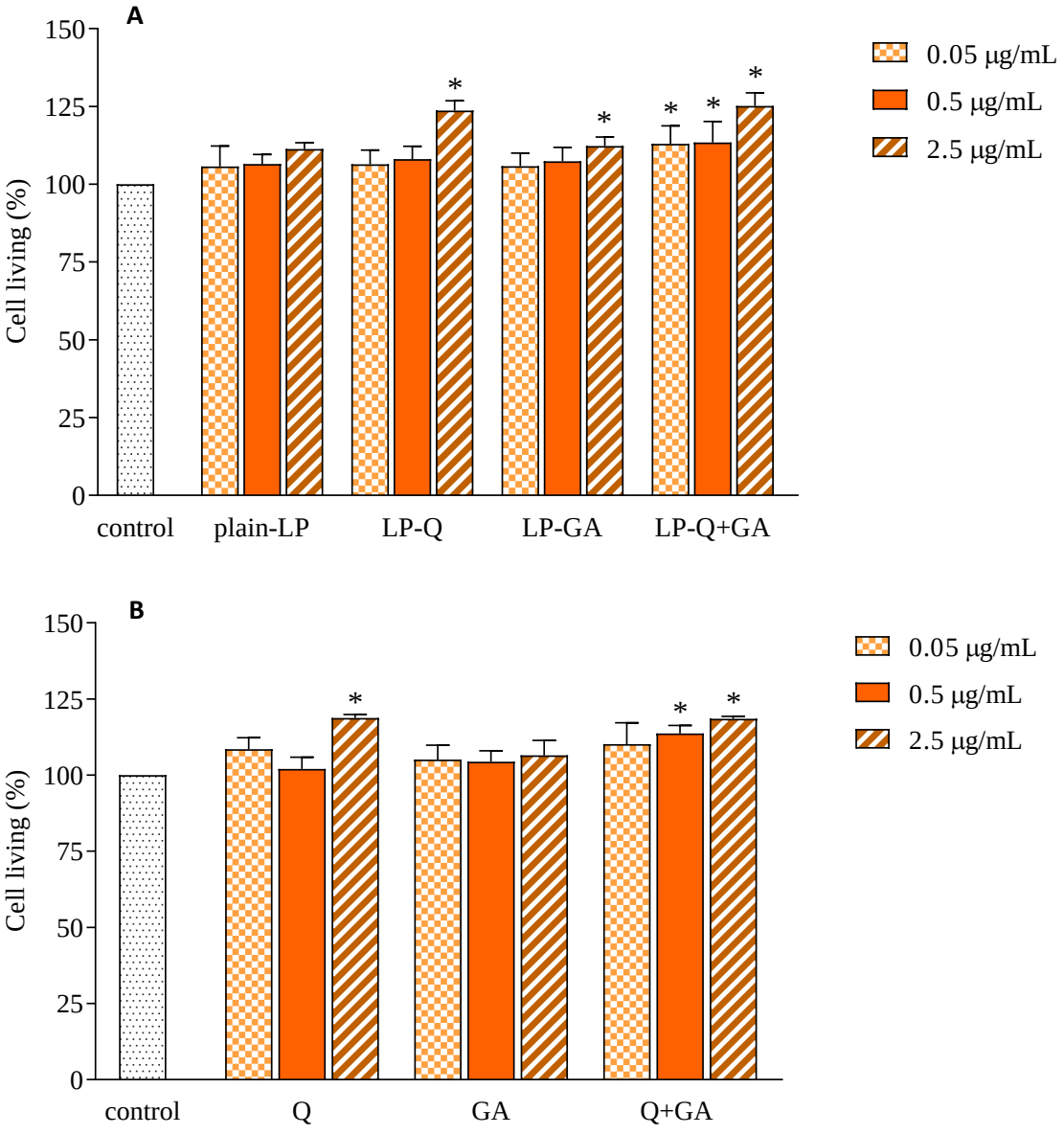

Figure VI.8 – Effect of (A) liposomal and (B) free polyphenols on RAW 264.7 cell viability compared to viability of untreated cells (100%) (mean ± SD, $n = 3$). The statistical significance was calculated with respect to control; * $p < 0.01$.

VI.4.2.4 Antifungal potential

Polyphenols not only possess useful biological properties, but also show few or no side effects and toxicity and thus may be promising natural molecules to challenge the problem of antifungal drug resistance. *Candida albicans* is the most frequent species isolated from

women suffering from candidiasis and accounts for 80-90% of total vaginal fungal infections [Sobel 2007]. For this reason, the antifungal activity of polyphenol-containing liposomes and free polyphenols was evaluated against *C. albicans* ATCC 10231.

Firstly, IC_{50} values were microscopically determined (*table VI.5*) and, as expected, plain-LP made only with SPC had no effect [Andersen 2017].

Although Q is known to possess antibacterial activity, neither free quercetin nor LP-Q were active in inhibiting *Candida* growth.

On the contrary, the two liposomal formulations containing GA considerably hampered *Candida* growth in the concentration range between 31 and 63 μg/mL after 24 h of treatment (*figure VI.9*). Free GA displayed a similar behaviour, indicating that liposomes effectively retained the antifungal activity of this phenolic acid.

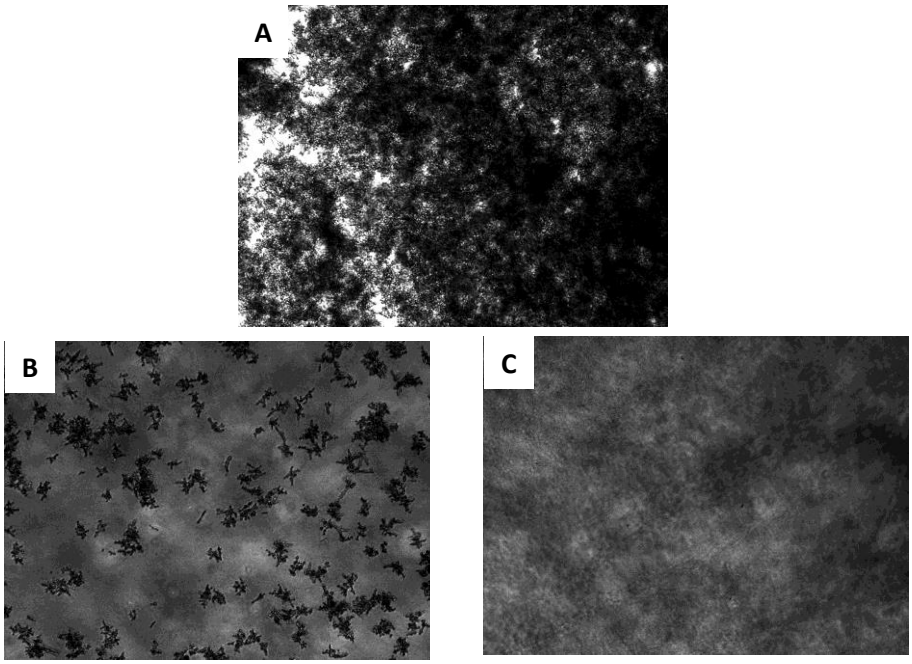

Figure VI.9 – Effect of LP-Q+GA on *C. albicans* growth (2000×): (A) control; (B) *Candida* treated with LP-Q+GA 31 μg/mL (IC_{50}); (C) *Candida* treated with LP-Q+GA 125 μg/mL (MLD).

To better clarify the antifungal effect of liposomes, aliquots of samples exhibiting less than 50% of growth were spotted onto agar plates and MLD were determined after 24 h of incubation. Free GA, as well as liposomes comprising GA, showed fungicidal effects at concentration higher than 125 μg/mL (*table VI.5*), demonstrating that LP-GA and LP-Q+GA were efficient in counteracting *Candida* proliferation.

Our findings were consistent with previous results obtained by Gehrke et al. [Gehrke 2013] and Özçelik et al. [Özçelik 2011] on the activity towards *C. albicans* ATCC 10231 exerted by pure GA isolated from *Schinus lentiscifolius* extract and standard GA

purchased from Sigma-Aldrich, respectively.

Table VI.5 – Anti *Candida* activity of liposomal formulations reported as IC_{50} (minimal concentration that inhibits 50% or more of visible growth) and MLD (minimal lethal dose) (mean ± SD, $n = 3$). na: not active.

	IC_{50} (μg/mL)	MLD (μg/mL)
LP-Q	na	na
LP-GA	31-63	125
LP-Q+GA	31-63	125
plain-LP	na	na
free Q	na	na
free GA	31-63	125

These promising findings could be further explored for localized treatment of infections and/or inflammation. By combining two or more polyphenols within a single nanocarrier, it would be possible to achieve efficient multitargeted treatment.

VI.5 Conclusions

In the present work, we successfully developed innovative liposomal formulations containing two polyphenols, namely Q and GA. The two molecules were formulated alone or in combination for the treatment of vaginal diseases, in particular we focused on candidiasis in an attempt to overcome the urgent problem of drug resistance.

All liposomal formulations had a desired size of about 200 nm. Liposomes containing both Q and GA were able to encapsulate Q at high level and to favour the entrapment of GA with respect to LP-GA. Moreover, they resulted stable over time and provided a sustained release of polyphenols.

LP-Q+GA displayed a strong anti-oxidant activity due to the synergism between the two entrapped polyphenols. Q and GA delivered together also showed the best anti-inflammatory profile and all formulations resulted not cytotoxic to cells.

Finally, LP-Q+GA efficiently counteract the growth of *C. albicans*, the most frequent etiological agent of VVC, due to the presence of GA that exerted a fungicidal activity.

VI.6 References

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VII. Final conclusions and future perspectives

In the present work different strategies to counteract vaginal diseases were taken into account. In particular, we focused on the delivery of well-established drugs (chlorhexidine and econazole nitrate) through innovative systems, as well as on the formulation of non-conventional active agents (probiotics cells and polyphenols) potentially able to oppose vaginal infections. Moreover, in order to improve the vaginal health *status*, both local and oral administration routes have been explored.

In the first paper, we developed probiotic formulations containing a strain (*Bifidobacterium breve* BC204) isolated from the vaginal swab of a healthy woman. We firstly verified that *B. breve* BC204 possessed excellent probiotic properties, such as strong antimicrobial activity against a plethora of microorganisms responsible for urogenital diseases and gastrointestinal infections, good ability to adhere to Caco-2 intestinal cells and intrinsic moderate resistance to gastric acidity. Secondly, we demonstrated that the appropriate choice of excipients, together with the application of the two-step procedure consisting of microencapsulation followed by tablet production, allowed to obtain oral formulations with optimal features. This approach revealed to be effective and reliable, since final dosage forms provided high loading and survival of *B. breve* BC204, as well as a delayed release and mucoadhesive abilities, which are required to assure the presence of an adequate amount of living probiotic cells at the treatment site. As a perspective, the efficacy of proposed oral formulations could be strengthened by combining two or more beneficial strains able to exert different health promoting activities, thus improving the global probiotic effect after oral intake. Moreover, animal models could be useful to better clarify the behaviour of tablets after administration and the impact of probiotic intake on the composition of gut and vaginal ecosystems.

Considering the growing interest for probiotic formulations in the nutraceutical field, the feasibility of adapting the entire productive process at large scale should also be investigated.

In the second paper, the freeze-drying technique was applied to produce novel lyophilized polymeric matrices for the local delivery of chlorhexidine, a disinfectant commonly employed to prevent and treat vaginal infections. The cationic nature of chlorhexidine allowed the ionic interaction with anionic polymers, giving rise to solid complexes. We took advantage of this to prepare freeze-dried matrices containing either the isolated complex or the complex along with free drug and polymers.

We then highlighted that the selection of the polymer and the preparative method permitted to modulate matrix ability to hydrate, adhere to vaginal mucosa and release the drug over the time. In particular, the matrix based on hyaluronic acid turned out to have the best properties, such as a flexible structure and excellent ability to hydrate and consequently adhere to vaginal mucosa. Moreover, it successfully provided the sustained release of a greater amount of chlorhexidine with respect to formulations obtained with other polymers. These aspects are important to guarantee a higher retention time of the drug inside the vaginal cavity, thus reducing daily dose frequency and consequently improving patient compliance. Finally, we found that hyaluronic acid, besides being an optimal excipient, was also able to enhance the antimicrobial activity of the formulation, especially against *Candida* spp. which usually exhibit less susceptibility to chlorhexidine. Taking into account the vaginal administration, we may consider applying the described technique to produce final formulations with different shapes, for example conical inserts instead of cylindrical matrices, in order to favour the *in vivo* application. The cytocompatibility with human vaginal epithelium should also be assessed.

The last two papers focused on the development of nanosystems based on lipid vesicles for treatment of candidiasis.

In the third paper, econazole nitrate was locally delivered in phosphatidylcholine vesicles containing a biosurfactant, which was selected as "green" alternative excipient over chemical surfactants. This work required a first effort to extract the biosurfactant from a vaginal probiotic strain (*L. crispatus* BC9) previously isolated in our laboratory, and to characterized it. We proved that the isolated biosurfactant possessed a peptide-like structure and promising technological qualities, such as good emulsifying properties and a high surface activity together with a low critical micelle concentration. We then employed the film rehydration and extrusion method to obtain mixed vesicles containing biosurfactant for the delivery of an antifungal drug. It is worth to note that mixed vesicles prepared with the biosurfactant showed improved characteristics compared to those prepared with a synthetic surfactant (Tween 80), such as higher encapsulation efficiency and mucoadhesion ability, as well as a sustained release of econazole. Notably, mixed vesicles containing biosurfactant significantly improved the ability of econazole to eradicate *Candida albicans* biofilm, leading to an almost complete dispersion of preformed fungal biofilm. This finding is of particular interest considering that the capacity of *Candida* spp. to form biofilm is often associated with recurrent infections and resistance to common treatments.

To further enhance the activity of mixed vesicles, a conventional antifungal drug (like econazole nitrate) could be coupled with a different biosurfactant able to exert antimicrobial activities itself. In this regard, a screening of biosurfactants produced by other probiotic strains is desirable. A deeper chemical characterization of isolated biosurfactant would also be useful for the optimization of delivery systems.

Furthermore, stability of mixed vesicles at different storage conditions and vaginal environment should be verified. The incorporation of mixed vesicles in a suitable gel vehicle should be considered to further evaluate their applicability in vaginal drug delivery.

In the fourth paper, we moved the attention to the possible employment of natural substances to replace or support conventional antifungal drugs. In particular, we successfully developed innovative multi active substance loaded liposomal formulations containing quercetin and gallic acid, either as a single substance or in combination, for the treatment of vaginal diseases, in particular vulvovaginal candidiasis. The simultaneous vaginal delivery of two polyphenols serves a dual purpose of eradicating the infection and alleviating itchiness and soreness usually associated with candidiasis.

Liposomes containing both quercetin and gallic acid had optimal size for vaginal delivery and were able to encapsulate high level of quercetin, as well as favour the entrapment of gallic acid. Moreover, they remained stable over time and provided a controlled release of both polyphenols. Interestingly, we demonstrated that quercetin and gallic acid synergistically exerted anti-oxidant effects, and the strong anti-oxidant activities were retained also after incorporation inside liposomes. Furthermore, the concomitant delivery of quercetin and gallic acid provided the better anti-inflammatory profile while remaining nontoxic.

Finally, the proposed liposomal formulation efficiently inhibited the growth of *C. albicans*; specifically, the effect can be attributed to the presence of gallic acid and its fungicidal activity.

Further testing could be useful to explore possible antimicrobial effects against other relevant vaginal pathogens. Moreover, considering the results obtained in the previous paper, the anti-biofilm activity of proposed formulations should be elucidated.

Finally, the evaluation of the liposomal formulations' safety in healthy and infected animals is of course highly fascinating.