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# Développement d'un gel vaginal à base de poloxamer 407, d'alginate de sodium et de *Lactobacillus crispatus* pour la prévention de la gonococcie

Kakwokpo N'Guessan

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# Développement d'un gel vaginal à base de poloxamer 407, d'alginate de sodium et de *Lactobacillus crispatus* pour la prévention de la gonococcie

Thèse de doctorat de l'Université Félix Houphouët-Boigny et de l'Université Paris-Saclay  
préparée à l'Université Paris-Sud

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Thèse présentée et soutenue à Châtenay-Malabry, le Jeudi 13 Décembre 2018, par

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## **DEDICACE**

A mon exceptionnel époux Yannick

A mes amours Michel-élie et Samuel-élie

A mon frère Didier et toute ma merveilleuse famille

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In memoriam à ma belle-mère N' Guessan Cécile Guiaya



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## TABLE DES MATIERES

ABRÉVIATIONS .....	13
INTRODUCTION GENERALE .....	15
<b><u>Première partie : TRAVAUX ANTERIEURS</u></b>	
INTRODUCTION.....	23
<b>Chapitre I : GONOCOCCIE : AVANCEES RECENTES ET PROBLEMES ACTUELS</b>	
.....	25
Introduction-Résumé .....	27
<b>Projet de publication : Recent advances, current issues and some contributions in the treatment and the prevention of gonorrhoea.....</b>	<b>29</b>
1. INTRODUCTION.....	31
2. EPIDEMIOLOGY .....	31
3. PATHOGENIC AGENT .....	32
4. TRANSMISSION .....	33
5. PATHOGENESIS MECHANISMS .....	34
6. SYMPTOMS .....	36
7. EVOLUTION OF THE TREATMENTS AND RESISTANCE DEVELOPMENT ..	37
8. MECHANISM OF RESISTANCE .....	41
9. EFFICIENCY OF THE PREVENTION MEANS .....	42
9.1. Vaccine.....	42
9.2. Male condom.....	44
9.3. Female condom and vaginal microbicide.....	44
10. PERSPECTIVE TO PREVENT OR TREAT GONORRHEA.....	46
10.1. Recent clinical trials on antibiotics .....	46
10.2. Herbal medicines.....	47
10.3. Bacteriotherapy .....	48
11. CONCLUSION .....	49
<b>Chapitre II : SYSTEMES DESTINES AU TRAITEMENT ET A LA PREVENTION DE LA GONOCOCCIE : INTERET DES FORMULATIONS A BASE DE LACTOBACILLES .....</b>	
.....	<b>63</b>
Introduction-Résumé .....	<b>65</b>
<b>Projet de publication : Formulations for the treatment and the prevention of gonorrhoea: interest of <i>lactobacilli</i> based systems.....</b>	<b>67</b>
1. INTRODUCTION.....	69

2.	WOMAN NORMAL GENITAL TRACT MICROBIOTA.....	70
3.	<i>LACTOBACILLI</i> MECHANISMS TO CONTROL INFECTIONS.....	71
3.1.	Enhancement of the epithelial barrier .....	72
3.2.	Adherence to epithelial surface .....	72
3.3.	Autoaggregation and coaggregation.....	73
3.4.	Acid production.....	73
3.5.	Hydrogen peroxide production.....	73
3.6.	Production of antimicrobial substances.....	74
3.7.	Production of biosurfactants.....	74
3.8.	Stimulation of the immune system.....	75
4.	<i>LACTOBACILLI</i> AS PROBIOTICS FOR THE PREVENTION AND THE TREATMENT OF UROGENITAL INFECTIONS .....	76
5.	<i>LACTOBACILLI</i> AS POTENTIAL PROBIOTICS FOR THE PREVENTION OF GONORRHEA.....	84
6.	VAGINA A PROMISING ROUTE FOR THE TREATMENT AND THE PREVENTION OF GONORRHEA.....	85
6.1.	Vaginal characteristics to take into account for the development of a vaginal formulation.....	85
6.2.	Vaginal forms.....	87
7.	CURRENT HURDLES TO THE USE OF <i>LACTOBACILLI</i> AGAINST GONORRHEA.....	89
8.	CONCLUSION.....	93

**Chapitre III : INTERET D'UN HYDROGEL CONTENANT *LACTOBACILLUS CRISPATUS* POUR LA PREVENTION DE LA GONOCOCCIE ..... 107**

1.	INTRODUCTION.....	109
2.	CHOIX DE LA VOIE VAGINALE .....	109
3.	INTERET DE <i>LACTOBACILLUS CRISPATUS</i> POUR LA PREVENTION DE LA GONOCOCCIE.....	110
4.	INTERET DE LA FORME "HYDROGEL" POUR LA VOIE VAGINALE .....	112
5.	LES GELS THERMOSENSIBLES A BASE DE POLOXAMERS : PROPRIETES PHYSICOCHIMIQUES ET APPLICATIONS PHARMACEUTIQUES.....	114
6.	PROPRIETES PHYSICOCHIMIQUES DE L'ALGINATE ET SES APPLICATIONS PHARMACEUTIQUES.....	118
7.	MELANGE ALGINATE - POLOXAMER.....	122
8.	CONCLUSION .....	126

**Deuxième partie : TRAVAUX EXPERIMENTAUX**

<b>INTRODUCTION.....</b>	<b>137</b>
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**Chapitre IV : DEVELOPPEMENT ET CARACTERISATION D'UN HYDROGEL A BASE DE POLOXAMER 407 ET D'ALGINATE DE SODIUM DESTINE A UNE ADMINISTRATION VAGINALE..... 139**

**Introduction- résumé ..... 141**

**Projet de publication : Investigation of a gel containing poloxamer 407 and sodium alginate as a platform for vaginal administration.....143**

1. INTRODUCTION.....	145
2. MATERIALS AND METHODS .....	146
2.1. Materials.....	146
2.2. Characterization of sodium alginate.....	146
2.3. Hydrogel preparation.....	147
2.4. Hydrogels diluted by simulated vaginal fluid .....	148
2.5. Alginate ionic gelling capacity.....	148
2.6. Rheological characterization .....	149
2.7. Texture Profile Analysis at 25°C .....	150
2.8. Expulsion evaluation from a vaginal device .....	150
3. RESULTS AND DISCUSSION .....	151
3.1. Sodium alginate characterization .....	151
3.2. Gel preparation and selection of the <i>in vitro</i> conditions .....	153
3.3. Sodium alginate formulations .....	153
3.4. Characterization of poloxamer 407 formulations and of their mixtures with alginate ... ..	156
3.5. Expulsion ability from a device and texture profile analysis .....	161
3.6. Microstructure of P21.5A1 .....	163
4. CONCLUSION .....	164

**Chapitre V : FORMULATION D'UN HYDROGEL A BASE DE POLOXAMER 407, D'ALGINATE DE SODIUM ET DE *L. CRISPATUS* DESTINE A INHIBER LA CROISSANCE DE *NEISSERIA GONORRHOEAE* ..... 167**

**Introduction- résumé ..... 168**

**Projet de publication : Characterization and *in vitro* evaluation of a vaginal gel containing *lactobacillus crispatus* for the prevention of gonorrhoea.....173**

1. INTRODUCTION.....	173
2. MATERIALS AND METHODS .....	175
2.1. Materials.....	175
2.2. Study of <i>Lactobacillus crispatus</i> .....	176
2.3. Purity control and counting of <i>L. crispatus</i> .....	177
2.4. Hydrogel preparation.....	177
2.5. Preparation of hydrogels diluted by simulated vaginal fluid .....	178

2.6.	Rheological characterization of the hydrogels .....	179
2.7.	pH measurement of the gels .....	179
2.8.	Assessment of the distribution of <i>L. crispatus</i> within the gel .....	179
2.9.	Assessment of the influence of gel state .....	180
2.10.	Stability studies of the gel P21.5A1L.....	180
2.11.	Inhibition of the growth of <i>N. gonorrhoeae</i> .....	180
3.	RESULTS AND DISCUSSION .....	181
3.1.	Characterization of <i>L. crispatus</i> .....	182
3.2.	Preparation and rheological characterization of gels .....	186
3.3.	Distribution and viability of <i>L. crispatus</i> in the gel .....	189
3.4.	Influence of the storage conditions .....	190
3.5.	Efficacy of inhibition of <i>N. gonorrhoeae</i> .....	194
4.	CONCLUSION .....	194
<b>Chapitre VI : MANIPULATIONS COMPLEMENTAIRES.....</b>		<b>201</b>
1.	ETUDE PRELIMINAIRE DE L'ORGANISATION D'UN GEL A BASE DE POLOXAMER 407, D'ALGINATE DE SODIUM ET DE <i>LACTOBACILLUS CRISPATUS</i> PAR MICROSCOPIE CONFOCALE .....	203
1.1.	Introduction .....	203
1.2.	Matériel et méthodes .....	204
1.3.	Résultats et discussion.....	205
1.4.	Conclusion.....	207
2.	INTRODUCTION DU MRS DANS LE GEL : AVANTAGES ET INCONVENIENTS.....	208
2.1.	Introduction .....	208
2.2.	Matériel et méthodes .....	208
2.3.	Résultats et discussion.....	209
2.4.	Conclusion.....	219
<b>DISCUSSION GENERALE.....</b>		<b>221</b>
1.	CHOIX ET CARACTERISATION DE <i>L. CRISPATUS</i> .....	223
2.	POLYMERES .....	225
3.	MELANGE LACTOBACILLES, POLOXAMER 407 ET ALGINATE .....	226
<b>CONCLUSION GENERALE ET PERSPECTIVES.....</b>		<b>231</b>

# ABRÉVIATIONS

API : Active Pharmaceutical Ingredient

A : sodium alginate

ATCC : american type collection center

CDC: center for disease control

CFU : colony forming unit

G' : elastic modulus

G'' : viscous modulus

HIV: human immunodeficiency virus

IL : interleukin

IM : intramuscular

Ig : immunoglobulin

IST : Infection sexuellement transmissible

IV : intravenous

*L.* : *Lactobacillus*

*L. crispatus* : *Lactobacillus crispatus*

LOS : lipo-oligosaccharide

MAPK : mitogen-activated protein kinase

MIC : Minimal Inhibitory Concentration

MRS : Man-Rogosa-Sharpe

NF-B : nuclear factor kappa B

*N. gonorrhoeae* : *Neisseria gonorrhoeae*

NIAID : National Institute of Allergy and Infectious Diseases

OMS : Organisation Mondiale de la Santé

OMV : outer membrane vesicles

PAMPs : pathogen-associated molecular patterns

PBS : phosphate buffer solution

pH : potentiel d'hydrogène

pH<sub>i</sub> : initial pH

P407 : poloxamer 407

POE : poly(oxyéthylène)

POP : poly(oxypropylène)

PEO : poly(ethylene oxyde)

PPO : poly(propylene oxyde)

STI : sexually transmitted infection

SVF : simulated vaginal fluid

T<sub>gel</sub> : température de gélification ou gelation temperature

TLR : toll-like receptor

TNF : tumor necrosis factor

USA : United States of America

WHO : World Health Organization

# **INTRODUCTION GENERALE**





Les infections sexuellement transmissibles (IST) peuvent être d'origine bactérienne (chlamydie, gonococcie, syphilis), virale (infection au papillomavirus humain, herpès, infection au virus de l'immunodéficience humaine (VIH)) ou parasitaire (trichomonase) (WHO, 2013). Les IST bactériennes sont guérissables. Toutefois, si elles ne sont pas diagnostiquées ni traitées, elles peuvent entraîner de graves complications et des séquelles à long terme pour les femmes, telles que des infections génitales hautes, des grossesses extra-utérines et des fausses couches. Non traitées chez la femme enceinte, elles augmentent le risque de morbidité pouvant aller jusqu'à la cécité et le décès du nouveau-né. De plus, non traitées, la gonococcie et la chlamydie peuvent provoquer la stérilité tant chez les hommes que chez les femmes (WHO, 2013). Ces IST peuvent également multiplier par 2 ou 3, voire plus, le risque de contracter le VIH (WHO, 2012, 2013).

Les IST bactériennes peuvent généralement être guéries par antibiothérapie. Malheureusement, des germes responsables de la chlamydie et de la syphilis ont développé de l'antibio-résistance. Mais cette résistance est beaucoup moins répandue comparée à celle observée avec le germe responsable de la gonococcie, le gonocoque (WHO, 2013). L'apparition de souches de gonocoque multi-résistantes a progressivement fait place à l'apparition de souches résistantes au traitement actuel de dernière intention, les céphalosporines à spectre étendu que sont la céfixime (voie orale) ou la ceftriaxone (voie injectable) (WHO, 2013). Aujourd'hui, des souches de gonocoque ne réagissant à aucun des antibiotiques connus, existants sur le marché, ont déjà été mises en évidence (WHO, 2017). Parallèlement, cette infection ne cesse de se propager à travers le monde. Selon les estimations de l'Organisation Mondiale de la Santé (OMS) réalisées en 2016, chaque année, 78 millions de personnes sont infectées par le gonocoque dont 35,2 millions dans la région du Pacifique occidental, 11,4 millions dans la région de l'Asie du Sud-Est, 11,4 millions dans la région africaine, 11,0 millions dans la région des Amériques, 4,7 millions dans la région européenne et 4,5 millions dans la région de la Méditerranée orientale (WHO, 2016). L'OMS rapporte même que ces chiffres sont sous-estimés par rapport à l'ampleur réelle de ce problème mondial, du fait de l'existence de formes asymptomatiques. L'OMS mentionne également que l'une des raisons de l'augmentation des cas de gonococcie dans le monde ces dernières années est la baisse de l'utilisation des préservatifs. Pourtant le préservatif est jusqu'à présent le seul moyen efficace de prévention contre la gonococcie lorsqu'il est correctement et régulièrement utilisé.

La recherche et le développement pour la gonococcie est relativement peu développée. Parmi toutes les nouvelles molécules, seulement 3 nouveaux antibiotiques candidats que sont la

solithromycine (essai de phase III); le zoliflodacin (essai de phase II); et le gepotidacin (essai de phase II) sont en phase de développement clinique (Fernandes and Martens, 2017). La mise au point de nouveaux antibiotiques n'est pas très attrayante pour les sociétés pharmaceutiques car ces traitements sont administrés uniquement pendant de courtes périodes contrairement aux médicaments contre les maladies chroniques. De plus, ces antibiotiques peuvent devenir moins efficaces à mesure que la résistance se développe, ce qui signifie un perpétuel recommencement (Fernandes and Martens, 2017). S'orienter vers de nouvelles approches afin d'améliorer la prévention serait une alternative.

Dans ce contexte, nous proposons de concevoir un nouveau moyen de prévention. En effet, les lactobacilles sont des bactéries commensales, prédominantes dans la flore vaginale saine qui luttent contre les infections *in vivo*. Leur présence et leur prédominance dans le vagin sont associées à une diminution de la susceptibilité de la femme aux infections urogénitales et d'infections sexuellement transmissibles. L'utilisation des lactobacilles pour le traitement et/ou la prévention des infections urogénitales telles que les infections récurrentes des voies urinaires, les bactérioses vaginales et les mycoses vaginales, est une approche en plein développement (Pascual et al., 2008, Reid, 2002, Reid et al., 2003, 2004) qui a déjà conduit à des produits sur le marché. Certains lactobacilles ont démontré une activité sur le gonocoque *in vitro* (Amant et al., 2002; Muench et al., 2009; Osset et al., 2001; Saigh et al., 1978) et *ex vivo* sur une muqueuse vaginale de porc (Breshears et al., 2015). Par ailleurs, la femme est la principale cible des IST, ce qui peut influencer la santé du nouveau-né. Ainsi, disposer d'un système qu'elle est capable de manipuler elle-même serait un atout considérable dans la gestion de la gonococcie. Ce système serait préférentiellement destiné à la voie vaginale qui est la voie d'administration la plus intéressante dans la prévention des IST puisqu'elle est la principale porte d'entrée des agents pathogènes. Les hydrogels sont des supports d'administration de substances actives. Ils ont une bonne acceptabilité, sont faciles à développer pour les formulateurs et faciles d'utilisation par les femmes. Un gel, surtout thermogélifiant, contenant des lactobacilles semble donc une approche attractive pour traiter ou prévenir la gonococcie par la voie vaginale. Il pourra se présenter sous une forme liquide pour faciliter son administration et son étalement et gélifier dans le vagin pour prolonger son temps de résidence.

Ce projet a été réalisé dans le cadre d'une thèse en co-tutelle entre l'Université Félix Houphouët-Boigny (Abidjan, Côte d'Ivoire) et l'Université Paris-Sud de l'Université Paris-Saclay (Chatenay-Malabry, France) sous la co-direction des Professeurs Armand Koffi et

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Ce manuscrit est rédigé en français avec certains chapitres sous formes d'articles destinés à publication rédigés en anglais. Dans une première partie intitulée travaux antérieurs, nous essaierons de faire l'état des lieux des connaissances sur la gonococcie, en particulier la structure du germe, son mécanisme d'infection et de développement des résistances, et les perspectives en matière de prévention et de traitement (**chapitre I**). Nous nous intéresserons ensuite (i) à l'utilisation des lactobacilles pour traiter et prévenir les infections et leur mécanisme d'action, (ii) à la voie vaginale et (iii) à la forme galénique hydrogel (**chapitre II**). Le **chapitre III** traitera des propriétés physico-chimiques des polymères utilisés pour la formulation dont le poloxamer 407 et l'alginate de sodium, de leur utilisation et de leurs applications en pharmacie. Dans une deuxième partie expérimentale, nous présenterons les aspects physico-chimiques du mélange de polymères (**chapitre IV**), les aspects de caractérisation de la souche de lactobacille choisie, ainsi que les aspects de formulation et de caractérisation (**chapitre V**). Quelques manipulations complémentaires en termes de pistes d'optimisation du système feront l'objet du **chapitre VI**. Nous terminerons par une discussion générale de nos travaux de recherches et par les perspectives de développement qui en sont issues.

## Références

Amant, D.C.S., Valentin-Bon, I.E., and Jerse, A.E. (2002). Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. *Infect. Immun.* *70*, 7169–7171.

Breshears, L.M., Edwards, V.L., Ravel, J., and Peterson, M.L. (2015). *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. *BMC Microbiol.* *15*.

Fernandes, P., and Martens, E. (2017). Antibiotics in late clinical development. *Biochem. Pharmacol.* *133*, 152–163.

Muench, D.F., Kuch, D.J., Wu, H., Begum, A.A., Veit, S.J., Pelletier, M.-E., Soler-García, Á.A., and Jerse, A.E. (2009). Hydrogen peroxide-producing *Lactobacilli* inhibit gonococci in vitro but not during experimental genital tract infection. *J. Infect. Dis.* *199*, 1369–1378.

Osset, J., Bartolomé, R.M., García, E., and Andreu, A. (2001). Assessment of the capacity of *Lactobacillus* to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells. *J. Infect. Dis.* *183*, 485–491.

Saigh, J.H., Sanders, C.C., and Sanders, W.E. (1978). Inhibition of *Neisseria gonorrhoeae* by aerobic and facultatively anaerobic components of the endocervical flora: evidence for a protective effect against infection. *Infect. Immun.* *19*, 704–710.

WHO (2012). Global incidence and prevalence of selected curable STIs-2008 (Geneva).

WHO (2013). Infections sexuellement transmissibles.

WHO (2016). WHO | WHO guidelines for the treatment of *Neisseria gonorrhoeae*.

WHO (2017). Augmentation des cas de gonorrhée résistante aux antibiotiques, de nouveaux médicaments sont nécessaires (Genève).

## **Première partie : TRAVAUX ANTERIEURS**



## INTRODUCTION

La première partie de ce manuscrit consacrée aux travaux antérieurs est organisée en trois chapitres.

**Le premier chapitre** est rédigé en anglais, sous la forme d'un article de revue destiné à être soumis à publication. Ce chapitre présente l'état de l'art sur la gonococcie, la structure de l'agent pathogène responsable, son mécanisme d'infection et de développement des résistances. Il souligne les progrès récents et les problèmes actuels de la prise en charge de cette infection sexuellement transmissible. Il discute également les traitements et moyens de prévention de la gonococcie ainsi que les perspectives en la matière.

**Le deuxième chapitre** également rédigé en anglais, sous la forme d'un article de revue destiné à la publication, montre l'intérêt de l'utilisation des lactobacilles pour la prévention et/ou le traitement de la gonococcie. Cette revue bibliographique, par ailleurs, justifie le choix d'une souche de lactobacilles (*Lactobacillus crispatus*) en raison de ses propriétés probiotiques et de son activité sur *Neisseria gonorrhoeae*. Des formulations à base de probiotiques pour la voie urogénitale sur le marché et celles faisant l'objet de travaux de recherche sont recensées. Ce chapitre, en outre, décrit les caractéristiques de la voie vaginale, ses avantages, ses inconvénients et les formes galéniques adaptées à une administration par cette voie.

**Le troisième chapitre**, rédigé en français, présente les caractéristiques des formulations thermosensibles à base de poloxamer 407 et celles de l'alginate de sodium, discute leurs propriétés physico-chimiques et leurs applications en pharmacie, dans le but de justifier le choix de ces excipients pour nos travaux expérimentaux.





**CHAPITRE I : GONOCOCCIE : AVANCEES RECENTES ET  
PROBLEMES ACTUELS**



## Introduction-Résumé

La gonococcie est une infection sexuellement transmissible (IST) bactérienne causée par *Neisseria gonorrhoeae* (*N. gonorrhoeae*), appelé gonocoque. Chaque année, environ 78 millions de nouvelles personnes la contractent dans le monde (WHO, 2016). Le plus souvent, cette infection est asymptomatique, de sorte que les cas signalés sont sous-estimés par rapport à l'ampleur réelle de l'infection. La gonococcie est la deuxième IST bactérienne la plus fréquente après l'infection par *Chlamydia trachomatis* (Kumar et al., 2005; Pearson et al., 2004; Rompalo, 2004; WHO, 2016). La gonococcie peut avoir des conséquences graves pouvant aller jusqu'à l'infertilité chez l'adulte (Arya et al., 1980; Grodstein et al., 1993) et la cécité chez le nouveau-né (Galega et al., 1984; Laga et al., 1986). Par ailleurs, la gonococcie facilite la transmission du virus de l'immunodéficience humaine (VIH) en fragilisant les barrières mécaniques contre l'infection et en augmentant l'inflammation, en augmentant le nombre de copies de l'ARN du VIH (CDC, 1998; Cohen, 2004; Fleming and Wasserheit, 1999; Jarvis and Chang, 2012; Wasserheit, 1992). Comparée aux deux autres IST bactériennes (chlamydie et syphilis), on note une augmentation très importante de la résistance aux antibiotiques dans le cas de la gonococcie, et bien plus, une résistance aux traitements de dernière intention (ceftriaxone et cefixime) (Lefebvre et al., 2018). Il n'existe actuellement pas de vaccins car la grande variabilité antigénique du gonocoque rend difficile leur développement. Il n'existe pas non plus de nouvelles molécules sur le marché pouvant être utilisées pour le traitement de la gonococcie. Le seul moyen de prévention efficace qui est le préservatif est malheureusement peu ou mal utilisé. Pour toutes ces raisons, la gonococcie est un problème majeur de santé publique.

Ce chapitre a pour objectif de décrire, dans un premier temps, l'agent pathogène responsable de la gonococcie, ses voies de transmission, sa pathogénicité, et ses manifestations. Nous présentons ensuite l'évolution des traitements et leurs limites ainsi que l'évolution des résistances aux antibiotiques. Nous abordons les moyens de prévention, et décrivons les mécanismes de résistances rendant difficile le développement de vaccins. Tout cela montre les difficultés liées au traitement et à la prévention de cette infection. Nous terminerons par les perspectives en la matière.

## Références

Arya, O.P., Taber, S.R., and Nsanze, H. (1980). Gonorrhoea and female infertility in rural Uganda. *Am. J. Obstet. Gynecol.* 138, 929–932.

CDC (1998). HIV prevention through early detection and treatment of other sexually transmitted diseases -- United States recommendations of the advisory committee for HIV and STD prevention. *47 (RR 12)*, 1–24.

Cohen, M.S. (2004). HIV and sexually transmitted diseases: lethal synergy. *Top. HIV Med. Publ. Int. AIDS Soc. USA 12*, 104–107.

Fleming, D.T., and Wasserheit, J.N. (1999). From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. *Sex. Transm. Infect. 75*, 3–17.

Galega, F.P., Heymann, D.L., and Nasah, B.T. (1984). Gonococcal ophthalmia neonatorum: the case for prophylaxis in tropical Africa. *Bull. World Health Organ. 62*, 95–98.

Grodstein, F., Goldman, M.B., and Cramer, D.W. (1993). Relation of tubal infertility to history of sexually transmitted diseases. *Am. J. Epidemiol. 137*, 577–584.

Jarvis, G.A., and Chang, T.L. (2012). Modulation of HIV transmission by *Neisseria gonorrhoeae*: molecular and immunological aspects. *Curr. HIV Res. 10*, 211–217.

Kumar, V., Abbas, A., and Fausto, N. (2005). *Robbins and Cotran pathologic basis of disease* (Philadelphia: Saunders).

Laga, M., Plummer, F.A., Nzanze, H., Namaara, W., Brunham, R.C., Ndinya-Achola, J.O., Maitha, G., Ronald, A.R., D’Costa, L.J., and Bhullar, V.B. (1986). Epidemiology of ophthalmia neonatorum in Kenya. *Lancet Lond. Engl. 2*, 1145–1149.

Lefebvre, B., Martin, I., Demczuk, W., Deshaies, L., Michaud, S., Labbé, A.-C., Beaudoin, M.-C., and Longtin, J. (2018). Ceftriaxone-Resistant *Neisseria gonorrhoeae*, Canada, 2017 (2018). *Emerging Infectious Disease journal - CDC*, 24 (2).

Pearson, R., Ferri, F., Jones, R., and Opal, S. (2004). *Gonorrhea*. St Louis: Elsevier;

Rompalo, A. (2004). *Gonorrhea*. In: Rake RE, Bope ET (Conn’s current therapy. Philadelphia: Saunders).

Wasserheit, J.N. (1992). Epidemiological synergy. Interrelationships between human immunodeficiency virus infection and other sexually transmitted diseases. *Sex. Transm. Dis. 19*, 61–77.

WHO (2016). WHO | WHO guidelines for the treatment of *Neisseria gonorrhoeae*.

## PROJET DE PUBLICATION

# RECENT ADVANCES, CURRENT ISSUES AND SOME CONTRIBUTIONS IN THE TREATMENT AND THE PREVENTION OF GONORRHEA

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### Abstract

The infection by *Neisseria gonorrhoeae* called gonorrhea is a sexually transmitted infection. Nowadays, it is a major public health concern due to the increasing resistance to antibiotics and the difficulties to develop a vaccine. This review is a critical study of the mechanisms of this infection, and of the evolution of its treatments and prevention means. It aims to underline the recent advances, the current hurdles in the treatment and the prevention of gonorrhea and the new strategies that are currently being developed.

### Keywords

Gonorrhea, prevention, treatment, *Neisseria gonorrhoeae*, antibio-resistance, vaccine.

## **Abbreviations**

API, Active Pharmaceutical Ingredient; ATCC, American Type Collection Center; CDC, Center for Disease Control; CFU, Colony Forming Unit; HIV, Human Immunodeficiency Virus; IL, interleukin; IM, intramuscular; IV, intravenous; Ig, immunoglobulin; *L.*, *Lactobacillus*; LOS, lipo-oligosaccharide; MIC, Minimal Inhibitory Concentration; NIAID, National Institute of Allergy and Infectious Diseases; *N. gonorrhoeae*, *Neisseria gonorrhoeae*; OMV, outer membrane vesicles; STI, Sexually Transmitted Infection; TLR, toll-like receptor; TNF, Tumor Necrosis Factor; USA, United States of America; WHO, World Health Organization.

## 1. INTRODUCTION

Gonorrhea is a sexually transmitted infection (STI) caused by *Neisseria gonorrhoeae* (*N. gonorrhoeae*). About 78 million new cases occurred each year in the world. More often this infection is asymptomatic, so the reported cases are underestimated compared to the real disease burden. Gonorrhea is the second most common bacterial STI after the infection by *Chlamydia trachomatis* (WHO, 2016). It can cause severe damages such as infertility (Grodstein et al., 1993) both in men and women and blindness in newborns (Laga et al., 1986). Moreover, gonorrhea, as all STI, facilitates the transmission of human immunodeficiency virus (HIV) by breaching of the mechanical barriers to infection, increasing inflammation, and enhancing the HIV RNA copy number (Cohen, 2004; Jarvis and Chang, 2012). In the early 2000s, *N. gonorrhoeae* became resistant to ciprofloxacin, a fluoroquinolone which up to then was used as the first line treatment of uncomplicated urogenital gonorrhea. Resistance to antibiotics is progressing. Nowadays, the emergence of resistance to third-generation cephalosporins that are current recommended treatments, mainly to oral cefixime, but also more recently to injectable ceftriaxone, has led to therapeutic impasse (WHO, 2017). Moreover, there is currently neither vaccine nor new drug emerging on the market. Thus, gonorrhea has become a major public health issue, and it is urgent and crucial to reinforce prevention by developing new strategies. In this article we review the majority of the data from published studies on gonorrhea. This will help to understand the current issues caused by gonorrhea, the mechanisms of infection, the evolution of the treatments and the prevention means, in order to give the basis for future research orientations.

## 2. EPIDEMIOLOGY

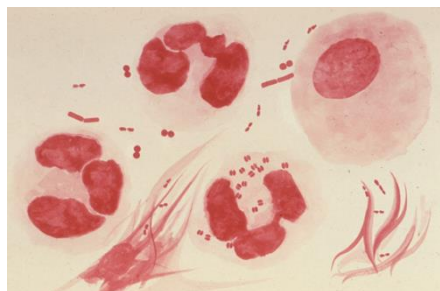
In 2012, the WHO reported that the estimated number of new cases of gonorrhea in the world was 87.7 million in 2005, and has increased to 106.1 million in 2008 (WHO, 2012). In 2016, the WHO reported that 78 million new cases occurred in 2012 among adolescents and adults aged 15–49 years worldwide with a global incidence rate of 19 per 1000 females and 24 per 1000 males (WHO, 2016). The prevalence of gonorrhea for the year 2013 was about one hundred million new cases (Newman et al., 2015; Vos et al., 2015). Therefore, each year, about one hundred million new persons become infected. This prevalence is underestimated due to the existence of asymptomatic forms mainly in women.



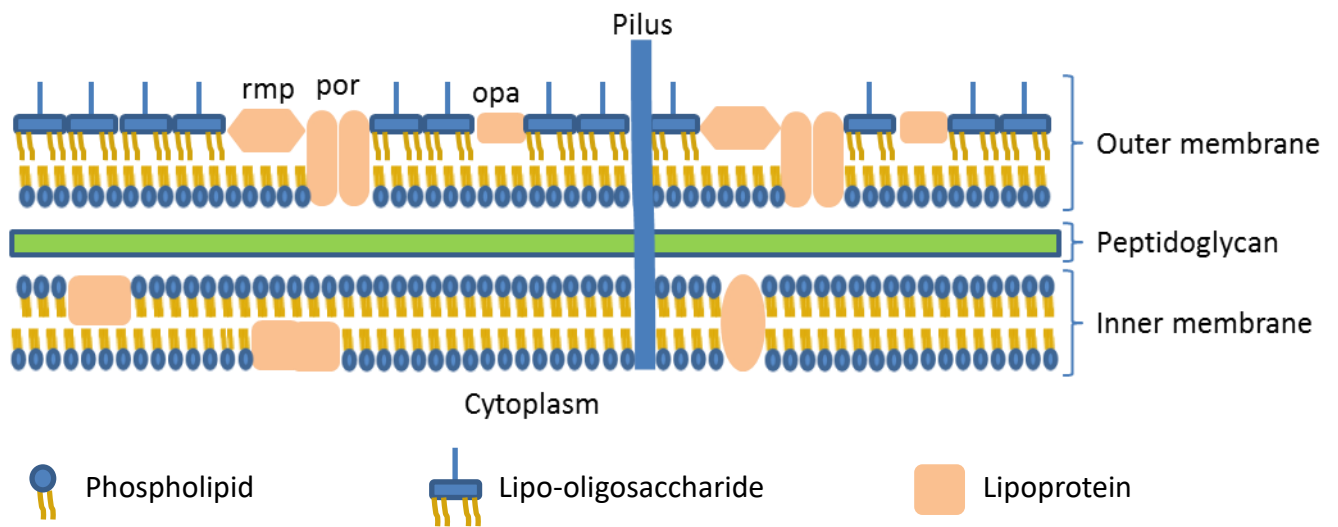
### 3. PATHOGENIC AGENT

Gonorrhea is caused by a bacterium called *Neisseria gonorrhoeae* (*N. gonorrhoeae*). *N. gonorrhoeae* is a Gram-negative diplococcus (**Fig. 1**) that is found in humans only. This non-motile bacterium survives poorly outside the human body and is sensitive to silver nitrate, drying, light and oxidation. It looks like a pair of coffee beans adjoined and flattened on the adjoining sides (**Fig. 1**) and measures 0.7-1  $\mu\text{m}$ . It is an intra or extra-cellular bacterium (**Fig. 1**). *N. gonorrhoeae* is an aerobic bacterium with stringent nutritional requirements. It can grow best on enriched media such as lysed sheep's blood agar called "chocolate" agar or selective medium called Thayer-Martin medium in the presence of 5 % of carbon dioxide ( $\text{CO}_2$ ). *N. gonorrhoeae* envelope consists in an inner membrane (that is composed of two phospholipid leaflets and different lipoproteins) surrounded by a thin peptidoglycan cell wall. This peptidoglycan wall is surrounded by an outer membrane (that consists of two leaflets, the inner leaflet being composed of one phospholipid layer and the outer leaflet of lipooligosaccharide (LOS) and different proteins) (Morse, 1978; Virji, 2009) (**Fig. 2**). Its surface is negatively charged and hydrophobic. Regarding *N. gonorrhoeae* antigenic structures and virulence factors (Virji, 2009) (**Fig. 2**), there are (i) *pili*, that help the bacterium to adhere to cells, (ii) LOS, an endotoxin, (iii) IgA1 protease that splits and inactivates immunoglobulin A (IgA), and (iv) 3 types of outer membrane proteins:

- porin proteins or protein I (*por*) that affect the permeability and penetration of antibiotics into the organism. They promote the intracellular survival of *N. gonorrhoeae*;
- opacity proteins or protein II (*opa*) that increase the binding of *N. gonorrhoeae* to the epithelial cells and promote its entry into cells;
- reduction-modifiable proteins or protein III (*rmp*) that protect other surface antigens from bactericidal antibodies. They are associated with protein I.



**Figure 1.** An urethral exudate containing *Neisseria gonorrhoeae* from a patient with gonococcal urethritis after Gram-staining showing typical intracellular Gram-negative diplococci, and pleomorphic extracellular Gram-negative organisms from the public health image library of CDC (CDC/Dr. Norman, 1974).



**Figure 2.** Schematic representation of the *Neisseria gonorrhoeae* surface structure, showing the major components of this wall (inner- and outer-membrane, peptidoglycan) and its antigenic structure (*pili*, *por*, *opa*, *rmp*, LOS) depicted from the publications of Edwards and Apicella, 2004, and Virji, 2009.

#### 4. TRANSMISSION

Gonorrhoea is commonly transmitted through sexual contact, from infected vagin to penis or mouth or from infected penis to vagin, mouth and anus. Gonorrhoea can also be transmitted from mother to child during delivery (De Schryver and Meheus, 1990). The bacterium then develops in the cervix, uterus, fallopian tubes and urethra of women or urethra of men and in mouth, throat, eyes and anus.

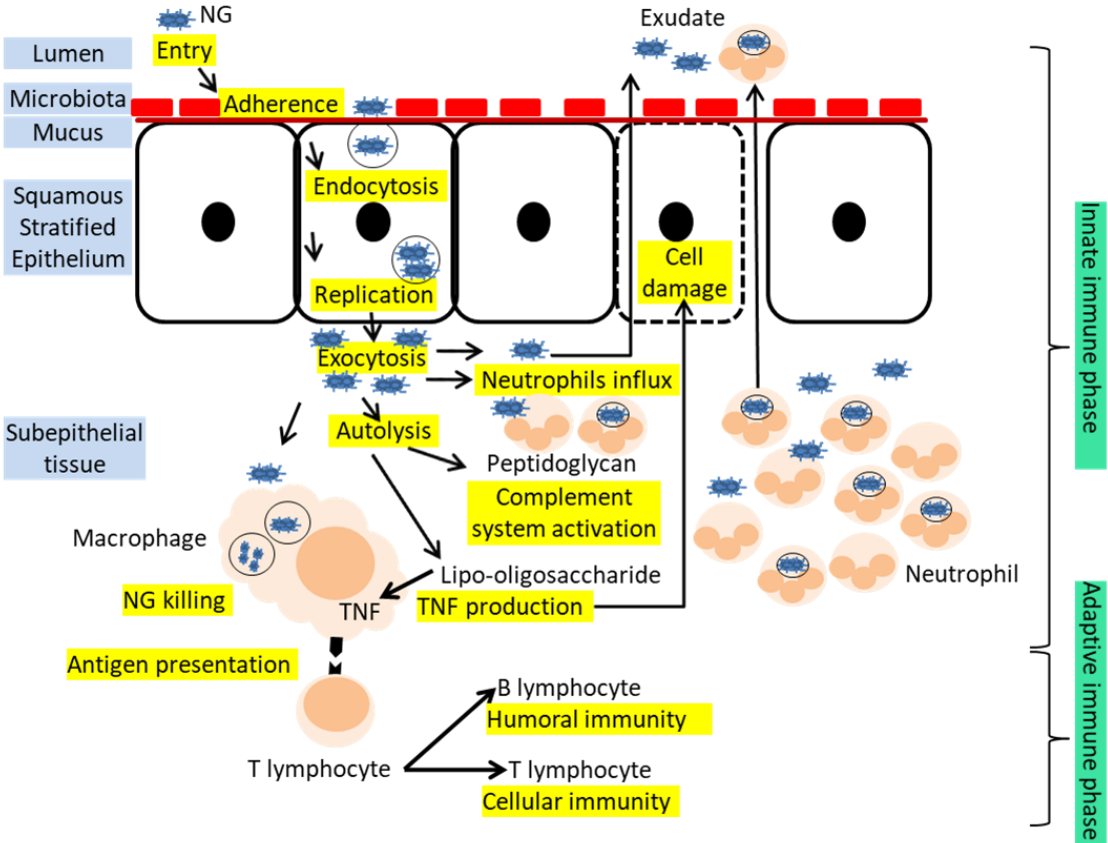
Lin et al. (1998) assessed the transmission of *N. gonorrhoeae* among men with urethritis and their female sex partners. They showed that *N. gonorrhoeae* auxotype (class defined by checking *N. gonorrhoeae* requirements for specific nutrients in chemically defined media) and serovar (class defined by checking *N. gonorrhoeae* antigenic properties using serologic methods) were similar both in a man and his female partner in 98 % of the cases (Lin et al., 1998). This result confirmed the sexual transmission of *N. gonorrhoeae*.

Moreover, *N. gonorrhoeae* transmission is more efficient from an infected man to a woman during vaginal intercourse and the rate increases after repeated exposure (Rompalo, 2004). It is independent of ejaculation. It was demonstrated that the difference likely reflects the volume of the inoculum of *N. gonorrhoeae* deposits on a susceptible mucosal surface as well as the inherent efficiency of internal deposition of infective secretions (Handsfield and Sparling, 2004).

## 5. PATHOGENESIS MECHANISMS

Host barriers to infection consist of innate and adaptive immune defense (Kumar et al., 2005; Mims et al., 2001). Innate immune defense mechanism exists before infection and responds rapidly to pathogens while adaptive immune responses are stimulated by exposure to pathogens and increase with successive exposures. The innate defense mechanisms include the intact mucosa surface as physical barrier to infection, phagocytic cells (macrophages, neutrophils), natural killer cells, and plasma proteins like complement system proteins (multimolecular complex that possesses cytolytic activity and forms the membrane attack complex (Janeway et al., 2001) and other mediators of inflammatory responses called cytokines (tumor necrosis factor (TNF) as TNF- $\alpha$ , interleukins (IL)), chemokines and interferons (Janeway et al., 2001b). When *N. gonorrhoeae* enters the host, it first adheres to the epithelial cells microvillousities by its *pili* which bind to CD46, a complement-regulatory protein expressed by all human nucleated cells (Kumar et al., 2005). *N. gonorrhoeae* opa proteins reinforce this adherence and promote *N. gonorrhoeae* penetration into epithelial cells by endocytosis (Bessen and Gotschlich, 1986). Then, *N. gonorrhoeae* replicates and forms a vacuole that is transported to the base of the cell, where the bacterium is released by exocytose into subepithelial tissue (**Fig. 3**). An influx of neutrophils associated with inflammation occurs at this site. Bacterial lipo-oligosaccharide and peptidoglycan are released by autolysis of cells. Bacterial polysaccharides activate the host's alternative complement pathway, while LOS stimulates the production of TNF which causes cell damage (McGee et al., 1992) and creates breaches in epithelial integrity through which the bacteria can pass. *N. gonorrhoeae* divides every 20-30 minutes, which leads to the formation of a submucosal abscess and accumulation of exudate in lumen (**Fig. 3**). However, this innate inflammatory response with neutrophil influx cannot avoid the infection. Some pathogenic *N. gonorrhoeae* can evade phagocytosis by neutrophils in three ways: by preventing their binding to the surface of neutrophil; by limiting the deposition of opsonic antibody or complement on bacterial surface and by varying antigenic surface structures to evade humoral immunity (Criss and Seifert, 2012). Therefore, adaptive immunity mediated by T and B lymphocytes and their products (T cytotoxic, antibodies) (Janeway et al., 2001b) helps avoid infection. Unfortunately, for some unknown reasons, many *gonococci* are able to survive and persist inside neutrophils with two consequences: the residence within phagosomes which protect pathogens from humoral immune surveillance, and the protection from potential toxic sources of cell-mediated immunity such as cytotoxic T lymphocytes because neutrophils are not

supposed to be antigen-presenting cells. Furthermore, the pathogenic *N. gonorrhoeae* can modulate the apoptosis programs of neutrophils and increase their life span (Criss and Seifert, 2012; Urban et al., 2006; Virji, 2009). Thus, neutrophils may carry viable bacteria to a secondary site such as the upper genital tract or the bloodstream resulting in dissemination (Boulton and Gray-Owen, 2002; Criss and Seifert, 2012). In addition, *N. gonorrhoeae* has the genetic capacity to generate much more diversity in the antigens of the surface *pili*, LOS, and less frequently antigens of protein 1. This helps *N. gonorrhoeae* to escape the host protective immune response. It can also secrete an IgA1 protease that can directly inactivate some classes of secretory IgA in mucosal secretions that prevent IgA-mediated opsonization of the bacteria to block phagocytosis and prevent complement mediated lysis. Then, it prolongs infection and prevents immunity to reinfection (Criss and Seifert, 2012; Urban et al., 2006). All these mechanisms facilitate the development of gonococcal infection and limit the efficacy of the prevention means such as vaccines.



**Figure 3.** Schematic depiction of the mechanism of infection by *Neisseria gonorrhoeae* reconstructed by summarizing the results of Virji (2009), and Criss and Seifert (2012).

## 6. SYMPTOMS

About 80 % of infected women and less than 10 % of infected men are free of symptoms (Handsfield and Sparling, 2004; Pearson et al., 2004). So, the infection by *N. gonorrhoeae* is more often asymptomatic in women while more often symptomatic in men.

In women, when symptoms occur, the incubation period is 7-21 days, and symptoms may result in vaginal discharge associated with vaginal bleeding and pain. Without suitable treatment, complications such as pelvic inflammatory disease (including low abdominal pain, fever and anorexia) can occur and increase the risk of chronic pelvic pain, mucopurulent cervicitis, cystitis, ectopic pregnancy, postpartum endometriosis or infertility. Women with no or mild symptoms of gonorrhea are still at risk for developing these serious complications. Infants born to mothers who have untreated or mild gonorrhea are at high risk of infection. The most severe symptoms in children are conjunctival infection called gonococcal ophthalmia neonatorum and sepsis, which can include arthritis and meningitis. Conjunctivitis is usually an acute illness that manifests 2–5 days after birth but can rapidly lead to the perforation of the globe of the eyes and blindness (CDC, 2015). Less severe manifestations in children include rhinitis, vaginitis and urethritis.

In men, symptoms are burns when they urinate associated with pain, penile mucopurulent discharge and urethritis. Symptoms frequently appear 2-5 days after infection but sometimes can be asymptomatic up to 30 days (Gaydos et al., 2006). Complications are epididymitis, orchitis (unilateral testicular pain, swelling and fever).

Rarely, both in men and women, *N. gonorrhoeae* can invade the bloodstream leading to gonococemia and can be disseminated to the joints (hands, wrists, and knees are the joints that are the most infected), the cardiovascular system or the skin. Dissemination results in myalgia, arthralgia, polyarthritis, tenosynovitis, dermatitis (papular, vesicular, or pustular, skin lesions) (Jain et al., 2007), occasionally perihepatitis (Lee et al., 2015), rarely meningitis (Anan and Culik, 1989) and endocarditis (Bunker and Kerr, 2015).

Humans who practice orogenital or anal sex can develop signs and symptoms of pharyngitis, tonsillitis, or proctitis, mainly men who have sex with men. Gonococcal proctitis can be painful with purulent rectal discharge; bleeding may occur and bowel movements may be painful. Most gonococcal infections of the pharynx are asymptomatic but can be relatively common in some populations (Mimiaga et al., 2008) and are more difficult to eradicate than are the infections at urogenital and anorectal sites (Ota et al., 2009).

## 7. EVOLUTION OF THE TREATMENTS AND RESISTANCE DEVELOPMENT

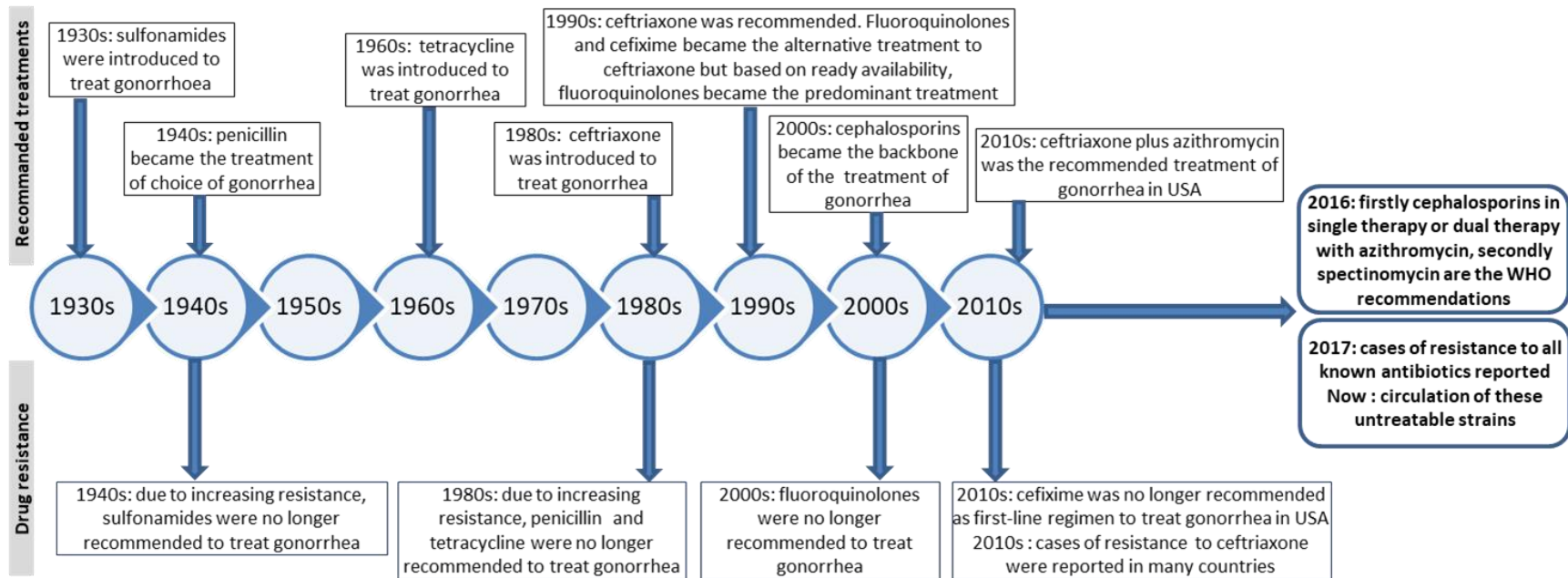
*N. gonorrhoeae* has progressively become resistant to previously and currently recommended antibiotics for the treatment of gonorrhea (**Fig. 4**).

Sulfonamides such as sulfanilamide (Van Slyke et al., 1941), sulfapyridine (Van Slyke et al., 1941) were used for gonococcal treatment after their introduction in 1936, but their effectiveness was short-lived because of the rapid emergence of gonococcal strains resistant by 1945 (Dunlop, 1949). Nevertheless, the association sulfamethoxazole and trimethoprim continued to be used (Lawrence et al., 1973). Penicillin became the recommended antibiotic treatment for the next 40 years (Sternberg and Turner, 1944), but progressively penicillin-resistant strains had emerged. (Amies, 1967; Phillips, 1976). As a result, the recommended dose of intramuscular procaine penicillin was increased from 50 000 units in 1945 to 4.8 million units by the early 1970s and it was associated with probenecid to increase its efficiency (CDC, 1974). Unfortunately, penicillin-resistant strains became widespread. In the early 1960s, spectinomycin was synthesized and commercialized as a specific gonorrhea treatment. However, some cases of resistance to spectinomycin were observed from 1967 to 1987 in different countries (Ison et al., 1983; Boslego et al., 1987). Therefore, it was recommended as an alternative treatment and was frequently used in the cases of resistance to penicillin in order to avoid rapid development of resistance after wide use. In 1985, ceftriaxone became a recommended treatment of uncomplicated gonococcal infections (CDC, 1985). At the same time (1980s), tetracycline resistance was spreading (Morse et al., 1986) to such extent that tetracycline was no longer a reasonable choice. By 1989, resistance to penicillin was so widespread that penicillin became ineffective. Ciprofloxacin became an alternative treatment option to ceftriaxone that was the recommended treatment for gonorrhea in the USA (CDC, 1989). By 1993, on the basis of data showing high effectiveness, safety, ready availability, and convenience as single dose therapies, oral fluoroquinolones (ciprofloxacin, ofloxacin or levofloxacin) were recommended as oral treatments of gonorrhea, as was the oral third-generation cephalosporin cefixime. Nevertheless, quinolone-resistant *N. gonorrhoeae* was identified in Hawaii in 1991. At about the same time (Patrick et al., 1995) and even more by 2000, quinolone-resistant *N. gonorrhoeae* strains were increasingly observed in the world (Azariah and Perkins, 2007; Berglund et al., 2002; CDC, 2002; Donegan et al., 2006; Farhi et al., 2007; Gordon et al., 1996; Ivens et al., 2000; Iverson et al., 2004; Kolader et al., 2004; Llanes et al., 2001; Moodley et al., 2006). As a result, fluoroquinolones were no longer recommended for treating gonorrhea in the countries with

reported cases of quinolone-resistant *N. gonorrhoeae*. In 2016, WHO stated that quinolones were no longer recommended for the treatment of gonorrhea due to the reported high level of resistance (WHO, 2016), leaving cephalosporins and spectinomycin as the only remaining antibiotics available for the treatment of gonorrhea since the early 2000s (WHO, 2016). The latest guidelines of the WHO for the treatment of different types of gonococcal infection are reported in **Table 1** (WHO, 2016). Briefly, cephalosporins in single therapy or dual therapy with azithromycin were the first line, and spectinomycin was the second line of the WHO recommendations. These recommended treatments could be applied to all adults and adolescents (10–19 years of age), including people living with HIV, sex workers, men who have sex with men, transgender persons and pregnant women. Nevertheless, they had to be adapted to the local data on resistance. Specific recommendations were also provided for prophylaxis and treatment of ophthalmia neonatorum (WHO, 2016). Otherwise, the proposed molecules might have some limitations. Regarding cefixime, it did not provide as high, nor as sustained bactericidal blood levels as ceftriaxone (CDC, 2012), and had a limited effectiveness on pharyngeal gonorrhea (CDC, 2012). So, cefixime was not preferred for a global treatment. In addition, already during the 2006–2013 period, the minimum concentrations of cefixime and cefixime needed to inhibit *in vitro* growth of the *N. gonorrhoeae* strains circulating in the USA and in many other countries had increased (Ohnishi et al., 2011; Cámara et al., 2012; CDC, 2014). Some treatment failures with cefixime or other oral cephalosporins were reported (Lo et al., 2008; Yokoi et al., 2007; Muratani et al., 2001). As a result, CDC no longer recommended the routine use of cefixime as a first-line treatment for the treatment of gonorrhea in the USA (CDC, 2012). Only one treatment, dual treatment with ceftriaxone and azithromycin, was recommended. In other hand, frequent use of azithromycin by 1990s for the treatment of STI such as chlamydia and/or gonorrhea had increased resistance of *N. gonorrhoeae* to azithromycin in many countries (Cole et al., 2011; Katz et al., 2012; Palmer et al., 2008; Starnino et al., 2009, 2012). Regarding spectinomycin, WHO recommended this drug for persons who cannot tolerate cephalosporins. When available, spectinomycin was an effective alternative for the treatment of urogenital and anorectal infection, but this drug had already showed poor efficacy against pharyngeal infection (Moran and Levine, 1995) and is expensive. Therefore, ceftriaxone remained the last antimicrobial known to be highly effective in a single dose for the treatment of gonorrhea at all anatomic sites of infection (CDC, 2015). Unfortunately, some cases of ceftriaxone treatment failures for pharyngeal infections had been observed (Chen et al., 2013; Golparian et al., 2014), lessening this ceftriaxone advantage. The notifications of reduced susceptibility

of *N. gonorrhoeae* induced the increase of ceftriaxone dose from 250 mg to 500 mg or even 1000 mg. Gonorrhea strains resistant to ceftriaxone are now spreading (Lefebvre et al., 2018). Even worse, cases of infection that no known antibiotic on the market can treat have been reported (WHO, 2017). This resistance of *N. gonorrhoeae* to antibiotics is very worrying and there is an urgent need of new treatments.





**Figure 4.** History of the development of *Neisseria gonorrhoeae* resistance showing the timeline of the recommended treatments (top) and the timeline of the drug resistance (below). *Neisseria gonorrhoeae* has become resistant to every antibiotic used to treat it. Depicted according to the publications of CDC (2016) and Unemo and Shafer (2011).

**Table 1.** Current treatments of gonorrhoea (CDC, 2015; WHO, 2016).

Type of gonococcal infection	Dual therapy	Single therapy
Uncomplicated genital and anorectal gonococcal infections	Single dose of ceftriaxone 250 mg intramuscular (IM) plus 1 g of oral azithromycin Or Single dose of oral cefixime 400 mg plus 1 g of oral azithromycin	Ceftriaxone 250 mg IM Or Cefixime 400 mg orally Or Spectinomycin 2 g IM
Uncomplicated oropharyngeal gonococcal infections	Idem	Ceftriaxone 250 mg IM as a single dose
Gonococcal conjunctivitis in neonates	None	Ceftriaxone 50 mg/kg (maximum 150 mg) IM Or Kanamycin 25 mg/kg (maximum 75 mg) IM Or Spectinomycin 25 mg/kg (maximum 75 mg)
Ocular prophylaxis in newborns	None	Topical application to both eyes immediately after birth of Tetracycline hydrochloride 1 % eye ointment Or Erythromycin 0.5 % eye ointment Or Povidone iodine 2.5 % solution (water-based) Or Silver nitrate 1 % solution Or Chloramphenicol 1 % eye ointment
Arthritis and arthritis-dermatitis syndrome	Ceftriaxone 1 g IM or intravenous (IV) every 24 hours plus azithromycin 1 g orally in a single dose Alternative treatment cefotaxime 1 g IV every 8 hours or ceftizoxime 1 g IV every 8 hours plus azithromycin 1 g orally in a single dose at least for 7 days	None
Gonococcal meningitis and endocarditis	Ceftriaxone 1-2 g IV every 12-24 hours and azithromycin 1 g orally in a single dose. Therapy for meningitis should be continued with recommended parenteral therapy for 10–14 days. Parenteral antimicrobial therapy for endocarditis should be administered for at least 4 weeks	None
Rare disseminated gonococcal infection in newborns	None	Ceftriaxone 25–50 mg/kg/day IV or IM in a single daily dose for 7 days, with a duration of 10–14 days Or Cefotaxime 25 mg/kg IV or IM every 12 hours for 7 days, with a duration of 10–14 days if meningitis is documented.

## 8. MECHANISM OF RESISTANCE

Unemo and Shafer (2014) and Patel et al. (2011) gave a detailed description of the mechanisms of resistance of *N. gonorrhoeae*. Briefly, *N. gonorrhoeae* can quickly adapt to survive in hostile environments. It has an extraordinary natural ability to modify its genome. Indeed, it can allow the transfer of partial or whole genes during all its life cycle and also it can use all types of known mutations for genomic changes. *N. gonorrhoeae* has developed almost all known physiological mechanisms of resistance to antibiotics: (i) the modification of the target of the antibiotic, preventing its fixation, (ii) the synthesis of bacterial enzymes modifying or inactivating the antibiotics (iii) the decrease of *N. gonorrhoeae* permeability to the antibiotics by changes in porin proteins, (iv) the use of a new metabolic pathway replacing

the pathway inhibited by the antibiotics, (v) the active mechanism of efflux of antibiotics, (vi) protecting the target enzyme by binding to another protein. These mechanisms may be mediated by chromosomal or plasmidic genes. Chromosome resistance is spontaneous. It appears in the absence of the antibiotic. It is stable and is transmitted during the bacterial division. The mutated DNA is replicated and transmitted to the daughter bacteria which will present the new characteristics appeared by mutation of the parent bacteria. Plasmids are DNA molecules, most often circular, different from chromosomal DNA, capable of autonomous replication, hosted in the cytoplasm of the bacterium. Plasmids can be transferred between bacteria of the same species or different species. Antibiotic resistance genes of *N. gonorrhoeae* are generally located in the chromosome. Only the genes responsible for the resistance to penicillin and tetracycline are plasmidics.

*N. gonorrhoeae* also can use polyamines (spermine and spermidine) found in the male genital tract to coat their surfaces, making them more resistant to complement-mediated killing by normal human serum and cationic antimicrobial peptides. *N. gonorrhoeae* has the ability to form biofilms that are more resistant to antibiotics than their planktonic counterparts. The asymptomatic *N. gonorrhoeae* also are widespread and the capacity to identify them in routine is still lacking, while they can transfer resistant genes and influence the resistance development.

## **9. EFFICIENCY OF THE PREVENTION MEANS**

To abstain from sexual intercourse or to be in a long-term mutually monogamous relationship with an uninfected partner are the surest ways to avoid the transmission of gonorrhea. However, they are difficult to control. Consequently, mechanical and chemical prevention means are required.

### **9.1 Vaccine**

The first vaccines to prevent the infection by *N. gonorrhoeae* were whole cell vaccines. In 1974, a crude killed whole cell vaccine was studied in a controlled experiment in Inuvik, Canada. The vaccine elicited only mild reactions in humans, and induced an antibody response in over 90 % of vaccine recipients but there was no evidence for protection. It was not further developed (Greenberg, 1975; Greenberg et al., 1974; Zhu et al., 2011). In 1976, an analogous vaccine made from a pilated *N. gonorrhoeae* strain was tested in chimps by investigators from the CDC in Atlanta, with good serum bactericidal and immunofluorescent

antibody responses. This vaccine resulted in a very similar protection to that observed after natural immune clearance of infection in chimpanzee, requiring a 1000-fold larger inoculum to infect vaccinated animals (Arko et al., 1976; Zhu et al., 2011). Due to the presence of high inflammatory LOS in whole cell vaccines, they were not further developed.

The next vaccine generation was *pili* vaccine. In 1982, a purified *pili* vaccine was able to protect human volunteers from experimental urethral infection after parenteral immunization (Brinton et al., 1982; Zhu et al., 2011). This vaccine resulted in antibody response in serum and the production of specific local mucosal antibody in genital secretions that blocked adherence of piliated *N. gonorrhoeae* to various cells *in vitro* (McChesney et al., 1982). However, in 1985, Tramont and Boslego showed that although *pili* vaccines were effective in some animal studies, they had a limited efficacy in human studies due to lack of broad cross reactivity of the vaccines with strains expressing antigenically variant *pili* (Tramont and Boslego, 1985). A large-scale trial of a purified gonococcal vaccine composed of a single *pilus* type was carried out in high-risk USA military personnel stationed in Korea, using a smaller intradermal dose different from the intramuscular ones used in the initial proof-of-concept experiment. Nevertheless, it also failed to protect men against gonococcal urethritis (Boslego et al., 1991).

Since then, other vaccine candidates have been studied. Researchers tried to get immunization with gonococcal outer membrane vesicles (OMV) due to the potential of OMV to induce an immune response against several different conformationally correct components on the bacterial surface and by analogy to successful meningococcal OMV vaccines (Plante et al., 2000; Zhu et al., 2005), but none had advanced to clinical trial. Other researchers tried recombinant proteins vaccines (Matsuka et al., 1998; Thomas et al., 2006). Another approach was to use genetic vaccines (Thomas et al., 2006; Zhu, et al., 2004; Zhu et al., 2005, 2011). Jerse et al. (2014) summarized the future challenges in vaccine against gonorrhoea. Recently, in 2016, Zielke et al. (2016) combined genomic and proteomic approach to identify promising gonococcal antigens as potential vaccine targets. Very recently, in 2017, Petousis-Harris et al. have showed the efficacy of a meningococcal vaccine on patients with gonorrhoea (Petousis-Harris et al., 2017). Vaccines to prevent gonorrhoea are still under development, but currently, there is no vaccine on the market.

## **9.2. Male condom**

Barlow (1977) showed that the correct use of condom was associated with a significantly lower probability of acquiring gonorrhea ( $p < 0.001$ ) and a significantly higher chance of being no sexually transmitted disease diagnosed ( $P < 0.0005$ ). Therefore, he gave the proof of the efficacy of condoms. Warner et al. (2006) in a review of 45 published studies on the efficiency of condom confirmed its efficacy in most of the studies. Its correct and consistent use is the key of its efficacy. In addition, condoms are relatively cheap and have no side effect. Unfortunately, condoms are badly and little used. Its disadvantage is that it can disrupt sexual enjoyment.

## **9.3. Female condom and vaginal microbicide**

To avoid difficulties to negotiate safe sex for women, trend is to move toward female-controlled prevention methods (female condom or vaginal microbicides). The female condom is a mechanic prevention means that has many advantages such as availability, efficacy, little reported disruption of sexual enjoyment, safety. However, it is expensive, difficult to use and it requires partner's agreement. Compared to condoms, microbicides are expected to interfere less with intimacy and sexual pleasure, and be more discrete. They do not necessitate partner's cooperation or knowledge. Women would not have to negotiate their use, as they often must do with condoms. Microbicides might also make it possible someday for women to protect themselves from STI, while still allowing them to get pregnant if they wish.

Vaginal microbicides are rings, gels, films, or inserts that can be applied inside the vagina to kill or neutralize viruses and bacteria (Abdel-Aleem, 2011). Their aim is to protect the women against STI by providing chemical, biological, and/or physical barriers to infection. Almost all the vaginal microbicides developed were focused on the prevention of HIV. The first generation of microbicides was detergent based products such as the nonoxynol-9 that demonstrated efficiency to disrupt the integrity of HIV *in vitro*. Roddy et al., (2002), in a randomized controlled trial, comparing both use of nonoxynol-9 gel and condom to alone use of condom for the prevention of the transmission of urogenital gonococcal and chlamydial infection, showed that nonoxynol-9 gel did not protect against these infections. The phase III clinical trial of nonoxynol-9 based products showed that they were neither safe nor effective against HIV because they could disrupt the cervico-vaginal epithelia and render women more vulnerable to all pathogens (Hillier et al., 2005). Hence, their use as microbicides was

abandoned. The next generation of microbicides after surfactants was polyanions such as CarraGuard, PRO 2000 or cellulose sulphate. In other hand, based on the observation that low pH (<4.5) could inactivate HIV (O'Connor et al., 1995), acid-buffering gels were also developed to be used to lower vaginal pH, prevent neutralization by semen and inactivate HIV (Mayer et al., 2001). Spencer et al. (2004) showed the efficacy of seven formulated agents that are CarraGuard, Ushercell, [poly]sodium 4-styrene sulfonate (T-PSS), PRO 2000, ACIDFORM, cellulose acetate phthalate (CAP), and BufferGel, in the protection against *N. gonorrhoeae* infection, by using a mouse model of *N. gonorrhoeae* genital tract infection. Fletcher and Shattock (2008) also discovered that long chain polyanionic compounds could prevent the HIV entry into target cells *in vitro* through non-specific inhibition of viral binding to target cells. Unfortunately, the use of these new generations of microbicides (polyanionic gels and buffered gel) were inefficacious in clinical trials (Ramjee et al., 2010). The recent studies have been devoted to chemicals already known as active agents including those currently used for the treatment. For example, a vaginal gel containing the antiretroviral tenofovir and a vaginal ring containing dapivirine (Baeten et al., 2016) were in Phase III trials. Both systems conferred modest level of protection. More recently, McKinnon et al. (2018) showed that the vaginal inflammation lessened the effectiveness of tenofovir gel. A ring containing tenofovir or tenofovir plus levonorgestrel is in phase I trial (Thurman et al., 2018). Generally, microbicides are well-accepted (Abdulai et al., 2012; Carballo-Diéguez et al., 2012; Gafos et al., 2010; Montgomery et al., 2010; Stadler and Saethre, 2011; Valley et al., 2012; Woodsong and Holt, 2015). Microbicide gels remain the preferred choice for the first line of microbicide product development. The potential reasons are the ease to develop a vaginal gel, the extensive work reported in the literature using this dosage form, the existence of several vaginal semi-solid products in the market and their possibility to be self-administered. Vaginal microbicides are administered by vaginal route for a direct activity as the sexual transmission is predominant in STI. The use of chemicals in prevention could not be applied to the prevention of gonorrhea due to the resistance issue. Thus, there is still a need to develop efficient vaginal microbicides.

Some data emphasized the importance of the vaginal microbiome in the success of vaginal microbicide (Fichorova et al., 2011). Klatt et al. (2017) showed that tenofovir gel was 3 times more effective in the 407 women with a *Lactobacillus*-dominant vaginal microbiome compared with 281 women whose vaginal microbiome had  $\leq 50\%$  *Lactobacillus*, suggesting that the bacterial communities rich in *Lactobacillus* may improve efficacy of topical microbicide gels. *Lactobacillus* activity should be more explored.

## 10. PERSPECTIVE TO PREVENT OR TREAT GONORRHEA

Face to the ability of *N. gonorrhoeae* to rapidly develop resistance, the development of a powerful preventive mean, effective on all types of *N. gonorrhoeae* is necessary. This part reports recent clinical trials performed on antibiotics and presents alternative strategies such as herbal medicines or probiotics that should be explored.

### 10.1. Recent clinical trials on antibiotics

In a recent randomized clinical multicenter trial, dual treatment of uncomplicated urogenital gonorrhea with single doses of oral gemifloxacin (a fourth-generation quinolone) 320 mg plus oral azithromycin 2 g was associated with cure rates of 99.5 %. and dual treatment with single doses of intramuscular gentamicin 240 mg plus oral azithromycin 2 g cured 100 % of cases (Kirkcaldy et al., 2014). This trial was not powered to provide reliable estimates of the efficacy of these treatments for treatment of rectal or pharyngeal infection, but both treatments cured all the few extragenital infections among the participants of the study. Either of these treatments might be considered as alternative treatment options in the presence of cephalosporin allergy. However, gastrointestinal adverse events might limit their use: 7.7 % of patients treated with gemifloxacin plus azithromycin and 3.3% of patients treated with gentamicin plus azithromycin vomited within 1 hour of medication administration. They were discontinued from the study and retreated with ceftriaxone and azithromycin (CDC, 2015; Kirkcaldy et al., 2014). The high risk of possible adverse events when taking 2 g of azithromycin oral dose was already reported (Handsfield et al., 1994).

More recently, both the National Institute of Allergy and Infectious Diseases (NIAID) and Center for Disease Control (CDC) have published clinical trials. In 2015, a Phase I trial was studying high dose of cefixime (800 mg, 1200 mg) with extended duration of treatment. This study aimed to evaluate the safety and optimal dosage of cefixime to treat reduced susceptible *N. gonorrhoeae* (NIAID, 2015). In 2016, a Phase II trial studying the safety and efficacy of an investigational antimicrobial drug, developed by AstraZeneca (now Entasis Therapeutics) was conducted to treat gonorrhea. Patients received an oral dose of the AstraZeneca drug, ETX0914, or ceftriaxone, a cephalosporin antibiotic administered by injection. The preliminary results showed that ETX0914 was safe and effective, indicating it was a promising candidate for advanced clinical studies (NIAID, 2016). Many new molecules have been studying, but only 3 new candidate antibiotics are in the clinical development phase:

solithromycin (phase III trial); zoliflodacin (phase II trial); and gepotidacin (phase II trial) (Fernandes and Martens, 2017). The phase III clinical trial of solithromycin has been successful.

## 10.2. Herbal medicines

Herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredients. They have been used in order to prevent or treat diseases for a long time everywhere in the world (Frass et al., 2012). They have gained popularity because of their advantages. Indeed, plants typically contain mixtures of different phytochemicals, main and secondary components. Compared to pharmacological drugs in modern medicine, the phytochemicals work together catalytically and synergistically to produce a combined effect that surpasses the total activity of the individual component (Vickers et al., 2001). The combined actions of phytochemicals tend to increase the activity of the main component(s) by speeding up or slowing down its assimilation after administration in the body. In addition, the diversity of components might result in additive beneficial effect, all interesting for the treatment. For example, a single plant may contain antibacterial and antifungal tannins that act as natural antibiotics, but also bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swellings and pain, phenolic compounds that can act as an antioxidant and venotonics, diuretic substances that enhance the elimination of waste products and toxins, and alkaloids that enhance mood and give a sense of well-being. Otherwise, it is postulated that secondary components might increase the stability of the active component(s), minimize the rate of undesired adverse side effects, and have an additive, potentiating, or antagonistic effect or induce better patient tolerance. Therefore, the medical use of crude and/or standardized extracts as opposed to isolated single compound is gaining much more interest in the scientific community. It is also postulated that that they are relatively less expensive and the risk of resistance of bacteria seems to be low. WHO encourages African member states to promote and integrate traditional medical practices in their health system and developed WHO traditional medicine strategy 2014-2023. About the effectiveness of herbal medicines against gonorrhoea, Vermani and Garg (2002), studied herbal medicines for sexually transmitted diseases and made a review of several plants having scientifically proven activity against them, particularly against gonorrhoea. Some scientifically proven plants using *in vitro* activity test against *N. gonorrhoeae* are summarized in **Table 2**. All these studies indicate the huge potential of



plants as an effective mean for prevention and treatment of gonorrhoea. Herbal medicines should be more explored to demonstrate their safety, their effectiveness and their low cost, their reduced risk of gonococcal resistance before being proposed as an alternative to antibiotherapy to treat or prevent gonorrhoea. *In vivo* efficacy should be proved. The challenges remain the reproducibility of this strategy because the composition and the effectiveness of plants often vary according to the place or the period of harvest and the climate during the growth of the plants.

**Table 2.** Scientific published plants with effectiveness on *Neisseria gonorrhoeae* *in vitro*.

Authors	Name (part used)
(Cáceres et al., 1995)	<i>Bixa Orellana</i> (bark), <i>Parmentiera edulis</i> (fruits), <i>Diphysa robinoides</i> (leaves), <i>Eupatorium odoratum</i> (leaves), <i>Gliricidia sepium</i> (leaves), <i>Physalis angulate</i> (leaves), <i>Piper aduncum</i> (leaves), <i>Prosopis juliflora</i> (leaves), <i>Casimiroa edulis</i> (root), <i>Clematis dioica</i> (whole plant)
(Silva et al., 2002)	<i>Terminalia macroptera</i> (leaves)
(Arbonnier, 2002)	<i>Carica papaya</i> (roots, leaves), <i>Dichrostachys cinerea</i> (ryes), <i>Tamarindus indica</i> (ryes), <i>Ziziphus mucronata</i> (rye), <i>Acacia sieberiana</i> (ryes), <i>Anthocleista procera</i> (roots), <i>Burkea africana</i> (ryes), <i>Cissus quadrangularis</i> (twigs), <i>Citrus aurantiaca</i> (leaves), <i>Citrus grandis</i> (leaves), <i>Citrus limon</i> (leaves), <i>Citrus reticulata</i> (leaves), <i>Citrus sinensis</i> (leaves), <i>Combretum aculeatum</i> (leaves), <i>Crossopheryx febrifuga</i> (rye), <i>Feretia apodanthera</i> (roots), <i>Gardenia erubescens</i> (roots), <i>Holarrhena floribunda</i> (roots, leaves), <i>Leptademia hastata</i> (leaves, latex), <i>Mitragyna inermis</i> (ryes, twigs), <i>Paullina pinnata</i> (roots), <i>Pavetta corymbosa</i> (roots), <i>Rhus natalensis</i> (roots, leaves), <i>Saba comorensis</i> (roots), <i>Spondias monbin</i> (leaves), <i>Terminalia albida</i> (ryes), <i>Uvaria chamae</i> (roots), <i>ziziphus mauritiana</i> (leaves)
(Shokeen et al., 2005)	<i>Ocimum sanctum</i> (leaves), <i>Drynaria quercifolia</i> (leaves), <i>Annona squamosal</i> (seeds, leaves)
(Chomnawang et al., 2009)	<i>Coscinium fenestratum</i> (stem)
(Shokeen et al., 2009)	<i>Adhatoda vasica</i> (roots), <i>Albizia lebbeck</i> (stem bark), <i>Drynaria peregrine</i> (stem bark), <i>Michelia champaca</i> (stem bark), <i>Alangium salviifolium</i> (seeds), <i>Anethum sowa</i> (fruits), <i>Azadirachta indica</i> (leaves), <i>Cedrela toona</i> (leaves), <i>Eugenia camaldulensis</i> (leaves), <i>Euphorbia hirta</i> (whole plant), <i>Phyllanthus fraternus</i> (whole plant), <i>Plumeria rubra</i> (leaves, stem, stem bark), <i>Ricinus communis</i> (stem, roots), <i>Salvadora persica</i> (leaves, stem bark), <i>Solanum tuberosum</i> (tubers), <i>Urginea indica</i> (bulbs)
(Otto et al., 2014)	<i>Cassia alata</i> (leaves and roots)

### 10.3. Bacteriotherapy

Bacteriotherapy consists in using harmless bacteria called probiotics to displace pathogenic organisms. It has become an attractive alternative to antibiotherapy. Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). The most important *genera* of Gram-positive bacteria used extensively as probiotics are *Lactobacillus* and *Bifidobacterium*. Currently, the use of *Lactobacillus* is increasing in the area of urogenital infections, while *Bifidobacterium* is not (Pascual et al., 2008; Reid, 2002; Reid et al., 2003, 2004). *Lactobacilli* can adhere to epithelial cells, compete with pathogens for adhesion sites or produce surfactants to inhibit

pathogens adhesion, co-aggregate with pathogens to prevent their spreading, produce antimicrobials substances such as acids, hydrogen peroxide a bacteriocins to inhibit pathogens growth, and stimulate the immune system. *Lactobacilli* have been already developed as probiotics for the treatment of urogenital infections such as recurrent urinary tract infections, bacterial vaginoses, and yeast vaginoses. Some are already on the market. Indeed, *Lactobacilli* are commensal, predominant in the normal vaginal flora and fight against infections. Their presence and dominance in the vagina are associated with a reduced risk of urogenital infections and sexually transmitted infections. Some *Lactobacilli* have demonstrated an activity on *N. gonorrhoeae in vitro* (Amant et al., 2002) and probiotic properties. Therefore, restoring the normal vaginal ecosystem by the administration of *Lactobacilli* with probiotic properties and effectiveness on *N. gonorrhoeae* might be a valuable approach to treat or prevent gonorrhea.

## 11. CONCLUSION

Multi-drug resistant strains of *N. gonorrhoeae* have emerged in the world increasing the risk of treatment failure. Nowadays, gonorrhea has become nearly impossible to treat. Most of time, the infection is asymptomatic but transmissible. The development of new chemical active ingredients is reduced, and it is feared that *N. gonorrhoeae* becomes resistant to the new antibiotics in development. Therefore, this review enables to understand the major mechanisms involved in the infection development to inspire new researches. Innovative prevention means are needed and other strategies such as herbal medicine, bacteriotherapy, should be evaluated. Several plants were shown to inhibit *N. gonorrhoeae in vitro*. However, their effectiveness should be demonstrated *in vivo*. The use of probiotics could also be another valuable strategy as they have already demonstrated their efficacy in the prevention of urogenital infections.

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## References

- Abdel-Aleem, H. (2011). Vaginal microbicides: An overview. *Middle East Fertil. Soc. J.* *16*, 14–18.
- Abdulai, M.A., Baiden, F., Adjei, G., Afari-Asiedu, S., Adjei, K., Tawiah, C., and Newton, S. (2012). An assessment of the likely acceptability of vaginal microbicides for HIV prevention among women in rural Ghana. *BMC Womens Health* *12*, 40.
- Amant, D.C.S., Valentin-Bon, I.E., and Jerse, A.E. (2002). Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. *Infect. Immun.* *70*, 7169–7171.
- Amies, C.R. (1967). Development of resistance of gonococci to penicillin. An eight-year study. *Can Med Assoc J* *96*, 33–35.
- Anan, T.J., and Culik, D.A. (1989). *Neisseria gonorrhoeae* dissemination and gonococcal meningitis. *J. Am. Board Fam. Pract.* *2*, 123–125.
- Arbonnier, M. (2002). Arbres, arbustes et lianes des zones sèches d’Afrique de l’Ouest (Editions Quae).
- Arko, R.J., Duncan, W.P., W. Jerry, B., Peacock, W.L., and Tomizawa, T. (1976). Immunity in infection with *Neisseria gonorrhoeae*: duration and serological response in the chimpanzee. *J. Infect. Dis.* *133*, 436–440.
- Arya, O.P., Taber, S.R., and Nsanze, H. (1980). Gonorrhoea and female infertility in rural Uganda. *Am. J. Obstet. Gynecol.* *138*, 929–932.
- Ashford, W., Golash, R., and Henning, V. (1976). Penicillinase producing *Neisseria gonorrhoeae*. *Lancet ii*, 657–658.
- Ashford, W., Potts, D., Adams, H., English, J., Johnson, S., Biddle, J., Thornsberry, C., and Jaffe, H. (1981). Spectinomycin-resistant penicillinase producing *Neisseria gonorrhoeae*. *Lancet ii* 1035–1037.
- Azariah, S., and Perkins, N. (2007). Risk factors and characteristics of patients with gonorrhoea presenting to Auckland sexual health service, New Zealand. *N. Z. Med. J.* *120*, U2491.
- Baeten, J.M., Palanee-Phillips, T., Brown, E.R., Schwartz, K., Soto-Torres, L.E., Govender, V., Mgodli, N.M., Matovu Kiweewa, F., Nair, G., Mhlanga, F., et al. (2016). Use of a vaginal ring containing dapivirine for HIV-1 prevention in women. *N. Engl. J. Med.* *375*, 2121–2132.
- Barlow, D. (1977). The condom and gonorrhoea. *The Lancet* *310*, 811–813.
- Barry, P.M., and Klausner, J.D. (2009). The use of cephalosporins for gonorrhoea: The impending problem of resistance. *Expert Opin. Pharmacother.* *10*, 555–577.
- Bentley, M.E., Fullem, A.M., Tolley, E.E., Kelly, C.W., Jogelkar, N., Srirak, N., Mwafulirwa, L., Khumalo-Sakutukwa, G., and Celentano, D.D. (2004). Acceptability of a microbicide among women and their partners in a 4-country phase i trial. *Am. J. Public Health* *94*, 1159–1164.
- Berglund, T., Unemo, M., Olcén, P., Giesecke, J., and Fredlund, H. (2002). One year of *Neisseria gonorrhoeae* isolates in Sweden: the prevalence study of antibiotic susceptibility shows relation to the geographic area of exposure. *Int. J. STD AIDS* *13*, 109–114.

Bessen, D., and Gotschlich, E.C. (1986). Interactions of gonococci with HeLa cells: attachment, detachment, replication, penetration, and the role of protein II. *Infect. Immun.* *54*, 154–160.

Boslego, J.W., Tramont, E.C., Takafuji, E., Diniega, B., Mitchell, B., Small, J., and Khan, W. (1987). Effect of spectinomycin use on the prevalence of spectinomycin-resistant and penicillinase-producing *Neisseriagonorrhoeae*. *N.Engl.J.Med* *317*, 272–278.

Boslego, J.W., Tramont, E.C., Chung, R.C., Mcchesney, D.G., Ciak, J., Sadoff, J.C., Piziak, M.V., Brown, J., Brinton, C.C.J., Wood, S.W., et al. (1991). Efficacy trial of a parenteral gonococcal pilus vaccine in men. *Vaccine* *9* 154–16.

Boulton, I.C., and Gray-Owen, S.D. (2002). Neisserial binding to CEACAM1 arrests the activation and proliferation of CD4+ T lymphocytes. *Nat. Immunol.* *3*, 229–236.

Brinton, C.C., Wood, S.W., Brown, A., Labik, A.M., Bryan, J.R., Lee, S.W., Polen, S.E., Tramon, E.C., Sadof, J., and Zollinger, W. (1982). The development of a Neisserial pilus vaccine for gonorrhea and meningococcal meningitis. *Semin. Infect. Dis.* *IV*, 140–159.

Bunker, D., and Kerr, L.D. (2015). Acute myopericarditis likely secondary to disseminated gonococcal infection. *Case Rep. Infect. Dis.* *2015*, e385126.

Cáceres, A., Menéndez, H., Méndez, E., Cohobón, E., Samayoa, B.E., Jauregui, E., Peralta, E., and Carrillo, G. (1995). Antigonorrhoeal activity of plants used in Guatemala for the treatment of sexually transmitted diseases. *J. Ethnopharmacol.* *48*, 85–88.

Cámara, J., Serra, J., Ayats, J., Bastida, T., Carnicer-Pont, D., Andreu, A., and Ardanuy, C. (2012). Molecular characterization of two high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. *J. Antimicrob. Chemother.* *67*, 1858–1860.

Carballo-Diéguéz, A., Giguere, R., Curtis, C., Beatrice, A.C., Jessica, K., Greg, Z., Mabrugaña, M., Cheng-Shiun, L., and McGowan, I. (2012). Acceptability of the candidate microbicide vivagel® and two placebo gels among ethnically diverse, sexually active young women participating in a phase 1 microbicide study. *AIDS Behav.* *16*, 1761–1774.

Carnicer-Pont, D., Smithson, A., Fina-Homar, E., and Bastida, M.T. (2012). First cases of *Neisseria gonorrhoeae* resistant to ceftriaxone in Catalonia, Spain, mAY 2011. *Enfermedades Infecc. Microbiol. Clínica* *30*, 218–219.

CDC (1974). CDC recommended treatment schedules. *Morb Mortal Wkly Rep* *23*, 341–342.

CDC (1985). STD Treatment Guidelines. *MMWR Suppl.* *34*, 75S-108S.

CDC (1989). Sexually transmitted diseases treatment guidelines. *MMWR Morb Mortal Wkly Rep* *38*, 1–43.

CDC (1998). HIV prevention through early detection and treatment of other sexually transmitted diseases -- United States recommendations of the advisory committee for HIV and STD Prevention. *47 (RR 12)*, 1–24.

CDC (2002). Increases in fluoroquinolone-resistant *Neisseria gonorrhoeae*--Hawaii and California, 2001. *MMWR Morb. Mortal. Wkly. Rep.* *51*, 1041–1044.

CDC (2012). Update to CDC's sexually transmitted diseases treatment guidelines, 2010: oral cephalosporins no longer a recommended treatment for gonococcal infections. *MMWR Morb. Mortal Wkly Rep* *61*, 590–594.

CDC (2014). Four treatment failures of pharyngeal gonorrhoea with ceftriaxone (500 mg) or cefotaxime (500 mg).

CDC (2015). Gonococcal Infections - 2015 STD Treatment Guidelines.

CDC (2016). Latest data on Antibiotic Resistant Gonorrhoea.

CDC/Dr. Norman, J. (1974). Picture 3693.

Chen, Y.M., Stevens, K., Tideman, R., Zaia, A., Tomita, T., Fairley, C.K., Lahra, M., Whiley, D., and Hogg, G. (2013). Failure of 500 mg of ceftriaxone to eradicate pharyngeal gonorrhoea, Australia. *J. Antimicrob. Chemother.* 68, 1445–1447.

Chomnawang, M.T., Trinapakul, C., and Gritsanapan, W. (2009). In vitro antigonococcal activity of *Coscinium fenestratum* stem extract. *J. Ethnopharmacol.* 122, 445–449.

Cohen, M.S. (2004). HIV and sexually transmitted diseases: lethal synergy. *Top. HIV Med. Publ. Int. AIDS Soc. USA* 12, 104–107.

Cokkinis, A., and McElligott, G. (1938). Sulfanilamide in gonorrhoea. An analysis of 633 cases. *Lancet* 355–362.

Cole, M.J., Unemo, M., Hoffmann, S., Chisholm, S.A., Ison, C.A., and van de Laar, M.J. (2011). The European gonococcal antimicrobial surveillance programme, 2009. *Euro Surveill* 16, pii: 19995.

Criss, A.K., and Seifert, H.S. (2012). A bacterial siren song: intimate interactions between *Neisseria* and neutrophils. *Nat. Rev. Microbiol.* 10, 178–190.

Darroch, J., and Frost, J. (1999). Women's interest in vaginal microbicides. *Fam Plann Perspect* 31, 16–23.

De Schryver, A., and Meheus, A. (1990). Epidemiology of sexually transmitted diseases: the global picture. *Bull. World Health Organ.* 68, 639–654.

Deguchi, T., Yasuda, M., Yokoi, S., Ishida, K.-I., Ito, M., Ishihara, S., Minamidate, K., Harada, Y., Tei, K., Kojima, K., et al. (2003). Treatment of uncomplicated gonococcal urethritis by double-dosing of 200 mg cefixime at a 6-h interval. *J. Infect. Chemother. Off. J. Jpn. Soc. Chemother.* 9, 35–39.

Dillon, J.-A.R., Ruben, M., Li, H., Borthagaray, G., Márquez, C., Fiorito, S., Galarza, P., Portilla, J.L., León, L., Agudelo, C.I., et al. (2006). Challenges in the control of gonorrhoea in South America and the Caribbean: monitoring the development of resistance to antibiotics. *Sex. Transm. Dis.* 33, 87–95.

Dillon, J.-A.R., Trecker, M.A., Thakur, S.D., and Gonococcal Antimicrobial Surveillance Program Network in Latin America and Caribbean 1990-2011 (2013). Two decades of the gonococcal antimicrobial surveillance program in South America and the Caribbean: challenges and opportunities. *Sex. Transm. Infect.* 89 *Suppl* 4, iv36-41.

Donegan, E.A., Wirawan, D.A.N., Muliawan, P., Schachter, J., Moncada, J., Parekh, M., and Knapp, J. (2006). Fluoroquinolone-resistant *Neisseria gonorrhoeae* in Bali, Indonesia: 2004. *Sex Transm Dis* 33, 625–629.

Dunlop, E.M.C. (1949). Gonorrhoea and the sulphonamides. *Br J Vener Dis* 25, 81–83.

Edwards, J.L., and Apicella, M.A. (2004). The molecular mechanisms used by *Neisseria gonorrhoeae* to initiate infection differ between men and women. *Clin. Microbiol. Rev.* 17, 965–981.

Farhi, D., Gerhardt, P., Falissard, B., Poupet, H., Poyart, C., and Dupin, N. (2007). Increasing rates of quinolone-resistant *Neisseria gonorrhoeae* in Paris, France. *J Eur Acad Dermatol Venereol* 21, 818–821.

Fichorova, R.N., Yamamoto, H.S., Delaney, M.L., Onderdonk, A.B., and Doncel, G.F. (2011). Novel vaginal microflora colonization model providing new insight into microbicide mechanism of action. *MBio* 2.

Fleming, D.T., and Wasserheit, J.N. (1999). From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. *Sex. Transm. Infect.* 75, 3–17.

Fletcher, P.S., and Shattock, R.J. (2008). PRO-2000, an antimicrobial gel for the potential prevention of HIV infection. *Curr. Opin. Investig. Drugs Lond. Engl.* 2000 9, 189–200.

Frass, M., Strassl, R.P., Friehs, H., Müllner, M., Kundi, M., and Kaye, A.D. (2012). Use and acceptance of complementary and alternative medicine among the general population and medical personnel: a systematic review. *Ochsner J.* 12, 45–56.

Gafos, M., Mzimela, M., Sukazi, S., Pool, R., Montgomery, C., and Elford, J. (2010). Intravaginal insertion in KwaZulu-Natal: sexual practices and preferences in the context of microbicide gel use. *Cult. Health Sex.* 12, 929–942.

Galarza, P., Abad, R., Canigia, L., Buscemi, L., Pagano, I., Oviedo, C., and Vázquez, J. (2010). New mutation in 23S rRNA gene associated with high level of azithromycin resistance in *Neisseria gonorrhoeae*. *Antimicrob.AgentsChemother* 54, 1652–1653.

Galega, F.P., Heymann, D.L., and Nasah, B.T. (1984). Gonococcal ophthalmia neonatorum: the case for prophylaxis in tropical Africa. *Bull. World Health Organ.* 62, 95–98.

Gaydos, C.A., Kent, C.K., Rietmeijer, C.A., Willard, N.J., Marrazzo, J.M., Chapin, J.B., Dunne, E.F., Markowitz, L.E., Klausner, J.D., Ellen, J.M., et al. (2006). Prevalence of *Neisseria Gonorrhoeae* among men screened for Chlamydia Trachomatis in four United States cities, 1999-2003. *Sex. Transm. Dis.* 33, 314–319.

Golparian, D., Ohlsson, A.K., Janson, H., Lidbrink, P., Richtner, T., Ekelund, O., Fredlund, H., and Unemo, M. (2014). Four treatment failures of pharyngeal gonorrhoea with ceftriaxone (500 mg) or cefotaxime (500 mg). *Eurosurveillance* 19, pii: 20862.

Gordon, S., Carolyn, L., Knapp, C., Longworth, D., Hall, G., and Washington, J. (1996). The emergence of *Neisseria gonorrhoeae* with decreased susceptibility to ciprofloxacin in Cleveland, Ohio: epidemiology and risk factors. *Ann Intern Med* 125(6), 46.

Greenberg, L. (1975). Field trials of a gonococcal vaccine. *J. Reprod. Med.* 14, 34–36.

Greenberg, L., Diena, B.B., Ashton, F.A., Wallace, R., Kenny, C.P., Znamirovski, R., Ferrari, H., and Atkinson, J. (1974). Gonococcal vaccine studies in Inuvik. *Can. J. Public Health Rev. Can. Sante Publique* 65, 29–33.

Grodstein, F., Goldman, M.B., and Cramer, D.W. (1993). Relation of Tubal Infertility to History of Sexually Transmitted Diseases. *Am. J. Epidemiol.* 137, 577–584.

Han, L., Lv, F., Xu, P., Zhang, G., Juniper, N.S., and Wu, Z. (2009). Microbicide acceptability among female sex workers in Beijing, China: Results from a Pilot Study. *J. Womens Health* 18, 1377–1384.

Handsfield, H., and Sparling, P. (2004). *Gonococcal Infections.* (Goldman: Cecil textbook of medicine. Philadelphia: Saunders).

Handsfield, H.H., Dalu, Z.A., Martin, D.H., Douglas, J.M., McCarty, J.M., and Schlossberg, D. (1994). Multicenter trial of single-dose azithromycin vs. ceftriaxone in the treatment of uncomplicated gonorrhea. Azithromycin gonorrhea study group. *Sex. Transm. Dis.* *21*, 107–111.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* *11*, 506–514.

Hillier, S., Moench, T., Shattock, R., Black, R., and Reichelderfer, P. (2005). In vitro and in vivo: the story of nonoxynol 9. *Veronese Fm J Acquir Immune Defic Syndr* *39(1)*, 1-8.

Holmes, W.R., Maher, L., and Rosenthal, S.L. (2008). Attitudes of men in an Australian male tolerance study towards microbicide use. *Sex. Health* *5*, 273–278.

Ison, C.A. (2011). Biology of *Neisseria gonorrhoeae* and the clinical picture of infection. in sexually transmitted infections and sexually transmitted diseases, G.E. Gross, and S.K. Tying, eds. (Springer Berlin Heidelberg), pp. 77–90.

Ison, C., Littleton, K., Shannon, K., Easmon, C., and Phillips, I. (1983). Spectinomycin resistant gonococci. *Br Med J* *287*, 1827–1829.

Ivens, D., Martin, I., and Ison, C. (2000). *Neisseria gonorrhoeae* in a London sexually transmitted infection clinic not fully sensitive to quinolones: are isolates imported and how effective is ciprofloxacin as a first-line therapy? *Int J STD AIDS* *11*, 774–776.

Iverson, C.J., Wang, S.A., Lee, M.V., Ohye, R.G., Trees, D.L., Knapp, J.S., Effler, P.V., O’connor, N.P., and Levine, W.C. (2004). Fluoroquinolone resistance among *Neisseria gonorrhoeae* isolates in Hawaii, 1990-2000: role of foreign importation and increasing endemic spread. *Sex. Transm. Dis.* *31*, 702–708.

Jack, D.L., Dodds, A.W., Anwar, N., Ison, C.A., Law, A., Frosch, M., Turner, M.W., and Klein, N.J. (1998). Activation of complement by mannose-binding lectin on isogenic mutants of *Neisseria meningitidis* serogroup B. *J. Immunol. Baltim. Md* *160*, 1346–1353.

Jain, S., Win, H.N., Chalam, V., and Yee, L. (2007). Disseminated gonococcal infection presenting as vasculitis: a case report. *J. Clin. Pathol.* *60*, 90–91.

Janeway, C.A.J., Travers, P., Walport, M., and Shlomchik, M.J. (2001b). Principles of innate and adaptive immunity.

Janeway, C.A.J., Travers, P., Walport, M., and Shlomchik, M.J. (2001). The complement system and innate immunity.

Jarvis, G.A., and Chang, T.L. (2012). Modulation of HIV Transmission by *Neisseria gonorrhoeae*: molecular and immunological aspects. *Curr. HIV Res.* *10*, 211–217.

Jarvis, G.A., and Vedros, N.A. (1987). Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. *Infect. Immun.* *55*, 174–180.

Jerse, A.E., Bash, M.C., and Russell, M.W. (2014). Vaccines against gonorrhea: current status and future challenges. *Vaccine* *32*, 1579–1587.

Kahler, C.M., Martin, L.E., Shih, G.C., Rahman, M.M., Carlson, R.W., and Stephens, D.S. (1998). The (alpha2-->8)-linked polysialic acid capsule and lipooligosaccharide structure both contribute to the ability of serogroup B *Neisseria meningitidis* to resist the bactericidal activity of normal human serum. *Infect. Immun.* *66*, 5939–5947.

Kampmeier, R. (1983). Introduction of sulfonamide therapy for gonorrhea. *Sex Transm Dis* 10, 81–84.

Katz, A.R., Komeya, A.Y., Soge, O.O., Kiaha, M.I., Lee, M.V.C., Wasserman, G.M., Maningas, E.V., Whelen, A.C., Kirkcaldy, R.D., Shapiro, S.J., et al. (2012). *Neisseria gonorrhoeae* with high-level resistance to azithromycin: case report of the first isolate identified in the United States. *Clin. Infect. Dis.* 54, 841–843.

Kirkcaldy, R.D., Weinstock, H.S., Moore, P.C., Philip, S.S., Wiesenfeld, H.C., Papp, J.R., Kerndt, P.R., Johnson, S., Ghanem, K.G., and Hook, E.W. (2014). The efficacy and safety of gentamicin plus azithromycin and gemifloxacin plus azithromycin as treatment of uncomplicated gonorrhea. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 59, 1083–1091.

Klatt, N.R., Cheu, R., Birse, K., Zevin, A.S., Perner, M., Noël-Romas, L., Grobler, A., Westmacott, G., Xie, I.Y., Butler, J., et al. (2017). Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* 356, 938–945.

Kolader, M., Peerbooms, P., Vader, P., van Bergen, J., Fennema, J., and de Vries, H. (2004). The rise in fluoroquinolone-resistant *Neisseria gonorrhoeae* among people attending the Municipal Health Service's clinic for sexually transmitted diseases in Amsterdam, the Netherlands; cefotaxime now first-choice treatment for uncomplicated gonorrhea. *Ned Tijdschr Geneesk* 148, 2129–2132.

Kubanov, A., Frigo, N., Kubanov, A., Sidorenko, S., Lesnaya, I., Polevshikova, S., Solomka, V., Bukano, N., Domeika, M., and Unemo, M. (2010). The Russian gonococcal antimicrobial susceptibility programme (RU-GASP) – national resistance prevalence in 2007 and 2008, and trends during 2005-2008.

Kumar, V., Abbas, A., and Fausto, N. (2005). Robbins and Cotran pathologic basis of disease (Philadelphia: Saunders).

Laga, M., Plummer, F.A., Nzanze, H., Namaara, W., Brunham, R.C., Ndinya-Achola, J.O., Maitha, G., Ronald, A.R., D'Costa, L.J., and Bhullar, V.B. (1986). Epidemiology of ophthalmia neonatorum in Kenya. *Lancet Lond. Engl.* 2, 1145–1149.

Lahra, M.M., Lo, Y.-R., and Whiley, D.M. (2013). Gonococcal antimicrobial resistance in the Western Pacific Region. *Sex. Transm. Infect.* 89 Suppl 4, iv19-23.

Lawrence, A., Phillips, I., and Nicol, C. (1973). Various Regimens of Trimethoprim-Sulfamethoxazole used in the treatment of gonorrhea. *J. Infect. Dis.* 128, S673–S678.

Lee, M.H., Byun, J., Jung, M., Yang, J.J., Park, K.-H., Moon, S., Lee, H.J., and Lee, M.S. (2015). Disseminated gonococcal infection presenting as bacteremia and liver abscesses in a healthy adult. *Infect. Chemother.* 47, 60–63.

Lefebvre, B., Martin, I., Demczuk, W., Deshaies, L., Michaud, S., Labbé, A.-C., Beaudoin, M.-C., and Longtin, J. (2018). Ceftriaxone-Resistant *Neisseria gonorrhoeae*, Canada, 2017 - Emerging Infectious Disease journal - CDC. 24. (2).

Lewis, D. (2010). The gonococcus fights back: is this time a knock out? *Sex Transm Infect* 86, 415–421.

Lin, J.S., Donegan, S.P., Heeren, T.C., Greenberg, M., Flaherty, E.E., Haivanis, R., Su, X.H., Dean, D., Newhall, W.J., Knapp, J.S., et al. (1998). Transmission of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* among men with urethritis and their female sex partners. *J. Infect. Dis.* 178, 1707–1712.



Llanes, R., Sosa, J., Guzmán, D., Gutiérrez, Y., Llop, A., and Ricardo O (2001). *Neisseria gonorrhoeae* resistant to ciprofloxacin: first report in Cuba. *Sex. Transm. Dis.* 28(2), 82–83.

Lo, J.Y.C., Ho, K.M., Leung, A.O.C., Tiu, F.S.T., Tsang, G.K.L., Lo, A.C.T., and Tapsall, J.W. (2008). Cefibuten resistance and treatment failure of *Neisseria gonorrhoeae* infection. *Antimicrob. Agents Chemother.* 52, 3564–3567.

Mahoney, J., Van Slyke, C., and Wolcott, R. (1941). Sulfathiazole treatment of gonococcal infections in men and women. Results in 360 patients. *Vener Inf* 22, 425–431.

Mahoney, J., Ferguson, C., Buchholtz, M., and van Slyke, C. (1943). The use of penicillin sodium in the treatment of sulfonamide-resistant gonorrhea in men. A preliminary report. *Am J Gonorr Vener Dis* 27.

Mantell, J.E., Morar, N.S., Myer, L., and Ramjee, G. (2006). “We have our protector”: misperceptions of protection against HIV among participants in a microbicide efficacy trial. *Am. J. Public Health* 96, 1073–1077.

Martin, J.E., Jr, Lester, A., Price, E.V., and Schmale, J.D. (1970). Comparative study of gonococcal susceptibility to penicillin in the United States, 1955–1969. *J Infect Dis* 122, 459–461.

Matsuka, Y.V., Dilts, D.A., Hoiseth, S., and Arumugham, R. (1998). Characterization of a subunit structure and stability of the recombinant porin from *Neisseria gonorrhoeae*. *J. Protein Chem.* 17, 719–728.

Mayer, K.H., Peipert, J., Fleming, T., Fullem, A., Moench, T., Cu-Uvin, S., Bentley, M., Chesney, M., and Rosenberg, Z. (2001). Safety and tolerability of BufferGel, a novel vaginal microbicide, in women in the United States. *Clin. Infect. Dis.* 32, 476–482.

McChesney, D., Tramont, E.C., Boslego, J.W., Ciak, J., Sadoff, J., and Brinton, C.C. (1982). Genital antibody response to a parenteral gonococcal *pilus* vaccine. *Infect. Immun.* 36, 1006–1012.

McGee, Z.A., Clemens, C.M., Jensen, R.L., Klein, J.J., Barley, L.R., and Gorby, G.L. (1992). Local induction of tumor necrosis factor as a molecular mechanism of mucosal damage by gonococci. *Microb. Pathog.* 12, 333–341.

McKinnon, L.R., Liebenberg, L.J., Yende-Zuma, N., Archary, D., Ngcapu, S., Sivo, A., Nagelkerke, N., Garcia Lerma, J.G., Kashuba, A.D., Masson, L., et al. (2018). Genital inflammation undermines the effectiveness of tenofovir gel in preventing HIV acquisition in women. *Nat. Med.* 24, 491–496.

Mimiaga, M.J., Mayer, K.H., Reisner, S.L., Gonzalez, A., Dumas, B., Vanderwarker, R., Novak, D.S., and Bertrand, T. (2008). Asymptomatic gonorrhea and chlamydial infections detected by nucleic acid amplification tests among Boston area men who have sex with men. *Sex. Transm. Dis.* 35, 495–498.

Mims, C., Nash, A., and Stephen, J. (2001). *Mims’ Pathogenesis of infectious disease* (San Diego, CA, Academic Press).

Montgomery, C.M., Gafos, M., Lees, S., Morar, N.S., Mweemba, O., Ssali, A., Stadler, J., Pool, R., and the MDP team (2010). Re-framing microbicide acceptability: findings from the MDP301 trial. *Cult. Health Sex.* 12, 649–662.

Moodley, P., Martin, I., Pillay, K., Ison, C., and Sturm, A. (2006). Molecular epidemiology of recently emergent ciprofloxacin-resistant *Neisseria gonorrhoeae* in South Africa. *Sex. Transm. Dis.* 33, 357–360.

Moran, J.S., and Levine, W.C. (1995). Drugs of choice for the treatment of uncomplicated gonococcal infections. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 20 Suppl 1, S47-65.

Morse, S.A. (1978). The biology of the gonococcus. *CRC Crit. Rev. Microbiol.* 7, 93–189.

Morse, S.A., Johnson, S.R., Biddle, J.W., and Roberts, M.C. (1986). High-level tetracycline resistance in *Neisseria gonorrhoeae* is result of acquisition of streptococcal tetM determinant. *Antimicrob. Agents Chemother.* 30, 664–670.

Muratani, T., Akasaka, S., Kobayashi, T., Yamada, Y., Inatomi, H., Takahashi, K., and Matsumoto, T. (2001). Outbreak of cefozopran (penicillin, oral cepheps, and aztreonam)-resistant *Neisseria gonorrhoeae* in Japan. *Antimicrob. Agents Chemother.* 45, 3603–3606.

Newman, L., Rowley, J., Hoorn, S.V., Wijesooriya, N.S., Unemo, M., Low, N., Stevens, G., Gottlieb, S., Kiarie, J., and Temmerman, M. (2015). Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. *PLOS ONE* 10, e0143304.

NIAID (2015). The Pharmacokinetics of Extended Duration High-dose Cefixime for the Decreased Susceptibility of *Neisseria Gonorrhoeae*: A Phase I Pilot Study. [ClinicalTrials.gov](http://ClinicalTrials.gov).

NIAID (2016). Randomized, Open-label Phase 2 Study of Oral AZD0914 in the Treatment of Gonorrhea. [ClinicalTrials.gov](http://ClinicalTrials.gov).

O'Connor, T.J., Kinchington, D., Kangro, H.O., and Jeffries, D.J. (1995). The activity of candidate virucidal agents, low pH and genital secretions against HIV-1 in vitro. *Int. J. STD AIDS* 6, 267–272.

Ohnishi, M., Saika, T., Hoshina, S., Iwasaku, K., Nakayama, S., Watanabe, H., and Kitawaki, J. (2011). Ceftriaxone-Resistant *Neisseria gonorrhoeae*, Japan. *Emerg. Infect. Dis.* 17, 148–149.

Oriel, J.D. (1994). *The Scars of Venus: A History of Venereology* (Springer-Verlag).

Ota, K.V., Fisman, D.N., Tamari, I.E., Smieja, M., Ng, L.-K., Jones, K.E., Diprima, A., and Richardson, S.E. (2009). Incidence and treatment outcomes of pharyngeal *Neisseria gonorrhoeae* and Chlamydia trachomatis infections in men who have sex with men: a 13-year retrospective cohort study. *Clin. Infect. Dis.* 48, 1237–1243.

Otto, R.B., Ameso, S., and Onegi, B. (2014). Assessment of antibacterial activity of crude leaf and root extracts of *Cassia alata* against *Neisseria gonorrhoea*. *Afr. Health Sci.* 14, 840–848.

Palmer, H.M., Young, H., Winter, A., and Dave, J. (2008). Emergence and spread of azithromycin-resistant *Neisseria gonorrhoeae* in Scotland. *J. Antimicrob. Chemother.* 62, 490–494.

Pantelic, M., Kim, Y.-J., Bolland, S., Chen, I., Shively, J., and Chen, T. (2005). *Neisseria gonorrhoeae* kills carcinoembryonic antigen-related cellular adhesion molecule 1 (CD66a)-expressing human B cells and inhibits antibody production. *Infect. Immun.* 73, 4171–4179.

Pascual, L.M., Daniele, M.B., Ruiz, F., Giordano, W., Pájaro, C., and Barberis, L. (2008). *Lactobacillus rhamnosus* L60, a potential probiotic isolated from the human vagina. *J. Gen. Appl. Microbiol.* 54, 141–148.

Patel, A.L., Chaudhry, U., Sachdev, D., Sachdeva, P.N., Bala, M., and Saluja, D. (2011). An insight into the drug resistance profile & mechanism of drug resistance in *Neisseria gonorrhoeae*. *Indian J. Med. Res.* 134, 419–431.

Patrick, D., Shaw, C., and Rekart, M. (1995). *Neisseria gonorrhoeae* with decreased susceptibility to ciprofloxacin in British Columbia: an imported phenomenon. *Can Common Rep* 21, 137–139.

Pearson, R., Ferri, F., Jones, R., and Opal, S. (2004). Gonorrhoea.

Percival, A., Rowlands, J., Corkill, J., Alergant, C., Arya, O., Rees, E., and Annels, A. (1976). Penicillinase-producing gonococci in Liverpool. *Lancet ii*, 1379–1382.

Petousis-Harris, H., Paynter, J., Morgan, J., Saxton, P., McArdle, B., Goodyear-Smith, F., and Black, S. (2017). Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. *Lancet Lond. Engl.* 390, 1603–1610.

Phillips, I. (1976). Beta-lactamase producing penicillin-resistant gonococcus. *Lancet ii*, 656–657.

Plante, M., Jerse, A., Hamel, J., Couture, F., Rioux, C.R., Brodeur, B.R., and Martin, D. (2000). Intranasal immunization with gonococcal outer membrane preparations reduces the duration of vaginal colonization of mice by *Neisseria gonorrhoeae*. *J. Infect. Dis.* 182, 848–855.

Ramjee, G., Kamali, A., and McCormack, S. (2010). The last decade of microbicide clinical trials in Africa: from hypothesis to facts. *AIDS Lond. Engl.* 24 Suppl 4, S40-49.

Reid, G. (2002). Probiotics for urogenital health. *Nutr. Clin. Care Off. Publ. Tufts Univ.* 5, 3–8.

Reid, G., Charbonneau, D., Erb, J., Kochanowski, B., Beurman, D., Poehner, R., and Bruce, A.W. (2003). Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunol. Med. Microbiol.* 35, 131–134.

Reid, G., Burton, J., and Devillard, E. (2004). The rationale for probiotics in female urogenital healthcare. *MedGenMed Medscape Gen. Med.* 6, 49.

Reyn, A., Korner, B., and Bentzon, M.W. (1958). Effects of Penicillin, Streptomycin, and Tetracycline on *N. gonorrhoeae* isolated in 1944 and in 1957\*. *Br. J. Vener. Dis.* 34, 227–239.

Roberts, M.C., Wagenvoort, J.H., van Klingeren, B., and Knapp, J.S. (1988). tetM- and beta-lactamase-containing *Neisseria gonorrhoeae* (tetracycline resistant and penicillinase producing) in The Netherlands. *Antimicrob. Agents Chemother.* 32, 158.

Roddy, R.E., Zekeng, L., Ryan, K.A., Tamoufé, U., and Tweedy, K.G. (2002). Effect of Nonoxynol-9 Gel on Urogenital Gonorrhoea and Chlamydial Infection: A Randomized Controlled Trial. *JAMA* 287, 1117–1122.

Rompalo, A. (2004). Gonorrhoea. In: Rake RE, Bope ET (Conn's current therapy. Philadelphia: Saunders).

Shokeen, P., Ray, K., Bala, M., and Tandon, V. (2005). Preliminary studies on activity of *Ocimum sanctum*, *Drynaria quercifolia*, and *Annona squamosa* against *Neisseria gonorrhoeae*. *Sex. Transm. Dis.* 32, 106–111.

- Shokeen, P., Bala, M., and Tandon, V. (2009). Evaluation of the activity of 16 medicinal plants against *Neisseria gonorrhoeae*. *Int. J. Antimicrob. Agents* 33, 86–91.
- Silva, O., Ferreira, E., Pato, M., Caniãsa, M., and Gomes, E.T. (2002). In vitro anti-*Neisseria gonorrhoeae* activity of *Terminalia macroptera* leaves. *FEMS Microbiol. Lett.* 217, 271–274.
- Skerlev, M., and Čulav-Košćak, I. (2014). Gonorrhea: New challenges. *Clin. Dermatol.* 32, 275–281.
- Söderholm, N., Vielfort, K., Hultenby, K., and Aro, H. (2011). Pathogenic *Neisseria* hitchhike on the uropod of human neutrophils. *PloS One* 6, e24353.
- Spencer, S.E., Valentin-Bon, I.E., Whaley, K., and Jerse, A.E. (2004). Inhibition of *Neisseria gonorrhoeae* genital tract infection by leading-candidate topical microbicides in a mouse model. *J. Infect. Dis.* 189, 410–419.
- Stadler, J., and Saethre, E. (2011). Blockage and flow: intimate experiences of condoms and microbicides in a South African clinical trial. *Cult. Health Sex.* 13, 31–44.
- Starnino, S., Stefanelli, P., and *Neisseria gonorrhoeae* Italian Study Group (2009). Azithromycin-resistant *Neisseria gonorrhoeae* strains recently isolated in Italy. *J. Antimicrob. Chemother.* 63, 1200–1204.
- Starnino, S., GASP-LAC Working Group, Galarza, P., Carvalho, M., Benzaken, A., Ballesteros, A., Cruz, O., Hernandez, A., Carbajal, J., Borthagaray, G., et al. (2012). Retrospective analysis of antimicrobial susceptibility trends (2000–2009) in *Neisseria gonorrhoeae* isolates from countries in Latin America and the Caribbean shows evolving resistance to ciprofloxacin, azithromycin and decreased susceptibility to ceftriaxone. *Sex Transm Dis* 39, 813–821.
- Sternberg, T., and Turner, T. (1944). The treatment of sulfonamide-resistant gonorrhea with penicillin sodium. Results in 1686 cases. *JAMA* 126, 157–163.
- Tapsall, J.W., Ndowa, F., Lewis, D.A., and Unemo, M. (2009). Meeting the public health challenge of multidrug- and extensively drug-resistant *Neisseria gonorrhoeae*. *Expert Rev. Anti Infect. Ther.* 7, 821–834.
- Thomas, C.E., Zhu, W., Van Dam, C.N., Davis, N.L., Johnston, R.E., and Sparling, P.F. (2006). Vaccination of mice with gonococcal TbpB expressed *in vivo* from venezuelan equine encephalitis viral replicon particles. *Infect. Immun.* 74, 1612–1620.
- Thurman, A.R., Schwartz, J.L., Brache, V., Clark, M.R., McCormick, T., Chandra, N., Marzinke, M.A., Stanczyk, F.Z., Dezzutti, C.S., Hillier, S.L., et al. (2018). Randomized, placebo controlled phase I trial of safety, pharmacokinetics, pharmacodynamics and acceptability of tenofovir and tenofovir plus levonorgestrel vaginal rings in women. *PLOS ONE* 13, e0199778.
- Tramont, E.C., and Boslego, J.W. (1985). Pilus vaccines. *Vaccine* 3, 3–10.
- Uhle, C., Latowsky, L., and Knight, F. (1941). Gonorrhea urethritis in the male. Treatment with sulfapyridine and sulfathiazole. *JAMA* 117, 247–249.
- Unemo, M., and Shafer, W.M. (2011). Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. *Ann. N. Y. Acad. Sci.* 1230, E19–28.
- Unemo, M., and Shafer, W.M. (2014). Antimicrobial Resistance in *Neisseria gonorrhoeae* in the 21st Century: Past, Evolution, and Future. *Clin. Microbiol. Rev.* 27, 587–613.

Unemo, M., Golparian, D., Nicholas, R., Ohnishi, M., Galloway, A., and Sednaoui, P. (2012a). High-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: novel penA mosaic allele in a successful international clone causes treatment failure. *Antimicrob. Agents Chemother.* *56*, 1273–1280.

Unemo, M., Golparian, D., Potocnik, M., and al (2012b). Treatment failure of pharyngeal gonorrhoea with internationally recommended first-line ceftriaxone verified in Slovenia, September 2011. *Euro Surveill* *17*, pii: 20200.

Unemo, M., Golparian, D., and Hellmark, B. (2014). First three *Neisseria gonorrhoeae* isolates with high-level resistance to azithromycin in Sweden: a threat to currently available dual-antimicrobial regimens for treatment of gonorrhea? *Antimicrob. Agents Chemother.* *58*, 624–625.

Urban, C.F., Lourido, S., and Zychlinsky, A. (2006). How do microbes evade neutrophil killing? *Cell. Microbiol.* *8*, 1687–1696.

Vallely, A., Fitzgerald, L., Fiya, V., Aeno, H., Kelly, A., Sauk, J., Kupul, M., Neo, J., Millan, J., Siba, P., et al. (2012). Intravaginal practices and microbicide acceptability in Papua New Guinea: implications for HIV prevention in a moderate-prevalence setting. *BMC Res. Notes* *5*, 613.

Van Slyke, C., Wolcott, R., and Mahoney, J. (1941). The chemotherapy of gonococcal infections. *JAMA* *116*, 276–280.

Van Slyke, C., Arnold, R., and Buchholtz, M. (1943). Penicillin therapy in sulfonamide-resistant gonorrhea in men. *Am J Public Health Nations Health* *33*, 1392–1394.

Vermani, K., and Garg, S. (2002). Herbal medicines for sexually transmitted diseases and AIDS. *J. Ethnopharmacol.* *80*, 49–66.

Vickers, A., Zollman, C., and Lee, R. (2001). Herbal medicine. *West. J. Med.* *175*, 125–128.

Virji, M. (2009). Pathogenic Neisseriae: surface modulation, pathogenesis and infection control. *Nat. Rev. Microbiol.* *7*, 274–286.

Vos, T., Barber, R.M., Bell, B., Bertozzi-Villa, A., Biryukov, S., Bolliger, I., Charlson, F., Davis, A., Degenhardt, L., Dicker, D., et al. (2015). Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the global burden of disease study 2013. *The Lancet* *386*, 743–800.

Warner, L., Stone, K.M., Macaluso, M., Buehler, J.W., and Austin, H.D. (2006). Condom use and risk of gonorrhea and Chlamydia: a systematic review of design and measurement factors assessed in epidemiologic studies. *Sex. Transm. Dis.* *33*, 36–51.

Wasserheit, J.N. (1992). Epidemiological synergy. Interrelationships between human immunodeficiency virus infection and other sexually transmitted diseases. *Sex. Transm. Dis.* *19*, 61–77.

WHO (2012). Global incidence and prevalence of selected curable STIs-2008 (Geneva).

WHO (2016). WHO | WHO guidelines for the treatment of *Neisseria gonorrhoeae*.

WHO (2017). Augmentation des cas de gonorrhée résistante aux antibiotiques, de nouveaux médicaments sont nécessaires (Genève).

Willcox, R.R. (1970). A survey of problems in the antibiotic treatment of gonorrhoea. With special reference to South-East Asia. *Br. J. Vener. Dis.* 46, 217–242.

Woodsong, C., and Holt, J.D.S. (2015). Acceptability and preferences for vaginal dosage forms intended for prevention of HIV or HIV and pregnancy. *Adv. Drug Deliv. Rev.* 92, 146–154.

Yokoi, S., Deguchi, T., Ozawa, T., and al. (2007). Threat to cefixime treatment for gonorrhea. *Emerg Infect Dis* 13:1275.

Zhu, W., Thomas, C.E., and Sparling, P.F. (2004). DNA Immunization of mice with a plasmid encoding *Neisseria gonorrhoeae* PorB protein by intramuscular injection and epidermal particle bombardment. *Vaccine* 22, 660–669.

Zhu, W., Thomas, C.E., Chen, C.-J., Van Dam, C.N., Johnston, R.E., Davis, N.L., and Sparling, P.F. (2005). Comparison of immune responses to gonococcal PorB delivered as outer membrane vesicles, recombinant protein, or Venezuelan equine encephalitis virus replicon particles. *Infect. Immun.* 73, 7558–7568.

Zhu, W., Chen, C.-J., Thomas, C.E., Anderson, J.E., Jerse, A.E., and Sparling, P.F. (2011). Vaccines for Gonorrhea: Can We Rise to the Challenge? *Front. Microbiol.* 2, 124.

Zielke, R.A., Wierzbicki, I.H., Baarda, B.I., Gafken, P.R., Soge, O.O., Holmes, K.K., Jerse, A.E., Unemo, M., and Sikora, A.E. (2016). Proteomics-driven Antigen Discovery for Development of Vaccines Against Gonorrhea. *Mol. Cell. Proteomics MCP* 15, 2338–2355.



**CHAPITRE II : SYSTEMES DESTINES AU TRAITEMENT  
ET A LA PREVENTION DE LA GONOCOCCIE : INTERET  
DES FORMULATIONS A BASE DE LACTOBACILLES**





## Introduction-Résumé

Les lactobacilles sont des bactéries Gram-positif, naturellement présentes dans la muqueuse vaginale (Cribby et al., 2009). Ils sont prédominants dans le vagin et jouent un rôle protecteur contre les infections vaginales (Boris and Barbés, 2000). En effet, ces lactobacilles jouent un rôle dans (i) le maintien de l'acidité du vagin, (ii) la compétition avec les pathogènes pour l'adhérence aux cellules épithéliales ou exclusion stérique, (iii) la production de biosurfactants pour inhiber les adhésines des pathogènes, (iv) la production d'acides organiques, de peroxyde d'hydrogène, de bactériocines pour inhiber la croissance des pathogènes, (v) la coagréation pour empêcher l'invasion de la lumière vaginale par le pathogène. Malheureusement, leur quantité et leur composition peut varier sous l'influence de plusieurs facteurs augmentant ainsi le risque d'infections vaginales particulièrement d'infection par le gonocoque (Wiesenfeld et al., 2003). Des études ont montré que la gonococcie est généralement associée à un déséquilibre des lactobacilles vaginaux. Leur contribution pour juguler la gonococcie serait donc une option à analyser, face aux difficultés actuelles de traitement et de prévention de la gonococcie, dont le germe responsable est devenu résistant à tous les antibiotiques connus sur le marché. Etant donné que les femmes sont les plus vulnérables (ONUSIDA, 2018) en ce qui concerne les infections sexuellement transmissibles dont cette infection, il apparaît alors judicieux de s'intéresser à l'utilisation possible des lactobacilles par les femmes afin de lutter contre la gonococcie. Etant donné que la voie vaginale est la principale voie de transmission de la gonococcie, il serait intéressant de développer un outil de prévention à action locale, administrable par cette voie dont le but serait d'empêcher l'adhérence du gonocoque aux cellules épithéliales de la muqueuse vaginale.

Ce chapitre a pour objectif de décrire la flore vaginale et les mécanismes d'action des lactobacilles, dans un premier temps, afin de comprendre l'intérêt des lactobacilles. Dans un second temps, il fait un état des lieux de l'approche probiotique qui consiste en l'administration de bactéries naturellement présentes dans l'organisme en quantité suffisante pour obtenir un effet bénéfique pour la santé de la sphère urogénitale. Le genre *Lactobacillus* est le plus étudié comme probiotique pour cette application. Certains lactobacilles sont même sur le marché. Par ailleurs, ce chapitre fait une étude critique des souches pouvant être utilisées pour la prévention ou le traitement de la gonococcie. Nous allons aussi décrire les avantages et les inconvénients de la voie vaginale, l'anatomie et la physiologie du vagin, ainsi

que les formes galéniques contenant des lactobacilles administrables par cette voie. Nous terminerons par les verrous à lever pour l'utilisation des lactobacilles contre la gonococcie.

## Références

Boris, S., and Barbés, C. (2000). Role played by *Lactobacilli* in controlling the population of vaginal pathogens. *Microbes Infect.* 2, 543–546.

Cribby, S., Taylor, M., and Reid, G. (2009). Vaginal microbiota and the use of probiotics. *Interdiscip. Perspect. Infect. Dis.* 2008, e256490.

ONUSIDA (2018). Résumé de l'étude de facteurs de vulnérabilité des filles et des femmes face aux IST/SIDA dans la région Atsinanana (ONUSIDA, SECNLS - 2011).

Wiesenfeld, H.C., Hillier, S.L., Krohn, M.A., Landers, D.V., and Sweet, R.L. (2003). Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. *Clin. Infect. Dis.* 36, 663–668.

**PROJET DE PUBLICATION**

**FORMULATIONS FOR THE TREATMENT AND THE  
PREVENTION OF GONORRHEA: INTEREST OF  
*LACTOBACILLI* BASED SYSTEMS**

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**Abstract**

The infection by *Neisseria gonorrhoeae* called gonorrhea is a sexually transmitted infection. Nowadays, it is a major public health concern due to the increasing resistance to antibiotics and the difficulties to develop a vaccine. The use of *Lactobacilli* as probiotics is a new approach that could be a valuable strategy. *Lactobacilli* are commensal in the vagina and fight naturally against infections. The presence and dominance of *Lactobacilli* in the vagina is associated with a reduced risk of urogenital infections such as yeast vaginitis, bacterial vaginosis, and urinary tract infections. Sexually transmitted infections are associated with *Lactobacilli* depletion. Therefore, restoring the normal vaginal ecosystem by the administration of *Lactobacilli* with probiotic properties and activity on *Neisseria gonorrhoeae* appears to be a valuable approach to treat or prevent gonorrhea. The vaginal route appears to be suitable for a direct activity of *Lactobacilli* on

the infection site. This review aims to underline the interest of the use of *Lactibacilli* to prevent or treat gonorrhoea, and the hurdles to overcome. It also gives the state of art about the vaginal route for the administration of such formulations.

### **Keywords**

Vaginal microbiota, *Lactobacillus*, vaginal administration, formulation, gonorrhoea.

### **Abbreviations**

ATCC, American Type Collection Center; HIV, Human Immunodeficiency Virus; *L.*, *Lactobacillus*; MAPK, mitogen-activated protein kinase; NF- $\kappa$ B, nuclear factor kappa B; *N. gonorrhoeae*, *Neisseria gonorrhoeae*; PAMPs, pathogen-associated molecular patterns; STI, Sexually Transmitted Infection; TLR, toll-like receptor; TNF, Tumor Necrosis Factor; WHO, World Health Organization.

## 1. INTRODUCTION

*Neisseria gonorrhoeae* (*N. gonorrhoeae*) is the bacterium responsible of gonorrhea, the second most common bacterial sexually transmitted infection (STI) after the infection by *Chlamydia trachomatis* (WHO, 2016). *N. gonorrhoeae* is transmitted by direct sexual contact and the vagina is the main way of its transmission. Most of time, the infection is asymptomatic in women increasing its propagation. *N. gonorrhoeae* is responsible of many cases of infertility (Grodstein et al., 1993) both in men and women, blindness in newborns (Laga et al., 1986), and facilitates the transmission of human immunodeficiency virus (Cohen, 2004; Jarvis and Chang, 2012). There is currently neither vaccines nor new drugs emerging on the market, while some untreatable strains are circulating. *N. gonorrhoeae* successively, adheres to the epithelial cells, penetrates into, multiplies, creates inflammation and weakens the epithelial barrier. It can persist inside neutrophils or modify their surface antigens to evade immune system. It can also produce some proteases to avoid the development of immunity to reinfection. *N. gonorrhoeae* can modify the antibiotics binding sites, synthesize enzymes to modify or inactivate the antibiotics, modify its surface porin proteins to reduce the antibiotics permeability, use a new metabolic pathway to replace the one inhibited by the antibiotics, use the active mechanism of efflux of antibiotics, and protect the target enzyme by binding to another protein (**Chapter I**). These mechanisms of pathogenicity and resistance help *N. gonorrhoeae* to survive. Thus, gonorrhea has become a major public health concern.

*Lactobacilli* are commensal, predominant bacteria of the normal vaginal microbiota. On the one hand, they are involved in maintaining the vaginal acidity and naturally fight against infections by combining different mechanisms. Beerepoot et al. (2012), in a randomized double-blind trial in post-menopausal women with recurrent urinary tract infections, showed that unlike trimethoprim-sulfamethoxazole, *Lactobacilli* did not increase antibiotic resistance in pathogens. The use of *Lactobacilli* has emerged in the field of the prevention or the treatment of urogenital infections (Pascual et al., 2008; Reid, 2002; Reid et al., 2003, 2004). *Lactobacilli* formulations have been developed for the treatment of recurrent urinary tract infections, bacterial vaginosis, and yeast vaginosis. Some are already on the market for this application. Therefore, restoring the normal vaginal microbiota by the administration of *Lactobacilli* with probiotic properties and activity on *N. gonorrhoeae* appears to be an attractive option to treat or prevent gonorrhea although there is currently no *Lactobacilli* formulation in the market for this indication. Some

*Lactobacilli* strains have demonstrated an activity on *N. gonorrhoeae* *in vitro* (Amant et al., 2002), *ex vivo* (Breshears et al., 2015) and probiotic properties. As women are at particularly high risk for STI due to their biological, economical and social vulnerabilities (ONUSIDA, 2018), it is especially important to focus on them. An effective, convenient to use, non-irritant, non-toxic and with low price, woman-controlled tool is necessary. A vaginal formulation containing *Lactobacilli* effective on *N. gonorrhoeae* could be a valuable strategy to prevent or treat gonorrhea.

This review describes the vaginal microbiota and the mechanisms of action of *Lactobacilli*. It presents the state of art of the use of *Lactobacilli* to treat or prevent urogenital infections, and more specially of *Lactobacilli* that are able to inhibit *N. gonorrhoeae*. Then, it gives an overview of the vaginal route characteristics, advantages and inconvenients, and dosage forms for the administration of vaginal systems. Finally, current hurdles to overcome for using *Lactobacilli* as probiotics to treat or prevent gonorrhea are presented.

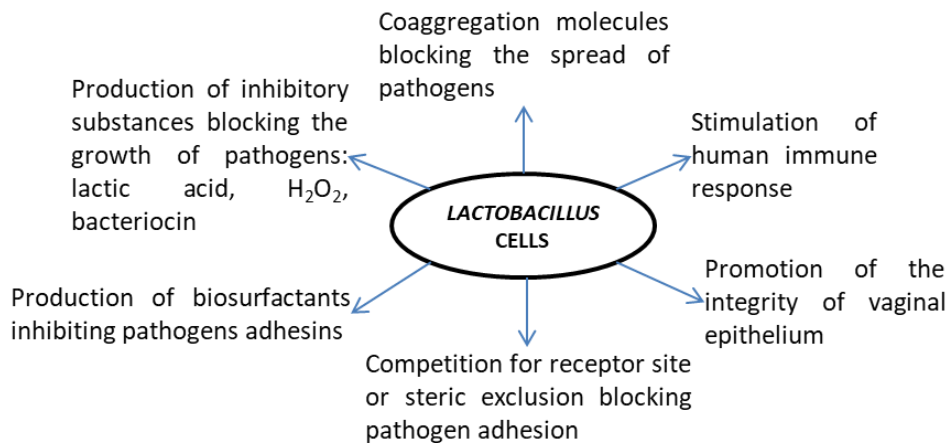
## 2. WOMAN NORMAL GENITAL TRACT MICROBIOTA

The tissues of the upper genital tract (ovaries, fallopian tubes, and uterus) are generally considered to be sterile. The introduction of bacteria into these tissues is typically associated with identifiable diseases (Heinonen et al., 1985; Rampersaud et al., 2012). On the contrary, the tissues of the lower genital tract (cervix, vagina, and vulva) are colonized by a microbiota. The cervical microbiota is similar to the vaginal microbiota, except that bacterial loads are lower (Ling et al., 2011; Vasquez et al., 2002). The vaginal microbiota is predominantly constituted by *Lactobacillus* (L.) species, that are Gram-positive, rod-shaped bacteria. They are *L. iners*, *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. casei*, *L. vaginalis*, *L. delbrueckii*, *L. salivarius*, *L. reuteri*, and *L. rhamnosus*. They have been described to colonize the vagina with varying degrees (Cribby et al., 2009). There are some interindividual variabilities (Vasquez et al., 2002) and geographical variations in the composition of the normal vaginal *Lactobacillus* microbiota. In healthy premenopausal women, *Lactobacilli* are present in quantities of  $10^7$  to  $10^8$  CFU/g of vaginal fluid (Boris and Barbés, 2000; Farage et al., 2010). Other bacterial species such as *Gardnerella vaginalis*, *Peptostreptococcus* spp., *Prevotella* spp., *Escherichia coli*, *Streptococcus agalactiae*, and *Enterococcus faecalis* are present in limited

quantities in a healthy vagina. Normally, the vaginal microbiota of healthy women can fight pathogens. Particularly, the *Lactobacillus* genus offer protection against urogenital tract diseases (Boris and Barbés, 2000). In addition, there is a correlation between loss and disturbance of the vaginal *Lactobacilli* and increased risk of genital infections including STI such as Human Immunodeficiency Virus HIV (Frank et al., 2012; Martin et al., 1999), Human Papilloma Virus HPV (Gao et al., 2013; Lee et al., 2013), herpes simplex virus type 2 HSV-2 (Cherpes et al., 2003), *Trichomonas vaginalis* (Brotman et al., 2012), *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (Wiesenfeld et al., 2003). This disturbance can be due to antibiotic use, sexual activity, menstruation, pregnancy, douching, hormonal alterations, and all contraception methods (Farage et al., 2010; Witkin et al., 2007). Both the predominance of *Lactobacilli* in the genital microbiota of healthy women and the missing of *Lactobacilli* in patients who develop urogenital tract infection have led to a focus on *Lactobacilli*, in order to understand their mechanisms of action.

### 3. LACTOBACILLI MECHANISMS TO CONTROL INFECTIONS

Postulated mechanisms of *Lactobacilli* against pathogens include direct inhibition by the production of inhibitory compounds or indirectly by the prevention of the pathogens adherence and spreading, the stimulation of immune system and/or reinforcement of the epithelial barrier function (Fig. 1).



**Figure 1.** *Lactobacilli* mechanisms controlling infections according to Reid (2001) and Reid and Burton (2002).



### 3.1. Enhancement of the epithelial barrier

The cervico-vaginal epithelium protected by the mucous layer and the natural antimicrobial components represent a barrier to pathogens. As for the intestinal barrier, *Lactobacilli* are able to influence many of the components of this epithelial barrier function either by decreasing cells apoptosis or by increasing mucin production. However, the mechanisms are not fully understood. The hypotheses are (i) the prevention of cytokine-induced epithelial damage by inhibiting tumor necrosis factor (TNF) (Yang et al., 2002), (ii) the possible enhancing of the expression of genes involved in tight junction signaling (Anderson et al., 2010) or genes encoding adherence junction proteins (Hummel et al., 2012), (iii) the increase of mucins (major macromolecular constituents of epithelial mucus) expression that interferes with pathogenic invasion and adherence (Caballero-Franco et al., 2007; Kim et al., 2008; Mack et al., 2003; Mattar et al., 2002), (iv) the initiation of the repair of the barrier function after damage by enhancing the expression and redistribution of tight junction proteins, reducing epithelium permeability to pathogens (Parassol et al., 2005) or mucosal regeneration (Caballero-Franco et al., 2007). Thus, the reinforcement of this barrier by a formulation that contains *Lactobacilli* and is able to cover the epithelium may help prevent gonorrhea.

### 3.2. Adherence to epithelial surface

*Lactobacilli* are able (i) to produce adhesins to fix vaginal cells, (ii) to adhere due to Van der Waals forces and electrostatics forces (Bohbot, 2008), (iii) to use one of the proteins of the vaginal fluid called fibronectine to adhere. Larsen et al. (2007) showed that the adhesion of *Lactobacilli* could be enhanced with the presence of calcium ions (Larsen et al., 2007). The adherence of *Lactobacilli* to epithelial cells and mucus varies among species and strains. This adherence is essential (i) to colonize the genital tract (Rose et al. (2012) showed the colonization of vaginal epithelial cells after adherence to them), (ii) to form a biofilm protecting the vaginal mucosa (Lepargneur and Rousseau, 2002), and (iii) to inhibit the growth and adhesion of pathogens (Reid et al., 2001). The inhibition of the adhesion of urogenital pathogens to cervico-vaginal epithelium by *Lactobacilli* are based on different mechanisms, such as steric exclusion, competition for receptor sites, and displacement of adhered pathogens (Zárate and Nader-Macias, 2006). The steric exclusion principle states that the huge number or size of *Lactobacilli* adherent

to cells may cover receptor sites in a non-specific manner avoiding pathogens adhesion. The competitive exclusion principle states that two species cannot occupy the same sites on epithelial cells, so *Lactobacilli* may occupy specific binding sites which would otherwise be available for pathogens. Regarding displacement, adherent pathogens may be removed from their fixation sites by *Lactobacilli*.

### **3.3. Autoaggregation and coaggregation**

*Lactobacilli* could auto-aggregate to form a biofilm resistant to pathogens adherence or co-aggregate with pathogens directly to inhibit their spread (Salas-Jara et al., 2016; Ventolini, 2015).

### **3.4. Acid production**

Acid production has long been known to be deleterious for some pathogens, not only killing viruses such as HIV, rotavirus and influenza virus, but also by displacing some pathogens from surfaces. *Lactobacilli*, the primary source of lactic acid (Boskey et al., 2001), acidify the vagina with lactic acid (Boskey et al., 1999) that is a potent, broad-spectrum bactericide and virucide. Lactic acid is able to inactivate *N. gonorrhoeae* (Graver and Wade, 2011). The resulting low pH may also increase the oxido-reduction potential. For every 1.0 unit decrease in pH, the redox potential increases by 60 mV (Jacob, 1970). *N. gonorrhoeae* is sensitive to oxidation but may develop defenses against oxidative stress (Seib et al., 2006). Therefore, the more *Lactobacilli* decrease the pH, the more efficient they are on *N. gonorrhoeae*.

### **3.5. Hydrogen peroxide production**

Some *Lactobacilli* can produce hydrogen peroxide ( $H_2O_2$ ) under permissive *in vitro* conditions (Martín and Suárez, 2010). The presence of  $H_2O_2$ -producing *Lactobacilli* in the vagina is associated with a reduced risk of infections (Mijac et al., 2006; Vallor et al., 2001) compared to “non- $H_2O_2$ -producing” *Lactobacilli*. From these results, the  $H_2O_2$  production by vaginal *Lactobacilli* may provide significant protection against infections.

### 3.6. Production of antimicrobial substances

*Lactobacilli* interfere with pathogens and prevent epithelial invasion by inducing host cells to produce peptides and by direct production of defensins, bacteriocins and bacteriocin-like substances, those increase the host defense to pathogens.

First, *Lactobacilli* induce the expression of antimicrobial peptides such as  $\beta$ -defensins by the host cells such as the neutrophils and the epithelial cells. The human  $\beta$ -defensin-2 (h $\beta$ D2) produced by cells during innate host defense can also serve as a signal mediating the initiation, mobilization and amplification of adaptive immune responses (Oppenheim et al., 2003; Yang et al., 2000, 2002). The h $\beta$ D2 is directly highly effective on a wide variety of bacteria, fungi and some viruses. These peptides provide the first line of host defense against infection by acting as “natural antibiotics” (Lehrer and Ganz, 2002; Thoma-Uszynski et al., 2001).

Second, *Lactobacilli* have been showed to directly suppress pathogens growth through the release of a variety of antimicrobial peptides such as defensins and bacteriocins. Bacteriocins are proteinaceous, bactericidal substances synthesized by bacteria, which usually have a narrow spectrum of activity, inhibiting strains of the same or closely related species (Tagg et al., 1976). They can afford a direct activity against specific pathogens without disturbing the beneficial commensal microbiota (Kjos et al., 2011). *Lactobacilli* can also produce bacteriocin-like substances that are antagonistic substances which are incompletely defined or do not fit the typical criteria of bacteriocins. They tend to have a broader spectrum of activity than bacteriocins. A great number of bacteriocin-like substances have been reported to be produced by *Lactobacilli*, inhibiting a wide range of both Gram-positive and -negative bacteria (Rammelsberg and Radler, 1990; Silva et al., 1987). Ocaña et al. (1999) characterized a bacteriocin-like substance produced by vaginal *L. salivarius* with activity against *N. gonorrhoeae*. There is still a need to elucidate the *in vivo* antagonist activity of bacteriocin and bacteriocin-like substances.

### 3.7. Production of biosurfactants

*Lactobacilli* may protect the host by the production of biosurfactants that are glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids (Rodrigues and Teixeira, 2010) with a tendency to accumulate at interfaces, especially at liquid-air interfaces (Velraeds et al., 1996). Biosurfactants can inhibit the growth of pathogens and

avoid their adhesion on epithelial cells (Gudina et al., 2010). Biosurfactants demonstrated various advantages when compared to chemical surfactants: they displayed a low toxicity, a high biodegradability and a high effectiveness at extreme temperatures or pH values (Rodrigues and Teixeira, 2010).

### **3.8. Stimulation of the immune system**

The innate immune response detects a wide range of molecular patterns that are commonly found on pathogens, but are foreign to mammals, called pathogen-associated molecular patterns (PAMPs). Macrophages express a set of pattern recognition receptors comprising various toll receptors (TLR), whose ligands include lipopolysaccharides as *N. gonorrhoeae* lipooligosaccharides. TLR ligation leads to the activation of the nuclear factor kappa B (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK) pathways (Faure et al., 2000; Muzio et al., 1998), the enhancement of phagocytosis, the production of reactive oxygen species and the release of cytokines, and lipid mediators that coordinate and amplify the local inflammatory response (Takeda, Guha). The adaptive immune response requires the recognition of specific molecular structures and depends on the generation of a large number of antigen receptors such as T-cell receptors and immunoglobulins. When T-cells recognize a foreign antigen presented to them, they initiate responses specifically targeting this antigen, including a direct attack against antigen-bearing cells by cytotoxic T-cells, stimulation of the production of antibodies by B-cells and induction of a local inflammatory response. T-cells can differentiate into at least two subtypes of T helper cells (Th). Th1 cells produce some cytokines such as interferon-gamma (IFN- $\gamma$ ), which can stimulate the macrophage to increase its production of a large number of mediators, including reactive oxygen species, lipid species and pro-inflammatory cytokines. About Th2 cells, they can stimulate humoral immunity by elaborating a number of cytokines that stimulate B-cell maturation into antibody-producing plasma cells and promote B-cell class-switching to increase the production of immunoglobulin E antibodies (IgE). Th2 cells can also facilitate the recruitment and activation of mast cells, another effector of allergic reactions and contributor to chronic inflammation in various pathological conditions. Th2 cells can attenuate the inflammatory response by producing cytokines with anti-inflammatory properties such as interleukin-10 (IL-10) to reduce inflammation.

*Lactobacilli*, observed in various physiological systems, may motivate the proliferation of immune cells (Imani Fooladi et al., 2015), increase the cytotoxicity of natural killer cells (Imani Fooladi et al., 2015; Iwabuchi et al., 2012; Soltan-Dallal et al., 2012), induce or increase the production of interferon- $\gamma$  (Imani Fooladi et al., 2015; Iwabuchi et al., 2012; Soltan-Dallal et al., 2012) and the expression of Th1 cytokines (Imani Fooladi et al., 2015), decrease the production of IL-4 (Imani Fooladi et al., 2015), increase the production of IL-12 (Iwabuchi et al., 2012; Soltan-Dallal et al., 2012). The stimulation of the immune system by vaginal *Lactobacilli* is not a well-documented mechanism. Rose et al. (2012) used vaginal epithelial multilayers treated with toll-like receptor (TLR) agonists to stimulate the TLR. After their treatment with *L. crispatus* ATCC 33820, interleukins IL-6 and IL-8 expression was significantly reduced. Also, *L. crispatus* ATCC 33820 and to lesser extent *L. jensenii* ATCC 25258 could induce a significant reduction of TNF secretion as well as some pro-inflammatory chemokines (Rose et al., 2012). The role of the vaginal isolates *L. rhamnosus* GR-1 and *L. reuteri* RC-14, in the prevention of *Candida albicans* vulvo-vaginitis by the stimulation and the modulation of the vaginal immune responses (suppression of TLR2, TLR6, IL-8, and TNF expression) was also reported (Wagner and Johnson, 2012).

#### **4. LACTOBACILLI AS PROBIOTICS FOR THE PREVENTION AND THE TREATMENT OF UROGENITAL INFECTIONS**

The concept of restoring the *Lactobacillus* content of the vaginal microbiota as a barrier to prevent infection originated from the Canadian urologist Andrew Bruce in the early 1970s (Reid and Bruce, 2001; Reid et al., 2003). Since then, extensive researches have showed that some *Lactobacillus* strains are able to colonize the vagina after their administration (Cadieux et al., 2002), to remain in the vagina for several months after insertion (Cadieux et al., 2002; Reid et al., 1994), and to reduce the risk of urinary tract infection, yeast vaginitis (Reid et al., 1995), and bacterial vaginosis (Reid et al., 2001). The administration of *Lactobacilli* can restore the vaginal flora and prevent urogenital infections. *Lactobacilli* may be used as a single strain or a mixture of several strains in the formulations. Several *Lactobacilli* formulations are already on the market (**Table 1**). Their content varies between  $10^5$  and  $10^{10}$  *Lactobacilli* by unit. They are often used to restore a healthy vaginal flora. Their storage conditions are usually between  $+4^{\circ}\text{C}$  and  $+8^{\circ}\text{C}$ , but

always below 25°C. They can be stored for about 2 years but for much of them the storage time is not explicit. They are mainly lyophilized and presented as symbiotic preparations *i.e.* as a mixture of probiotics with nourishing substances called prebiotics such as fructooligosaccharides, lactose, inulin, maltodextrin. Clinical trials are in progress to give the proof of their efficacy in human (**Table 2**). Three *Lactobacilli* have emerged in the prevention and treatment of urogenital infections: *L. rhamnosus*, *L. crispatus* and *L. fermentum* (Reid, 1999) (**Tables 1 and 2**). Strains selection had been based on *in vitro* tests and the source of the strains (Burton et al., 2003; Reid and Bruce, 2001; Vasquez et al., 2002). Some properties had to be fulfilled by *Lactobacilli* to establish them as effective probiotic organisms. They included the ability to (i) exclude or reduce pathogenic adherence, (ii) persist and multiply, (iii) produce acids, and bacteriocins antagonistic to pathogen growth, (iv) resist vaginal microbicides, including spermicides, (v) be safe and therefore non-invasive, non-carcinogenic, and non-pathogenic, and (vi) coaggregate and form a normal, balanced flora (Reid et al., 1999; Salminen et al., 1996; Sobel, 1999). Regarding these properties *L. rhamnosus* and *L. fermentum* had been more studied than *L. crispatus*. There are no published data on *L. rhamnosus* and *L. fermentum* efficacy on *N. gonorrhoeae*. However, it was showed that *L. crispatus* inhibits the infectivity of *N. gonorrhoeae* (Amant et al., 2002). In addition, Vielfort et al. (2008) investigated how *Lactobacilli* and *Gonococci* competed for adherence to human epithelial cervical cells and showed that *L. crispatus* was able to reduce gonococcal adherence while *L. rhamnosus* was not. *L. rhamnosus* and *L. fermentum* originate from the intestinal tract, while *L. crispatus* originates from the vagina.

One strain called *L. crispatus* CTV-05 had been well studied as regards its adherence to vaginal epithelial cells *in vitro* (Kwok et al., 2006) and its ability to colonize the vagina in the majority of studied women (Antonio and Hillier, 2003). Another study from Antonio et al. (2009) evaluating gelatin capsules containing *L. crispatus* CTV-05 for safety and vaginal colonization in 90 young women aged 14–21 years, showed that 90 % of participants who lacked *L. crispatus* colonization at enrollment were successfully colonized by CTV-05. The authors suggested that exposure to semen, vaginal intercourse, and the presence of *Lactobacilli* of the same species at enrollment might lessen the colonization by the probiotics. They also suggested that *L. crispatus* CTV-05 was safe (Antonio et al., 2009). Vallor et al (2001) demonstrated that 23 out of 57 (40 %) of women of their study initially naturally colonized by vaginal H<sub>2</sub>O<sub>2</sub>-producing *L. crispatus* or *L. jensenii* remained colonized over 8 months. Pavlova et al. (2002) determined the species of

*Lactobacilli* that colonize the vagina and compared them with those found in food and the environment. Their results showed that most encountered vaginal isolates were *L. crispatus*, *L. jensenii* and *L. gasseri* and few vaginal isolates matched food or environmental *Lactobacillus* species. Therefore, *L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii* seem to be specific to the human vagina (Pavlova et al., 2002; Ravel et al., 2011; Zhou et al., 2007, 2010), where they could produce copious amounts of lactic acid and a protective low pH (3.5–4) (Ravel and Brotman, 2016). They are the predominant strains of the vaginal *Lactobacilli* (Wijgert et al., 2014). Among these four predominant and specific vaginal strains, *L. crispatus* is the most commonly and frequently occurring *Lactobacillus* species of the healthy vaginal flora of women including pregnant women. Its number is reduced in genital diseases. *L. crispatus* is ubiquitous from all continents as evidenced by clinical studies performed worldwide (Aagaard et al., 2012; Antonio et al., 1999; Benning et al., 2014; Mitchell et al., 2013; Pavlova et al., 2002; Zhou et al., 2007). Moreover, *L. crispatus* promotes the stability of the normal vaginal microbiota while *L. gasseri*, and/or *L. iners* are more conducive to the occurrence of abnormal vaginal microbiota. *L. crispatus*-dominated vaginal microbiota might change to a *L. iners*-dominated vaginal microbiota but is less likely to change directly to a dysbiotic state, and conversely (Gajer et al., 2012; Verstraelen et al., 2009). *L. crispatus* inhibits the infectivity of not only *N. gonorrhoeae* (Amant et al., 2002), but other pathogens such as *Chlamydia trachomatis* (Nardini et al., 2016), bacterial and yeast pathogens (*Escherichia coli*, *Candida albicans*, *Candida glabrata*, and *Gardnerella vaginalis*) (Hütt et al., 2016). Therefore, *L. crispatus* could simultaneously prevent or treat different urogenital infections.

*L. crispatus* appears to be very interesting as a probiotic to prevent urogenital infections, due to its properties, its origin, its efficacy on several urogenital pathogens and the presence of some species already on the market.

**Table 1.** Some probiotic formulations on the market

Route	Dosage form	Strains	Composition - Trade name	Storage	Indication	Laboratory
Oral	Powder	<i>L. helveticus</i> laiffi L10, <i>L. rhamnosus</i> R0011	<i>L. helveticus</i> laiffi L10, <i>L. rhamnosus</i> R0011, purified water, fructose, atomized cranberry juice powder containing 7 % of proanthocyanidin; Carrier: magnesium hydroxide, tricalcium phosphate; Preservatives: potassium sorbate, sodium benzoate; Natural strawberry flavor GYNEBIOTIC Pro®  Double compartment bottle with <i>Lactobacillus</i> in the bottle cap and cranberry powder in the bottle.	Refrigerator	To restore the normal vaginal flora during pregnancy, menopause, and antibiotic treatment  For the intimate comfort and the balance of the urogenital flora	ARAGAN
Capsule		<i>L. acidophilus</i> , <i>L. rhamnosus</i>	Capsule of <i>L. acidophilus</i> and <i>L. rhamnosus</i> and tablet of 36 mg of proanthocyanudines GYNEBIOTIC® cranberry BIO	Refrigerator	To protect and restore the vaginal flora	ARAGAN
		<i>L. rhamnosus</i> , <i>L. acidophilus</i> , <i>L. reuteri</i> , <i>L. crispatus</i>	<i>Lactobacilli</i> 5.10 <sup>9</sup> / capsule Maltodextrin, Emulsifier: magnesium stearate; Acidifying agent: ascorbic acid. Capsule : hypromellose Dyes: Candurin, red Allura AC. OROGYN ®	Shelt from heat	To preserve the vaginal flora	CDD
		<i>L. rhamnosus</i> GR-1, <i>L. reuteri</i> RC-14	Glucose, <i>Lactobacilli</i> , Maltodextrin; Excipient : Hydroxypropylmethylcellulose, Cellulose ; Anti-caking agent: Silicon dioxide, Magnesium salts of fatty acids, Dye: Titanium dioxide FEMIBION ® floreintime	Refrigerator	To restore and maintain the balance of the vaginal flora	MERCK
		<i>L. brevis</i> , <i>L. acidophilus</i> , <i>L. rhamnosus</i>	<i>Lactobacilli</i> 2.5x10 <sup>9</sup> / capsule Fructooligosaccharides 20 mg; Capsule: gelatin; Antioxidant: ascorbic acid; Feedstock: maltodextrin; Stabilizer: magnesium stearate PROGYN ®	15-25°C	To maintain the balance of the vaginal flora  To prevent bacterial and yeast vaginoses	PHACOBEL



Table 1. Continued

Route	Dosage form	Strains	Composition - Trade name	Storage	Indication	Laboratory
Oral	Capsule	<i>L. rhamnosus</i> GR-1, <i>L. reuteri</i> RC-14	<i>Lactobacilli</i> 5x10 <sup>9</sup> /capsule Milk, Dextrose anhydrate, Potato starch, Microcrystalline cellulose, vegetable Magnesium stearate, Beef gelatin, Titanium dioxide FEM-DOPHILUS®	Refrigerator	To restore and maintain the balance of flora	JARROW FORMULAS
Vaginal	Tablet	<i>L. casei</i> var <i>rhamnosus</i> <i>döderleini</i>	Prolonged release tablet  GYNOPHILUS® LP	< 25°C	To maintain the balance of the vaginal flora, used after a local antibiotic or antifungal treatment or used at the same time as an oral antibiotic treatment	BESINS INTERNATIONAL
		<i>L. acidophilus</i>	<i>L. acidophilus</i> : 10 <sup>9</sup> /tablet Lactic acid 60 %: 15 mg Dry extract of <i>Krameriatriandra</i> containing 15 % of neolignans 1 mg GYNEBIOTIC® intima	Refrigerator	To restore the normal vaginal flora during pregnancy, menopause and antibiotic treatment For the intimate comfort and the balance of the urogenital flora	ARAGAN
	Capsule	<i>L. casei</i> var <i>rhamnosus</i> <i>döderleini</i>	Freeze-dried <i>Lactobacillus</i> 10 <sup>9</sup> /g Estriol Progesterone; For lyophilization: lactose monohydrate, potato starch, sodium thiosulfate, sodium glutamate; Capsule shell composition: gelatin, titanium dioxide TROPHIGIL®	< 25°C	To restore the normal vaginal flora and relieve vaginal dryness, vaginal itching, atrophic vulvovaginitis due to estrogen deficiency	BESINS INTERNATIONAL
		<i>L. acidophilus</i> , <i>L. casei</i> , <i>L. rhamnosus</i> , <i>L. delbrueckii</i> ssp. <i>bulgaricus</i> and <i>S. thermophilus</i>	<i>Lactobacilli</i> 4.10 <sup>9</sup> /capsule Ascorbic acid, gelatin, magnesium stearate, maltodextrin PROBIOSAT® Vaginal	Refrigerator	To restore and maintain a normal vaginal flora Used as adjuvant for the treatment of vaginal infections	BERRY NATURALS
		<i>L. casei</i> var <i>rhamnosus</i> <i>döderleini</i>	<i>Lactobacillus</i> 10 <sup>9</sup> /g estriol, progesterone Sodium glutamate, lactose monohydrate, potato starch, sodium thiosulfate, gelatin, titanium dioxide FLORGYNAL® gelules	< 25°C	To be used in the case of atrophic vulvovaginitis due to estrogen deficiency	BIOSE PROBIONOV

Table 1. Continued

Route	Dosage form	Strains	Composition - Trade name	Storage	Indication	Laboratory
Vaginal	Capsule	<i>L. crispatus</i> , <i>L. reuteri</i> and <i>L. rhamnosus</i>	Sodium hyaluronate 5 mg, burdock 50 mg, Lyophilized <i>Lactobacilli</i> > 2.10 <sup>9</sup> / capsule Maltodextrin, vegetable magnesium stearate Gelatin, Titanium dioxide MYCORESS®	< 25°C	Restoration of natural flora after antifungal treatment or recurrent mycoses	BIOES
Vaginal	Capsule	<i>L. rhamnosus</i> (PB01™), <i>L. gasseri</i> (EB01™)	<i>Lactobacilli</i> > 10 <sup>8</sup> / capsule Anhydrous glucose, lactitol monohydrate, corn starch, hydroxypropylmethylcellulose, titanium dioxide and magnesium stearate MEGIGYNE ®	-	To restore and maintain the balance of the vaginal flora	SAFORELLE
		<i>L. plantarum</i> P17630	<i>L. plantarum</i> , corn starch, mannitol, crosslinked sodium carboxymethylcellulose, vegetable magnesium stearate HYDRALIN FLORA®	< 25°C	To restore and maintain the balance of the vaginal flora	BAYER
		<i>L. casei</i> var. <i>rhamnosus</i> <i>döderlein</i> (LCR 35)	Lyophilized <i>Lactobacillus</i> > 10 <sup>9</sup> /g Lactose monohydrate GYNOPHILUS®	< 25°C	To maintain the balance of the vaginal flora, used after a local antibiotic or antifungal treatment or used at the same time as an oral antibiotic treatment	BESINS INTERNATIONAL
Suppository		<i>L. gasseri</i> and <i>L. acidophilus</i>	Freeze-dried <i>Lactobacilli</i> > 10 <sup>9</sup> / suppository, calcium lactate, inulin, solidified fat SYMBIOVAG®	Refrigerator	To stabilize the vaginal environment	SYMBIOPHARM
Periodic pad		<i>L. fermentum</i> , <i>L. casei</i> var. <i>rhamnosus</i> , <i>L. gasseri</i>	lyophilized <i>Lactobacilli</i> Pad: 100 % viscose not treated with chlorine Cord: 100 % cotton External layer: 100 % polypropene. FLORGYNAL® tampon probiotique  Pad with applicator	< 25°C	To be used during menstruation To reduce vaginal discomfort: itching, irritation ... To protect the vagina against bacterial imbalances To restore the natural flora To improve healing in addition to anti-infectious treatment	SAFORELLE

**Table 2.** Some probiotic formulations studied in clinical trials for the prevention or the treatment of urogenital infections

Route	Form	Composition	Infection	Outcome	Source	
Oral	Capsule	Freeze-dried <i>L. rhamnosus</i> GR-1 and <i>L. reuteri</i> RC-14	Bacterial vaginosis	Prevention of bacterial vaginosis among women living with HIV	(Hummelen et al., 2010)	
		Gelatin capsules of freeze-dried <i>L. rhamnosus</i>	Bacterial vaginosis	Colonization, restoration and maintenance of a normal vaginal flora.	(Reid et al., 2001)	
		GR-1 and <i>L. reuteri</i> (>10 <sup>9</sup> /strain/capsule viable)	Yeast vaginitis	Reduction of bacteria and yeast infection	(Reid et al., 2003)	
		5.10 <sup>9</sup> CFU <i>L. acidophilus</i> PBS066 (40 mg as lyophilized), 5.10 <sup>9</sup> CFU <i>L. reuteri</i> PBS072 (30 mg as lyophilized), 320 mg inulin, 5 mg silica, 5 mg talc or 5.10 <sup>9</sup> CFU <i>L. plantarum</i> PBS067 (12 mg as lyophilized), 5.10 <sup>9</sup> CFU <i>L. rhamnosus</i> PBS070 (20 mg as lyophilized), 5.10 <sup>9</sup> CFU <i>Bifidobacterium animalis subsp. lactis</i> PBS075 (60 mg as lyophilized), 298 mg inulin, 5 mg silica, 5 mg talc	Vaginal candidiasis	<i>L. plantarum</i> was not as efficient as other strains in colonizing the vaginal mucosa after oral intake.	(Mezzasalma et al., 2017)	
Vaginal	Suppository	<i>L. crispatus</i> GAI 98322 (1.0 × 10 <sup>8</sup> CFU per one suppository)	Urinary tract infection	Reduction in the recurrence of bacterial urinary tract infections	(Uehara et al., 2006)	
		<i>L. crispatus</i> (Lactin-V; Osel)	Recurrent urinary tract infection	Reduction of the occurrence of urinary tract infection in premenopausal women	(Czaja et al., 2007; Stapleton et al., 2011)	
			Bacterial vaginosis	Colonization of women with bacterial vaginosis, The product was well tolerated and accepted by women	(Hemmerling et al., 2009, 2010)	
		Glycerol suppositories of <i>Lactobacillus</i> GG	Reccurent Candidal vulvovaginitis	Improvement of women vaginal symptoms, reduction of vaginal erythema and discharge	(Hilton et al., 1995)	
	Tablet		<i>L. brevis</i> (CD2), <i>L. salivarius</i> (FV2) and <i>L. plantarum</i> (FV9)	Bacterial vaginosis	Treatment of bacterial vaginosis Restoration of a healthy vaginal flora Prevention of sperm lipid peroxidation, thus preservation of sperm motility and viability	(Maggi et al., 2000) (Mastromarino et al., 2009) (Barbonetti et al., 2011)
			Lyophilized <i>L. acidophilus</i> (≥10 <sup>7</sup> CFU/tablet) Oestriol (0.03 mg/tablet) (Gynoflor®)	Bacterial vaginosis	Cure of bacterial vaginosis of pre-menopausal women and recolonization of the vagina	(Parent et al., 1996)
				Vacterial vaginosis, candidiasis, trichomoniasis	Enhancement of the restoration of the vaginal flora by the administration of live <i>Lactobacilli</i> in combination with low dose oestriol.after anti-infective treatment	(Ozkinay et al., 2005)
		<i>L. rhamnosus</i> (Normogin®)	Bacterial vaginosis	A long-term (24 months) treatment of women affected by bacterial vaginosis lead to the restoration of the vaginal physiological pH associated with a reduction of the intensity of bacterial vaginosis symptoms	(Rossi et al., 2010)	

**Table 2.** Continued

Vaginal	Tampon	Freeze-dried <i>L. gasseri</i> , <i>L. casei</i> var. <i>rhamnosus</i> and <i>L. fermentum</i>	Bacterial vaginosis	Administration of the tampon after an open treatment with vaginal clindamycin ovules. No improvement in the cure rate by this antibiotic.	(Eriksson et al., 2005)
	Capsule	<i>L. rhamnosus</i> GR-1 and <i>L. reuteri</i> RC-14	Bacterial vaginosis	Effective cure of bacterial vaginosis using this <i>Lactobacilli</i> formulation (90 %)	(Anukam et al., 2006)
		<i>L. crispatus</i>	Bacterial vaginosis	Women great interest in this intravaginal application for the treatment of bacterial vaginosis, Infection prevention	(Marrazzo et al., 2006)
		<i>L. rhamnosus</i>	Bacterial vaginosis	Stabilization of the vaginal ecosystem and reduction of the recurrence of bacterial vaginosis	(Marcone et al., 2010)
	Sanitary napkins and pantliners	<i>Lactobacillus</i> strains		Lactic acid production	(Carlucci et al., 2009; Husmark and Gustafson, 2007)

## 5. LACTOBACILLI AS POTENTIAL PROBIOTICS FOR THE PREVENTION OF GONORRHEA

Regarding the inhibition of *N. gonorrhoeae*, Saigh et al. (1978) showed that the most active inhibitors of *N. gonorrhoeae* from endocervical flora were *Streptococci*, *Staphylococci*, and *Lactobacilli*. The inhibition of *N. gonorrhoeae in vitro* had been reported for *L. acidophilus* in 1994 (Zheng et al., 1994). Amant et al. (2002) demonstrated that *L. acidophilus* ATCC 4356, *L. crispatus* ATCC 33197, *L. jensenii* ATCC 25258, *L. gasseri* ATCC 33323 inhibited *N. gonorrhoeae* at low pH (5.8 - <7), but that *L. crispatus* and *L. jensenii* inhibited *N. gonorrhoeae* at neutral pH (7) too, suggesting that both strains may protect more effectively than *L. acidophilus* ATCC 4356 and *L. gasseri* ATCC 33323. Breshears et al. (2015) showed the inhibition of *N. gonorrhoeae ex vivo*.

To study exclusion or reduction of pathogenic adherence, Vielfort et al. (2008) analysed the early interactions between *Lactobacilli* and *N. gonorrhoeae*, and investigated how they competed for adherence to human epithelial cervical cells. They showed that *L. crispatus*, *L. gasseri* and *L. reuteri* were able to reduce gonococcal adherence while *L. rhamnosus* was not. In addition, *N. gonorrhoeae* and *Lactobacilli* formed mixed microcolonies and these complexes detached frequently from the epithelial cell surface reducing gonococcal colonization (Vielfort et al., 2008). Other strains *L. gasseri* ATCC 33323 and *L. gasseri* ATCC 9857 have been showed to reduce gonococcal adherence to epithelial cells and their invasion. They were able to displace adherent *N. gonorrhoeae* from epithelial cells, suggesting their potential use as post-exposure prophylaxy (Spurbeck and Arvidson, 2008, 2010). *L. gasseri* does not co-aggregate with *N. gonorrhoeae* neither in cell culture medium supernatant nor at the cell surface (Spurbeck and Arvidson, 2008). The persistence and multiplication after administration to women of these strains that inhibit *N. gonorrhoeae* or its adherence have not been explored yet, neither their resistance to vaginal microbicides, including spermicides. Safety assessment studies have performed these strains. Among all these strains studied to inhibit *N. gonorrhoeae*, *L. crispatus* appears to be very promising. Administrating *L. crispatus* might help in the prevention of gonorrhea. It could be very interesting to develop a probiotic formulation containing *L. crispatus* intended for a vaginal administration.

## **6. VAGINA A PROMISING ROUTE FOR THE TREATMENT AND THE PREVENTION OF GONORRHEA**

All the recommended treatments of gonorrhoea have been administered either orally or by injection. The issue is that, after administration by oral and parenteral route, the active pharmaceutical ingredients are poorly absorbed in the vagina, where they are supposed to exhibit their pharmacological function. This leads to the development of resistances. About the treatment of yeast vaginitis and bacterial vaginosis, the oral dosage form is associated to a vaginal administration form with interesting results. The majority of *Lactobacilli* formulations were initially developed for oral intake and it has been hypothesized that ingested *Lactobacilli* could migrate from the gut to rectum and ascend from the rectal skin to the vagina (Morelli et al., 2004). However, this way is indirect and too long. Now, trend is to develop *Lactobacilli* formulations for direct vaginal administration to enhance their efficacy and reduce the period between the administration and the action. The vaginal route has some advantages. The dosage forms are easy to apply. The administration is not painful like by parenteral route and there is no risk of vomiting drugs as sometimes observed by oral route with chemical active pharmaceutical ingredient. Prolonged dosage form with continuous release can be administered, improving users' compliance. Vaginal dosage forms can be inserted and easily removed. Repeated administration is possible. The presence of a dense network of blood vessels makes the vagina an excellent route for drug delivery when both local and systemic effects are foreseen (Hussain and Ahsan, 2005; Richardson and Illum, 1992; Vermani and Garg, 2000; Weber et al., 2005). Moreover, the vaginal route allows avoiding the hepatic first pass. As gonorrhoea is mainly spread through sexual transmission, the vagina is therefore an interesting route for gonorrhoea prevention.

### **6.1. Vaginal characteristics to take into account for the development of a vaginal formulation**

#### **6.1.1. Vaginal anatomy and histology**

Human vagina is a part of the female genital tract. It is a fibromuscular tube of about 10 cm in length which extends from the vulva to the cervix (Van De Graaff, 2001). From outside to inside, the vaginal wall consists of four layers: the *tunica adventicia*, the muscular coat, the *lamina propria*, and the nonkeratinized stratified squamous epithelial layer (Burkitt et al., 1994). The *tunica adventicia* is made of loose connective tissue, whereas in the muscular coat,

there are smooth elastic fibers. All these confer an excellent elasticity to the vagina. The *lamina propria* is a connective tissue rich in blood and lymphatic vessels draining drugs to the internal iliac vein, thus avoiding the first hepatic pass (Richardson and Illum, 1992; Van De Graaff, 2001). The thickness of the vaginal epithelial cell layer varies between 200 and 300  $\mu\text{m}$  (Sjöberg et al., 1988). This thickness is not really influenced by menstrual cycle but is strongly dependent on age and hormonal levels especially estrogen level (Song et al., 2004). This low variability is interesting for the drug membrane passage because some drug absorption can be affected by the thickness of the vaginal epithelium. The vagina is innervated by two types of nerve supply: a peripheral one, which primarily supplies to the lower quarter of the vagina and makes it a highly sensible area and an autonomic fiber one that responds to stretch and are not very sensitive to pain. This explains why women do not feel discomfort when using continuous intravaginal drug delivery systems (Alexander et al., 2004; Choudhury et al., 2011).

### **6.1.2. Vaginal physiology**

The surface of the vaginal wall is covered by numerous cross folds, often called *rugae* that increase the surface area of the vaginal wall and provide distensibility and support. So *rugae* provide a large surface of absorption to drugs. These *rugae* are covered by a viscoelastic gel called vaginal mucus. Mucus contains organic and inorganic salts, mucin, proteins, carbohydrates, urea and fatty acids (Vermani and Garg, 2000). Its composition and its physical characteristics change according to the different phases of the menstrual cycle. During the follicular phase, mucus is thinner, easily penetrable and is associated to a thick and cohesive epithelium due to estrogen effect. In luteal phase, mucus is thicker and associated to desquamation of the epithelium surface that renders it thin, loose and porous due to progesterone effect. Mucus production rate is higher during ovulatory phase. It protects the vaginal mucosa of the external environment and ensures lubrication (Junginger et al., 2002). This mucus layer is also the site of the interactions between the vaginal mucosa and the administered active pharmaceutical ingredients (API) (Khanvilkar et al., 2001). It could then be exploited for bioadhesive drug delivery systems to avoid leakage after administration (Woodley, 2001). Moreover, the mucus is able to capture foreign particles and remove them, so it could serve as a permeability barrier to prospective drug candidates (Hussain and Ahsan, 2005).

Although the vagina does not possess any gland, it secretes a fluid called vaginal fluid (Paavonen, 1983). The vaginal fluid contains the cervical mucus, desquamated vaginal cells, plasma transudate, leucocytes, carbohydrates, amino acids, aliphatic acids, proteins, enzymes, enzymes inhibitors and immunoglobulins (IgA, for example) (Burgos and Roig, 1978; Chien and Lee, 2002; Rohan and Sassi, 2009; Wagner and Levin, 1978). The mass of the vaginal fluid is estimated between 0.5 and 0.75 g on average every time and its production is about 6 g/day (Owen and Katz, 1999; Owen et al., 2000). The vaginal fluid volume, viscosity and pH may have either positive or negative impacts on vaginal drug absorption (Hussain and Ahsan, 2005). If its volume increases, drugs may be removed from vaginal cavity during the flow of the fluid, reducing the contact time and thus the absorption. Regarding its pH, since many drugs are weak electrolytes, the vaginal pH may change their degree of ionization and modify their release profile from the drug delivery systems, and so affect their absorption (Hussain and Ahsan, 2005). Normally, the vaginal pH is acidic, varying between 3.5 and 5.5. It is modified by vaginal secretions, after sexual intercourse, during menstruation and after menopause. This pH is maintained by *Lactobacilli*, the predominant bacteria of the healthy vaginal flora, which transformed glycogen into lactic acid. The *Lactobacilli* of the vaginal flora control this microbiota by competing with other microorganisms for adherence to epithelial cells and by producing antimicrobial compounds such as organic acids which lowers the vaginal pH, hydrogen peroxide, bacteriocin-like substances and possibly biosurfactants to control the population of vaginal pathogens (Boris and Barbés, 2000). In addition, the vagina contains enzymes that may affect the absorption of proteins (Chien and Lee, 2002). The normal vagina is anaerobic with pressures of O<sub>2</sub> and CO<sub>2</sub> respectively lower and higher than atmospheric levels, though menstruation increases oxygen pressure (Hill et al., 2005). All these characteristics are to be considered in the development of vaginal formulations.

## 6.2. Vaginal forms

Vaginal formulations can be administered in (i) liquid forms: solutions, emulsions and suspensions, (ii) semi-solid forms: creams, ointments and gels, (iii) solid forms: tablets, capsules, films, and (iv) devices such as rings (Vermani and Garg, 2000). On the market, formulations such as contraceptives, labor inducers, vaginal infections treatments, local menopausal syndroms treatments are commonly available in the following dosage forms: cream, gel, tablet, capsule, foam, ovule, ointment, suppository, tampon, douche, and vaginal



ring. In recent studies, because of the risk of leakage reducing the retention and contact time, the liquid forms are less developed. However, vaginal rings and semi-solid forms raise a great interest. In fact, vaginal rings are positioned in the upper third of the vagina especially susceptible to infection, near the cervico-vaginal junction. They do not interfere with coitus and are expected to release the API in a controlled manner, in the vaginal lumen over a long period up to several months. Vaginal rings are generally made of a polymer matrix (silicone, thermoplastic materials, ethylene vinylacetate copolymer) containing drug substances (Malcolm et al., 2010).

The advantages of semi-solid forms are their tolerance, their feasibility and low cost of production (Pilcher, 2004). They are expected to cover the vaginal mucosa (Hussain and Ahsan, 2005). The development of systems that are administrated in the body as liquids or semi-solids and undergo a transition to solidify *in situ* has emerged as a very appealing strategy to ensure patient compliance and comfort. Therefore, hydrogels and especially thermosensitive ones gained a particular attention in research. They are the most frequently studied semi-solid dosage forms.

Hydrogels are gels in which the dispersion medium is an aqueous solution. Hydrogels are generally colorless, odorless, tasteless and can be used in combination with vaginal devices or condoms (Pilcher, 2004). Hydrogels offer several other advantages such as hydrophilicity, biocompatibility, good distribution and retention, appropriate drug release and acceptability by patients. Hydrogels have been mostly used for the treatment of vaginal dryness and local delivery of antimicrobial drugs, microbicides, contraceptives and labor inducers. Hydrogels have also been used for a potential systemic delivery of hormones, vaccines, proteins and peptides. However, an applicator is needed to reach the cervico-vaginal junction (Hussain and Ahsan, 2005). Preformed gels are more and more replaced by so called *in-situ* forming hydrogels that can be administered as a liquid before transforming to a gel inside the body, due to specific physiological triggers such as pH, temperature or ions. The temperature sensitive approach can be advantageous for the vaginal application as it does not require any external material for inducing gelation. Several polymers have been studied for this purpose: polysaccharides such as cellulose-derivatives or chitosan mixed with glycerophosphate, *N*-isopropylacrylamide copolymers, poly(ethylene oxide-*b*-propylene oxide-*b*-ethylene oxide) (poloxamers and their copolymers, poly(ethylene oxide)/(d,l-lactic acid-*co*-glycolic acid) copolymers (Ruel-Gariépy and Leroux, 2004). The most widely studied temperature-sensitive polymers are poloxamers. Poloxamers are triblock copolymers composed of a central hydrophobic chain of poly (propylene oxide) and two hydrophilic side chains of poly

(ethylene oxide). Upon increasing the temperature, if their concentration is high enough, such copolymers can self-assemble into micelles in aqueous solution. The micelles subsequently organize themselves in a micellar cubic phase leading to a gelation of the system (Dumortier et al., 2006).

To solve the problem of leakage and messy effect of the gels, the trend is to develop mucoadhesive hydrogels. Mucoadhesive hydrogels are able to adhere to the biological tissues or the mucus layer of the tissue allowing intimacy of contact, to improve drug absorption and to prolong residence time at the site of administration. Mucohesion necessitates mucoadhesive compounds (Justin-Temu et al., 2004; Valenta, 2005). Poly(acrylate), chitosan, cellulose-derivatives, hyaluronic acid and derivatives, pectin, starch, poly(ethylene glycol), carrageenan, alginate and gelatin are examples of mucoadhesive polymers (Acarturk, 2009; Andrews et al., 2009; Valenta, 2005).

Researchers have developed vaginal hydrogels that are both temperature sensitive and mucoadhesive. Such systems gelify in response to the temperature change from ambient to body temperature facilitating their spreadability on the mucosa, and persist in the vagina by adhesion (Aka-Any-Grah et al., 2010; Cellesi, 2012; das Neves and Bahia, 2006; Roy et al., 2001; Ruel-Gariépy and Leroux, 2004).

The vaginal route is very interesting for the administration of formulations intended to treat or prevent infections. However, it may show some limits such as the risks of local irritation, or microbiota deterioration as well as the reduction of the efficacy of the drugs sensitive to the vaginal pH. In addition, the efficiency of the drugs may be influenced by sexual intercourse (Brache et al., 2009; Hurst et al., 2008; Merriam et al., 2015) and personal hygiene. Some gender specificity may also influence the efficacy of some drugs: pregnancy (Krauer and Krauer, 1977; Loebstein et al., 1997; Sachdeva et al., 2009), menstruation discharge, and menopause.

## **7. CURRENT HURDLES TO THE USE OF *LACTOBACILLI* AGAINST GONORRHEA**

*Lactobacilli* are predominant in the healthy vaginal flora and greatly impact the vaginal health (see **section 2**), some *Lactobacilli* have showed their interest for urogenital probiotic applications (see **sections 3 and 4**) and can inhibit *N. gonorrhoeae* (see **section 5**). For all these reasons they are particularly interesting for the development of probiotics for the

prevention of gonorrhoea. However, they should combine several of the mechanisms of inhibition of pathogens. To this respect *L. crispatus* appears to be very interesting as a probiotic to prevent urogenital infections (see **sections 4 and 5**), especially for the prevention of gonorrhoea. Several hurdles must however be overcome before using them as a mean of prevention against gonorrhoea.

First of all, the acceptability by the patients of such formulations containing *Lactobacilli* should be evaluated.

Concerning strain itself, a beneficial effect attributed to one strain cannot be assumed to be provided by another strain, even if it belongs to the same species (Ibnou-Zekri et al., 2003; Sanders, 2008). Thus, the probiotic effects of the chosen *Lactobacilli* for the formulation development should be clearly demonstrated, particularly its efficacy on *N. gonorrhoeae*. In addition, some strains are not well suited to colonize the vagina. For example, a commercial probiotic strain *L. rhamnosus* GG, was apparently not able to colonize the vagina nor was the *L. acidophilus* strain studied (Colodner et al., 2003), maybe explaining why both strains failed to prevent recurrence of urinary tract infections (Baerheim et al., 1994; Kontiokari et al., 2001). Therefore, the ability to colonize the vagina should be evaluated in early stage of probiotics development at the same time as the identification of the chosen strain and the proof of its activity. Probiotics optimal dose should also be determined.

In 2002, a joint working group of the FAO and the WHO drafted guidelines for the evaluation of probiotics in food (FAO/WHO, 2002) that was slightly modified by the consensus statement of 2014 (Hill et al., 2014). In short, these guidelines included: (i) to identify and characterize the organism to the genus and species level with internationally accepted methods (genotypic and phenotypic methods), (ii) to have a clear and consistent strain designation (international culture collection name), (iii) to show probiotic benefits such as the production of bacteriocins, acids, and hydrogen peroxide, and the ability to inhibit adhesion of pathogens both *in vitro* and *in vivo*, (iv) to establish safety *in vitro* and/or in animal study and human studies by minimum tests including antibiotic resistance patterns, metabolic activities, toxin production, hemolytic activity, infectivity in immunocompromised animal models, side effects in humans, and adverse incidents in consumers, (v) to establish efficacy in double blind, randomized, placebo-controlled phase 2 human trials. After these evaluation steps, a formulation can be called probiotic. A phase 3 effectiveness trial to compare the probiotic to standard means of prevention or treatment should then be conducted. The guidelines also included requirements for labeling of the probiotic products with strain specification, minimum number of viable bacteria at the end of shelf life, storage conditions, which would

be helpful to the consumers to safeguard their own interest. As there are currently no guidelines for medicinal probiotics, these guidelines may help for the evaluation of probiotic intended for medical use. Nevertheless, some criteria of these guidelines such as the adherence, hydrogen peroxide production and the viability of the strains are still controversial. Some studies on the interaction between *Lactobacilli* and *N. gonorrhoeae* confirmed this controversy. In fact, the number of adherent bacteria did not correlate to the level of protection against gonococcal infection (Vielfort et al., 2008). That means that this mechanism is not so crucial in the development of the probiotic formulation. Regarding H<sub>2</sub>O<sub>2</sub> production, some studies have showed that its production by vaginal *Lactobacilli* is implausible as a mechanism of protection *in vivo*, since (i) the hypoxic condition of the vagina precludes bacterial production of H<sub>2</sub>O<sub>2</sub> because the production of hydrogen peroxide requires aerobic conditions, (ii) even if it is produced, the high antioxidant capacity of cervico-vaginal fluid blocks the microbicidal activity of H<sub>2</sub>O<sub>2</sub>, and (iii) H<sub>2</sub>O<sub>2</sub> is more toxic to vaginal *Lactobacilli* than to the seventeen species responsible of bacterial vaginoses (O'Hanlon et al., 2010, 2011). Graver and Wade (2011) showed that during anaerobic growth, the inhibition of *N. gonorrhoeae* by the vaginal *Lactobacillus* tested was primarily due to acidification, suggesting that those seeking to select *Lactobacillus* for probiotic use should address the acidification potential of candidate *Lactobacillus* to prevent gonorrhea. As regards the viability of the strains, in the development of the probiotic formulations, it is stated that the selected probiotics should be able to survive during industrial processing and remain viable and stable during storage. However, some strains are able to be as beneficial when alive or dead, so the viability is not always a limiting property (Mottet and Michetti, 2005; Ouwehand et al., 2000; Reid et al., 1995). Freeze-drying bacterial cultures is often used to increase their shelf life as well as their stability during storage at room temperature (Zárate and Nader-Macías, 2006). These dried bacteria can then be incorporated into several dosage forms for vaginal application. The challenge is that probiotic formulations maintain their properties during long term storage. In the absence of guidelines to evaluate *Lactobacilli* as probiotics to prevent or treat gonorrhea, in view of these studies, adherence, hydrogen peroxide production and the viability of the strains can be evaluated but should not be limiting criteria.

Furthermore, the usual animal models able to mimic gonorrhea are mice, guinea pig, chimpanzee (Arko, 1989; Chandler and Kraus, 1976; Lucas et al., 1971). However, *N. gonorrhoeae* is a human obligatory bacterium. Thus, after given the proof of the concept, and the efficacy of the formulation *in vitro* and in animals, products should be used in patients. Only human clinical trials can provide the definitive answer to whether or not *Lactobacilli* can

be efficient as probiotics for the prevention of gonorrhoea particularly the efficacy and the security of using them.

The evaluation of the security is crucial even if *Lactobacilli* are generally considered as safe. Some clinical trials comparing the use of some *Lactobacillus* strains to the use of antibiotics for the prevention of urinary tract infections showed no side effect with *Lactobacilli* (Brumfitt et al., 1992; Brumfitt et al., 1983; Reid et al., 1995), but safety should be more explored because some adverse events have been reported (Hemmerling et al., 2010). These authors, in a phase 2a study of *L. crispatus* CTV-05 (LACTIN-V) administered by a vaginal applicator, showed the occurrence of mild and moderate adverse events such as vaginal discharge, abdominal pain, dysuria, pollakiuria, vaginal odor and genital pruritus. No serious adverse events occurred and no deep epithelial disruption was seen during colposcopic evaluation.

By mixing different species, the efficacy could be the addition of the one of each strain (Famularo et al., 2001; Gardiner et al., 2002). However, some preliminary studies should demonstrate this complementarity before.

Furthermore, the incorporation of substances that may nourish *Lactobacilli* in formulations could enhance their efficacy. Prebiotics could stimulate the growth, the activity, or both, of endogenous *Lactobacilli* that already reside in the vagina. The combination of probiotics and prebiotics could also improve the survival of the probiotic organisms, because their specific substrate is readily available for their metabolism. It could be advantageous to the host that the probiotic and the prebiotic are offered at the same time. Nowadays, there is a great interest in the mixture of probiotics and prebiotics called synbiotics. However, prebiotics alone have the potential to optimize, maintain and restore the flora of the vaginal ecosystem (Al-Ghazzewi and Tester, 2016). Some reports have promoted prebiotics application in vaginal health (Al-Ghazzewi and Tester, 2016), but their efficacy on gonorrhoea was not studied. **Table 3** gathers the main steps that should be followed for the selection of probiotics intended for the prevention of gonorrhoea.

**Table 3.** Steps for the development of a vaginal probiotic formulation intended for the prevention of gonorrhoea (FAO/WHO, 2002; Hill et al., 2014; Reid et al., 1999; Salminen et al., 1996; Sobel, 1999).

Isolation of a strain	<ul style="list-style-type: none"> <li>▪ To extract the strain from the human vagina</li> <li>▪ To identify and characterize the organism to the genus and species level with internationally accepted methods (genotypic and phenotypic methods)</li> <li>▪ To have a clear and consistent strain designation (international culture collection name)</li> </ul>
Security aspect	<ul style="list-style-type: none"> <li>▪ To establish safety <i>in vitro</i> and/or in animal study and human studies by minimum tests including antibiotic resistance patterns, metabolic activities, toxin production, hemolytic activity, infectivity in immunocompromised animal models, side effects in humans, and adverse incidents in consumers</li> </ul>
Functional aspect	<ul style="list-style-type: none"> <li>▪ To show probiotic benefits such as the production of bacteriocins and acids</li> <li>▪ To show ability to inhibit the growth, the adhesion and the spread of pathogens both <i>in vitro</i> and <i>in vivo</i></li> <li>▪ To determine the effective dosage</li> <li>▪ To show ability to colonize the vagina</li> <li>▪ To show ability to co-aggregate pathogens, to auto-aggregate and form biofilm</li> </ul>
Technological aspect	<ul style="list-style-type: none"> <li>▪ To show stability during the process</li> <li>▪ To show stability within the formulation</li> <li>▪ To show preservation of the functional properties in the formulation</li> <li>▪ To show a prolonged release profile in the vagina</li> <li>▪ To show formulation efficacy</li> </ul>
Clinical evidence of usefulness	<ul style="list-style-type: none"> <li>▪ To establish efficacy in double blind, randomized, placebo-controlled phase 2 human trials</li> <li>▪ To compare the probiotic to standard means of prevention or treatment in a phase 3 effectiveness trial</li> </ul>

## 8. CONCLUSION

Gonorrhoea is a major public health issue. The emergence of resistance to antibiotics and the difficulties to develop vaccines call for alternative solutions. One promising approach is the use of *Lactobacilli* recognized to be an important defense against infection. They are naturally hosted by the vagina and may be introduced in sufficient quantity by a dosage form. Therefore, they would restore the normal microbiota and enhance the natural defense mechanisms, to induce a protection towards infections. Many concomitant properties are required to confer this protection. They have been identified, but they have not yet been fully elucidated. *Lactobacilli* are lacking in some cases of urogenital tract infections. So, the probiotic approach to restore the vaginal ecosystem and fight against urogenital infections is increasing in this field. Probiotics are generally considered to be safe and are already widely used. However, more work is required to isolate strains with scientifically proven efficacy on *N. gonorrhoeae*. Then, such strains could be incorporated into vaginal formulations to prevent gonorrhoea. *L. crispatus* has showed to fulfil many criteria. Its characterization as well as its efficacy on *N. gonorrhoeae* should however continue to be fully assessed. Some usual formulations such as tablets, capsules, suppositories containing *Lactobacilli* have already showed their efficacy for the vaginal route and may be a support. However, even if there are

few published data on the other vaginal forms such as semi-solid forms containing *Lactobacilli*, it could be very interesting to assess them.

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### References

- Aagaard, K., Riehle, K., Ma, J., Segata, N., Mistretta, T.-A., Coarfa, C., Raza, S., Rosenbaum, S., Van den Veyver, I., Milosavljevic, A., et al. (2012). A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. *PloS One* 7, e36466.
- Acarturk, F. (2009). Mucoadhesive vaginal drug delivery systems. *recent pat. drug deliv. Formul.* 3, 193–205.
- Aka-Any-Grah, A., Bouchemal, K., Koffi, A., Agnely, F., Zhang, M., Djabourov, M., and Ponchel, G. (2010). Formulation of mucoadhesive vaginal hydrogels insensitive to dilution with vaginal fluids. *Eur. J. Pharm. Biopharm.* 76, 296–303.
- Alexander, N.J., Baker, E., Kaptein, M., Karck, U., Miller, L., and Zampaglione, E. (2004). Why consider vaginal drug administration? *Fertil. Steril.* 82, 1–12.
- Al-Ghazzewi, F. h., and Tester, R. f. (2016). Biotherapeutic agents and vaginal health. *J. Appl. Microbiol.* 121, 18–27.
- Amant, D.C.S., Valentin-Bon, I.E., and Jerse, A.E. (2002). Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. *Infect. Immun.* 70, 7169–7171.
- Anderson, R.C., Cookson, A.L., McNabb, W.C., Park, Z., McCann, M.J., Kelly, W.J., and Roy, N.C. (2010). *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. *BMC Microbiol.* 10, 316.
- Andrews, G.P., Laverty, T.P., and Jones, D.S. (2009). Mucoadhesive polymeric platforms for controlled drug delivery. *Eur. J. Pharm. Biopharm.* 71, 505–518.
- Antonio, M.A.D., and Hillier, S.L. (2003). DNA fingerprinting of *Lactobacillus crispatus* strain CTV-05 by repetitive element sequence-based PCR analysis in a pilot study of vaginal colonization. *J. Clin. Microbiol.* 41, 1881–1887.
- Antonio, M.A.D., Hawes, S.E., and Hillier, S.L. (1999). The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J. Infect. Dis.* 180, 1950–1956.
- Antonio, M.A.D., Meyn, L.A., Murray, P.J., Busse, B., and Hillier, S.L. (2009). Vaginal colonization by probiotic *Lactobacillus crispatus* CTV-05 is decreased by sexual activity and endogenous *Lactobacilli*. *J. Infect. Dis.* 199, 1506–1513.

- Anukam, K.C., Osazuwa, E., Osemene, G.I., Ehigiagbe, F., Bruce, A.W., and Reid, G. (2006). Clinical study comparing probiotic *Lactobacillus* GR-1 and RC-14 with metronidazole vaginal gel to treat symptomatic bacterial vaginosis. *Microbes Infect.* 8, 2772–2776.
- Arko, R.J. (1989). Animal models for pathogenic *Neisseria* species. *Clin. Microbiol. Rev.* 2, S56–S59.
- Arya, O.P., Taber, S.R., and Nsanze, H. (1980). Gonorrhoea and female infertility in rural Uganda. *Am. J. Obstet. Gynecol.* 138, 929–932.
- Baerheim, A., Larsen, E., and Digranes, A. (1994). Vaginal application of *Lactobacilli* in the prophylaxis of recurrent lower urinary tract infection in women. *Scand. J. Prim. Health Care* 12, 239–243.
- Barbonetti, A., Cinque, B., Vassallo, M.R.C., Mineo, S., Francavilla, S., Cifone, M.G., and Francavilla, F. (2011). Effect of vaginal probiotic lactobacilli on in vitro-induced sperm lipid peroxidation and its impact on sperm motility and viability. *Fertil. Steril.* 95, 2485–2488.
- Beerepoot, M.A.J., Riet, G. ter, Nys, S., Wal, W.M. van der, Borgie, C.A.J.M. de, Reijke, T.M. de, Prins, J.M., Koeijers, J., Verbon, A., Stobberingh, E., et al. (2012). *Lactobacilli* vs antibiotics to prevent urinary tract infections: A randomized, double-blind, noninferiority trial in postmenopausal women. *Arch. Intern. Med.* 172, 704–712.
- Benning, L., Golub, E.T., Anastos, K., French, A.L., Cohen, M., Gilbert, D., Gillevet, P., Munyazesa, E., Landay, A.L., Sikaroodi, M., et al. (2014). Comparison of lower genital tract microbiota in HIV-infected and uninfected women from Rwanda and the US. *PloS One* 9, e96844.
- Bohbot, J.-M. (2008). Les sécrétions vaginales. *Pelvi-Périnéologie* 3, 19–24.
- Boris, S., and Barbés, C. (2000). Role played by *Lactobacilli* in controlling the population of vaginal pathogens. *Microbes Infect.* 2, 543–546.
- Boskey, E.R., Telsch, K.M., Whaley, K.J., Moench, T.R., and Cone, R.A. (1999). Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. *Infect. Immun.* 67, 5170–5175.
- Boskey, E.R., Cone, R.A., Whaley, K.J., and Moench, T.R. (2001). Origins of vaginal acidity: high d/l lactate ratio is consistent with bacteria being the primary source. *Hum. Reprod.* 16, 1809–1813.
- Brache, V., Croxatto, H., Kumar, N., Sitruk-Ware, R., Cochón, L., Schiappacasse, V., Sivin, I., Muñoz, C., Maguire, R., and Faundes, A. (2009). Effect of sexual intercourse on the absorption of levonorgestrel after vaginal administration of 0.75 mg in Carraguard® gel: a randomized, cross-over, pharmacokinetic study. *Contraception* 79, 150–154.
- Breshears, L.M., Edwards, V.L., Ravel, J., and Peterson, M.L. (2015). *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. *BMC Microbiol.* 15.
- Brotman, R.M., Bradford, L.L., Conrad, M., Gajer, P., Ault, K., Peralta, L., Forney, L.J., Carlton, J.M., Abdo, Z., and Ravel, J. (2012). Association between *Trichomonas vaginalis* and vaginal bacterial community composition among reproductive-age women. *Sex. Transm. Dis.* 39, 807–812.



Brumfitt, W., Hamilton-Miller, J.M., Gargan, R.A., Cooper, J., and Smith, G.W. (1983). Long-term prophylaxis of urinary infections in women: comparative trial of trimethoprim, methenamine hippurate and topical povidone-iodine. *J Urol* 130, 1110–1114.

Brumfitt, W., Hamilton-Miller, J.M., Walker, S., and Roberts, D. (1992). Cefaclor as a prophylactic agent for recurrent urinary infections: a comparative trial with macrocrystalline nitrofurantoin. *Drugs Exp. Clin. Res.* 18, 239–244.

Burgos, M., and Roig, e V.-L.C.E. (1978). Ultrastructure of the vaginal mucosa. E E Hafez T N Evans Eds *Hum. Reprod. Med. Hum. Vagina* North Holl. Publ. N. Y. 63–93.

Burkitt, H., Young, B., and Heath, J.. (1994). *Histologia Funcional.* 3rd Ed Guanab. Koogan Rio Jan. RJ 353.

Burton, J.P., Cadieux, P.A., and Reid, G. (2003). Improved understanding of the bacterial vaginal microbiota of women before and after probiotic instillation. *Appl. Environ. Microbiol.* 69, 97–101.

Caballero-Franco, C., Keller, K., De Simone, C., and Chadee, K. (2007). The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292, G315-322.

Cadieux, P., J., Burton, G., Gardiner, I., Braunstein, A., Bruce, C.Y.K., and Reid., G. (2002). Lactobacillus strains and vaginal ecology. *JAMA* 287 1940–1941.

Carlucci, G., Di, C.A., Pescr, A., and Talone, D. (2009). Articles comprising lactic acid producing microorganisms.

CDC (1998). HIV Prevention through early detection and treatment of other sexually transmitted diseases -- United States recommendations of the advisory committee for HIV and STD prevention. 47 (*RR 12*), 1–24.

Cellesi, F. (2012). Thermoresponsive hydrogels for cellular delivery. *Ther. Deliv.* 3, 1395–1407.

Chandler, F.W., and Kraus, S.J. (1976). Animal model of human disease. Gonorrhea. *Am. J. Pathol.* 82, 437–440.

Cherpes, T.L., Meyn, L.A., Krohn, M.A., Lurie, J.G., and Hillier, S.L. (2003). Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 37, 319–325.

Cherpes, T.L., Hillier, S.L., Meyn, L.A., Busch, J.L., and Krohn, M.A. (2008). A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. *Sex. Transm. Dis.* 35, 78–83.

Chien, Y., and Lee, C.. (2002). Drug delivery, vaginal route. *Enycl. Pharm. Technol.* 2nd Ed 961–985.

Choudhury, A., Das, S., and Kar, M. (2011). A Review on Novelty and Potentiality of Vaginal Drug Delivery. *Int. J. PharmTech Res.* 3, 1033–1044.

Cohen, M.S. (2004). HIV and sexually transmitted diseases: lethal synergy. *Top. HIV Med. Publ. Int. AIDS Soc. USA* 12, 104–107.

Colodner, R., Edelstein, H., Chazan, B., and Raz, R. (2003). Vaginal colonization by orally administered Lactobacillus rhamnosus GG. *Isr. Med. Assoc. J. IMAJ* 5, 767–769.

Cribby, S., Taylor, M., and Reid, G. (2009). Vaginal Microbiota and the Use of Probiotics. *Interdiscip. Perspect. Infect. Dis.* 2008, e256490.

Czaja, C.A., Stapleton, A.E., Yarova-Yarovaya, Y., and Stamm, W.E. (2007). Phase I Trial of a *Lactobacillus crispatus* Vaginal Suppository for Prevention of Recurrent Urinary Tract Infection in Women. *Infect. Dis. Obstet. Gynecol.* 2007.

Dumortier, G., Grossiord, J.L., Agnely, F., and Chaumeil, J.C. (2006). A review of poloxamer 407 pharmaceutical and pharmacological characteristics. *Pharm. Res.* 23, 2709–2728.

Eriksson, K., Carlsson, B., Forsum, U., and Larsson, P.-G. (2005). A double-blind treatment study of bacterial vaginosis with normal vaginal lactobacilli after an open treatment with vaginal clindamycin ovules. *Acta Derm. Venereol.* 85, 42–46.

Famularo, G., Pieluigi, M., Coccia, R., Mastroiacovo, P., and Simone, C.D. (2001). Microecology, bacterial vaginosis and probiotics: perspectives for bacteriotherapy. *Med. Hypotheses* 56, 421–430.

FAO/WHO (2002). Food and Agricultural Organization of the United Nations and World Health Organization. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food.

Farage, M., Miller, K., and Sobel, J. (2010). Dynamics of the vaginal ecosystem—hormonal influences. *Infect. Dis. Res. Treat.* 3, 1–15.

Faure, E., Equils, O., Sieling, P.A., Thomas, L., Zhang, F.X., Kirschning, C.J., Polentarutti, N., Muzio, M., and Arditi, M. (2000). Bacterial lipopolysaccharide activates NF- $\kappa$ B through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. *J. Biol. Chem.* 275, 11058–11063.

Fleming, D.T., and Wasserheit, J.N. (1999). From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. *Sex. Transm. Infect.* 75, 3–17.

Frank, D.N., Manigart, O., Leroy, V., Meda, N., Valéa, D., Zhang, W., Dabis, F., Pace, N.R., Van de Perre, P., and Janoff, E.N. (2012). Altered vaginal microbiota are associated with perinatal mother-to-child transmission of HIV in African women from Burkina Faso. *J. Acquir. Immune Defic. Syndr.* 1999 60, 299–306.

Gajer, P., Brotman, R.M., Bai, G., Sakamoto, J., Schütte, U.M.E., Zhong, X., Koenig, S.S.K., Fu, L., Ma, Z.S., Zhou, X., et al. (2012). Temporal dynamics of the human vaginal microbiota. *Sci. Transl. Med.* 4, 132ra52.

Galega, F.P., Heymann, D.L., and Nasah, B.T. (1984). Gonococcal ophthalmia neonatorum: the case for prophylaxis in tropical Africa. *Bull. World Health Organ.* 62, 95–98.

Gao, W., Weng, J., Gao, Y., and Chen, X. (2013). Comparison of the vaginal microbiota diversity of women with and without human papillomavirus infection: a cross-sectional study. *BMC Infect. Dis.* 13, 271.

Gardiner, G.E., Heinemann, C., Bruce, A.W., Beuerman, D., and Reid, G. (2002). Persistence of *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 but Not *L. rhamnosus* GG in the human vagina as demonstrated by randomly amplified polymorphic DNA. *Clin. Diagn. Lab. Immunol.* 9, 92–96.

Graver, M.A., and Wade, J.J. (2011). The role of acidification in the inhibition of *Neisseria gonorrhoeae* by vaginal *Lactobacilli* during anaerobic growth. *Ann. Clin. Microbiol. Antimicrob.* 10, 8.

Grodstein, F., Goldman, M.B., and Cramer, D.W. (1993). Relation of tubal infertility to history of sexually transmitted diseases. *Am. J. Epidemiol.* 137, 577–584.

Gudina, E., Rocha, V., Teixeira, J.A., and Rodrigues, L. (2010). Antimicrobial and antiadhesive properties of a biosurfactant isolated from *Lactobacillus paracasei* ssp. *paracasei* A20. *Applied Microbiol. I* 50, 419–424.

Gupta, K., Stapleton, A.E., Hooton, T.M., Roberts, P.L., Fennell, C.L., and Stamm, W.E. (1998). Inverse association of H<sub>2</sub>O<sub>2</sub>-producing *Lactobacilli* and vaginal *Escherichia coli* colonization in women with recurrent urinary tract infections. *J. Infect. Dis.* 178, 446–450.

Heinonen, P.K., Teisala, K., Punnonen, R., Miettinen, A., Lehtinen, M., and Paavonen, J. (1985). Anatomic sites of upper genital tract infection. *Obstet. Gynecol.* 66, 384–390.

Hemmerling, A., Harrison, W., Schroeder, A., Park, J., Korn, A., Shiboski, S., and Cohen, C.R. (2009). Phase 1 dose-ranging safety trial of *Lactobacillus crispatus* CTV-05 for the prevention of bacterial vaginosis. *Sex. Transm. Dis.* 36, 564–569.

Hemmerling, A., Harrison, W., Schroeder, A., Park, J., Korn, A., Shiboski, S., Foster-Rosales, A., and Cohen, C.R. (2010). Phase 2a study assessing colonization efficiency, safety, and acceptability of *Lactobacillus crispatus* CTV-05 in women with bacterial vaginosis. *Sex. Transm. Dis.* 37, 745–750.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514.

Hill, D.R., Brunner, M.E., Schmitz, D.C., Davis, C.C., Flood, J.A., Schlievert, P.M., Wang-Weigand, S.Z., and Osborn, T.W. (2005). In vivo assessment of human vaginal oxygen and carbon dioxide levels during and post menses. *J. Appl. Physiol. Bethesda Md* 99, 1582–1591.

Hillier, S.L., Krohn, M.A., Klebanoff, S.J., and Eschenbach, D.A. (1992). The Relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women: *Obstet. Gynecol.* 79, 369–373.

Hilton, E., Rindos, P., and Isenberg, H.D. (1995). *Lactobacillus* GG vaginal suppositories and vaginitis. *J. Clin. Microbiol.* 33, 1433.

Hummel, S., Veltman, K., Cichon, C., Sonnenborn, U., and Schmidt, M.A. (2012). Differential targeting of the E-Cadherin/ $\beta$ -Catenin complex by Gram-positive probiotic *Lactobacilli* improves epithelial barrier function. *Appl. Environ. Microbiol.* 78, 1140–1147.

Hummelen, R., Changalucha, J., Butamanya, N.L., Cook, A., Habbema, J.D.F., and Reid, G. (2010). *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 to prevent or cure bacterial vaginosis among women with HIV. *Int. J. Gynaecol. Obstet. Off. Organ Int. Fed. Gynaecol. Obstet.* 111, 245–248.

Hurst, B., Jones, A., Elliot, M., Marshburn, P., and Matthews, M. (2008). Absorption of vaginal estrogen cream during sexual intercourse: a prospective, randomized, controlled trial. *J. Reprod. Med.* 53, 29–32.

Husmark, U., and Gustafson, I. (2007). Hygiene product comprising probiotic composition.

Hussain, A., and Ahsan, F. (2005). The Vagina as a route for the systemic drug delivery. *J. Controlled Release* 103, 301–313.

Hütt, P., Lapp, E., Štšepetova, J., Smidt, I., Taelma, H., Borovkova, N., Oopkaup, H., Ahelik, A., Rööp, T., Hoidmets, D., et al. (2016). Characterisation of probiotic properties in human vaginal lactobacilli strains. *Microb. Ecol. Health Dis.* 27.

Ibnou-Zekri, N., Blum, S., Schiffrin, E.J., and von der Weid, T. (2003). Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties in vitro. *Infect. Immun.* 71, 428–436.

Imani Fooladi, A.A., Yazdi, M.H., Pourmand, M.R., Mirshafiey, A., Hassan, Z.M., Azizi, T., Mahdavi, M., and Soltan Dallal, M.M. (2015). Th1 Cytokine production induced by *Lactobacillus acidophilus* in BALB/c Mice bearing transplanted breast tumor. *Jundishapur J. Microbiol.* 8.

Iwabuchi, N., Yonezawa, S., Odamaki, T., Yaeshima, T., Iwatsuki, K., and Xiao, J.-Z. (2012). Immunomodulating and anti-infective effects of a novel strain of *Lactobacillus paracasei* that strongly induces interleukin-12. *FEMS Immunol. Med. Microbiol.* 66, 230–239.

Jacob, H.E. (1970). Redox potential. *Methods Microbiol* 2, 91–123.

Jarvis, G.A., and Chang, T.L. (2012). Modulation of HIV transmission by *Neisseria gonorrhoeae*: molecular and immunological aspects. *Curr. HIV Res.* 10, 211–217.

Junginger, H., Thanou, M., and Verhoef, J.. (2002). Mucoadhesive hydrogels in drug delivery. *Encycl. Pharm. Technol.* 1848–1863.

Justin-Temu, M., Damian, F., Kinget, R., and Van Den Mooter, G. (2004). Intravaginal gels as drug delivery systems. *J. Womens Health* 2002 13, 834–844.

Khanvilkar, K., Donovan, M.D., and Flanagan, D.R. (2001). Drug transfer through mucus. *Adv. Drug Deliv. Rev.* 48, 173–193.

Kim, Y., Kim, S.-H., Whang, K.-Y., Kim, Y.-J., and Oh, S. (2008). Inhibition of *Escherichia coli* O157:H7 attachment by interactions between lactic acid bacteria and intestinal epithelial cells. *J. Microbiol. Biotechnol.* 18, 1278–1285.

Kjos, M., Borrero, J., Opsata, M., Birri, D.J., Holo, H., Cintas, L.M., Snipen, L., Hernandez, P.E., Nes, I.F., and Diep, D.B. (2011). Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria. *Microbiology* 157, 3256–3267.

Klebanoff, S., Hillier, S., Eschenbach, D., and Waltersdorph, A. (1991). Control of the microbial flora of the vagina by H<sub>2</sub>O<sub>2</sub>-generating lactobacilli. *J Infect Dis* 164, 94–100.

Kontiokari, T., Sundqvist, K., Nuutinen, M., Pokka, T., Koskela, M., and Uhari, M. (2001). Randomised trial of cranberry-lingonberry juice and *Lactobacillus* GG drink for the prevention of urinary tract infections in women. *BMJ* 322, 1571.

Krauer, B., and Krauer, F. (1977). Drug kinetics in pregnancy. *Clin. Pharmacokinet.* 2, 167–181.

Kumar, V., Abbas, A., and Fausto, N. (2005). Robbins and Cotran pathologic basis of disease (Philadelphia: Saunders).

- Kwok, L., Stapleton, A.E., Stamm, W.E., Hillier, S.L., Wobbe, C.L., and Gupta, K. (2006). Adherence of *Lactobacillus crispatus* to vaginal epithelial cells from women with or without a history of recurrent urinary tract infection. *J. Urol.* *176*, 2050–2054.
- Laga, M., Plummer, F.A., Nzanze, H., Namaara, W., Brunham, R.C., Ndinya-Achola, J.O., Maitha, G., Ronald, A.R., D'Costa, L.J., and Bhullar, V.B. (1986). Epidemiology of ophthalmia neonatorum in Kenya. *Lancet Lond. Engl.* *2*, 1145–1149.
- Larsen, N., Nissen, P., and Willats, W.G.T. (2007). The effect of calcium ions on adhesion and competitive exclusion of *Lactobacillus ssp.* and *E. Coli* O138. *Int J Food Microbiol* *114*, 113–119.
- Lee, J.E., Lee, S., Lee, H., Song, Y.-M., Lee, K., Han, M.J., Sung, J., and Ko, G. (2013). Association of the Vaginal Microbiota with Human Papillomavirus Infection in a Korean Twin Cohort. *PLOS ONE* *8*, e63514.
- Lehrer, R.I., and Ganz, T. (2002). Defensins of vertebrate animals. *Curr Opin Immunol* *14*, 96–102.
- Lepargneur, J.P., and Rousseau, V. (2002). [Protective role of the Doderlein flora]. *J. Gynecol. Obstet. Biol. Reprod. (Paris)* *31*, 485–494.
- Ling, Z., Liu, X., Chen, X., Zhu, H., Nelson, K., Xia, Y., al, and Microb Eco (2011). Diversity of cervicovaginal microbiota associated with female lower genital tract infections. *61*, 704–714.
- Loebstein, R., Lalkin, A., and Koren, G. (1997). Pharmacokinetic changes during pregnancy and their clinical relevance. *Clin. Pharmacokinet.* *33*, 328–343.
- Lucas, C.T., Chandler, F., Martin, J.E., and Schmale, J.D. (1971). Transfer of gonococcal urethritis from man to chimpanzee: An animal model for gonorrhea. *JAMA* *216*, 1612–1614.
- Mack, D.R., Ahrne, S., Hyde, L., Wei, S., and Hollingsworth, M.A. (2003). Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells in vitro. *Gut* *52*, 827–833.
- Maggi, L., Mastromarino, P., Macchia, S., Brigidi, P., Pirovano, F., Matteuzzi, D., and Conte, U. (2000). Technological and biological evaluation of tablets containing different strains of *Lactobacilli* for vaginal administration. *Eur. J. Pharm. Biopharm.* *50*, 389±395.
- Malcolm, R.K., Edwards, K.-L., Kiser, P., Romano, J., and Smith, T.J. (2010). Advances in microbicide vaginal rings. *Antiviral Res.* *88*, S30–S39.
- Marcone, V., Rocca, G., Lichtner, M., and Calzolari, E. (2010). Long-term vaginal administration of *Lactobacillus rhamnosus* as a complementary approach to management of bacterial vaginosis. *Int. J. Gynecol. Obstet.* *110*, 223–226.
- Marrazzo, J.M., Cook, R.L., Wiesenfeld, H.C., Murray, P.J., Busse, B., Krohn, M., and Hillier, S.L. (2006). Women's satisfaction with an intravaginal *Lactobacillus* capsule for the treatment of bacterial vaginosis. *J. Womens Health* *2002* *15*, 1053–1060.
- Martín, R., and Suárez, J.E. (2010). Biosynthesis and degradation of H<sub>2</sub>O<sub>2</sub> by vaginal lactobacilli. *Appl. Environ. Microbiol.* *76*, 400–405.
- Martin, H.L., Richardson, B.A., Nyange, P.M., Lavreys, L., Hillier, S.L., Chohan, B., Mandaliya, K., Ndinya-Achola, J.O., Bwayo, J., and Kreiss, J. (1999). Vaginal *Lactobacilli*, Microbial Flora, and Risk of Human Immunodeficiency Virus Type 1 and Sexually Transmitted Disease Acquisition. *J. Infect. Dis.* *180*, 1863–1868.

Mastromarino, P., Macchia, S., Meggiorini, L., Trinchieri, V., Mosca, L., Perluigi, M., and Midulla, C. (2009). Effectiveness of Lactobacillus-containing vaginal tablets in the treatment of symptomatic bacterial vaginosis. *Clin. Microbiol. Infect.* *15*, 67–74.

Mattar, A.F., Teitelbaum, D.H., Drongowski, R.A., Yongyi, F., Harmon, C.M., and Coran, A.G. (2002). Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. *Pediatr. Surg. Int.* *18*, 586–590.

Merriam, K.S., Leake, K.A., Elliot, M., Matthews, M.L., Usadi, R.S., and Hurst, B.S. (2015). Sexual absorption of vaginal progesterone: A randomized control trial. *Int. J. Endocrinol.* *2015*, e685281.

Mezzasalma, V., Manfrini, E., Ferri, E., Boccarusso, M., Di Gennaro, P., Schiano, I., Michelotti, A., and Labra, M. (2017). Orally administered multispecies probiotic formulations to prevent uro-genital infections: a randomized placebo-controlled pilot study. *Arch. Gynecol. Obstet.* *295*, 163–172.

Mijac, V., Dukić, S., Opavski, N., Dukić, M., and Ranin, L. (2006). Hydrogen peroxide producing lactobacilli in women with vaginal infections. *Eur. J. Obstet. Gynecol. Reprod. Biol.* *129*, 69–76.

Mitchell, C., Balkus, J.E., Fredricks, D., Liu, C., McKernan-Mullin, J., Frenkel, L.M., Mwachari, C., Luque, A., Cohn, S.E., Cohen, C.R., et al. (2013). Interaction between lactobacilli, bacterial vaginosis-associated bacteria, and HIV Type 1 RNA and DNA genital shedding in U.S. and Kenyan Women. *AIDS Res. Hum. Retroviruses* *29*, 13–19.

Morelli, L., Zonenenschain, D., Del Piano, M., and Cognein, P. (2004). Utilization of the intestinal tract as a delivery system. *Journal of Clinical Gastroenterology. J. Clin. Gastroenterol.* *38*, 107–110.

Mottet, C., and Michetti, P. (2005). Probiotics: Wanted Dead or Alive. *Dig Liver Dis* *37*, 3–6.

Muzio, M., Natoli, G., Saccani, S., Levrero, M., and Mantovani, A. (1998). The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J. Exp. Med.* *187*, 2097–2101.

Nardini, P., Ñahui Palomino, R.A., Parolin, C., Laghi, L., Foschi, C., Cevenini, R., Vitali, B., and Marangoni, A. (2016). Lactobacillus crispatus inhibits the infectivity of Chlamydia trachomatis elementary bodies, in vitro study. *Sci. Rep.* *6*, 29024.

das Neves, J., and Bahia, M.F. (2006). Gels as vaginal drug delivery systems. *Int. J. Pharm.* *318*, 1–14.

Ocaña, V.S., Holgado, A.A.P. de R., and Nader-Macías, M.E. (1999). Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain. *Appl. Environ. Microbiol.* *65*, 5631–5635.

O’Hanlon, D.E., Lanier, B.R., Moench, T.R., and Cone, R.A. (2010). Cervicovaginal fluid and semen block the microbicidal activity of hydrogen peroxide produced by vaginal lactobacilli. *BMC Infect. Dis.* *10*, 120.

O’Hanlon, D.E., Moench, T.R., and Cone, R.A. (2011). In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. *BMC Infect. Dis.* *11*, 200.

ONUSIDA (2018). Résumé de l'étude de facteurs de vulnérabilité des filles et des femmes face aux IST/SIDA dans la région Atsinanana (ONUSIDA, SECNLS - 2011).

Oppenheim, J., Biragyn, A., Kwak, L., and Yang, D. (2003). Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *Ann. Rheum. Dis.* 62, ii17–ii21.

Ouwehand, A.C., Tolkkio, S., Kulmala, J., Salminen, S., and Salminen, E. (2000). Adhesion of inactivated probiotic strains to intestinal mucus. *Appl. Microbiol.* 31, 82 ~ 86.

Owen, D.H., and Katz, D.F. (1999). A vaginal fluid simulant. *Contraception* 59, 91–95.

Owen, D.H., Peters, J.J., and Katz, D.F. (2000). Rheological properties of contraceptive gels. *Contraception* 62, 321–326.

Ozkinay, E., Terek, M.C., Yayci, M., Kaiser, R., Grob, P., and Tuncay, G. (2005). The effectiveness of live lactobacilli in combination with low dose oestriol (Gynoflor) to restore the vaginal flora after treatment of vaginal infections. *BJOG Int. J. Obstet. Gynaecol.* 112, 234–240.

Paavonen, J. (1983). Physiology and ecology of the vagina. *Scand J Infect Suppl* 40, 31–35.

Parassol, N., Freitas, M., Thoreux, K., Dalmasso, G., Bourdet-Sicard, R., and Rampal, P. (2005). *Lactobacillus casei* DN-114001 inhibits the increase in paracellular permeability of enteropathogenic *Escherichia coli*-infected T84 cells. *Res Microbiol* 156, 256–262.

Parent, D., Bossens, M., Bayot, D., Kirkpatrick, C., Graf, F., Wilkinson, F.E., and Kaiser, R.R. (1996). Therapy of bacterial vaginosis using exogenously-applied *Lactobacilli acidophili* and a low dose of estriol: a placebo-controlled multicentric clinical trial. *Arzneimittelforschung.* 46, 68–73.

Pascual, L.M., Daniele, M.B., Ruiz, F., Giordano, W., Pájaro, C., and Barberis, L. (2008). *Lactobacillus rhamnosus* L60, a potential probiotic isolated from the human vagina. *J. Gen. Appl. Microbiol.* 54, 141–148.

Pavlova, S.I., Kilic, A.O., Kilic, S.S., So, J.-S., Nader-Macias, M.E., Simoes, J.A., and Tao, L. (2002). Genetic diversity of vaginal *Lactobacilli* from women in different countries based on 16S rRNA gene sequences. *J. Appl. Microbiol.* 92, 451–459.

Pearson, R., Ferri, F., Jones, R., and Opal, S. (2004). Gonorrhoea.

Pilcher, H. (2004). Starting to gel. *Nature* 430, 138–140.

Rammelsberg, M., and Radler, F. (1990). Antibacterial polypeptides of *Lactobacillus* species. *J. Appl. Bacteriol.* 69, 177–184.

Rampersaud, R., Randis, T.M., and Ratner, A.J. (2012). Microbiota of the upper and lower genital tract. *Semin. Fetal. Neonatal Med.* 17, 51–57.

Ravel, J., and Brotman, R.M. (2016). Translating the vaginal microbiome: gaps and challenges. *Genome Med.* 8, 35.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., Stacey, L.M., Karlebach, shara, Gorle, R., Russell, J., Carol, O.T., et al. (2011). Vaginal microbiome. *Proc Natl Acad Sci U S A* 108, 4680–4687.

Reid, G. (1999). The scientific basis for probiotic strains of *Lactobacillus*. *Appl. Environ. Microbiol.* 65, 3763–3766.

Reid, G. (2001). Probiotic agents to protect the urogenital tract against infection. *Am. J. Clin. Nutr.* 73, 437s-443s.

Reid, G. (2002). Probiotics for urogenital health. *Nutr. Clin. Care Off. Publ. Tufts Univ.* 5, 3–8.

Reid, G., and Bruce, A.W. (2001). Selection of *Lactobacillus* strains for urogenital probiotic applications. *J. Infect. Dis.* 183, S77–S80.

Reid, G., and Burton, J. (2002). Use of *Lactobacillus* to prevent infection by pathogenic bacteria. *Microbes Infect.* 4, 319–324.

Reid, G., Cook, R.L., and Bruce, A.W. (1987). Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. *J. Urol.* 138, 330–335.

Reid, G., Millsap, K., and Bruce, A.W. (1994). Implantation of *Lactobacillus casei* var *rhamnosus* into vagina. *Lancet* 344 1229.

Reid, G., Bruce, A., and Taylor, M. (1995). Instillation of *Lactobacillus* and stimulation of indigenous organisms to prevent recurrence of urinary tract infections. *Microecol* 23, 32–45.

Reid, G., Smeianov, V., and Bruce, A.W. (1999). Biotherapeutic agents to prevent cystitis in women. *GWELmer LMcFarland CSurawicz Ed Biotherapeutic Agents Infect. Dis. Humana Press Inc Totowa NJ* - 245–261.

Reid, G., Beuerman, D., Heinemann, C., and Bruce, A.W. (2001). Probiotic *Lactobacillus* dose required to restore and maintain a normal vaginal flora. *FEMS Immunol. Med. Microbiol.* 32, 37–41.

Reid, G., Charbonneau, D., Erb, J., Kochanowski, B., Beuerman, D., Poehner, R., and Bruce, A.W. (2003). Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunol. Med. Microbiol.* 35, 131–134.

Reid, G., Burton, J., and Devillard, E. (2004). The rationale for probiotics in female urogenital healthcare. *MedGenMed Medscape Gen. Med.* 6, 49.

Richardson, J., and Illum, L. (1992). The vaginal route of peptide and protein drug delivery. *Adv Drug Deliv Rev* 8, 341–366.

Rodrigues, L.R., and Teixeira, J.A. (2010). Biomedical and therapeutic applications of biosurfactants. *Adv Exp Med Biol* 672, 75–87.

Rohan, L.C., and Sassi, A.B. (2009). Vaginal drug delivery systems for HIV prevention. *AAPS J.* 11, 78–87.

Rompalo, A. (2004). Gonorrhea. In: Rake RE, Bope ET (Conn's current therapy. Philadelphia: Saunders).

Rose, W.A.I., McGowin, C.L., Spagnuolo, R.A., Eaves-Pyles, T.D., Popov, V.L., and Pyles, R.B. (2012). Commensal bacteria modulate innate immune responses of vaginal epithelial cell multilayer cultures. *PLOS ONE* 7, e32728.

Rossi, A., Rossi, T., Bertini, M., and Caccia, G. (2010). The use of *Lactobacillus rhamnosus* in the therapy of bacterial vaginosis. Evaluation of clinical efficacy in a population of 40 women treated for 24 months. *Arch. Gynecol. Obstet.* 281, 1065–1069.

Roy, S., Gourde, P., Piret, J., Désormeaux, A., Lamontagne, J., Haineault, C., Omar, R.F., and Bergeron, M.G. (2001). Thermoreversible gel formulations containing sodium lauryl



Sulfate or n-Lauroylsarcosine as Potential Topical Microbicides against Sexually Transmitted Diseases. *Antimicrob. Agents Chemother.* 45, 1671–1681.

Ruel-Gariépy, E., and Leroux, J.-C. (2004). In situ-forming hydrogels—review of temperature-sensitive systems. *Eur. J. Pharm. Biopharm.* 58, 409–426.

Sachdeva, P., Patel, B.G., and Patel, B.K. (2009). Drug Use in Pregnancy; a Point to Ponder! *Indian J. Pharm. Sci.* 71, 1–7.

Saigh, J.H., Sanders, C.C., and Sanders, W.E. (1978). Inhibition of *Neisseria gonorrhoeae* by aerobic and facultatively anaerobic components of the endocervical flora: evidence for a protective effect against infection. *Infect. Immun.* 19, 704–710.

Salas-Jara, M.J., Ilabaca, A., Vega, M., and García, A. (2016). Biofilm forming *Lactobacillus*: New challenges for the development of probiotics. *Microorganisms* 4.

Salminen, S., Isolauri, E., and Salminen, E. (1996). Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. *Antonie Van Leeuwenhoek* 70, 347–358.

Sanders, M.E. (2008). Probiotics: definition, sources, selection, and uses. *Clin. Infect. Dis.* 46 Suppl 2, S58-61; discussion S144-151.

Seib, K.L., Wu, H.-J., Kidd, S.P., Apicella, M.A., Jennings, M.P., and McEwan, A.G. (2006). Defenses against oxidative stress in *Neisseria gonorrhoeae*: a system tailored for a challenging environment. *Microbiol. Mol. Biol. Rev.* 70, 344–361.

Silva, M., Jacobus, N.V., Deneke, C., and Gorbach, S.L. (1987). Antimicrobial substance from a human *Lactobacillus* strain. *Antimicrob. Agents Chemother.* 31, 1231–1233.

Sjöberg, I., Cajander, S., and Rylander, E. (1988). Morphometric characteristics of the vaginal epithelium during the menstrual cycle. *Gynecol. Obstet. Invest.* 26, 136–144.

Sobel, J.D. (1999). Biotherapeutic agents as therapy for vaginitis. GW Elmer McFarland CSurawicz Ed Biotherapeutic Agents *Infect. Dis. Humana Press Inc Totowa* 221–244.

Soltan-Dallal, M., Yazdi, M., Holakuyee, M., Hassan, Z.M., Abolhassani, M., and Mahdavi, M. (2012). *Lactobacillus casei ssp. casei* induced Th1 cytokine profile and natural killer cells activity in invasive ductal carcinoma bearing mice. *Iran. J. Allergy Asthma Immunol.* 11, 183–189.

Song, Y., Wang, Y., Thakur, R., Meidan, V., and Michniak, M.B. (2004). Mucosal drug delivery: membranes, methodologies and applications. *Crit Rev Ther Drug Carr. Syst* 21, 195–256.

Spurbeck, R.R., and Arvidson, C.G. (2008). Inhibition of *Neisseria gonorrhoeae* epithelial cell interactions by vaginal *Lactobacillus* Species. *Infect. Immun.* 76, 3124–3130.

Spurbeck, R.R., and Arvidson, C.G. (2010). *Lactobacillus jensenii* surface-associated proteins inhibit *Neisseria gonorrhoeae* adherence to epithelial cells. *Infect. Immun.* 78, 3103–3111.

Stapleton, A.E., Au-Yeung, M., Hooton, T.M., Fredricks, D.N., Roberts, P.L., Czaja, C.A., Yarova-Yarovaya, Y., Fiedler, T., Cox, M., and Stamm, W.E. (2011). Randomized, placebo-controlled phase 2 trial of a *Lactobacillus crispatus* probiotic given intravaginally for prevention of recurrent urinary tract infection. *Clin. Infect. Dis.* 52, 1212–1217.

Strus, M., Brzychczy-Włoch, M., Gosiewski, T., Kochan, P., and Heczko, P.B. (2006). The in vitro effect of hydrogen peroxide on vaginal microbial communities. *FEMS Immunol. Med. Microbiol.* 48, 56–63.

Tagg, J.R., Dajani, A.S., and Wannamaker, L.W. (1976). Bacteriocins of Gram-positive bacteria. *Bacteriol. Rev.* 40, 722–756.

Thoma-Uszynski, S., Stenger, S., Takeuchi, O., Ochoa, M.T., Engele, M., Sieling, P.A., Barnes, P.F., Rollinghoff, M., Bolcskei, P.L., Wagner, M., et al. (2001). Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science* 291, 1544–1547.

Uehara, S., Monden, K., Nomoto, K., Seno, Y., Kariyama, R., and Kumon, H. (2006). A pilot study evaluating the safety and effectiveness of *Lactobacillus* vaginal suppositories in patients with recurrent urinary tract infection. *Int. J. Antimicrob. Agents* 28 Suppl 1, S30-34.

Valenta, C. (2005). The use of mucoadhesive polymers in vaginal delivery. *Adv. Drug Deliv. Rev.* 57, 1692–1712.

Vallor, A.C., Antonio, M.A.D., Hawes, S.E., and Hillier, S.L. (2001). Factors Associated with Acquisition of, or Persistent Colonization by, Vaginal *Lactobacilli*: Role of hydrogen peroxide production. *J. Infect. Dis.* 184, 1431–1436.

Van De Graaff, K. (2001). *Human Anatomy*, 6th ed. McGraw-Hill Educ.

Vasquez, A., Jakobsson, T., Ahrne, S., Forsum, U., and Molin, G. (2002). Vaginal *Lactobacillus* flora of healthy Swedish women. *J Clin Microbiol* 40, 2746–2749.

Velraeds, M.M., van der Mei, H.C., Reid, G., and Busscher, H.J. (1996). Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. *Appl. Environ. Microbiol.* 62, 1958–1963.

Ventolini, G. (2015). Vaginal *Lactobacillus*: biofilm formation, *in vivo* – clinical implications. *Int. J. Womens Health* 7, 243–247.

Vermani, null, and Garg, null (2000). The scope and potential of vaginal drug delivery. *Pharm. Sci. Technol. Today* 3, 359–364.

Verstraelen, H., Verhelst, R., Claeys, G., De Backer, E., Temmerman, M., and Vanechoutte, M. (2009). Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol* 9, 116.

Vielfort, K., Sjölander, H., Roos, S., Jonsson, H., and Aro, H. (2008). Adherence of clinically isolated lactobacilli to human cervical cells in competition with *Neisseria gonorrhoeae*. *Microbes Infect.* 10, 1325–1334.

Wagner, G., and Levin, R.J. (1978). Vaginal fluid. In E. S. E. Hafez and T. N. Evans (Eds.), *Human Reproductive Medicine: The Human Vagina*, North Holland Publishing, pp. 121–137.

Wagner, R.D., and Johnson, S.J. (2012). Probiotic lactobacillus and estrogen effects on vaginal epithelial gene expression responses to *Candida albicans*. *J. Biomed. Sci.* 19, 58.

Wasserheit, J.N. (1992). Epidemiological synergy. Interrelationships between human immunodeficiency virus infection and other sexually transmitted diseases. *Sex. Transm. Dis.* 19, 61–77.

Weber, J., Desai, K., and Darbyshire, J. (2005). The Development of vaginal microbicides for the prevention of HIV transmission. *PLoS Med.* 2.

WHO (2016). WHO | WHO guidelines for the treatment of *Neisseria gonorrhoeae*.

Wiesenfeld, H.C., Hillier, S.L., Krohn, M.A., Landers, D.V., and Sweet, R.L. (2003). Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 36, 663–668.

Wijgert, J.H.H.M. van de, Borgdorff, H., Verhelst, R., Crucitti, T., Francis, S., Verstraelen, H., and Jespers, V. (2014). The vaginal microbiota: what have we learned after a decade of molecular characterization? *PLOS ONE* 9, e105998.

Witkin, S.S., Linhares, I.M., and Giraldo, P. (2007). Bacterial flora of the female genital tract: function and immune regulation. *Best Pract. Res. Clin. Obstet. Gynaecol.* 21, 347–354.

Woodley, J. (2001). Bioadhesion: new possibilities for drug administration? *Clin. Pharmacokinet.* 40, 77–84.

Yan, F., Cao, H., Cover, T.L., Whitehead, R., Washington, M.K., and Polk, D.B. (2007). Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology* 132, 562–575.

Yang, D., Chen, Q., Chertov, O., and Oppenheim, J.J. (2000). Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. *J. Leukoc. Biol.* 68, 9–14.

Yang, D., Biragyn, A., Kwak, L.W., and Oppenheim, J.J. (2002). Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol.* 23, 291–296.

Zárate, G., and Nader-Macias, M.E. (2006). Influence of probiotic vaginal *Lactobacilli* on in vitro adhesion of urogenital pathogens to vaginal epithelial cells. *Lett. Appl. Microbiol.* 43, 174–180.

Zárate, G., and Nader-Macías, M.E.F. (2006). Viability and biological properties of probiotic vaginal *Lactobacilli* after lyophilization and refrigerated storage into gelatin capsules. *Process Biochem.* 41, 1779–1785.

Zheng, H.Y., Alcorn, T.M., and Cohen, M.S. (1994). Effects of H<sub>2</sub>O<sub>2</sub>-producing lactobacilli on *Neisseria gonorrhoeae* growth and catalase activity. *J. Infect. Dis.* 170, 1209–1215.

Zhou, X., Brown, C.J., Abdo, Z., Davis, C.C., Hansmann, M.A., Joyce, P., Foster, J.A., and Forney, L.J. (2007). Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME J.* 1, 121–133.

Zhou, X., Hansmann, M.A., Davis, C.C., Suzuki, H., Brown, C.J., Schütte, U., Pierson, J.D., and Forney, L.J. (2010). The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. *FEMS Immunol. Med. Microbiol.* 58, 169–181.

**CHAPITRE III : INTERET D'UN HYDROGEL CONTENANT  
*LACTOBACILLUS CRISPATUS* POUR LA PREVENTION DE  
LA GONOCOCCIE**



## 1. INTRODUCTION

Les hydrogels ont fait l'objet de plusieurs études pour la prévention et/ou le traitement des infections de la voie vaginale, surtout des infections sexuellement transmissibles. Face aux difficultés de traitement et de prévention de la gonococcie, dues respectivement au développement des résistances et à la baisse de l'utilisation du préservatif, notre équipe propose une stratégie basée sur des hydrogels probiotiques à base de *Lactobacillus crispatus*, de poloxamer 407 et d'alginate de sodium. Cette chapitre vise à faire une synthèse bibliographique permettant de justifier le choix de la forme hydrogel, des polymères et de *Lactobacillus crispatus* pour la prévention de la gonococcie.

## 2. CHOIX DE LA VOIE VAGINALE

Les infections sexuellement transmissibles continuent de nos jours à se propager. Dans de nombreux cas, elles ne sont pas diagnostiquées et conduisent éventuellement à des complications à long terme à l'instar de la stérilité (Chan, 2005). Les jeunes femmes âgées de 15 à 24 ans sont plus susceptibles d'être infectées par les IST que les jeunes hommes (UNFPA et al., 2015). Des études ont démontré que les préservatifs lorsqu'ils sont utilisés de manière correcte et systématique sont efficaces contre l'acquisition et la transmission d'IST (Matson et al., 2011; Sarkar, 2008). Malheureusement, dans de nombreuses sociétés, les femmes ont souvent peu ou pas la possibilité de négocier leur utilisation avec leurs partenaires. Les autres principaux obstacles à l'utilisation du préservatif sont : la peur que le/la partenaire soit méfiant(e) vis-à-vis de soi, la consommation d'alcool et la réduction du plaisir sexuel (Donta et al., 2014; Macharia et al., 2017; Mahlalela and Maharaj, 2015; Sahay et al., 2015; Sarkar, 2008; Tafuri et al., 2010). En conséquence, la stratégie basée sur l'utilisation constante du préservatif n'a pas atteint un niveau suffisamment élevé d'efficacité, même après de nombreuses années de promotion généralisée et intensive (Potts et al., 2008). Cela explique l'élaboration, la mise en œuvre et l'émergence ces dernières années de nouvelles stratégies préventives contrôlées par les femmes elles-mêmes, sûres, efficaces et acceptables. Ainsi, dans le cadre de nos travaux qui s'intéressent plus particulièrement à la gonococcie, notre stratégie est de proposer des solutions qui soient adaptées à la femme, pour une prise en charge efficiente de cette IST. L'analyse des symptômes de la gonococcie, de son mécanisme de transmission et de sa pathogénicité, réalisée dans le **chapitre I** a permis de mettre en évidence que cette infection se manifeste le plus souvent sous forme de brûlure mictionnelle

et d'écoulement purulent chez l'homme, tandis qu'elle est le plus souvent asymptomatique chez la femme. Ainsi, même si la femme ne présente aucun signe d'infection, elle peut tout de même la transmettre à son partenaire et aussi au nouveau-né. Toutes ces raisons font de la voie vaginale la principale voie sexuelle de transmission de la gonococcie. Il semble donc pertinent d'orienter notre stratégie vers la voie vaginale. L'étude du mécanisme de pathogénicité (**chapitre I**) nous a permis de comprendre le niveau de ce mécanisme sur lequel il serait intéressant d'agir pour prévenir l'infection. En effet, l'adhérence du gonocoque aux cellules épithéliales est la première étape déterminante et la seule étape qui se déroule dans la lumière vaginale. Pour la prévention de la transmission du gonocoque, notre proposition est de développer un système capable d'empêcher son adhérence aux cellules épithéliales. La voie vaginale est déjà une voie d'administration utilisée pour les antiseptiques, les antibiotiques, les antifongiques, les antiparasitaires, les hormones et les contraceptifs (Vidal, 2017). C'est la voie idéale pour une action locale au niveau du vagin. Les formes pharmaceutiques adaptées à cette voie sont nombreuses : crèmes vaginales, gels vaginaux, douches vaginales, capsules vaginales, ovules, comprimés vaginaux (Vidal, 2017).

### **3. INTERET DE *LACTOBACILLUS CRISPATUS* POUR LA PREVENTION DE LA GONOCOCCIE**

La microflore vaginale est dominée par des espèces de *Lactobacillus*. Plusieurs espèces de *Lactobacillus* telles que *L. iners*, *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. casei*, *L. vaginalis*, *L. delbrueckii*, *L. salivarius*, *L. reuteri* et *L. rhamnosus* ont été décrites pour coloniser le vagin à divers degrés (Cribby et al., 2009). D'autres espèces bactériennes telles que *Gardnerella vaginalis*, *Peptostreptococcus spp.*, *Prevotella spp.*, *Escherichia coli*, *Streptococcus agalactiae* et *Enterococcus faecalis* sont présentes en quantités limitées dans un vagin sain. La composition bactérienne vaginale varie d'une femme à une autre (Vasquez et al., 2002) et d'une région à une autre. L'utilisation d'antibiotiques, l'activité sexuelle, la menstruation, la grossesse, les douches vaginales, les hormones et toutes les méthodes de contraception sont susceptibles de perturber le microbiote vaginal (Farage et al., 2010; Witkin et al., 2007). Malheureusement, il existe une corrélation entre la perte et la perturbation des lactobacilles vaginaux et le risque élevé d'infections génitales, y compris les IST telles que le virus de l'immuno-déficience humaine (VIH) (Dols et al., 2012; Frank et al., 2012; Hummelen et al., 2010; Martin et al., 1999; Mitchell et al., 2009, 2013; Schellenberg et al., 2011; Sewankambo, et al., 1997; Spear et al., 2011, 2008), le

virus du papillome humain (Dols et al., 2012; Gao et al., 2013; Lee et al., 2013; Mitchell et al., 2012; Smith et al., 2012), le virus de l'herpès simplex de type 2 HSV-2 (Cherpes et al., 2003), *Trichomonas vaginalis* (Brotman et al., 2012), *Neisseria gonorrhoeae* et *Chlamydia trachomatis* (Wiesenfeld et al., 2003).

*L. crispatus* est une espèce de *Lactobacillus* naturellement présente dans le vagin, qui favorise la stabilité de la microflore vaginale normale. L'étude par vidéomicroscopie de la composition bactérienne de mucus, fraîchement prélevés chez des femmes, a montré que le piégeage des cellules virales était efficace dans le cas où le mucus était dominé par *L. crispatus* tandis que cela n'était pas le cas quand le mucus était dominé par *L. iners* ou *Gardnerella vaginalis* (Nunn et al., 2015). Ceci montre l'importance de la présence de *L. crispatus*.

*L. crispatus* inhibe plusieurs bactéries, levures et virus dont *Neisseria gonorrhoeae* (Amant et al., 2002; Breshears et al., 2015), *Chlamydia trachomatis* (Nardini et al., 2016), et *Escherichia coli*, *Candida albicans*, *Candida Glabrata* et *Gardnerella vaginalis* (Hütt et al., 2016). *L. crispatus* est spécifique au vagin humain (Pavlova et al., 2002; Ravel et al., 2011; Zhou et al., 2007, 2010). Il peut produire d'importantes quantités d'acide lactique et confère au vagin un pH de 3,5-4 qui est protecteur vis-à-vis du gonocoque et d'autres agents responsables d'une IST (Ravel and Brotman, 2016). Il est aussi capable de réduire l'adhérence du gonocoque aux cellules cervicales épithéliales humaines (Vielfort et al., 2008). Certains probiotiques à base de *L. crispatus* sont déjà commercialisés pour la prévention et/ou le traitement de bactérioses vaginales, mycoses vaginales et infections urinaires récurrentes. Par conséquent, l'administration de *L. crispatus* pourrait aider à prévenir la gonococcie.

En outre, Amant et al. (2002) ont démontré que *L. acidophilus* ATCC 4356, *L. crispatus* ATCC 33197, *L. jensenii* ATCC 25258, *L. gasseri* ATCC 33323 étaient capables d'inhiber le gonocoque à faible pH (5,8 - <7), mais *L. crispatus* et *L. jensenii* le pouvaient aussi à pH neutre (7). Ils ont suggéré que *L. crispatus* et *L. jensenii* peuvent protéger plus efficacement que *L. acidophilus* ATCC 4356 et *L. gasseri* ATCC 33323 car le pH vaginal qui normalement est acide peut tendre vers la neutralité dans certaines circonstances. Par ailleurs, Hütt et al., en 2016 ont montré que la production d'acide lactique de plusieurs souches vaginales de *L. crispatus* ( $15,6 \pm 2,8 \text{ mg.mL}^{-1}$ ) était supérieure à celle de plusieurs souches vaginales de *L. jensenii* ( $11,6 \pm 2,6 \text{ mg.mL}^{-1}$ ) mais qu'elle est spécifique à chaque souche. Les souches de *L. crispatus* présentaient une meilleure activité antagoniste contre *E. coli* et *Candida spp.* que *L. jensenii*. Ils ont donc suggéré qu'un candidat probiotique potentiel pourrait être trouvé parmi les souches de *L. crispatus*. Dans le cadre de nos travaux, notre choix s'est donc porté sur *L. crispatus* ATCC 33197 qui a été le plus étudié pour son activité antigonococcique. Le but



est de recoloniser la muqueuse vaginale, rétablir l'équilibre biologique et constituer une barrière microbiologique contre le gonocoque.

#### **4. INTERET DE LA FORME “HYDROGEL” POUR LA VOIE VAGINALE**

La forme galénique que nous avons retenue pour administrer *L. crispatus* est l'hydrogel vaginal. En effet, les hydrogels vaginaux suscitent un grand intérêt en recherche pour la prévention du VIH et des IST. Ils ont été étudiés précédemment dans les équipes partenaires de ce projet de thèse (Institut Galien Université Paris Sud (IGPS) et le département de Pharmacie Galénique Cosmétologie et législation de la faculté de pharmacie de l'Université Félix Houphouët-Boigny (PGCL)). En effet, un hydrogel à base d'un polymère thermogélifiant (le poloxamer 407) et d'un polymère mucoadhésif (l'hydroxypropylméthylcellulose) a été particulièrement étudié pour la voie rectale dans le traitement du paludisme chez l'enfant (Koffi et al., 2006, 2008). Les résultats prometteurs obtenus, tels que la libération prolongée et l'amélioration de la biodisponibilité de la quinine chez le lapin, ont favorisé des travaux ultérieurs visant à adapter cette formulation à la voie vaginale (Aka-Any-Grah et al., 2010). En effet, Aka-Any-Grah et al. (2010) ont développé des hydrogels à base d'une part de poloxamer 407 ou de mélange de deux poloxamer 407 et 188 et d'autre part de l'hydroxypropylméthylcellulose. Ces gels étaient insensibles à la dilution par le fluide vaginal simulé (Aka-Any-Grah et al., 2010). Ces gels étaient par ailleurs capable de véhiculer le peptide M48U1 et de former simultanément une barrière contre la diffusion du virus VIH jusqu'aux cellules épithéliales vaginales du macaque (Bouchemal et al., 2015). Des travaux sur les gels de poloxamer destinés à une application cutanée pour le traitement de l'ulcère de Buruli ou contenant d'autres molécules antipaludiques et destinés au traitement du paludisme sont également en cours.

Le choix de la forme galénique hydrogel résulte également de l'étude comparée des gels aux autres formes pharmaceutiques adaptés à la voie vaginale. En effet, les gels ont un risque de fuite plus faible que les formes liquides (douches vaginales) en raison de leur importante viscosité, ce qui augmente leur rétention et leur temps de contact avec la muqueuse vaginale. Les anneaux vaginaux, quant à eux, sont des dispositifs de diffusion de substances médicamenteuses. Ils sont généralement constitués d'une matrice polymère solide (silicone, matériaux thermoplastiques, copolymère d'éthylène et d'acétate de vinyle) (Malcolm et al., 2010) et laissent diffuser les molécules de faible masse molaire. Dans le cas de notre système, ce sont des bactéries qui doivent être libérées et la diffusion n'est pas un mécanisme possible

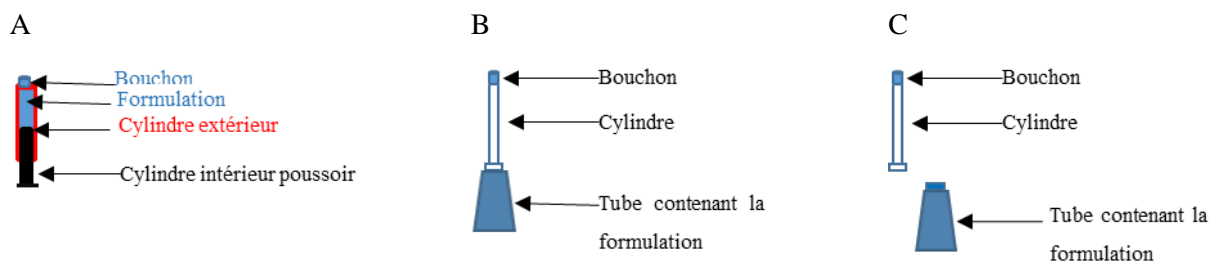
pour des particules de cette taille dans ce type de systems. Ainsi, ces anneaux ne conviennent pas à notre objectif. Concernant les comprimés, capsules et ovules, ils présentent l'avantage d'une bonne conservation. Toutefois, leur temps de délitement dans le vagin est parfois prolongé, et il existe un risque de répartition non homogène de la substance active. Pour toutes ces raisons, nous avons préféré les formes semi-solides qui devraient tapisser toute la muqueuse vaginale (Hussain and Ahsan, 2005) à l'image du mucus vaginal. En effet dans la partie la plus interne du vagin, le mucus forme un gel viscoélastique qui tapisse toute la muqueuse vaginale et constitue une barrière naturelle contre les microorganismes. En outre, parmi les formes semi-solides, les gels (monophasé) sont plus faciles à préparer que les crèmes qui contiennent une phase lipophile et une phase hydrophile. Parmi les gels, les hydrogels ont été retenus. En effet, les hydrogels sont des gels dans lesquels le milieu de dispersion est aqueux. Cette hydrophilie est un gros avantage pour éviter les salissures sur les vêtements. Les hydrogels sont généralement incolores, inodores, insipides et peuvent être utilisés en combinaison avec des dispositifs vaginaux ou des préservatifs (Pilcher, 2004). Ils offrent plusieurs autres avantages tels que la biocompatibilité, la bonne distribution et la mucoadhésion, la libération appropriée de la substance active et l'acceptabilité par les patientes.

Par ailleurs, les hydrogels trouvent déjà de nombreuses applications :

- pour l'immobilisation cellulaire : les hydrogels sont utilisés pour former des matrices comme support pour la croissance de cellules souches, de cellules bactériennes par adsorption, ou encapsulation ou pour l'administration de probiotiques (Cellesi, 2012; Chaluvadi et al., 2012; Willaert, 2011)
- dans l'ingénierie tissulaire : les hydrogels sont utilisés comme matériaux d'échafaudage pour le comblement, la régénération ou la réparation tissulaire (Maisani, 2017)
- pour la délivrance de substances actives (Cook and Brown, 2018).

Concernant la voie vaginale, les hydrogels ont été principalement formulés pour le traitement de la sécheresse vaginale et l'administration locale de médicaments antimicrobiens, de microbicides, de contraceptifs et d'inducteurs du travail lors d'un accouchement (Vidal, 2017). Les hydrogels ont également été étudiés pour la possibilité d'une administration systémique d'hormones (Almomen et al., 2015), de vaccins (Gordon et al., 2012), de protéines et de peptides (Hussain and Ahsan, 2005; Richardson and Illum, 1992) passant par la voie vaginale. Le développement d'un gel pour la voie vaginale nécessite la conception d'un applicateur car ce dernier est nécessaire pour atteindre la jonction cervico-vaginale (Hussain and Ahsan, 2005). La **figure 1** présente quelques schémas d'applicateurs. Les applicateurs constitués de

deux cylindres dont le cylindre externe contient la préparation et le cylindre interne constitue le piston avec un bouchon pour fermeture sont généralement utilisés pour les formes solides (**Fig. 1A**), mais pourraient être adaptés aux formes semi-solides. Généralement, pour les formes semi-solides, les applicateurs sont constitués d'un tube contenant la préparation, d'un cylindre pour une administration en profondeur et un bouchon. Ils peuvent exister en un ensemble continu (**Fig. 1B**) ou en deux parties à assembler avant usage que sont le tube d'une part et le cylindre d'autre part (**Fig. 1C**). La présentation en deux parties semble plus adaptée pour des préparations extemporanées.

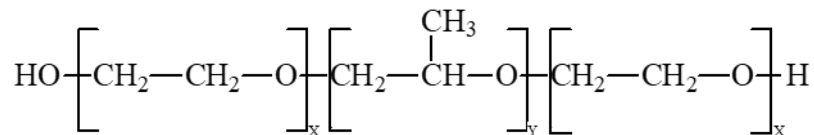


**Figure 1.** Description schématique d'applicateurs vaginaux sur le marché.

Les hydrogels préformés sont de plus en plus remplacés par des systèmes qui peuvent être administrés sous forme de liquide avant de se transformer en un gel à l'intérieur du corps, stimulés par des facteurs physiologiques spécifiques tels que le pH, la température et les ions. Les hydrogels sensibles à la température peuvent être avantageux pour l'application vaginale car ils ne nécessitent aucun composé supplémentaire externe pour induire la gélification.

## **5. LES GELS THERMOSENSIBLES A BASE DE POLOXAMERS : PROPRIETES PHYSICOCHIMIQUES ET APPLICATIONS PHARMACEUTIQUES**

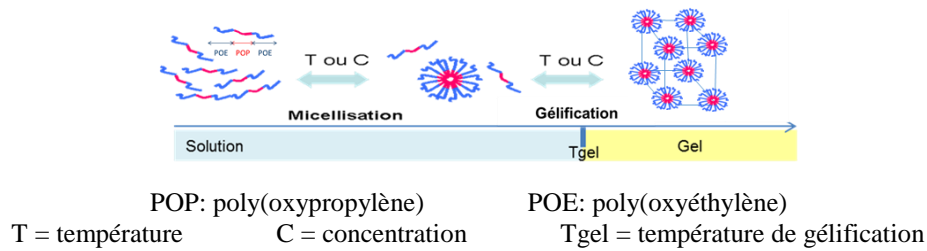
Les poloxamers sont des copolymères synthétiques triblocs composés de blocs de poly(oxyéthylène) (POE) et de poly(oxypropylène) (POP), soit une structure de type : POE-POP-POE (**Fig. 2**).



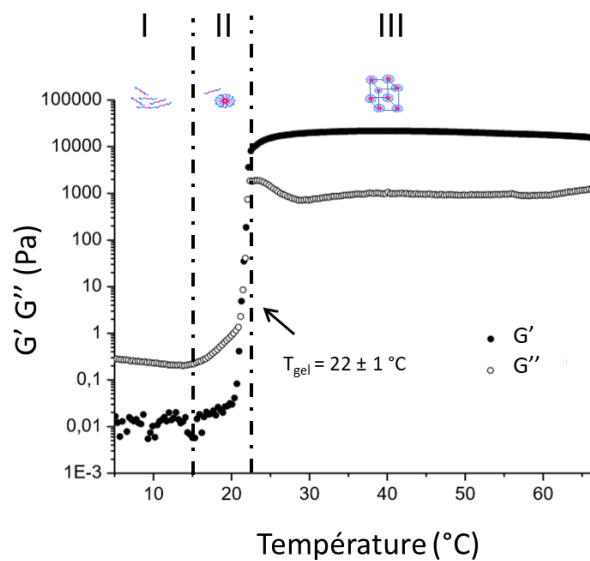
**Figure 2.** Formule chimique générale des poloxamers, x et y sont le nombre de répétition des unités d'oxyéthylène et d'oxypropylène.

Parmi les poloxamers, le poloxamer 407 et le poloxamer 188 sont les plus utilisés pour une application pharmaceutique. Le poloxamer 407 possède 95-105 unités oxyéthylène (OE), 54-60 unités oxypropylène (OP) et une masse molaire moyenne de 9840-14600 Da (Pharmacopée Européenne, 2018). Il est non-ionique, non toxique et administrable à l'Homme (Singh-Joy and McLain, 2008). Les solutions de poloxamer 407 sont autoclavables à 120 °C pendant 15 min à la pression de 1 bar, sans modifier significativement leur viscosité (Dimitrova et al., 2000; Dumortier et al., 2006; Misra, 2014). Il est facile de préparer des solutions de poloxamer 407 de concentration inférieure à 30 %. En solution diluée, le poloxamer 407 se comporte comme un tensioactif. A des concentrations supérieures ou égales à 16 % (m/m), le poloxamer 407 possède des propriétés thermogélifiantes qui sont réversibles. Cette propriété est caractérisée par la température de gélification en-dessous de laquelle la préparation est fluide et au-dessus de laquelle elle est semi-solide. En effet, à basse température et en solution diluée, le poloxamer 407 est sous la forme d'unimères. Au fur et à mesure que la température augmente, les unimères s'organisent en micelles en raison de la déshydratation de la partie hydrophobe formant ainsi des micelles sphériques à cœur hydrophobe (Alexandridis et al., 1994; Cao et al., 2005) (**Fig. 3**). Si la température est davantage augmentée et que la concentration en poloxamer 407 est supérieure à 16 %, ces micelles, dont la taille du cœur mesure environ 50 Å, peuvent s'organiser pour donner la structure ordonnée d'un gel de type cubique micellaire (Mortensen and Pedersen, 1993). La gélification du poloxamer est donc déclenchée par la température (Lenaerts et al., 1987). D'un point de vue rhéologique, cela se traduit par une brusque augmentation des modules élastique  $G'$  et visqueux  $G''$  (**Fig. 4**). La **Figure 4** décrit les différentes phases d'une courbe de rhéologie type lors de l'élévation de la température. Dans la phase I, la solution de poloxamer se comporte comme un liquide visqueux ( $G'' > G'$ ). Le poloxamer est sous forme d'unimères et les modules sont faibles.  $G''$  diminue légèrement avec la température car sous l'effet de l'augmentation de la température, il y a une déshydratation progressive des chaînes de poloxamer qui se trouvent alors en moins bon solvant et se contractent. La phase II est marquée par l'augmentation des modules témoignant de la formation des micelles. La température de gélification,  $T_{\text{gel}}$ , peut être définie comme la température de croisement des

modules élastiques et visqueux. Dans la phase III, le système se comporte comme un gel élastique ( $G' > G''$ ), car les micelles sont organisées sous forme d'un réseau tridimensionnel. Les valeurs des modules sont élevées. Une formulation à base de poloxamer 407 peut ainsi se présenter sous la forme liquide à température ambiante pour faciliter son administration et son étalement sur les muqueuses et gélifier à la température corporelle (Dumortier et al., 2006). Les gels de poloxamer 407 sont transparents, incolores et lavables à l'eau.



**Figure 3.** Mécanisme d'association des unimères de poloxamer conduisant à la micellisation et la gélfication d'après les informations recueillies dans la revue de Dumortier et al. (2006).



**Figure 4.** Courbe de rhéologie type décrivant la gélfication du poloxamer à 20 % (m/m) due à l'élévation de la température et exprimant les paramètres suivants : la température de gélfication ( $T_{gel}$ ), le module élastique ( $G'$ ) et le module visqueux ( $G''$ ) (Aka-Any-Grah et al., 2010).

L'incorporation de principes actifs et d'autres additifs peut fortement modifier la température de gélfication du poloxamer. L'ajout d'acides (principalement acides forts) et d'alcools (éthanol ou isopropanol) a tendance à diminuer la rigidité des gels et à augmenter la

température de gélification (Vadnere and Amidon, 1984). En revanche, l'incorporation de sels minéraux (NaCl, CaCl<sub>2</sub>, Na<sub>2</sub>SO<sub>4</sub>...) ou d'autres polymères comme le Carbopol®, l'alginate de sodium, la méthylcellulose et l'hydroxypropylméthylcellulose entraîne une diminution de la température de gélification (Das et al., 2010; Su et al., 2003).

Le poloxamer 407 a été utilisé en recherche dans des formulations pharmaceutiques injectables (Derakhshandeh et al., 2010), vaginales (Aka-Any-Grah et al., 2010; Liu et al., 2009; Rençber et al., 2017), oculaires (Dimitrova et al., 2000), rectales (Charrueau et al., 2001; Koffi et al., 2006), cutanées (Dumortier et al., 2006), nasales (Wang et al., 2017) et buccales (Zeng et al., 2014).

Veyries et al., en 2000 ont par ailleurs montré que le poloxamer 407 est capable d'inhiber l'adhésion de pathogènes à une surface de poly(méthylméthacrylate). Cette propriété anti-adhésive du poloxamer est non spécifique. Ces auteurs ont suggéré que la combinaison de poloxamer 407 avec un agent antimicrobien pourrait être une approche prometteuse pour prévenir l'infection de matériaux étrangers *in vivo* : un gel de poloxamer contenant des antibiotiques pourrait ainsi être utilisé pour enrober les prothèses au moment de la chirurgie afin de prévenir les infections. Le poloxamer, en ingénierie tissulaire, a été utilisé pour l'encapsulation de cellules (Gioffredi et al., 2016; Khattak et al., 2005; Sengupta et al., 2015; Suntornnond et al., 2017; Wagner et al., 2014).

Au vu de toutes ces propriétés, nous avons donc retenu le poloxamer 407 dans le cadre de notre étude. Notre hypothèse est que le poloxamer 407, par ses propriétés physico-chimiques, permettrait à la formulation de recouvrir la muqueuse vaginale et d'empêcher l'adhésion du gonocoque aux cellules épithéliales. De plus, *L. crispatus* serait incorporé dans la formulation de sorte que même si quelques gonocoques arrivaient à pénétrer dans le gel, ils seraient inhibés par l'activité de *L. crispatus*. Néanmoins, les gels de poloxamer ont de faibles propriétés mécaniques et mucoadhésive avec une vitesse de dissolution rapide dans les fluides biologiques, réduisant leur temps de résidence au site d'administration (Koffi et al., 2006). Pour améliorer ces propriétés, plusieurs stratégies ont été proposées :

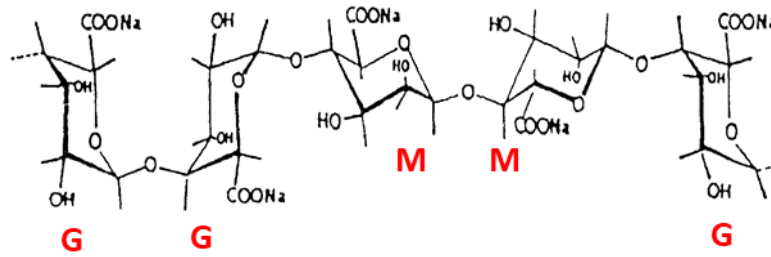
- Modification chimique des chaînes de polymère (Chen et al., 2011; Hsu et al., 2009)
- Mélange physique avec un polymère mucoadhésif (Huang et al., 2016; Ur-Rehman et al., 2011; Wang et al., 2017)
- Ajout des petites molécules (NaCl) qui peuvent renforcer la viscosité et la tenue mécanique (Choi et al., 1999; Ricci et al., 2005).

La méthode que nous avons retenue du fait de sa facilité à mettre en œuvre est le mélange du poloxamer 407 avec des polymères mucoadhésifs capables de former des enchevêtrements

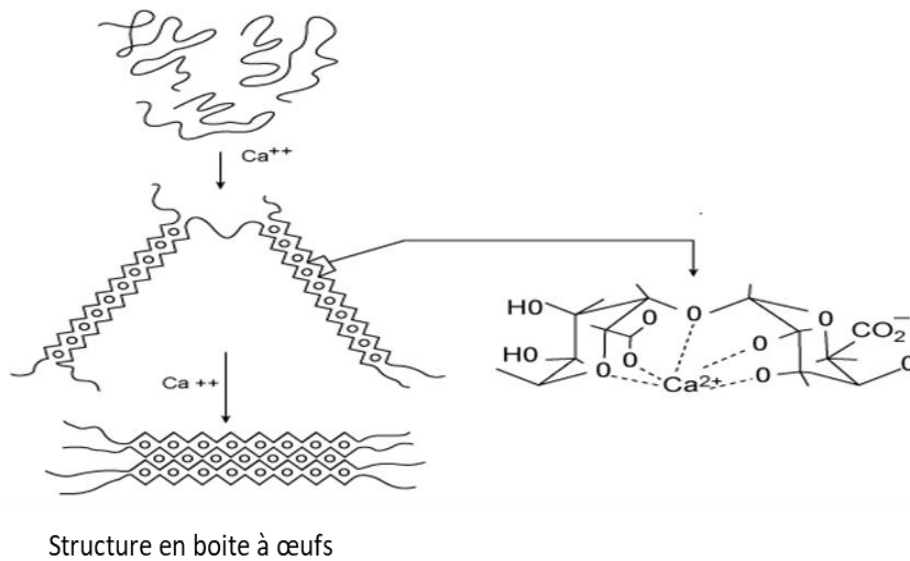
ou des liaisons non covalentes avec le mucus couvrant les tissus épithéliaux, prolongeant ainsi le temps de résidence *in vivo*. Il existe plusieurs polymères mucoadhésifs : les polyacrylates, le chitosane, les dérivés de la cellulose, l'acide hyaluronique, la pectine, l'amidon, le poly(éthylène glycol), le carraghénane, l'alginate et la gélatine (Acarturk, 2009; Andrews et al., 2009; Boddupalli et al., 2010). Certains travaux rapportent le développement de formulations destinées à la voie vaginale, constituées d'un mélange de poloxamer 407 et d'un polymère mucoadhésif tel que le chitosane (Ur-Rehman et al., 2011), l'hydroxypropylmethylcellulose (Aka-Any-Grah et al., 2010; Bilensoy et al., 2006; Deshkar et al., 2015; Karavana et al., 2012; Rençber et al., 2017; Tuğcu-Demiröz et al., 2013), le carbopol (Bilensoy et al., 2006), le polycarbophile (Chang et al., 2002), l'acide hyaluronique (Bilensoy et al., 2006), le carraghénane (Liu et al., 2009). Tous ces auteurs ont montré l'amélioration des propriétés mucoadhésives des gels de poloxamer en présence de ces polymères. Bouchemal et al. (2015) ont montré par vidéomicroscopie l'effet barrière du gel de poloxamer 407 et d'hydroxypropylmethylcellulose contre la diffusion de particules de polystyrène modifiées mimant la taille et la charge de surface du VIH-1.

## 6. PROPRIETES PHYSICOCHEMIQUES DE L'ALGINATE ET SES APPLICATIONS PHARMACEUTIQUES

Dans le cadre de nos travaux, nous nous sommes intéressés à l'alginate de sodium comme polymère mucoadhésif. L'alginate est un polysaccharide hydrophile, naturel, issu d'algues brunes (*Phaeophyceae*) ou de bactéries. Sa structure contient deux types de monomères, l'acide  $\beta$ -D-mannuronique (M) et l'acide  $\alpha$ -L-guluronique (G) qui sont organisés sous forme de blocs M-M, G-G et M-G (**Fig. 5**). La proportion de résidus mannuroniques par rapport aux résidus guluroniques (rapport noté M/G) ainsi que le nombre et la longueur des blocs M-M, G-G et M-G est très variable. Qin (2008) résume la composition en unité M et G d'alginate extraites d'algues couramment utilisées pour la production d'alginate commercialisé. L'alginate issu des tiges de *Laminaria hyperborean* renferme 60 % de blocs G-G contrairement aux autres alginates qui en renferment 14-31 % (Qin, 2008).



**Figure 5.** Formule chimique de l'alginate (Grasdalen et al., 1981).



**Figure 6.** Schéma de la gélification ionique de l'alginate avec les ions calcium (Braccini and Pérez, 2001).

Il existe plusieurs sels d'alginate (calcium, sodium, ammonium, and potassium). L'alginate de sodium est un polymère biodégradable, non-toxique et mucoadhésif. Il se dissout dans l'eau en formant des solutions colloïdales visqueuses. Il gélifie en présence d'ions divalents comme  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Mg}^{2+}$  et forme des hydrogels élastiques et non thermoréversibles (**Fig. 6**). L'utilisation de l'ion calcium est la plus fréquente. La formation de gels d'alginate est un processus assez complexe car il dépend de la masse molaire, de la répartition des blocs de monomères (M-M, M-G, G-G), de leur pourcentage en unité M ou G, de la source des ions calcium (chlorure, phosphate, lactate ou acétate de calcium) et des procédés de préparation du gel. En effet, les ions calcium s'associent préférentiellement aux fragments poly-G plutôt qu'aux fragments poly-M (Wang et al., 1993). Les blocs poly-G, en raison de leur



conformation spatiale, permettent une chélation plus énergétique des ions calcium (Braccini and Pérez, 2001). Ces derniers interagissent avec les fonctions carboxylates et les atomes d'oxygène des fonctions hydroxyles. Le gel d'alginate se présente sous la forme d'un enchaînement régulier dit de type « egg box » ou boîte à œufs avec des zones organisées reliées par les segments poly-M ou poly-(M-G) (**Fig. 6**). Lors de la gélification avec les ions calcium, le gel peut se présenter sous trois états physiques différents selon la concentration en ions calcium. A très faible concentration, il y a formation d'agrégats (microgels) qui provoquent une diminution de la viscosité de la solution d'alginate. Puis, à partir d'une certaine concentration, les macromolécules se réarrangent pour former un réseau tridimensionnel occupant tout l'échantillon. Si la concentration en calcium augmente encore, on observe un phénomène de synérèse (gel avec démixtion) dû à une diminution du taux de gonflement des chaînes d'alginate avec expulsion d'eau hors du réseau. Par ailleurs, la gélification de l'alginate dans une solution ne contenant que du chlorure de calcium est rapide, mal contrôlée et conduit à la formation d'un gel non homogène. Pour obtenir un gel plus homogène, il est possible d'ajouter du chlorure de sodium à la solution de chlorure de calcium qui sert à gélifier l'alginate, ainsi qu'à la solution d'alginate (Smidsrød and Skjåk-Braek, 1990). D'autre part, l'utilisation d'un tampon phosphate tel que l'hexamétophosphate de sodium pourrait ralentir le processus de gélification avec le chlorure de calcium. En effet, les groupes carboxylates de l'alginate et les groupes phosphates présents dans le tampon entrent en compétition avec les ions calcium (Lee and Mooney, 2012). De plus, le sulfate de calcium ( $\text{CaSO}_4$ ) et le carbonate de calcium ( $\text{CaCO}_3$ ), en raison de leur faible solubilité, peuvent ralentir la vitesse de gélification de l'alginate. Une solution d'alginate peut donc être mélangée avec du  $\text{CaCO}_3$ , qui n'est pas soluble dans l'eau à pH neutre. Le glucono- $\delta$ -lactone est ensuite ajouté à ce mélange alginate/ $\text{CaCO}_3$  pour dissocier les ions  $\text{Ca}^{2+}$  du  $\text{CaCO}_3$  en abaissant le pH. Les ions  $\text{Ca}^{2+}$  libérés déclenchent ensuite la gélification de la solution d'alginate de manière plus graduelle. Les gels d'alginate ainsi obtenus ont généralement une grande porosité qui facilite la diffusion des molécules et leur libération. La présence d'un agent chélatant tel que l'acide éthylènediaminetétraacétique (EDTA), le lactate, le citrate ou le phosphate augmente l'élimination des ions calcium de la matrice.

Le comportement rhéologique des gels d'alginate est influencé par la structure du polymère. En effet, la proportion des blocs poly-G et leur longueur conditionnent la formation des gels obtenus en présence de calcium ainsi que les propriétés mécaniques. Les segments G-G sont rigides et statiques alors que les segments M-M ou M-G sont flexibles. La présence de longs blocs G-G génèrent la formation d'un réseau rigide et statique, alors que les gels à faible taux

de guluronate et longs segments élastiques génèrent des structures dynamiques (Brus et al., 2017; Funami et al., 2009). Une contamination microbienne peut entraîner une dépolymérisation de l'alginate conduisant à une diminution de la viscosité, d'où la nécessité de sa stérilisation.

L'alginate trouve des applications en pharmacie, en cosmétologie, en ingénierie tissulaire et en industrie alimentaire. En pharmacie, il est utilisé dans les formes sèches pour l'obtention de comprimés à matrice hydrophile afin de contrôler la libération des ingrédients pharmaceutiques actifs ou comme agent désintégrant ou agent liant. Dans les formes liquides et pâteuses, il est utilisé comme agent épaississant et stabilisant. Il est aussi utilisé pour l'encapsulation de substances actives et de cellules. Les solutions d'alginate ont également un excellent pouvoir lubrifiant et cicatrisant. Certaines formulations destinées à la voie vaginale renferment de l'alginate : des comprimés (Abruzzo et al., 2013; El-Kamel et al., 2002), des films (Mishra et al., 2016; Rai et al., 2010), des hydrogels (Ferreira et al., 2017), des microsphères (Szekalska et al., 2015), et des microparticules (Martín-Villena et al., 2013).

Par ailleurs, Chaluvadi et al. (2012) ont étudié la survie de différents probiotiques (*Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*) dans des formulations symbiotiques composées de 10 mg.mL<sup>-1</sup> de fructo-oligosaccharides, d'inuline et de pecto-oligosaccharides dans une matrice d'alginate, après conservation sous aérobie à la température de 4 °C. Ils ont montré l'effet bénéfique de l'alginate sur la croissance des probiotiques avec l'inhibition simultanée de la colonisation du gros intestin par *Salmonella enteritidis*. Toutefois, l'activité métabolique après cette conservation était stressée. Au moins 10<sup>7</sup> UFC/mL de bactéries probiotiques ont survécu après 21 jours de conservation dans ce gel d'alginate. La survie était meilleure dans les gels symbiotiques additionnés de fructo-oligosaccharides, d'inuline et de pecto-oligosaccharides par rapport aux gels d'alginate seuls. Ils montrent ainsi que les prébiotiques protègent les bactéries probiotiques et prolongent leur durée de conservation dans les gels, dans des conditions aérobies réfrigérées (Chaluvadi et al., 2012). Le gel d'alginate pourrait ainsi former une matrice pouvant servir de support pour la croissance des lactobacilles d'étude.

L'alginate possède de bonnes propriétés mucoadhésives. Après son contact avec la muqueuse, il s'étale, s'hydrate grâce au fluide vaginal, gonfle et adhère grâce aux acides carboxyliques de sa structure qui créent des liaisons hydrogène avec les glycoprotéines de la mucine (Patil and Sawant, 2009). L'enchevêtrement avec le mucus est amélioré lorsque les chaînes sont dépliées. En milieu acide, l'alginate ne gonfle pas beaucoup, les chaînes ont plutôt tendance à s'agréger. Ainsi, l'alginate est moins mucoadhésif à pH acide. Dans le cas où l'alginate est

réticulé avec un cation divalent, sa flexibilité diminue, ce qui diminue ses propriétés de mucoadhésion (Patil and Sawant, 2009). Les propriétés mucoadhésives de l'alginate peuvent aussi être influencées par sa masse molaire : les chaînes d'alginate de faible masse molaire restent relativement rigides, et moins susceptibles de se lier à la mucine par rapport à celles de masse molaire élevée (Menchicchi et al., 2015). L'alginate est assez souvent associé à un autre polymère mucoadhésif tel que le chitosane (Abruzzo et al., 2013), la gélatine (Ferreira Almeida and Almeida, 2004) dérivés de la cellulose (Sirviö et al., 2014).

La mucoadhésion prolonge le temps de résidence des formulations, favorisant l'absorption et l'efficacité des actifs thérapeutiques. Bagul et al. (2009), comparant différents polymères mucoadhésifs, les ont classés dans l'ordre croissant suivant, en fonction de la force de mucoadhésion : gélatine, gomme dammar, gomme copal, éthylcellulose, alginate de sodium, gomme xanthane, chitosane, hydroxypropylméthylcellulose, carbopol.

## 7. MELANGE ALGINATE-POLOXAMER

Dans le cadre de notre travail, nous avons choisi de mélanger le poloxamer 407 et l'alginate de sodium. Le choix du mélange de l'alginate de sodium au poloxamer avait pour but d'obtenir un système mucoadhésif, pouvant potentiellement gélifier par deux mécanismes qui sont la thermogélification du poloxamer et la gélification ionique de l'alginate, étant donné que le fluide vaginal contient des ions calcium sous la forme d'hydroxyde de calcium à 0,22 g/L (Owen and Katz, 1999). La présence de groupes hydroxyles terminaux dans la structure du poloxamer, permet des mélanges physiques ou des greffages chimiques (Chen et al., 2011; Fang et al., 2009). Lin et al. (2004) ont proposé l'hypothèse que les molécules d'eau permettent des liaisons hydrogène entre les groupes carboxyles de l'alginate et les groupes éthers du poloxamer 407, pouvant conduire à la formation d'un réseau tridimensionnel, donc d'un gel. Ainsi, dans les conditions suivantes (pH 7,4, 37 °C), la répulsion ionique entre les groupes carboxyles chargés négativement peut produire une conformation d'alginate plus étirée et favoriser ainsi la formation de ces liaisons hydrogène avec les blocs de poly(oxyéthylène) du poloxamer, ce qui pourrait favoriser la formation du gel et renforcer ses propriétés mécaniques. Le **tableau 1** reporte les études publiées sur les mélanges d'alginate de sodium et de poloxamer 407. Il ressort que ces deux polymères sont compatibles. La gélification de l'un n'empêche pas la gélification de l'autre polymère.

**Tableau 1.** Publications reportant l'utilisation d'un mélange de poloxamer et d'alginate

Type de système	Concentration	Caractéristiques de l'alginate	Mécanisme de gélification	Principaux résultats - Application	Auteur
Solutions à base d'alginate et de poloxamer, pilocarpine	Alginate 0.5-3 % (m/m) : Poloxamer 407 12-16 % (m/m)	M/G = 1,56	Gélification <i>in situ</i> pour une administration ophtalmique	Administration ophtalmique de pilocarpine Evaluation à pH 4,0 et 25 °C d'une part et à pH 7,4 et 37 °C d'autre part. Les concentrations de cations (Na <sup>+</sup> (0,14 M) et Ca <sup>2+</sup> (0,0005 M)) en présence de fluide lacrymal ne sont pas suffisamment élevées pour que la solution d'alginate à 2 % (w / w) forme un gel fort. Les alginates avec des teneurs en guluronate de plus de 65 % forment des gels instantanément en présence de fluide lacrymal simulé, tandis que ceux avec des teneurs en guluronate faibles forment des gels faibles avec une cinétique relativement lente. La teneur en guluronate de l'alginate de sodium utilisé dans cette étude n'était que de 39 %. Cette étude a permis d'identifier le gel optimal pour une administration ophtalmique dans le but d'obtenir une gélification <i>in situ</i> et un gel résistant aux forces de cisaillement exercées par le battement des paupières.	Lin et al. (2004)
Microparticules d'alginate-poloxamer, terbutaline, sérumalbumine bovine	Alginate 3 % (m/m) : Poloxamer 407 3 % (m/m) dans la phase aqueuse en ratio 1 : 5, 3 : 3, 5 : 1 et 5 : 0	Non données	Emulsion hydrophile/lipophile gélifiée par du chlorure de calcium	Encapsulation de sulfate de terbutaline et de protéine telle que la sérumalbumine bovine	Moebus et al. (2009)
Microbilles poloxamer-alginate métoprolol	Concentration totale en polymère 2 % (m/v)	Non données	Gélification externe du mélange poloxamer et alginate dans du chlorure de calcium	Libération contrôlée du succinate de métoprolol	Parhi and Suresh (2015)

**Tableau 1. Suite**

Type de système	Concentration	Caractéristiques de l'alginate	Mécanisme de gélification	Principaux résultats - Application	Auteur
Mélange poloxamer - alginate-CaCl <sub>2</sub>	Non donnée	Non données	Gélification par du chlorure de calcium	Réduction des adhérences après la chirurgie du strabisme dans un modèle de lapin	Ryu et al. (2013)
Mélange poloxamer-alginate-CaCl <sub>2</sub>	Non donnée	Non données	Gélification par du chlorure de calcium	Prévenir l'adhérence postopératoire chez les chiens en formant une barrière sur la surface d'une plaie intra-abdominale après une chirurgie abdominale chez le chien	Kim (2015)
Poloxamer-alginate-CaCl <sub>2</sub> Lidocaine	Non donnée	Non données	Gélification par du chlorure de calcium	Propriétés analgésiques, anti-inflammatoires et antiadhésives du mélange chargé de lidocaïne dans un modèle de douleur incisionnelle chez le rat	Choi et al. (2017)
Mélanges physiques (A+P) ou des greffages chimiques (AP), selegiline	Alginate 0,63 % (m/v) Poloxamer 407 20 % (m/v) AP 15 % (m/v)	Alginate 1,2 10 <sup>5</sup> g/mol	Gélification par la température	Administration transdermique de la selegiline par le poloxamer seul, le mélange poloxamer 407-alginate, ou le poloxamer greffé sur l'alginate.  La température de gélification du mélange de poloxamer et d'alginate était similaire à celle du poloxamer seul. Celle du poloxamer greffé à l'alginate était plus grande que celle des deux systèmes.  Le mélange physique alginate et poloxamer conduit à une structure très poreuse avec des pores irréguliers interconnectés de tailles différentes variant de 10-20 µm de diamètre, alors que le greffage chimique du poloxamer à l'alginate se présente sous la forme d'un réseau semblable à une éponge avec des pores sphériques.	(Chen et al., 2011)
Hydrogel alginate, poloxamer, liposome	Alginate 0 - 2 % (m/m) Poloxamer 407 17-20 % (m/m)	Non données	Gélification interne de l'alginate. Ajouter la poudre de poloxamer et d'alginate à la solution de CaEDTA (acide éthylènediaminetétraacétique de calcium). Bien homogénéiser puis ajouter le GDL (glucono-δ-lactone).	L'alginate est compatible avec le poloxamer tant sous forme liquide que gélifiée.  L'alginate ne modifie pas significativement les propriétés élastiques du mélange de polymère. Une gélification à la fois du poloxamer par la température et de l'alginate par les ions est observée. Le réseau formé par l'alginate ne modifie pas l'organisation des micelles du poloxamer. Ces auteurs recommandent une concentration en alginate inférieure ou égale à 1% car la présence de l'alginate et du calcium diminue la température de gélification.  Les liposomes ne modifient pas les propriétés du mélange de polymères mais interfèrent dans la cinétique du mécanisme de gélification comme en témoigne la différence des pentes de G' et G''. Leur influence ne dépend ni de leur taille ni de leur potentiel zêta. Le poloxamer, gélifié, impose son comportement aux systèmes.	(Grassi et al., 2006)

**Tableau 1. Suite**

Type de système	Concentration	Caractéristiques de l'alginate	Mécanisme de gélification	Principaux résultats - Application	Auteur
Hydrogel alginate - poloxamer	Alginate 0.5-1 % (m/v) Poloxamer 407 5-30 % (m/v)	%G ≥ 60, M = 310 000 g.mol <sup>-1</sup>	Gélification interne de l'alginate avec le CaEDTA (acide éthylènediaminetétraacétique de calcium) et le GDL (glucono-δ-lactone). Ce gel a été trempé dans la solution de poloxamer pendant 7 jours	Les hydrogels d'alginate ont une gamme de tailles de pores pouvant aller de 5 à 200 nm. Les rayons hydrodynamiques des unimères de poloxamer 407 sont d'environ 2 nm, et les rayons de micelles de poloxamer 407 sont d'environ 11 nm. Ainsi, les chaînes de poloxamer peuvent diffuser librement au sein du réseau formé par l'alginate sans encombrement stérique étant donné que le mélange de polymères se fait à 4 ° C. La calorimétrie et la diffusion des neutrons ont permis de démontrer que la présence d'une matrice d'alginate n'empêche pas la formation de micelles de poloxamer ni l'auto-assemblage de micelles dans un réseau cubique. La rhéologie et la diffusion des neutrons ont démontré que l'hydrogel est thermogélifiant.	(White et al., 2013)
Hydrogel alginate-laponite-poloxamer	Alginate 0.75-1 % (m/v) Laponite 0.5-1 % (m/v) Poloxamer 407 5-30 % (m/v)	Non données	Gélification interne du mélange alginate-laponite avec le CaEDTA et le GDL. Ce gel est ensuite trempé dans la solution de poloxamer pendant 7 jours	Ces auteurs démontrent un effet synergique entre l'alginate, la laponite et le poloxamer. Le poloxamer 407 augmente l'élasticité de gels d'alginate – laponite. La variation de la concentration en alginate n'influence pas les modules rhéologiques. La présence de laponite et d'alginate n'interfère pas dans le mécanisme de gélification du poloxamer	(Hom and Bhatia, 2017)
Film de mélange alginate-poloxamer	Alginate 2 % (m/m) Poloxamer 407 18 % (m/m)	Alginate A <sub>1</sub> , M/G =1.49 M = 396 000 g.mol <sup>-1</sup> , Alginate A <sub>2</sub> , M/G =0.56 M = 419 000 g.mol <sup>-1</sup>	Gélification thermique suivie de gélification externe ionique par CuSO <sub>4</sub> .5H <sub>2</sub> O	Modélisation dans le but de prédire l'épaisseur, la dureté, et le délai d'obtention d'un film de poloxamer 407 et d'alginate.	(Barba et al., 2014)
Film alginate - poloxamer	Alginate 0,25-1 % (m/v) Poloxamer 407 5-30 % (m/v)	Non données	Gélification interne de l'alginate avec le CaEDTA et le GDL Ce gel, étalé dans une boîte de Pétri, a été trempé avec la solution de poloxamer pendant 7 jours	Formulation de films épais et larges, thermosensibles, élastiques pouvant être utilisés pour les pansements, l'administration de substances actives ou en ingénierie tissulaire.	(Quah et al., 2018)

## 8. CONCLUSION

Cette revue bibliographique permet donc de justifier le choix de la voie vaginale et le développement d'un hydrogel vaginal à base de poloxamer 407, d'alginate de sodium et de *L. crispatus* pour la prévention de la gonococcie. Cet hydrogel tapisserait la muqueuse vaginale, afin de piéger le gonocoque et l'empêcher d'adhérer aux cellules épithéliales (barrière physique). A cela s'ajouterait l'activité propre de *L. crispatus* vis-a-vis de *N. gonorrhoeae*. Les hydrogels thermosensibles sont particulièrement intéressants car ils peuvent se présenter sous une forme liquide afin de faciliter leur administration et leur étalement sur la muqueuse vaginale puis gélifier à la température corporelle. Le développement d'un hydrogel thermosensible constitue une stratégie intéressante pour une application biomédicale. Pour obtenir la thermogélification, le poloxamer 407, un polymère synthétique, thermosensible et amphiphile a été retenu pour nos travaux. Afin d'améliorer l'adhésion du gel à la muqueuse vaginale et prévenir ainsi les fuites, nous avons choisi d'ajouter un second polymère dit mucoadhésif à la formulation. Pour obtenir la mucoadhésion, l'alginate de sodium, un polymère mucoadhésif, anionique, naturel, biocompatible, biodégradable qui gélifie en présence d'ions calcium, a été retenu. L'association de ces deux polymères pourrait potentiellement permettre de combiner les deux mécanismes de gélification, notamment la gélification induite par la température du poloxamer et la gélification ionique de l'alginate.

## Références

- Abruzzo, A., Bigucci, F., Cerchiara, T., Saladini, B., Gallucci, M.C., Cruciani, F., Vitali, B., and Luppi, B. (2013). Chitosan/alginate complexes for vaginal delivery of chlorhexidine digluconate. *Carbohydr. Polym.* *91*, 651–658.
- Acarturk, F. (2009). Mucoadhesive Vaginal Drug Delivery Systems. *Recent Pat. Drug Deliv. Formul.* *3*, 193–205.
- Aka-Any-Grah, A., Bouchemal, K., Koffi, A., Agnely, F., Zhang, M., Djabourov, M., and Ponchel, G. (2010). Formulation of mucoadhesive vaginal hydrogels insensitive to dilution with vaginal fluids. *Eur. J. Pharm. Biopharm.* *76*, 296–303.
- Alexandridis, P., Holzwarth, J.F., and Hatton, T.A. (1994). Micellization of Poly(ethylene oxide)-Poly(propylene oxide)-Poly(ethylene oxide) Triblock Copolymers in Aqueous Solutions: Thermodynamics of Copolymer Association. *Macromolecules* *27*, 2414–2425.
- Almomen, A., Cho, S., Yang, C.-H., Li, Z., Jarboe, E.A., Peterson, C.M., Huh, K.M., and Janát-Amsbury, M.M. (2015). Thermosensitive progesterone hydrogel: A safe and effective new formulation for vaginal application. *Pharm. Res.* *32*, 2266–2279.
- Amant, D.C.S., Valentin-Bon, I.E., and Jerse, A.E. (2002). Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. *Infect. Immun.* *70*, 7169–7171.

Andrews, G.P., Lavery, T.P., and Jones, D.S. (2009). Mucoadhesive polymeric platforms for controlled drug delivery. *Eur. J. Pharm. Biopharm.* 71, 505–518.

Bagul, U., Gujar, K., Dhat, S., Aphale, S., and Bhavsar, M. (2009). In vitro study of mucoadhesive strength of polymers for mucoadhesive drug delivery systems. *Int. J. Curr. Pharm. Res.* 1.

Barba, A.A., Lamberti, G., Rabbia, L., Grassi, M., Larobina, D., and Grassi, G. (2014). Modeling of the reticulation kinetics of alginate/pluronic blends for biomedical applications. *Mater. Sci. Eng. C* 37, 327–331.

Bilensoy, E., Abdur Rouf, M., Vural, I., Šen, M., and Atilla Hincal, A. (2006). Mucoadhesive, thermosensitive, prolonged-release vaginal gel for clotrimazole:  $\beta$ -cyclodextrin complex. *AAPS PharmSciTech* 7.

Boddupalli, B.M., Mohammed, Z.N.K., Nath, R.A., and Banji, D. (2010). Mucoadhesive drug delivery system: An overview. *J. Adv. Pharm. Technol. Res.* 1, 381–387.

Bouchemal, K., Aka-Any-Grah, A., Dereuddre-Bosquet, N., Martin, L., Lievin-Le-Moal, V., Le Grand, R., Nicolas, V., Gibellini, D., Lembo, D., Poüs, C., et al. (2015). Thermosensitive and mucoadhesive pluronic-hydroxypropylmethylcellulose hydrogel containing the mini-CD4 M48U1 is a promising efficient barrier against HIV diffusion through macaque cervicovaginal mucus. *Antimicrob. Agents Chemother.* 59, 2215–2222.

Braccini, I., and Pérez, S. (2001). Molecular basis of  $\text{Ca}^{2+}$ -induced gelation in alginates and pectins: The Egg-box model revisited. *Biomacromolecules* 2, 1089–1096.

Breshears, L.M., Edwards, V.L., Ravel, J., and Peterson, M.L. (2015). *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. *BMC Microbiol.* 15.

Brotman, R.M., Bradford, L.L., Conrad, M., Gajer, P., Ault, K., Peralta, L., Forney, L.J., Carlton, J.M., Abdo, Z., and Ravel, J. (2012). Association between *Trichomonas vaginalis* and vaginal bacterial community composition among reproductive-age women. *Sex. Transm. Dis.* 39, 807–812.

Brus, J., Urbanova, M., Czernek, J., Pavelkova, M., Kubova, K., Vyslouzil, J., Abbrent, S., Konefal, R., Horský, J., Vetchy, D., et al. (2017). Structure and dynamics of alginate gels cross-linked by polyvalent ions probed via solid state NMR spectroscopy. *Biomacromolecules* 18, 2478–2488.

Cao, X., Xu, G., Li, Y., and Zhang, Z. (2005). Aggregation of poly(ethylene oxide)–poly(propylene oxide) block copolymers in aqueous solution: DPD Simulation Study. *J. Phys. Chem. A* 109, 10418–10423.

Cellesi, F. (2012). Thermoresponsive hydrogels for cellular delivery. *Ther. Deliv.* 3, 1395–1407.

Chaluvadi, S., Hotchkiss Jr., A. t., Call, J. e., Luchansky, J. b., Phillips, J. g., Liu, L., and Yam, K. I. (2012). Protection of probiotic bacteria in a synbiotic matrix following aerobic storage at 4 °C. *Benef. Microbes* 3, 175–187.

Chan, D.J. (2005). Factors affecting sexual transmission of HIV-1: Current evidence and implications for prevention. *Curr. HIV Res.* 3, 223–241.

Chang, J.Y., Oh, Y.-K., Kong, H.S., Kim, E.J., Jang, D.D., Nam, K.T., and Kim, C.-K. (2002). Prolonged antifungal effects of clotrimazole-containing mucoadhesive thermosensitive gels on vaginitis. *J. Control. Release.* 82, 39–50.



Charrueau, C., Tuleu, C., Astre, V., Grossiord, J.-L., and Chaumeil, J.-C. (2001). Poloxamer 407 as a thermogelling and adhesive polymer for rectal administration of short-chain fatty acids. *Drug Dev. Ind. Pharm.* 27, 351–357.

Chen, C.-C., Fang, C.-L., Al-Suwayeh, S.A., Leu, Y.-L., and Fang, J.-Y. (2011). Transdermal delivery of selegiline from alginate-pluronic composite thermogels. *Int. J. Pharm.* 415, 119–128.

Cherpes, T.L., Meyn, L.A., Krohn, M.A., Lurie, J.G., and Hillier, S.L. (2003). Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. *Clin. Infect. Dis.* 37, 319–325.

Choi, G.J., Kang, H., Hong, M.E., Shin, H.Y., Baek, C.W., Jung, Y.H., Lee, Y., Kim, J.W., Park, I.K., and Cho, W.J. (2017). Effects of a lidocaine-loaded poloxamer/alginate/CaCl<sub>2</sub> mixture on postoperative pain and adhesion in a rat model of incisional pain. *Anesth. Analg.* 125, 320–327.

Choi, H., Lee, M., Kim, M., and Kim, C. (1999). Effect of additives on the physicochemical properties of liquid suppository bases. *Int. J. Pharm.* 190, 13–19.

Cook, M.T., and Brown, M.B. (2018). Polymeric gels for intravaginal drug delivery. *J. Controlled Release* 270, 145–157.

Cribby, S., Taylor, M., and Reid, G. (2009). Vaginal microbiota and the use of probiotics. *Interdiscip. Perspect. Infect. Dis.* 2008, e256490.

Das, N., Madan, P., and Lin, S. (2010). Development and *in vitro* evaluation of insulin-loaded buccal Pluronic F-127 gels. *Pharm. Dev. Technol.* 15, 192–208.

Derakhshandeh, K., Fashi, M., and Seifoleslami, S. (2010). Thermosensitive pluronic® hydrogel: prolonged injectable formulation for drug abuse. *Drug Des. Devel. Ther.* 4, 255–262.

Deshkar, S.S., Patil, A.T., and Poddar, S.S. (2015). Development of thermosensitive gel of fluconazole for vaginal candidiasis. *Int. J. Pharm. Pharm. Sci.* 8, 391–398.

Dimitrova, E., Bogdanova, S., Mitcheva, M., Tanev, I., and Minkov, E. (2000). Development of model aqueous ophthalmic solution of indomethacin. *Drug Dev. Ind. Pharm.* 26, 1297–1301.

Dols, J.A.M., Reid, G., Kort, R., Schuren, F.H.J., Tempelman, H., Bontekoe, T.R., Korporaal, H., Van der Veer, E.M., Smit, P.W., and Boon, M.E. (2012). PCR-based identification of eight *Lactobacillus* species and 18 hr-HPV genotypes in fixed cervical samples of South African women at risk of HIV and BV. *Diagn. Cytopathol.* 40, 472–477.

Donta, B., Begum, S., and Naik, D.D. (2014). Acceptability of male condom: an Indian scenario., Acceptability of male condom: An Indian scenario. *Indian J. Med. Res. Indian J. Med. Res.* 140 *Suppl*, 140, S152, S152-6.

Dumortier, G., Grossiord, J.L., Agnely, F., and Chaumeil, J.C. (2006). A review of poloxamer 407 pharmaceutical and pharmacological characteristics. *Pharm. Res.* 23, 2709–2728.

El-Kamel, A., Sokar, M., Naggar, V., and Al Gamal, S. (2002). Chitosan and sodium alginate-based bioadhesive vaginal tablets. *AAPS PharmSci* 4, E44.

Fang, J.-Y., Hsu, S.-H., Leu, Y.-L., and Hu, J.-W. (2009). Delivery of cisplatin from Pluronic co-polymer systems: liposome inclusion and alginate coupling. *J. Biomater. Sci. Polym. Ed.* 20, 1031–1047.

Farage, M., Miller, K., and Sobel, J. (2010). Dynamics of the vaginal ecosystem—hormonal influences. *Infect. Dis. Res. Treat.* 3, 1–15.

Ferreira, N.N., Perez, T.A., Pedreiro, L.N., Prezotti, F.G., Boni, F.I., Cardoso, V.M. de O., Venâncio, T., and Gremião, M.P.D. (2017). A novel pH-responsive hydrogel-based on calcium alginate engineered by the previous formation of polyelectrolyte complexes (PECs) intended to vaginal administration. *Drug Dev. Ind. Pharm.* 43, 1656–1668.

Ferreira Almeida, P., and Almeida, A.J. (2004). Cross-linked alginate-gelatine beads: a new matrix for controlled release of pindolol. *J. Control. Release Off. J. Control. Release Soc.* 97, 431–439.

Frank, D.N., Manigart, O., Leroy, V., Meda, N., Valéa, D., Zhang, W., Dabis, F., Pace, N.R., Van de Perre, P., and Janoff, E.N. (2012). Altered vaginal microbiota are associated with perinatal mother-to-child transmission of HIV in African women from Burkina Faso. *J. Acquir. Immune Defic. Syndr.* 1999 60, 299–306.

Funami, T., Fang, Y., Noda, S., Ishihara, S., Nakauma, M., Draget, K.I., Nishinari, K., and Phillips, G.O. (2009). Rheological properties of sodium alginate in an aqueous system during gelation in relation to supermolecular structures and Ca<sup>2+</sup> binding. *Food Hydrocoll.* 23, 1746–1755.

Gao, W., Weng, J., Gao, Y., and Chen, X. (2013). Comparison of the vaginal microbiota diversity of women with and without human papillomavirus infection: a cross-sectional study. *BMC Infect. Dis.* 13, 271.

Gioffredi, E., Boffito, M., Calzone, S., Giannitelli, S.M., Rainer, A., Trombetta, M., Mozetic, P., and Chiono, V. (2016). Pluronic F127 hydrogel characterization and biofabrication in cellularized constructs for tissue engineering applications. *Procedia CIRP* 49, 125–132.

Gordon, S.N., Kines, R.C., Kutsyna, G., Ma, Z.-M., Hryniewicz, A., Roberts, J.N., Fenizia, C., Hidajat, R., Brocca-Cofano, E., Cuburu, N., et al. (2012). Targeting the vaginal mucosa with human papillomavirus pseudovirion vaccines delivering SIV DNA. *J. Immunol. Baltim. Md 1950* 188, 714–723.

Grasdalen, H., Larsen, B., and Smisrod, O. (1981). <sup>13</sup>C-n.m.r. studies of monomeric composition and sequence in alginate. *Carbohydr. Res.* 89, 179–191.

Grassi, G., Crevatin, A., Farra, R., Guarnieri, G., Pascotto, A., Rehimers, B., Lapasin, R., and Grassi, M. (2006). Rheological properties of aqueous pluronic-alginate systems containing liposomes. *J. Colloid Interface Sci.* 301, 282–290.

Hom, W.L., and Bhatia, S.R. (2017). Significant enhancement of elasticity in alginate-clay nanocomposite hydrogels with PEO-PPO-PEO copolymers. *Polymer* 109, 170–175.

Hsu, S.-H., Leu, Y.-L., Hu, J.-W., and Fang, J.-Y. (2009). Physicochemical characterization and drug release of thermosensitive hydrogels composed of a hyaluronic acid/pluronic f127 graft. *Chem. Pharm. Bull. (Tokyo)* 57, 453–458.

Huang, L., Shen, M., Li, R., Zhang, X., Sun, Y., Gao, P., Fu, H., Liu, H., He, Y., Du, Y., et al. (2016). Thermo-sensitive composite hydrogels based on poloxamer 407 and alginate and their therapeutic effect in embolization in rabbit VX2 liver tumors. *Oncotarget* 7, 73280.

Hummelen, R., Fernandes, A.D., Macklaim, J.M., Dickson, R.J., Chagalucha, J., Gloor, G.B., and Reid, G. (2010). Deep sequencing of the vaginal microbiota of women with HIV. *PLOS ONE* 5, e12078.

Hussain, A., and Ahsan, F. (2005). The Vagina as a route for the systemic drug delivery. *J. Controlled Release* 103, 301–313.

Hütt, P., Lapp, E., Štšepetova, J., Smidt, I., Taelma, H., Borovkova, N., Oopkaup, H., Ahelik, A., Rööp, T., Hoidmets, D., et al. (2016). Characterisation of probiotic properties in human vaginal *Lactobacilli* strains. *Microb. Ecol. Health Dis.* 27.

Karavana, S.Y., Rençbe, S., Şenyiğit, Z.A., and Baloğlu, E. (2012). A new in-situ gel formulation of itraconazole for vaginal administration. *Pharmacol. Amp Pharm.* 03, 417.

Khattak, S.F., Bhatia, S.R., and Roberts, S.C. (2005). Pluronic F127 as a cell encapsulation material: utilization of membrane-stabilizing agents. *Tissue Eng.* 11, 974–983.

Kim, J. (2015). An Assessment of the Application of poloxamer/sodium alginate/CaCl<sub>2</sub> mixture after abdominal surgery in dogs: Effects on postoperative adhesion and safety. thesis..

Koffi, A.A., Agnely, F., Ponchel, G., and Grossiord, J.L. (2006). Modulation of the rheological and mucoadhesive properties of thermosensitive poloxamer-based hydrogels intended for the rectal administration of quinine. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 27, 328–335.

Koffi, A.A., Agnely, F., Besnard, M., Kablan Brou, J., Grossiord, J.L., and Ponchel, G. (2008). In vitro and in vivo characteristics of a thermogelling and bioadhesive delivery system intended for rectal administration of quinine in children. *Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Pharm. Verfahrenstechnik EV* 69, 167–175.

Lee, K.Y., and Mooney, D. (2012). Alginate: properties and biomedical applications. *Prog Polym Sci* 37, 106–126.

Lee, J.E., Lee, S., Lee, H., Song, Y.-M., Lee, K., Han, M.J., Sung, J., and Ko, G. (2013). Association of the vaginal microbiota with human Papillomavirus infection in a Korean twin cohort. *PLOS ONE* 8, e63514.

Lenaerts, V., Triqueneaux, C., Quartern, M., Rieg-Falson, F., and Couvreur, P. (1987). Temperature-dependent rheological behavior of Pluronic F-127 aqueous solutions. *Int. J. Pharm.* 39, 121–127.

Lin, H.-R., Sung, K.C., and Vong, W.-J. (2004). In situ gelling of alginate/pluronic solutions for ophthalmic delivery of pilocarpine. *Biomacromolecules* 5, 2358–2365.

Liu, Y., Zhu, Y., Wei, G., and Lu, W. (2009). Effect of carrageenan on poloxamer-based in situ gel for vaginal use: Improved in vitro and in vivo sustained-release properties. *Eur. J. Pharm. Sci.* 37, 306–312.

Macharia, A.G., Kombe, Y., Mwaniki, P., and Habtu, M. (2017). Barriers of Condom Use among HIV Positive Women at Thika Level 5 Hospital, Kenya. *J. AIDS Clin. Res.* 8, 1–4.

Mahlalela, N.B., and Maharaj, P. (2015). Factors facilitating and inhibiting the use of female condoms among female university students in Durban, KwaZulu–Natal, South Africa. *Eur. J. Contracept. Reprod. Health Care* 20, 379–386.

Maisani, M. (2017). Conception et développement d'hydrogels pour l'ingénierie tissulaire appliquée au tissu osseux (Bordeaux).

Malcolm, R.K., Edwards, K.-L., Kiser, P., Romano, J., and Smith, T.J. (2010). Advances in microbicide vaginal rings. *Antiviral Res.* 88, S30–S39.

Martin, H.L., Richardson, B.A., Nyange, P.M., Lavreys, L., Hillier, S.L., Chohan, B., Mandaliya, K., Ndinya-Achola, J.O., Bwayo, J., and Kreiss, J. (1999). Vaginal *Lactobacilli*,

Microbial Flora, and Risk of Human Immunodeficiency Virus Type 1 and Sexually Transmitted Disease Acquisition. *J. Infect. Dis.* 180, 1863–1868.

Martín-Villena, M.J., Fernández-Campos, F., Calpena-Campmany, A.C., Bozal-de Febrer, N., Ruiz-Martínez, M.A., and Clares-Naveros, B. (2013). Novel microparticulate systems for the vaginal delivery of nystatin: Development and characterization. *Carbohydr. Polym.* 94, 1–11.

Matson, P.A., Adler, N.E., Millstein, S.G., Tschann, J.M., and Ellen, J.M. (2011). Developmental changes in condom use among urban adolescent females: Influence of partner context. *J. Adolesc. Health* 48, 386–390.

Menchicchi, B., Fuenzalida, J.P., Hensel, A., Swamy, M.J., David, L., Rochas, C., and Goycoolea, F.M. (2015). Biophysical analysis of the molecular interactions between polysaccharides and mucin.

Mishra, R., Joshi, P., and Mehta, T. (2016). Formulation, development and characterization of mucoadhesive film for treatment of vaginal candidiasis. *Int. J. Pharm. Investig.* 6, 47.

Misra, A. (2014). *Applications of Polymers in Drug Delivery* (Smithers Rapra).

Mitchell, C., Moreira, C., Fredricks, D., Paul, K., Caliendo, A.M., Kurpewski, J., Ingersoll, J., and Cu-Uvin, S. (2009). Detection of fastidious vaginal bacteria in women with HIV infection and bacterial vaginosis. *Infect. Dis. Obstet. Gynecol.* 2009, 236919.

Mitchell, C., Fredricks, D., Winer, R., and Koutsky, L. (2012). Effect of sexual debut on vaginal microbiota in a cohort of young women. *Obstet Gynecol* 120, 1306–1313.

Mitchell, C., Balkus, J.E., Fredricks, D., Liu, C., McKernan-Mullin, J., Frenkel, L.M., Mwachari, C., Luque, A., Cohn, S.E., Cohen, C.R., et al. (2013). Interaction between lactobacilli, bacterial vaginosis-associated bacteria, and HIV Type 1 RNA and DNA genital shedding in U.S. and Kenyan Women. *AIDS Res. Hum. Retroviruses* 29, 13–19.

Moebus, K., Siepmann, J., and Bodmeier, R. (2009). Alginate–poloxamer microparticles for controlled drug delivery to mucosal tissue. *Eur. J. Pharm. Biopharm.* 72, 42–53.

Mortensen, K., and Pedersen, J.S. (1993). Structural study on the micelle formation of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer in aqueous solution. *Macromolecules* 26, 805–812.

Nardini, P., Ñahui Palomino, R.A., Parolin, C., Laghi, L., Foschi, C., Cevenini, R., Vitali, B., and Marangoni, A. (2016). *Lactobacillus crispatus* inhibits the infectivity of *Chlamydia trachomatis* elementary bodies, *in vitro* study. *Sci. Rep.* 6, 29024.

Nunn, K.L., Wang, Y.-Y., Harit, D., Humphrys, M.S., Ma, B., Cone, R., Ravel, J., and Lai, S.K. (2015). Enhanced trapping of HIV-1 by human cervicovaginal mucus is associated with *Lactobacillus crispatus*-dominant microbiota. *MBio* 6, e01084-15.

Owen, D.H., and Katz, D.F. (1999). A vaginal fluid simulant. *Contraception* 59, 91–95.

Parhi, R., and Suresh, P. (2015). Alginate-poloxamer beads for controlled release of metoprolol succinate. *Turk. J. Pharm. Sci.* 12, 59–66.

Patil, S.B., and Sawant, K.K. (2009). Development, optimization and *in vitro* evaluation of alginate mucoadhesive microspheres of carvedilol for nasal delivery. *J. Microencapsul.* 26, 432–443.

Pavlova, S.I., Kilic, A.O., Kilic, S.S., So, J.-S., Nader-Macias, M.E., Simoes, J.A., and Tao, L. (2002). Genetic diversity of vaginal *Lactobacilli* from women in different countries based on 16S rRNA gene sequences. *J. Appl. Microbiol.* 92, 451–459.

Pharmacopée Européenne (2018). Poloxamères (Strasbourg, France.).

Pilcher, H. (2004). Starting to gel. *Nature* 430, 138–140.

Potts, M., Halperin, D.T., Kirby, D., Swidler, A., Marseille, E., Klausner, J.D., Hearst, N., Wamai, R.G., Kahn, J.G., and Walsh, J. (2008). Public health. Reassessing HIV prevention. *Science* 320, 749–750.

Qin, Y. (2008). Alginate fibres: an overview of the production processes and applications in wound management. *Polym. Int.* 57, 171–180.

Quah, S.P., Smith, A.J., Preston, A.N., Laughlin, S.T., and Bhatia, S.R. (2018). Large-area alginate/PEO-PPO-PEO hydrogels with thermoreversible rheology at physiological temperatures. *Polymer* 135, 171–177.

Rai, D., Maniruzzaman, M., and Boateng, J.S. (2010). Development and characterisation of sodium alginate and HPMC films for mucosal drug delivery. *Int. J. Biotechnol.*

Ravel, J., and Brotman, R.M. (2016). Translating the vaginal microbiome: gaps and challenges. *Genome Med.* 8, 35.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., Stacey, L.M., Karlebach, shara, Gorle, R., Russell, J., Carol, O.T., et al. (2011). Vaginal microbi -. *Proc Natl Acad Sci U A* 108, 4680–4687.

Rençber, S., Karavana, S.Y., Şenyiğit, Z.A., Eraç, B., Limoncu, M.H., and Baloğlu, E. (2017). Mucoadhesive in situ gel formulation for vaginal delivery of clotrimazole: formulation, preparation, and in vitro/in vivo evaluation. *Pharm. Dev. Technol.* 22, 551–561.

Ricci, E.J., Lunardi, L.O., Nanclares, D.M.A., and Marchetti, J.M. (2005). Sustained release of lidocaine from Poloxamer 407 gels. *Int. J. Pharm.* 288, 235–244.

Richardson, J., and Illum, L. (1992). The vaginal route of peptide and protein drug delivery. *Adv Drug Deliv Rev* 8, 341–366.

Ryu, W.Y., Jung, H.M., Roh, M.S., Kwon, Y.H., Jeung, W.J., Park, W.C., Rho, S.H., and Ahn, H.B. (2013). The effect of a temperature-sensitive poloxamer-alginate-CaCl<sub>2</sub> mixture after strabismus surgery in a rabbit model. *J. Am. Assoc. Pediatr. Ophthalmol. Strabismus JAAPOS* 17, 484–489.

Sahay, S., Deshpande, S., Bembalkar, S., Kharat, M., Parkhe, A., Brahme, R.G., Paranjape, R., Bollinger, R.C., and Mehendale, S.M. (2015). Failure to use and sustain male condom usage: lessons learned from a prospective study among men attending STI Clinic in Pune, India. *PLOS ONE* 10, e0135071.

Sarkar, N.N. (2008). Barriers to condom use. *Eur J Contracept Reprod Health Care* 13, 114–122.

Schellenberg, J.J., Links, M.G., Hill, J.E., Dumonceaux, T.J., Kimani, J., Jaoko, W., Wachihi, C., Mungai, J.N., Peters, G.A., Tyler, S., et al. (2011). Molecular definition of vaginal microbiota in East African commercial sex workers. *Appl. Environ. Microbiol.* 77, 4066–4074.

Sengupta, A., Dwivedi, N., Kelly, S.C., Tucci, L., Thadhani, N.N., and Prausnitz, M.R. (2015). Poloxamer surfactant preserves cell viability during photoacoustic delivery of molecules into cells. *Biotechnol. Bioeng.* *112*, 405–415.

Sewankambo, N., Gray, R.H., Wawer, M.J., Paxton, L., McNaim, D., Wabwire-Mangen, F., Serwadda, D., Li, C., Kiwanuka, N., Hillier, S.L., et al. (1997). HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. *Lancet* *350*, 546–550.

Singh-Joy, S.D., and McLain, V.C. (2008). Safety assessment of poloxamers 101, 105, 108, 122, 123, 124, 181, 182, 183, 184, 185, 188, 212, 215, 217, 231, 234, 235, 237, 238, 282, 284, 288, 331, 333, 334, 335, 338, 401, 402, 403, and 407, poloxamer 105 benzoate, and poloxamer 182 dibenzoate as used in cosmetics. *Int. J. Toxicol.* *27 Suppl 2*, 93–128.

Sirviö, J.A., Kolehmainen, A., Liimatainen, H., Niinimäki, J., and Hormi, O.E.O. (2014). Biocomposite cellulose-alginate films: promising packaging materials. *Food Chem.* *151*, 343–351.

Smidsrød, O., and Skjåk-Braek, G. (1990). Alginate as immobilization matrix for cells. *Trends Biotechnol.* *8*, 71–78.

Smith, B.C., McAndrew, T., Chen, Z., Harari, A., Barris, D.M., Viswanathan, S., Rodriguez, A.C., Castle, P., Herrero, R., Schiffman, M., et al. (2012). The cervical microbiome over 7 years and a comparison of methodologies for its characterization. *PLoS One* *7*, e40425.

Spear, G., Gilbert, D., Landay, A., Zariffard, R., French, A., Patel, P., and al (2011). Pyrosequencing of the genital microbiotas of HIV-seropositive and -seronegative women reveals *Lactobacillus iners* as the predominant *Lactobacillus* species. *Appl Env. Microbiol* *77*, 378–381.

Spear, G.T., Sikaroodi, M., Zariffard, M.R., Landay, A.L., French, A.L., and Gillevet, P.M. (2008). Comparison of the diversity of the vaginal microbiota in HIV-infected and HIV-uninfected women with or without bacterial vaginosis. *J. Infect. Dis.* *198*, 1131–1140.

Su, Y.-L., Wei, X.-F., and Liu, H.-Z. (2003). Effect of sodium chloride on association behavior of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer in aqueous solutions. *J. Colloid Interface Sci.* *264*, 526–531.

Suntornnond, R., Tan, E.Y.S., An, J., and Chua, C.K. (2017). A highly printable and biocompatible hydrogel composite for direct printing of soft and perfusable vasculature-like structures. *Sci. Rep.* *7*, 16902.

Szekalska, M., Winnicka, K., Czajkowska-Kośnik, A., Sosnowska, K., and Amelian, A. (2015). Evaluation of alginate microspheres with metronidazole obtained by the spray drying technique. *Acta Pol. Pharm.* *72*, 569–578.

Tafari, S., Martinelli, D., Germinario, C., and Prato, R. (2010). Determining factors for condom use: A survey of young Italian adults. *Eur. J. Contracept. Reprod. Health Care* *15*, 24–30.

Tuçcu-Demiröz, F., Acartürk, F., and Erdoğan, D. (2013). Development of long-acting bioadhesive vaginal gels of oxybutynin: formulation, in vitro and in vivo evaluations. *Int. J. Pharm.* *457*, 25–39.

UNFPA, OMS, and ONUSIDA (2015). Déclaration de position sur les préservatifs et la prévention du VIH, des autres infections sexuellement transmissibles et des grossesses non désirées.

Ur-Rehman, T., Tavelin, S., and Gröbner, G. (2011). Chitosan in situ gelation for improved drug loading and retention in poloxamer 407 gels. *Int. J. Pharm.* *409*, 19–29.

Vadnere, M., and Amidon, G. (1984). Thermodynamic studies on the gel - sol transition of some pluronic polyols. *Int. J. Pharm.* *207* – 218.

Vasquez, A., Jakobsson, T., Ahrne, S., Forsum, U., and Molin, G. (2002). Vagina. I Lactobacillus flora of healthy Swedish women. *J Clin Microbiol* *40*, 2746–2749.

Veyries, M.-L., Faurisson, F., Joly-Guillou, M.-L., and Rouveix, B. (2000). Control of Staphylococcal Adhesion to Polymethylmethacrylate and Enhancement of Susceptibility to Antibiotics by Poloxamer 407. *Antimicrob. Agents Chemother.* *44*, 1093–1096.

Vidal (2017). *Le Qictionnaire* (Paris: Vidal Eds).

Vielfort, K., Sjölander, H., Roos, S., Jonsson, H., and Aro, H. (2008). Adherence of clinically isolated lactobacilli to human cervical cells in competition with *Neisseria gonorrhoeae*. *Microbes Infect.* *10*, 1325–1334.

Wagner, M.A., Marks, W.H., and Bhatia, S.K. (2014). Hydrogel Encapsulation to Improve Cell Viability during Syringe Needle Flow. *J. Long. Term Eff. Med. Implants* *24*.

Wang, Y., Jiang, S., Wang, H., and Bie, H. (2017). A mucoadhesive, thermoreversible in situ nasal gel of geniposide for neurodegenerative diseases. *PLOS ONE* *12*, e0189478.

Wang, Z.-Y., Zhang, Q.-Z., Konno, M., and Saito, S. (1993). Sol–gel transition of alginate solution by the addition of various divalent cations: <sup>13</sup>C-nmr spectroscopic study. *Biopolymers* *33*, 703–711.

White, J.C., Saffer, E.M., and Bhatia, S.R. (2013). Alginate/PEO-PPO-PEO composite hydrogels with thermally-active plasticity. *Biomacromolecules* *14*, 4456–4464.

Wiesenfeld, H.C., Hillier, S.L., Krohn, M.A., Landers, D.V., and Sweet, R.L. (2003). Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. *Clin. Infect. Dis.* *36*, 663–668.

Willaert, R. (2011). *Cell Immobilization and Its Applications in Biotechnology*. pp. 313–367.

Witkin, S.S., Linhares, I.M., and Giraldo, P. (2007). Bacterial flora of the female genital tract: function and immune regulation. *Best Pract. Res. Clin. Obstet. Gynaecol.* *21*, 347–354.

Zeng, N., Dumortier, G., Maury, M., Mignet, N., and Boudy, V. (2014). Influence of additives on a thermosensitive hydrogel for buccal delivery of salbutamol: Relation between micellization, gelation, mechanic and release properties. *Int. J. Pharm.* *467*, 70–83.

Zhou, X., Brown, C.J., Abdo, Z., Davis, C.C., Hansmann, M.A., Joyce, P., Foster, J.A., and Forney, L.J. (2007). Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME J.* *1*, 121–133.

Zhou, X., Hansmann, M.A., Davis, C.C., Suzuki, H., Brown, C.J., Schütte, U., Pierson, J.D., and Forney, L.J. (2010). The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. *FEMS Immunol. Med. Microbiol.* *58*, 169–181.

## **Deuxième partie : TRAVAUX EXPERIMENTAUX**





## INTRODUCTION

La revue bibliographique a démontré l'intérêt de *L. crispatus* dans la prévention de la gonococcie et l'intérêt de la voie vaginale pour cette application. Parmi les formes destinées à cette voie, les hydrogels, bien qu'encore peu étudiés pour l'administration des probiotiques, sont apparus comme particulièrement intéressants pour délivrer les lactobacilles. L'objet de cette partie expérimentale a donc été d'étudier l'intérêt de cette approche pour le développement d'une forme vaginale pour la prévention de la gonococcie.

Après une première étape d'optimisation, en faisant varier les concentrations des polymères (poloxamer 407 et alginate de sodium), nous avons pu sélectionner une formulation (**projet d'article, chapitre IV**). Ensuite, nous avons caractérisé *L. crispatus* et nous l'avons incorporé dans cette formulation que nous avons caractérisée de façon plus approfondie par rhéologie et microscopie confocale et dont nous avons évalué l'efficacité *in vitro* sur *N. gonorrhoeae* (**projet d'article, chapitre V**). Enfin, des études complémentaires feront l'objet du **chapitre VI** de ce manuscrit.

Ces études ont été réalisées, d'une part, en conditions mimant les conditions d'administration, et d'autre part, en conditions mimant les conditions physiologiques (37 °C en présence de fluide vaginal simulé). En ce qui concerne les conditions d'administration, la température ambiante est de 19-20 °C en France et en Europe tandis qu'elle est de 25-30 °C en Côte d'Ivoire et en Afrique subsaharienne. Nous avons choisi les conditions ambiantes de la Côte d'Ivoire afin de mieux apprécier les propriétés des gels car le poloxamer étant thermosensible, s'il est possible d'administrer les gels dans ces conditions, il sera aussi possible de les administrer à des températures plus basses.



**CHAPITRE IV : DEVELOPPEMENT ET  
CARACTERISATION D'UN HYDROGEL A BASE DE  
POLOXAMER 407 ET D'ALGINATE DE SODIUM DESTINE  
A UNE ADMINISTRATION VAGINALE**



## Introduction- résumé

Le but de cette étude était de concevoir et de caractériser un hydrogel à base de poloxamer 407 et d'alginate de sodium comme une plateforme destinée à l'administration par la voie vaginale de produits préventifs ou curatifs. L'alginate de sodium a été choisi pour ses propriétés muco-adhésives et sa capacité à donner un gel ionique. Le poloxamer, quant à lui, a été sélectionné pour ses propriétés thermogélifiantes. La masse molaire, la conformation et le ratio M/G de l'alginate ont été préalablement déterminés. Différents mélanges de polymères ont été préparés. Leurs propriétés en écoulement, leur température de gélification, leurs propriétés viscoélastiques, leur capacité de gélification ionique ont été évaluées. Les formulations ont été caractérisées à 25 ° C pour mimer les conditions d'administration et à 37 °C, après dilution avec le fluide vaginal simulé, afin de mimer les conditions physiologiques. Cette étude a permis d'améliorer les connaissances sur les mélanges de poloxamer 407 et d'alginate de sodium. Elle a également permis de sélectionner un gel contenant 21,5 % de poloxamer 407 et 1 % d'alginate qui présentait des propriétés mécaniques adéquates pour assurer une administration vaginale et était résistant à la dilution par les sécrétions vaginales. Ce gel est destiné à être auto-administré par la femme grâce à un dispositif d'administration tel que décrit dans la **Figure 1** du **chapitre. 3**, mais en première intention, nous avons développé un test d'expulsion mené avec une seringue telle quelle sans aiguille ni canule pour simuler un tel dispositif d'administration.



## PROJET DE PUBLICATION :

# INVESTIGATION OF A GEL CONTAINING POLOXAMER 407 AND SODIUM ALGINATE AS A PLATFORM FOR VAGINAL ADMINISTRATION

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### Abstract

This study aimed to design a handy and easy-to-use hydrogel-based delivery system as a platform for the administration of relevant substances into the vagina. For this hydrogel, sodium alginate, a natural anionic mucoadhesive polymer and poloxamer 407, a temperature-sensitive polymer were selected. <sup>1</sup>H NMR spectroscopy and SEC-MALLS experiments were performed to characterize alginate. Then, alginate solutions (0.5-2 % w/w), poloxamer 407 solutions (16-21.5 % w/w) and their mixtures were prepared. Their flow properties, gelation temperature, viscoelastic properties, ionic gelation ability, administration ability were



assessed. This commercial alginate had a mannuronate-to-guluronate ratio of 1.24, a weight-average molecular weight of  $300\,000 \pm 2\%$  g.mol<sup>-1</sup>, a polydispersity index of  $1.8 \pm 3\%$ . Alginate solutions displayed a viscous behavior, and were not appropriate for vaginal administration when used alone. Poloxamer solutions and their mixtures with alginate were temperature-sensitive, shear-thinning, non-thixotropic and had an elastic behavior suitable for a vaginal administration. The gel containing 21.5 % of poloxamer 407 and 1 % of sodium alginate seemed promising due to (i) its low resistance to expulsion from a device and suitability for a self-administration, (ii) its rheological properties and its resistance to dilution with vaginal fluid, favorable for *in situ* gelation and prolonged vaginal residence.

### **Keywords**

Gel, poloxamer 407, rheology, sodium alginate, temperature-sensitive, vaginal fluid, viscoelastic properties.

### **Abbreviations**

A, sodium alginate; G', elastic modulus; G'', viscous modulus; P, poloxamer 407; SVF, simulated vaginal fluid, T<sub>gel</sub>, gelation temperature.

## 1. INTRODUCTION

The reduced effectiveness of antibiotic-based treatments due to antibiotic resistance calls for novel strategies for the prevention of genitourinary infections in women. As the vagina is the key route of contamination, the administration of products with effectiveness on the early stages of infections could be valuable. Therefore, researches have been focused on vaginal delivery systems (Chien and Lee, 2002; Choudhury et al., 2011; Cook and Brown, 2018). There are a variety of polymeric matrices that has been investigated for the vaginal administration, using different dosage forms (Cook and Brown, 2018; Mastromarino et al., 2002; Misra, 2014). Among them, hydrogel-based delivery systems and particularly thermo-responsive hydrogels seem attractive. Indeed, thermo-responsive hydrogels can be liquid at storage temperature and be easily administered as a liquid, whereas the gelation occurs at physiological temperature. Once gelled, such formulation has a high resistance to flow, which prolongs its residence time at the site of administration. Poloxamer 407, a temperature-sensitive amphiphilic triblock copolymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), has been widely investigated in the development of temperature-sensitive gels, thanks to its ability to undergo reversible thermal gelation (Charrueau et al., 2001; Dumortier et al., 2006). It is already widely used in the pharmaceutical field and is considered as a safe excipient. Poloxamer 407 is often mixed with a mucoadhesive polymer which may increase its adhesion to the mucus, so its residence time at the administration site (Aka-Any-Grah et al., 2010). In the literature, several mucoadhesive polymers have been studied for this purpose (Acarturk, 2009). A great interest was devoted to naturally occurring polysaccharides such as alginate due to their biocompatibility, biodegradability and non-toxicity (Lee and Mooney, 2012). Alginate is an anionic linear polysaccharide extracted from different brown seaweed (*Phaeophyceae*), consisting of (1-4)-linked blocks of poly- $\beta$ -D-mannuronic acid (M) and poly- $\alpha$ -L-guluronic acid (G) residues. Its M/G ratio as well as the arrangement of these residues can vary depending on its source (Grasdalen et al., 1981). Its physical properties is determined by its mannuronate-to-guluronate ratio (M/G) ratio, its sequences and its molecular weight. Sodium alginate can form mucoadhesive gels in the presence of divalent cations such as  $\text{Ca}^{2+}$ , which bind to G units to form an “egg-box” structure. Both poloxamer 407 and sodium alginate may be combined to obtain a solution with *in situ* gelation capacities (Lin et al., 2004), may be gelled with calcium chloride to obtain beads or capsules (Moebus et al., 2009; Parhi and Suresh, 2015), gelled by CaEDTA and glucono- $\delta$ -lactone to obtain hydrogels (Grassi et al., 2006; Hom and Bhatia, 2017; White

et al., 2013), or gelled both thermally and ionically to obtain a film (Barba et al., 2014; Quah et al., 2018). Both polymers may be mixed together or alginate may be grafted onto poloxamer (Chen et al., 2011).

The present study aims to develop a gel intended for an administration into the vagina by the women themselves. This gel should have optimized flow properties, gelation temperature and viscoelastic properties associated with low hardness and low expulsion force from a device to ensure its easy administration, and should maintain its gelation at 37 °C in the presence of vaginal fluid. The *in vivo* studies conducted by Owen et al. (2000) showed that shear rates in the vagina are due to movements of the vaginal epithelial surfaces, gravity and capillary flow. They are approximately 0.1 s<sup>-1</sup> at the time of initial spreading and can increase up to 100 s<sup>-1</sup> due to coitus. Mixtures of poloxamer 407 and sodium alginate were prepared by varying their concentrations in order to improve their mechanical properties to withstand these shear forces into the vagina and dilution with the vaginal fluid. The formulations were first investigated to ensure both thermal gelation with poloxamer 407 and ionic gelation with alginate. Then, they were characterized by rheological studies (*i*) at 25 °C to mimic the administration conditions, and (*ii*) at 37 °C, after dilution with the simulated vaginal fluid (SVF) in order to mimic the body conditions. Their ability to be easily expelled from a device and their textural profile were also assessed.

## **2. MATERIALS AND METHODS**

### **2.1. Materials**

Poloxamer 407 of pharmaceutical grade (Lutrol<sup>®</sup> F127) was purchased from BASF (Germany). According to the supplier, its average molecular weight (Mw) was 11774 g.mol<sup>-1</sup> (9840-14600 g.mol<sup>-1</sup>). Sodium alginate was purchased from Roth (France), lot N° 351172083. Bovine serum albumin and all other reagents were supplied by Sigma-Aldrich (France) and were of analytical grade. Sterile ultrapure water (18.2 MΩ, MilliQ, Millipore, France) was used.

### **2.2. Characterization of sodium alginate**

The molecular weight of sodium alginate was determined using size-exclusion chromatography (SEC) coupled online with multiangle light scattering (MALLS). The whole

line was equipped with a degasser (DGU-20A3, Shimadzu, Japan), a pump (LC10Ai, Shimadzu, Japan) with a flow rate of  $0.5 \text{ mL min}^{-1}$  (the eluent was  $0.1 \mu\text{m}$  filtered  $0.1 \text{ mol.L}^{-1}$   $\text{LiNO}_3$ ), an automatic injector (SIL-20A, Shimadzu, Japan), a stainless steel pre-filter ( $0.45 \mu\text{m}$ ), one pre-column (OHPAK SB-G), two analytical size-exclusion columns (OHPAK SB 804 HQ and 806 HQ), and three detectors: (i) a multi-angle light scattering (MALLS) detector (Dawn EOS, Wyatt Technology, CA, U.S.A.) fitted with a K5 cell of  $50 \mu\text{L}$ , with a red source (Ga-As 690 nm) of mW and 18 photodiodes (angles distributed around the cell), (ii) a differential refractive index (DRI) detector (RID 10A, Shimadzu, Japan), and (iii) a differential viscosimeter detector (Viscostar II, Wyatt Technology, CA, U.S.A.). The Astra 6.0.6.13 software package was set to collect and extrapolate data according to the known value of the differential refractive index,  $\text{dn/dC}$  ( $\text{dn/dC} = 0.14 \text{ mL.g}^{-1}$ ) (Colinet et al., 2009). The sodium alginate M/G ratio was determined by  $^1\text{H}$  NMR spectroscopy. It was performed in a 5 mm diameter tube at  $80 \text{ }^\circ\text{C}$ , on a Bruker Avance 400 spectrometer at 400 MHz. The chemical shift scale was calibrated on the basis of the solvent ( $\text{D}_2\text{O}$ ) peak. The acquisition time was 1 s and the number of scans was 128. The sample was previously partially hydrolyzed, freeze-dried, dissolved in  $\text{D}_2\text{O}$  and freeze-dried to replace exchangeable protons with deuterium, and finally dissolved in  $\text{D}_2\text{O}$  at  $10 \text{ mg/mL}$  for measurements as described by Jensen et al. (2015).

### 2.3. Hydrogel preparation

Hydrogels containing different ratios of poloxamer 407 (16-21.5 % w/w) and/or sodium alginate (0.5-2 % w/w) were prepared. Polymer solubilization was performed by using a mixer equipped with a turbine adapted to the mixing of viscous preparations (Rayneri-turbotest, Rayneri, France) under an agitation of 1000 rpm (Aka-Any-Grah et al., 2010). Sodium alginate solutions were prepared by progressive solubilization of the alginate powder in ultrapure water at room temperature. The poloxamer powder was gradually added either in ultrapure water or in alginate solution at  $4 \text{ }^\circ\text{C}$ . After complete dissolution, each formulation was equilibrated 48 h at  $4^\circ\text{C}$  to eliminate foam and air bubbles and was autoclaved ( $121 \text{ }^\circ\text{C}$ , 15 min). The compositions of the different formulations are summarized in **Table 1**.

**Table 1.** Formulations developed with alginate (A) and poloxamer (P)

Denomination	Alginate (% w/w)	Poloxamer (% w/w)
A0.5	0.5	
A1	1	
A1.5	1.5	
A2	2	
P16		16
P18.5		18.5
P21.5		21.5
P21.5A1	1	21.5

#### 2.4. Hydrogels diluted by simulated vaginal fluid

Vaginal fluid is composed by fluids that originate from uterus, cervix, and sometimes menstrual secretions and sperm (Owen and Katz, 1999). However, the human vaginal fluid quantity is limited, and it degrades rapidly once collected from its source. Thus, Owen et Katz (1999) had developed a simulated vaginal fluid (SVF) in order to test vaginal pharmaceutical forms. SVF was prepared by adding NaCl (3.51 g), KOH (1.4 g), Ca(OH)<sub>2</sub> (0.22 g), bovine serum albumin (0.018 g), lactic acid (2.00 g), acetic acid (1.00 g), glycerol (0.16 g), urea (0.4 g), and glucose (5.00 g) to 900 mL of ultrapure water. Their mixture was stirred mechanically until complete dissolution. The pH of the mixture was then adjusted to 4.5 using HCl 1N. The final volume was adjusted with water to 1 L. In this study, to mimic the maximum dilution of gels that was likely to occur after vaginal administration, 5 mL of each formulation were mixed with 0.75 mL of SVF by vortexing. The aim of this test was to assess the influence of the dilution on the thermogelation and the rheological properties of the formulations.

#### 2.5. Alginate ionic gelling capacity

The evaluation of ion activated gelation of alginate was performed in the presence of the calcium ions of SVF in order to identify the formulations suitable for use as *in situ* gelling systems. Gelling capacity of the formulations was determined by mixing the 5 g of formulations with 0.75 mL of SVF. Then, calcium carbonate was introduced into formulations at 0.03 and 0.05 w/w %. The gelation was assessed by the tube inversion method (Ur-Rehman et al., 2011).

## 2.6. Rheological characterization

Rheological studies aimed to determine the flow properties, the gelation temperatures and viscoelastic properties of the formulations. All the measurements were carried out on a rotational rheometer ARG2 (TA instruments, New Castle, USA). The geometry was an aluminum cone/plate (diameter 40 mm, angle 1° and cone truncation 28 μm) equipped with a solvent trap to limit evaporation during measurement. Temperature was controlled by Peltier diodes placed in the lower plate. Experiments were started after a 3-minute equilibration time. Three experimental conditions were investigated: 25 °C, 37 °C and 37 °C after dilution with SVF.

Regarding flow properties, the viscosity was recorded as a function of shear rate. The shear rate was increased gradually from 0.01 s<sup>-1</sup> to 1000 s<sup>-1</sup> over 3 min, maintain to 1000 s<sup>-1</sup> for 1 min and gradually decreased from 1000 s<sup>-1</sup> to 0.01 s<sup>-1</sup> over 3 min.

For oscillatory measurements, linear viscoelastic regions, where the elastic modulus (G') and the viscous modulus (G'') remained constant with stress were determined. The oscillatory measurements were then performed under a stress value belonging to the linear viscoelastic regime. G' (Eq. 1) is related to the elasticity or the energy stored and recovered in the material per deformation cycle, whereas G'' (Eq. 2) describes the viscous character or the energy dissipated during the cycle. The elastic modulus reflects the solid-like component of elastic behavior. The higher the G' value, the more pronounced the elastic character and conversely, the higher G'', the more pronounced the viscous properties.

$$G' = \frac{\tau_0}{\gamma_0} \cos \delta \quad (1)$$

$$G'' = \frac{\tau_0}{\gamma_0} \sin \delta \quad (2)$$

$$\tan \delta = \frac{G''}{G'} \quad (3)$$

$\tau_0$  and  $\gamma_0$  are the maximal amplitudes of the stress and the strain respectively.  $\delta$ , the phase angle, varies between 0° and 45° ( $\tan \delta < 1$ ,  $G' > G''$ ) for a rather elastic material and between 45° and 90° ( $\tan \delta > 1$ ,  $G'' > G'$ ) for a predominantly viscous material (Eq. 3). A temperature sweep from 5 °C to 45 °C was performed at a frequency of 1 Hz, with a heating rate of 1 °C/min. G' and G'' were measured as a function of temperature. This oscillatory experiment allowed to determine the sol–gel transition temperature ( $T_{\text{gel}}$ ) defined as the crossover of G'

and  $G''$  curves. In addition, a frequency sweep was performed from 0.01 to 50 Hz to determine the viscoelastic properties.

All rheological tests were realized in triplicates and results were expressed as mean  $\pm$  standard deviation. TRIOS software was used for data analysis.

## **2.7. Texture Profile Analysis at 25°C**

To predict performance of the formulations during product filling and the ease of removing the formulation from the packaging system, texture profile analysis (TPA) was performed with a TA1 texture analyser (TA instrument, Ametek, France) in TPA mode. 10 g of formulations were transferred into a 40 mL vial, taking care to avoid air bubbles introduction into samples. The samples were stored at 25 °C for 24 h before measurements. A plastic cylindrical analytical probe of uniform 12.7 mm diameter, 120 mm height was forced down into each sample at a defined rate of 1 mm.s<sup>-1</sup> over a defined depth of 10 mm at a temperature of 25 °C. Two cycles of compression separated by a delay period of 20 s were applied. The hardness, the cohesiveness and adhesiveness were determined from the force-time and the force-displacement curves obtained. Five replicates were performed for each sample.

## **2.8. Expulsion evaluation from a vaginal device**

The capacity of the gels to be expelled from a vaginal device was studied. A 5 mL plastic syringe Terumo® with 12 mm cylinder diameter, and a central tip of 3 mm×10 mm diameter×length without needle nor cannula was used to mimic such a vaginal applicator. For the test, a home-made device described previously by Burckbuchler et al. (2010) was coupled to a TA-XT2 Texture Analyzer (Stable MicroSystems, Godalming, UK) in compression mode which was equipped with a force transducer calibrated with a 30 kg sensor. The principle consisted in applying a displacement of 30 mm, at a speed of 2 mm.s<sup>-1</sup>, at 30 °C, to the plunger of the syringe previously filled with the gel and in measuring the resulting maximal expulsion force and the work required to expel formulations. This latter was determined from the area under the force-displacement curve recorded during plunger compression. All measurements were performed on six replicates.

### 3. RESULTS AND DISCUSSION

#### 3.1. Sodium alginate characterization

Characterization of sodium alginate by SEC/MALLS allowed to determine the weight-average molecular weight ( $M_w$ ), the number-average molecular weight ( $M_n$ ), the gyration radius ( $R_g$ ) and the intrinsic viscosity of sodium alginate in  $\text{LiNO}_3$  ( $0.1 \text{ mol.L}^{-1}$ ).

$M_w$  was  $300\,000 \pm 2\% \text{ g.mol}^{-1}$  and  $M_n$  was  $165\,000 \pm 3\% \text{ g.mol}^{-1}$ . The polydispersity index (PI) (**Eq. 4**) was  $1.8 \pm 3\%$ . It indicated a slight polymolecularity of the sodium alginate chains.

$$PI = \frac{M_w}{M_n} \quad (4)$$

In the conditions described above, alginate intrinsic viscosity  $[\eta]$  was  $1200 \pm 0.5\% \text{ mL.g}^{-1}$ . From  $[\eta]$ , the Mark-Houwink-Sakurada equation (**Eq. 5**) allowed to determine the “a” exponent which is related to the polymer conformation (Teraoka, 2002). K and a are constant parameters giving information for a defined alginate–solvent pair. Two “a” values were obtained: 0.6 corresponding to linear flexible expanded alginate, and 1 due to the stiffness of some hydrated chains. These results showed the presence of two types of chain conformations one flexible and one rigid.

$$[\eta] = K \cdot M_v^a \quad (5)$$

The hydrodynamic radius  $R_h$  of sodium alginate was  $36 \pm 1\% \text{ nm}$  determined by SEC/MALLS. From  $R_h$ , the radius of giration  $R_g$  was estimated according to **Eq. 6** (Teraoka, 2002) and was about 60 nm.

$$R_g \approx \frac{R_h}{0.6} \quad (6)$$

$R_g$  was used to calculate the critical chain overlap concentration of sodium alginate  $C^*$ .  $C^*$  characterizes the transition between the dilute regime and the semi-dilute unentangled regime.  $C^*$  was calculated according to **Eq. 7** (Teraoka, 2002) and was 0.06 % w/w.

$$C^* = \frac{M}{N_A \left(\frac{4}{3} \pi R_g^3\right)} \quad (7)$$



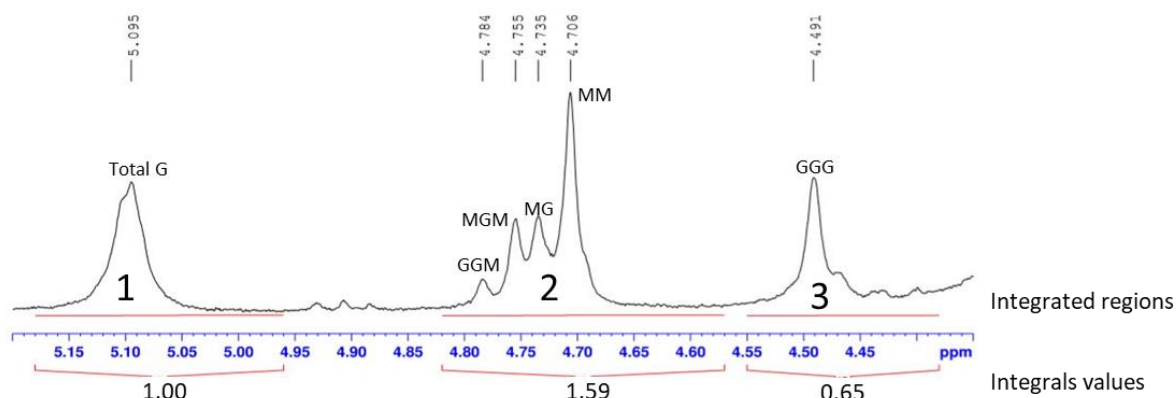
where  $M$  is the mass of the chain and  $N_A$  the Avogadro's number. Another method to estimate  $C^*$  is based on **Eq. 8** (Teraoka, 2002).-

$$C^* = \frac{1}{[\eta]} \quad (8)$$

With this equation, the obtained  $C^*$  was 0.08 % w/w. This latter value is of the same order of magnitude as the previous one, but should be more precise due to the presence of two types of chain conformations in the sample.

SEC/MALLS studies were realized in  $\text{LiNO}_3$  whose ionic strength was higher than water. In high ionic strength media, the conformation of polyelectrolyte coils such as alginate is more compact than in water, due to the screening of electrostatic interactions. Thus, in our formulation conditions (in water), the alginate chains are expected to be more extended, consequently,  $C^*$  should be lower than 0.08 % w/w.

**Figure 1** shows the  $^1\text{H}$  NMR spectrum obtained with sodium alginate. The regions 1 (4.96-5.18 ppm), 2 (4.57-4.82 ppm), 3 (4.38-4.55 ppm) were integrated to obtain respectively  $I_1$ ,  $I_2$ ,  $I_3$ . This allowed to calculate the alginate M/G ratio according to **Eq. 9** as described by Jensen et al. (2015).



**Figure 1.** Sodium alginate  $^1\text{H}$  NMR spectrum.

$$M/G = \frac{I_2 + I_3 - I_1}{I_1} \quad (9)$$

The M/G ratio was  $1.24 \pm 0.04$  that corresponded to 55 % of M units and 45 % of G units. This value was similar to the M/G ratio of alginate obtained from the fronds of *Laminaria*

*hyperborean* (Qin, 2008). It was higher than those of other parts of this algae or other *Laminaria* species (Fertah et al., 2014; Qin, 2008). Fertah et al. (2014) showed that M/G ratio value of 1.12 allowed the preparation of alginates suitable to form soft and elastic gels.

### **3.2. Gel preparation and selection of the in vitro conditions**

Different formulations were prepared by varying the concentration of each polymer. Sodium alginate was used at concentrations ranging from 0.5-2 % w/w, that belonged to the semi-diluted regime as these concentrations were higher than  $C^*$ . All formulations appeared to be homogeneous to the naked eye after storage and were translucent. However, A1.5, A2 and their corresponding mixtures with poloxamer 407 were so viscous that they were difficult to prepare. Thus, they were not considered for further studies.

As these formulations were intended for a vaginal administration, some particular conditions should be taken into account. Generally, two to five milliliters of topical vaginal products are applied in the vagina (Aka-Any-Grah et al., 2010). However, a volume of 5 mL could significantly better cover the human vaginal epithelium, immediately following its insertion, within the first 30 min and without sexual intercourse, compared to lower volumes (3 mL) (Barnhart et al., 2004). Therefore, a volume of 5 mL for each gel unit would be interesting to ensure a good epithelial coverage. Owen and Katz (1999) also showed that the volume of ambient vaginal fluid present at any time in the vagina was approximately 0.5–0.75 mL. Therefore, to mimic the maximum dilution of the formulations that was likely to occur after its application, 5 g of each formulation were mixed with 0.75 mL of SVF by vortexing. To explore the ability of the formulations to withstand vaginal fluid dilution, rheological studies were performed (i) at 37 °C without dilution with SVF and (ii) at 37°C after dilution with SVF to mimic complete dilution with SVF. Indeed, gels in the vagina should lie between those two extreme conditions.

### **3.3. Sodium alginate formulations**

In a first step, formulations containing only sodium alginate were studied. The effect of alginate concentration, of the dilution with SVF at 37 °C and of ionic calcium induced gelation were assessed.

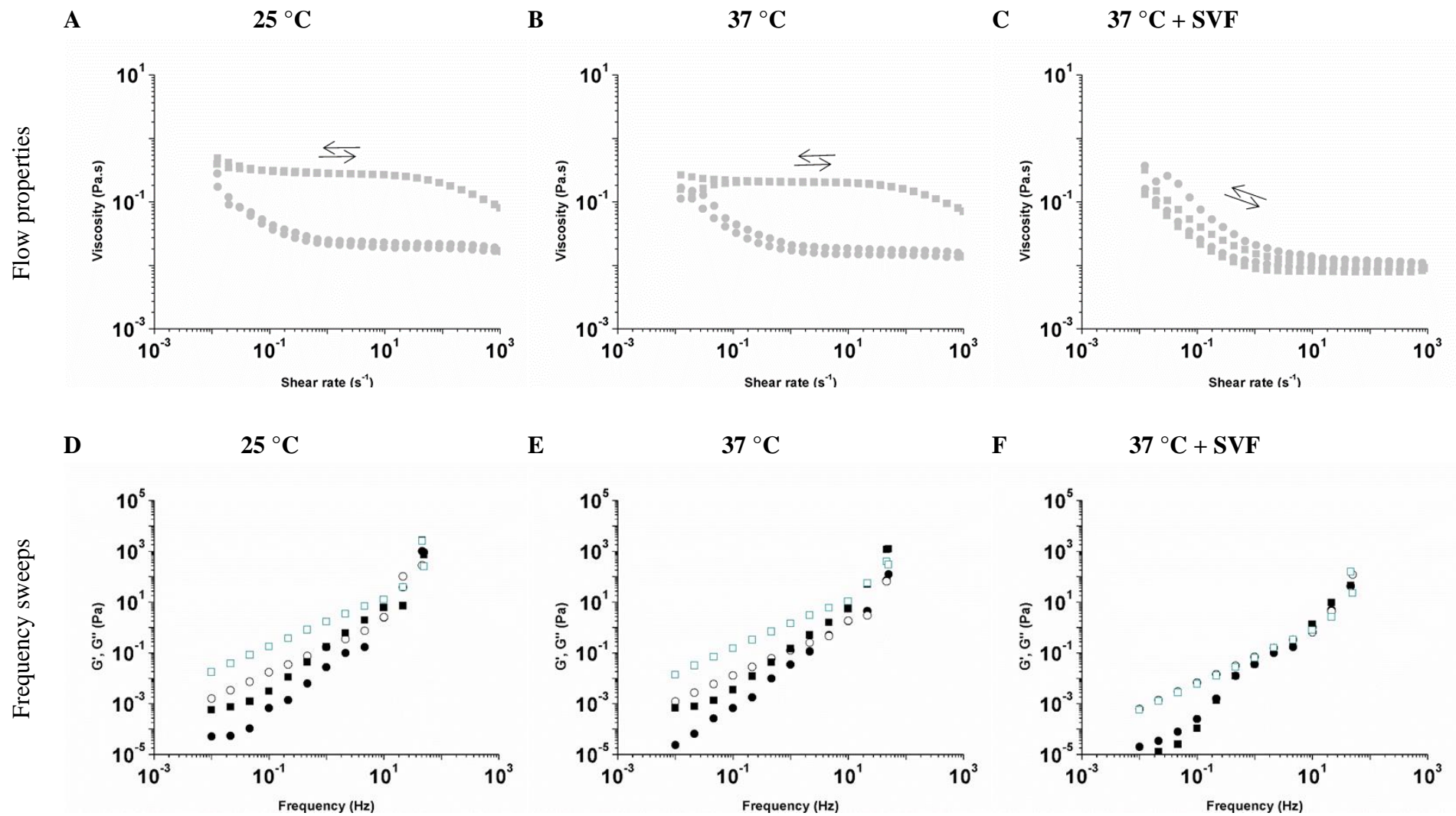
### ***Rheological behavior at 25 °C and at 37 °C***

The variations of the viscosity of alginate solutions as a function of shear rates at 25 °C showed an increase of viscosity with concentration. Both A0.5 and A1 were slightly shear-thinning (**Fig. 2A**). The formulations also displayed a non-thixotropic behavior showed by the overlay of downward and upward curves of the viscosity *versus* shear rates. The  $G'$  and  $G''$  moduli were frequency-dependent at 25 °C (**Fig. 2D**). The viscous modulus of A0.5 and A1 were respectively higher than their elastic moduli and the crossover was observed at high frequency (**Fig. 2D**). At the crossover frequency of A0.5 (10 Hz),  $G' = G'' = 2.5$  Pa, but at the crossover frequency of A1 (about 46 Hz),  $G' = G'' \approx 2600$  Pa. Alginate solutions had predominant viscous behavior.

The rheological behavior of sodium alginate at 37 °C was similar to the one of 25 °C (**Fig. 2B, E** compared to **Fig. 2A, D**).

### ***Rheological behavior at 37 °C after dilution with SVF***

Regarding the flow properties after dilution with SVF, A0.5 viscosity was similar to the results at 37 °C while the one of A1 was reduced (**Fig. 2B, C**). After dilution with SVF at 37 °C (**Fig. 2F**), the viscoelastic moduli of A0.5 were not modified but those of A1 were reduced compared to the results at 37 °C (**Fig. 2E**). In these conditions, the crossover frequency of both alginate solutions was 1 Hz, with  $G' = G'' = 0.1$  Pa.



**Figure 2.** Flow properties (A, B, C), frequency sweeps (D, E, F) at 25 °C (A, D), at 37 °C (B, E) and at 37 °C after dilution with simulated vaginal fluid (C, F) of alginate solutions A0.5 and A1: (●)  $\eta$  for A0.5, (●)  $G'$  for A0.5, (○)  $G''$  for A0.5, (■)  $\eta$  for A1, (■)  $G'$  for A1, (□)  $G''$  for A1.

### ***Gelling capacity in the presence of calcium ions***

The gelling capacity of alginate solutions at different concentrations was evaluated in the presence of human simulated vaginal fluid, in order to assess the possibility of ionic gelation *in situ*. Indeed, the human vaginal fluid contains  $0.003 \text{ mol.L}^{-1}$  of calcium ions from calcium hydroxide (Owen and Katz, 1999). As the other components of the vaginal fluid could interfere with the gelation, a calcium hydroxide solution corresponding to its concentration in SVF was also studied as a control. The gelling capacity of A0.5 and A1 in the presence of the maximum volume of vaginal fluid (0.75 mL) was assessed. None of the tested formulations gelled after the addition of SVF. Using the calcium hydroxide solution at the same concentration, volume and nature, simulating the calcium ion content of the SVF, no gelation was observed neither. To the best of our knowledge, there is no previous study reporting sodium alginate gelation induced by vaginal fluid. However, previous studies were performed with simulated tear fluid. Sodium alginate with a high G content ( $> 65 \%$ ) at 0.5-2 % (w/v) could gel instantaneously in the presence of calcium ions simulating their content in simulated tear fluid as well as in simulated tear fluid itself (Cohen et al., 1997; Lin et al., 2004). The tear fluid contains more calcium than SVF ( $0.02 \text{ mol.L}^{-1}$ ) (Marques et al., 2011). In the present study, both G content of alginate and calcium concentration in SVF were lower, respectively about 45 % and  $0.003 \text{ mol/L}$ . Thus, they were not sufficient to ensure a gelation. The dilution with SVF reduced the concentration of alginate in the system from 0.5 to 0.4 and from 1 to 0.9 (% w/w), still above  $C^*$ .

In situ ionic gelation of alginate under these conditions being difficult to reach, insoluble calcium ions such as calcium carbonate ( $\text{CaCO}_3$ ) was added in the formulation in order to increase calcium concentration. Thus, alginate solutions were mixed with  $\text{CaCO}_3$  and SVF was added to this mixture. Unfortunately, ionic gelation was not obtained neither. Brus et al., (2017) showed that hydrated M-rich blocks of alginate had higher flexibility, lower ability to bind alginate crosslinking ions, and had tendency to self-aggregate into nanodomains. Such behavior could also be involved with our alginate sample.

### **3.4. Characterization of poloxamer 407 formulations and of their mixtures with alginate**

Sodium alginate solutions had a viscous liquid behavior that was not suitable for a vaginal administration when used alone. Several studies have showed that the mixture of alginate with poloxamer may increase its mechanical properties (Quah et al., 2018; White et al., 2013). A1

was retained for the studies with poloxamer due to its better viscoelastic properties compared to A0.5. Poloxamer solutions were first studied in order to determine the optimal poloxamer concentration. Then, the optimized poloxamer solution was mixed with alginate at 1 %. Their flow, thermogelling, viscoelastic properties and their ionic gelation were assessed.

### ***Poloxamer solutions***

Poloxamer 407 solutions were studied at concentration ranging from 16 to 21.5 % w/w. **Figure 3** reports the results of poloxamer at 21.5 %. All poloxamer formulations displayed a shear-thinning behavior at 25 °C. This behavior was interesting for an easy administration with a device, due to the low flow resistance when applied at high shear conditions. The formulations also displayed a non-thixotropic behavior. As soon as the shear was removed, the viscosity recovered its initial value. The poloxamer 407 formulations were temperature-sensitive. Below the gelation temperature ( $T_{gel}$ ),  $G'$  was lower than  $G''$ . Above  $T_{gel}$ ,  $G'$  became higher than  $G''$  (**Fig. 3C**). Around  $T_{gel}$ ,  $G'$  and  $G''$  strongly increased with temperature. The gelation temperatures are gathered in **Table 2**. The one of P21.5 was the lowest ( $21 \pm 1$  °C) compared to  $25 \pm 1$  °C for P18.5 and  $32 \pm 1$  °C for P16 (**Table 2**).  $G'$  and  $G''$  moduli were also frequency-dependent (**Fig. 3E**). P21.5 had the highest viscoelastic moduli, and thus, lead to the most consistent gels (**Table 2**).

### ***Influence of dilution of poloxamer solutions with SVF***

All poloxamer formulations remained shear-thinning and non-thixotropic after dilution with SVF as exemplify by the **Figure 3B** for P21.5. P16 diluted by SVF lost its thermal gelation due to the concentration of poloxamer that became 13.9 %, which was less than the minimal concentration required to obtain a gelation. This diluted system was not characterized. The other poloxamer formulations diluted with SVF had a gelation temperature 4 °C higher than the non-diluted ones (**Table 2**). The dilution of P18.5 by SVF lead to a final poloxamer concentration of 16 % which is the minimal concentration to obtain a gelation. Therefore, 21.5 % appeared to be the optimal poloxamer concentration and was selected to prepare mixtures with alginate. With this sample, the final poloxamer concentration after dilution with SVF is 18.7 %.

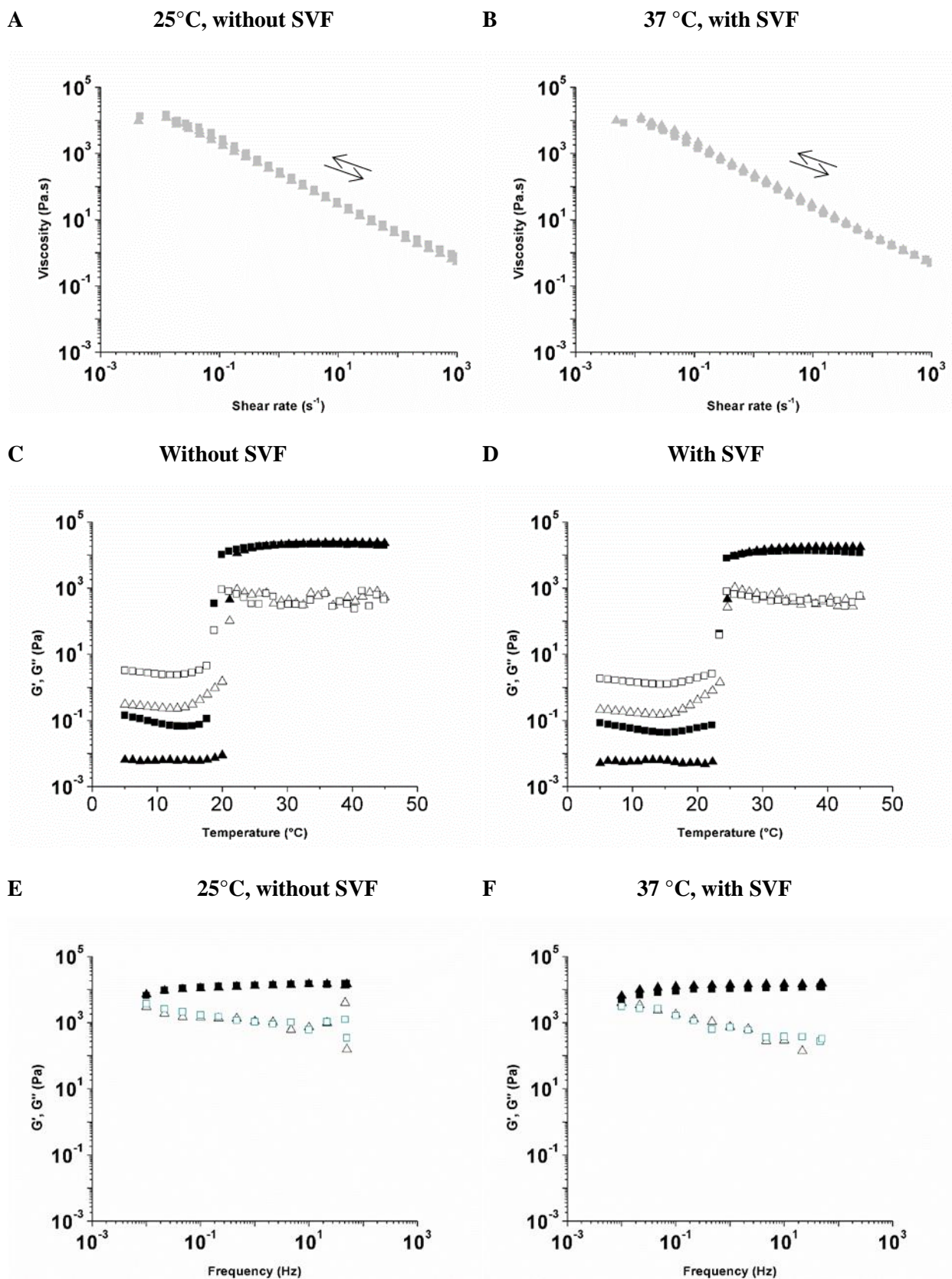
### ***Influence of alginate on poloxamer solutions and gels***

The mixture of poloxamer 407 with alginate (P21.5A1) was macroscopically homogeneous and translucent. The ethylene oxide groups of poloxamer 407 and sodium alginate chains

could interact by hydrogen bonds, which contributed to their miscibility in solution (Caykara et al., 2005; He et al., 2004). Moreover, the miscibility of neutral polymers with polyelectrolytes is also favored by the entropy of the counter ions of the polyelectrolyte.

P21.5A1 remained shear-thinning (**Fig 3A**), temperature-sensitive (**Fig. 3C**). The gelation temperatures of P21.5 and its mixture P21.5A1 were similar, showing that alginate did not significantly interfere with the poloxamer 407 gelation.  $T_{gel}$  was  $19 \pm 1$  °C for P21.5A1. The administration conditions are 19-20 °C in Europe while they are 25-30 °C in sub-Saharan Africa. Therefore, P21.5A1 might be in gel state when administered at room temperature or it may be stored in colder conditions if a liquid state is required for its administration. At temperatures below the gelation temperature, the viscoelastic moduli values of the mixture were higher than those of the poloxamer formulation. Therefore, sodium alginate chains contributed to the elasticity of the mixture thanks to the network they formed. Above the gelation temperature, the viscoelastic moduli of P21.5 and P21.5A1 with sodium alginate were not significantly different suggesting that at high temperatures poloxamer 407 imposed its behavior to the mixtures (**Fig. 3C**). These results are in accordance with those of Grassi et al. (2006) who showed that the properties of pure P407 at 20 % and its corresponding mixtures with alginate 1-2 % were largely different at low temperatures but similar at temperatures above gelation. They also demonstrated that the presence of alginate chains had a favorable influence on the structural process, providing a stabilizing framework for micelles caging and packing. However, once the structural process was complete, the properties of the systems almost coincided with those of poloxamer 407 alone.

In frequency sweeps, the elastic modulus was higher than the viscous modulus at all the studied frequencies (**Fig. 3E**) as both studied temperatures (25 °C et 37 °C) were above  $T_{gel}$ , so the formulations were in gel state. When the frequency increased the elastic modulus remained constant while the viscous modulus slightly decreased.



**Figure 3.** Flow properties (A, B), temperature sweep (C, D) displaying one data point out of ten, frequency sweep (E, F) of P21.5 and P21.5A1 as was (A, C, E) and after dilution with SVF (B, D, F): (▲)  $\eta$  for P21.5, (▲)  $G'$  for P21.5, ( $\Delta$ )  $G''$  for P21.5, (■)  $\eta$  for P21.5A1, (■)  $G'$  for P21.5A1, ( $\square$ )  $G''$  for P21.5A1.



**Table 2.** Gelation temperature, viscous and elastic moduli values at a frequency of 1 Hz for the poloxamer formulations and the poloxamer–alginate mixture.

Formulation	T <sub>gel</sub> (°C)	Conditions	G' (Pa)	G'' (Pa)
P16	32 ± 1	25 °C	(4.0 ± 0.7) 10 <sup>-3</sup>	0.4 ± 0.1
P18.4	25 ± 1		3.0 ± 0.2	40 ± 6
P21.5	21 ± 1		(12,7 ± 0.2).10 <sup>3</sup>	(1.1 ± 0.01).10 <sup>3</sup>
P21.5A1	19 ± 1		(13.5 ± 0.4).10 <sup>3</sup>	(1.0 ± 0.2).10 <sup>3</sup>
P18.5 + SVF	29 ± 1	37 °C	(8.4 ± 0.5).10 <sup>3</sup>	(0.9 ± 0.2).10 <sup>3</sup>
P21.5 + SVF	25 ± 1		(13.8 ± 0.2).10 <sup>3</sup>	(0.7 ± 0.1).10 <sup>3</sup>
P21.5A1 + SVF	24 ± 1		(10.5 ± 0.2).10 <sup>3</sup>	(0.7 ± 0.2).10 <sup>3</sup>

### *Influence of SVF on poloxamer and alginate mixture*

P21.5A1 remained shear-thinning and non-thixotropic after dilution with SVF (**Fig. 3B**). Flow experiments at 37°C in the presence of SVF were likely to be related to the residence time of the gel in the vagina, important property to ensure the therapeutic efficacy and to avoid leakage. Indeed, the shearing and dilution with SVF will influence the rheological properties of the formulations and, hence, their spreading and retention in the vagina. A good spreadability of P21.5A1 is expected due to its shear-thinning behavior. After dilution with SVF, the viscosities remained high, which is favorable to a prolonged residence time.

Dilution of P21.5 with SVF also increased the gelation temperature. T<sub>gel</sub> of P21.5A1 after dilution with SVF was similar to P21.5 sample diluted with SVF (**Fig. 3D**). Therefore, SVF had the same influence on both P21.5 and P21.5A1 gelation properties.

After dilution with SVF the poloxamer and its mixtures remained viscoelastic with G' that remained higher than G'' (**Table 2**). The elastic modulus value of the diluted mixture P21.5A1 with SVF was lower than the non-diluted one (**Fig 3E** compared to **Fig. 3F**). This could be explained by the decrease in polymer concentration, but also by the more compact conformation of alginate chains due to ionic strength increased, caused by SVF. Moreover, proteins and other electrolytes contained in SVF could also interact with the polymers and decrease the elasticity of the system.

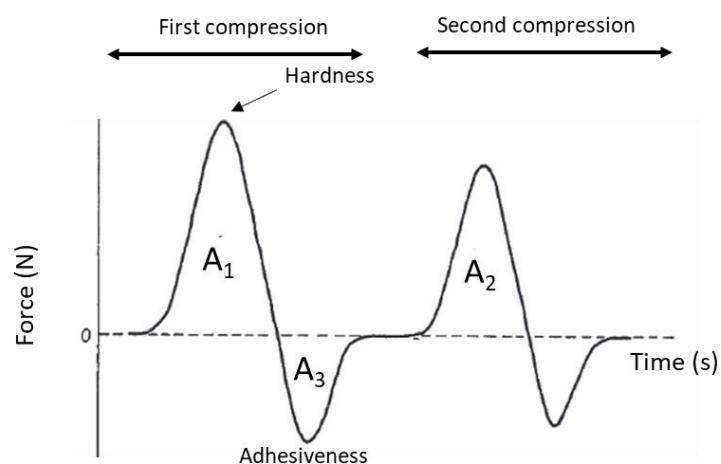
### *Addition of calcium carbonate in P21.5A1*

Ionic gelation of sodium alginate in P21.5A1 formulation was studied to assess if the presence of poloxamer could favor this ionic gelation. Calcium carbonate (0.03 and 0.05 %) was introduced into the gels. The gelling capacity was determined by diluting the gels with SVF in cold conditions to avoid poloxamer 407 gelation, and by visually observing the flow after tube

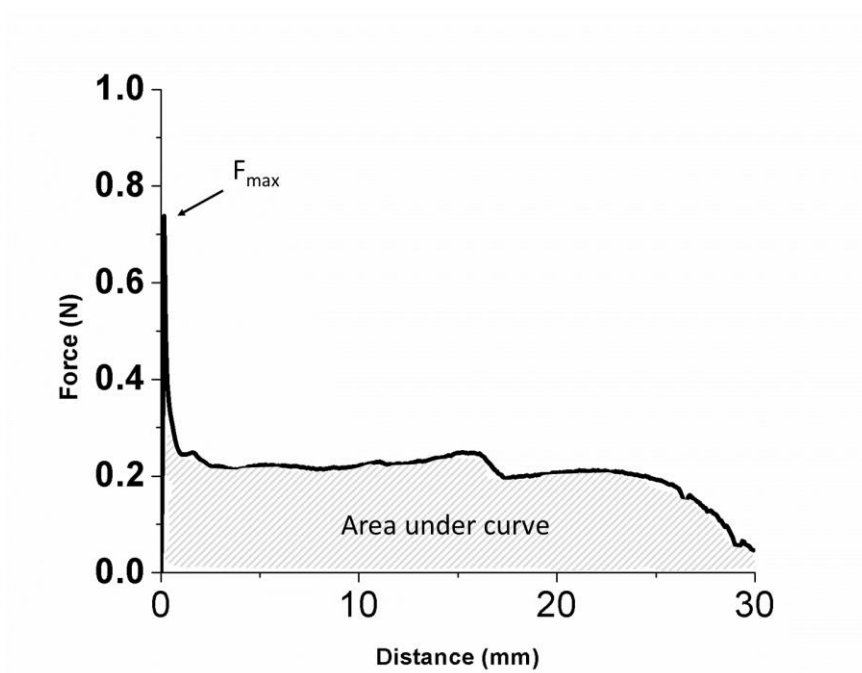
inversion. Unfortunately, the ionic gelation did not occur. Ionic gelation is dependent upon multiple parameters such as (i) the alginate M/G ratios, (ii) the type of ionic cross-linker, (iii) the influence of the other ions, proteins and electrolytes contained in the SVF, (iv) the concentration of alginate and the viscosity of the formulation, and (v) the ability of SVF to create a sufficiently acidic environment. The great variability on the alginate used in the published data, and the lack of alginate characterization in some cases did not allow us to determine the limit value of each parameter to obtain ionic gelation. Therefore, this approach was abandoned.

### 3.5. Expulsion ability from a device and texture profile analysis

Two practical administration tests, texture profile analysis (TPA) at 25°C and an expulsion test from a syringe without needle at 30° C were performed. TPA is frequently used to identify formulations that may be suitable for clinical application. The studied parameters were hardness, cohesiveness and adhesiveness that were obtained from the experimental force *versus* time curves (see typical graph in **Fig. 4**) and related force *versus* displacement curves. Hardness was the peak force during the first compression cycle, needed to deform the sample. In administration conditions, hardness should be low to allow the gel to be easily removed from the container and spread onto the mucosa. Cohesiveness was related to the effects of repeated shearing stresses on the structural properties of formulations and measures the reconstruction of the gel after application. High cohesiveness value provides full structural recovery following gel application. Adhesiveness was the work required to overcome the attractive forces between the surface of the probe and the surface of the sample when the probe was removed. In our study, the adhesiveness and cohesiveness values of the P21.5 were similar to those of P21.5A1 (**Table 3**). Similar maximal force and similar work to expel gels from the syringe were also obtained with both systems (**Table 3**). In syringeability tests, it is usually considered that the maximal force to exert on the plunger should be below 30 N for manual injection (El Kechai et al., 2015). Yet, maximal force values around or below 10 N are preferred. In our case, the maximal force was much smaller indicating an easy administration. Moreover, the values of the work to expel the formulations were below 13 mJ, despite both formulations were in gel state. This can be explained by the shear-thinning behavior of those formulations. There is not yet a consensus of adequate value of the work to expel the gels into the vagina, but these results might help to design a vaginal device to allow a handy and easy administration of the gels by women themselves.



**Figure 4.** Typical texture profile analysis (TPA) curve depicted from Chen (2009). Hardness: peak force during the first compression cycle. Adhesiveness: negative force area  $A_3$  for the first compression. Cohesiveness: ratio of positive force area during the second compression to that during the first compression ( $A_2/A_1$ ).



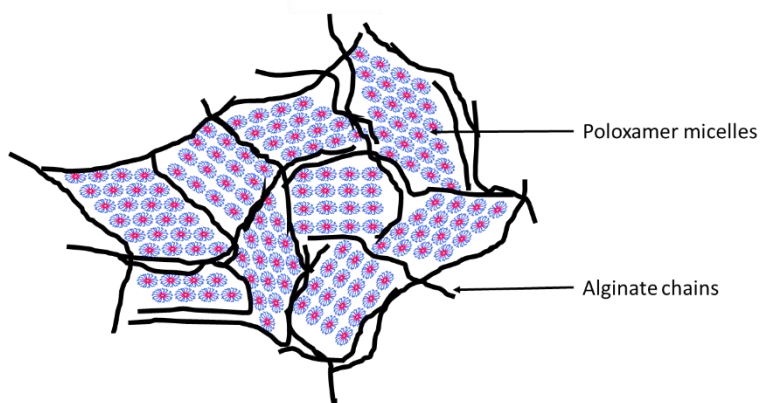
**Figure 5.** Force-distance profile for P21.5A1 in a 5 mL syringe, without needle nor cannula.

**Table 3.** Texture profile analysis and expulsion ability of P21.5 and P21.5A1.

Samples	Texture in TPA mode			Expulsion ability from a device	
	Adhesiveness (mJ)	Cohesiveness	Hardness (N)	Maximal force (N)	Work of expulsion (mJ)
P21.5	$4.81 \pm 0.21$	$0.89 \pm 0.04$	$0.59 \pm 0.03$	$0.8 \pm 0.1$	$6.1 \pm 1.5$
P21.5A1	$4.79 \pm 1.49$	$0.93 \pm 0.09$	$0.48 \pm 0.03$	$0.7 \pm 0.2$	$6.4 \pm 5.9$

### 3.6. Microstructure of P21.5A1

From the rheological results of P21.5 and P21.5A1 associated to the characterization of sodium alginate, it was possible to propose a macroscopic organization of both polymers in water. Indeed, in aqueous solution, some alginate chains were in extended conformation stabilized by hydrogen bonds and others were flexible due to the high M content. In addition, the viscoelastic moduli of P21.5A1 was higher than those of P21.5 when the systems were liquid suggesting that alginate chains created a network of entangled chains that provided some elasticity. In gel state, P21.5A1 viscoelastic moduli were similar to P21.5 suggesting that poloxamer micelles of P21.5A1 crystallized by forming the same clusters as P21.5. Therefore, the location of alginate chains could only be around the microdomains formed by the poloxamer micelles. **Figure 6** presents the organization that can be inferred for our mixture. This organization is almost similar to the observation by transmission electronic microscopy of a mixture of poloxamer and alginate after their  $T_{gel}$  reported by Abrami et al. (2014). White et al. (2013) using differential scanning calorimetry, small-angle neutron scattering and rheology proposed a similar organization of a mixture of ionic gelled alginate and thermally gelled poloxamer. Further microscopic studies such as small-angle X-ray scattering or small-angle neutron scattering should be performed to further characterize our systems, which do not contain calcium ions.



**Figure 6.** Schematic depiction of the mixture P21.5A1 showing the crystallized poloxamer micelles and alginate chains.

#### 4. CONCLUSION

In this work, hydrogels containing different concentrations of poloxamer 407 and sodium alginate and intended to be a platform for vaginal administration were developed. The results confirmed the macroscopic compatibility between both polymers. All the formulations containing P407 were temperature-sensitive. The optimal concentration of poloxamer to preserve the thermogelling properties after dilution with vaginal fluids was 21.5 % w/w. Adding 1 % w/w sodium alginate enhanced the formulation elasticity in liquid state. The mixture of P407 at 21.5 % w/w and sodium alginate at 1 % w/w was a temperature-sensitive system with increased elasticity and adequate rheological properties, although it was not possible to induce *in-situ* ionic gelation of sodium alginate in these conditions. Its rheological properties at 25 °C associated to the texture profile analysis and the evaluation of its ability to be expelled were all favorable to its administration. Its rheological properties at 37 °C after dilution with SVF were adequate to avoid leakage and ensure its spreading, making this gel a promising candidate for vaginal administration.

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#### References

- Abrami, M., D'Agostino, I., Milcovich, G., Fiorentino, S., Farra, R., Asaro, F., Lapasin, R., Grassi, G., and Grassi, M. (2014). Physical characterization of alginate-Pluronic F127 gel for endoluminal NABDs delivery. *Soft Matter* *10*, 729–737.
- Acarturk, F. (2009). Mucoadhesive Vaginal Drug Delivery Systems. *Recent Pat. Drug Deliv. Formul.* *3*, 193–205.
- Aka-Any-Grah, A., Bouchemal, K., Koffi, A., Agnely, F., Zhang, M., Djabourov, M., and Ponchel, G. (2010). Formulation of mucoadhesive vaginal hydrogels insensitive to dilution with vaginal fluids. *Eur. J. Pharm. Biopharm.* *76*, 296–303.
- Barba, A.A., Lamberti, G., Rabbia, L., Grassi, M., Larobina, D., and Grassi, G. (2014). Modeling of the reticulation kinetics of alginate/pluronic blends for biomedical applications. *Mater. Sci. Eng. C* *37*, 327–331.

Barnhart, K.T., Pretorius, E.S., Timbers, K., Shera, D., Shabbout, M., and Malamud, D. (2004). In vivo distribution of a vaginal gel: MRI evaluation of the effects of gel volume, time and simulated intercourse. *Contraception* 70, 498–505.

Brus, J., Urbanova, M., Czernek, J., Pavelkova, M., Kubova, K., Vyslouzil, J., Abbrent, S., Konefal, R., Horský, J., Vetchy, D., et al. (2017). Structure and dynamics of alginate gels cross-linked by polyvalent ions probed via solid state NMR spectroscopy. *Biomacromolecules* 18, 2478–2488.

Burckbuchler, V., Mekhloufi, G., Giteau, A.P., Grossiord, J.L., Huille, S., and Agnely, F. (2010). Rheological and syringeability properties of highly concentrated human polyclonal immunoglobulin solutions. *Eur. J. Pharm. Biopharm.* 76, 351–356.

Caykara, T., Demirci, S., Eroglu, M., and Güven, O. (2005). Poly(ethylene oxide) and its blends with sodium alginate. *Polymer* 46, 10750–10757.

Charrueau, C., Tuleu, C., Astre, V., Grossiord, J.-L., and Chaumeil, J.-C. (2001). Poloxamer 407 as a Thermogelling and Adhesive Polymer for Rectal Administration of Short-Chain Fatty Acids. *Drug Dev. Ind. Pharm.* 27, 351–357.

Chen, J. (2009). Food oral processing—A review. *Food Hydrocoll.* 23, 1–25.

Chen, C.-C., Fang, C.-L., Al-Suwayeh, S.A., Leu, Y.-L., and Fang, J.-Y. (2011). Transdermal delivery of selegiline from alginate-Pluronic composite thermogels. *Int. J. Pharm.* 415, 119–128.

Chien, Y., and Lee, C. (2002). Drug delivery, vaginal route. *Encycl. Pharm. Technol.* 2nd Ed 961–985.

Choudhury, A., Das, S., and Kar, M. (2011). A Review on Novelty and Potentiality of Vaginal Drug Delivery. *Int. J. PharmTech Res.* 3, 1033–1044.

Cohen, S., Lobel, E., Trevgoda, A., and Peled, Y. (1997). A novel in situ-forming ophthalmic drug delivery system from alginates undergoing gelation in the eye. *J. Controlled Release* 44, 201–208.

Colinet, I., Dulong, V., Hamaide, T., Le Cerf, D., and Picton, L. (2009). New amphiphilic modified polysaccharides with original solution behaviour in salt media. *Carbohydr. Polym.* 75, 454–462.

Cook, M.T., and Brown, M.B. (2018). Polymeric gels for intravaginal drug delivery. *J. Controlled Release* 270, 145–157.

Dumortier, G., Grossiord, J.L., Agnely, F., and Chaumeil, J.C. (2006). A review of poloxamer 407 pharmaceutical and pharmacological characteristics. *Pharm. Res.* 23, 2709–2728.

El Kechai, N., Bochot, A., Huang, N., Nguyen, Y., Ferrary, E., and Agnely, F. (2015). Effect of liposomes on rheological and syringeability properties of hyaluronic acid hydrogels intended for local injection of drugs. *Int. J. Pharm.* 487, 187–196.

Fertah, M., Belfkira, A., montassir Dahmane, E., Taourirte, M., and Brouillette, F. (2014). Extraction and characterization of sodium alginate from Moroccan *Laminaria digitata* brown seaweed. *Arab. J. Chem.*

Grasdalen, H., Larsen, B., and Smisrod, O. (1981). <sup>13</sup>C-N.M.R. studies of monomeric composition and sequence in alginate. *Carbohydr. Res.* 89, 179–191.

Grassi, G., Crevatin, A., Farra, R., Guarnieri, G., Pascotto, A., Rehimers, B., Lapasin, R., and Grassi, M. (2006). Rheological properties of aqueous Pluronic-alginate systems containing liposomes. *J. Colloid Interface Sci.* *301*, 282–290.

He, Y., Zhu, B., and Inoue, Y. (2004). Hydrogen bonds in polymer blends. *Prog. Polym. Sci.* *29*, 1021–1051.

Hom, W.L., and Bhatia, S.R. (2017). Significant enhancement of elasticity in alginate-clay nanocomposite hydrogels with PEO-PPO-PEO copolymers. *Polymer* *109*, 170–175.

Jensen, H.M., Larsen, F.H., and Engelsen, S.B. (2015). Characterization of alginates by nuclear magnetic resonance (NMR) and vibrational spectroscopy (IR, NIR, Raman) in combination with chemometrics. *Methods Mol. Biol. Clifton NJ* *1308*, 347–363.

Lee, K.Y., and Mooney, D. (2012). Alginate: properties and biomedical applications. *Prog Polym Sci* *37*, 106–126.

Lin, H.-R., Sung, K.C., and Vong, W.-J. (2004). In situ gelling of alginate/pluronic solutions for ophthalmic delivery of pilocarpine. *Biomacromolecules* *5*, 2358–2365.

Marques, M.R.C., Loebenberg, R., and Almukainzi, M. (2011). Simulated biological fluids with possible application in dissolution testing. *Dissolution Technol.* *18*, 15–28.

Mastromarino, P., Brigidi, P., Macchia, S., Maggi, L., Pirovano, F., Trinchieri, V., Conte, U., and Matteuzzi, D. (2002). Characterization and selection of vaginal *Lactobacillus* strains for the preparation of vaginal tablets. *J. Appl. Microbiol.* *93*, 884–893.

Misra, A. (2014). *Applications of Polymers in Drug Delivery* (Smithers Rapra).

Moebus, K., Siepmann, J., and Bodmeier, R. (2009). Alginate–poloxamer microparticles for controlled drug delivery to mucosal tissue. *Eur. J. Pharm. Biopharm.* *72*, 42–53.

Owen, D.H., and Katz, D.F. (1999). A vaginal fluid simulant. *Contraception* *59*, 91–95.

Owen, D.H., Peters, J.J., and Katz, D.F. (2000). Rheological properties of contraceptive gels. *Contraception* *62*, 321–326.

Parhi, R., and Suresh, P. (2015). Alginate-poloxamer beads for controlled release of metoprolol succinate. *Turk. J. Pharm. Sci.* *12*, 59–66.

Qin, Y. (2008). Alginate fibres: an overview of the production processes and applications in wound management. *Polym. Int.* *57*, 171–180.

Quah, S.P., Smith, A.J., Preston, A.N., Laughlin, S.T., and Bhatia, S.R. (2018). Large-area alginate/PEO-PPO-PEO hydrogels with thermoreversible rheology at physiological temperatures. *Polymer* *135*, 171–177.

Teraoka, I. (2002). *Polymer solutions: An introduction to physical properties* (New York, Etats-Unis: John Wiley).

Ur-Rehman, T., Tavelin, S., and Gröbner, G. (2011). Chitosan in situ gelation for improved drug loading and retention in poloxamer 407 gels. *Int. J. Pharm.* *409*, 19–29.

White, J.C., Saffer, E.M., and Bhatia, S.R. (2013). Alginate/PEO-PPO-PEO composite hydrogels with thermally-active plasticity. *Biomacromolecules* *14*, 4456–4464.

**CHAPITRE V : FORMULATION D'UN HYDROGEL A BASE  
DE POLOXAMER 407, D'ALGINATE DE SODIUM ET DE *L.*  
*CRISPATUS* DESTINE A INHIBER LA CROISSANCE DE  
*NEISSERIA GONORRHOEAE***





## Introduction- résumé

La résistance croissante de *Neisseria gonorrhoeae* à tous les traitements antibiotiques actuels et les difficultés liées aux moyens de prévention ont conduit à l'étude de méthodes alternatives pour prévenir cette infection sexuellement transmissible. Dans ce travail, une stratégie de prévention basée sur l'utilisation d'un gel vaginal contenant des lactobacilles a été évaluée. Une souche de *Lactobacillus crispatus* a été sélectionnée sur la base des données publiées concernant sa capacité à inhiber *Neisseria gonorrhoeae* et ses propriétés probiotiques ont été caractérisées. Un hydrogel thermosensible contenant 21,5 % de poloxamer 407, 1 % d'alginate de sodium et  $9 \log_{10}$  UFC/unité de gel de *Lactobacillus crispatus* a été mis au point. La température de gélification et les caractéristiques rhéologiques de cette formulation sont apparues favorables à une administration vaginale. La microscopie confocale a révélé des *Lactobacillus crispatus* viables dans le gel au bout de six mois de conservation à 4 °C et à 25 °C, mais ces bactéries n'étaient plus cultivables. La capacité des souches de lactobacilles à inhiber *Neisseria gonorrhoeae* a été observée avec le gel. Un tel système est donc prometteur pour la prévention de la gonococcie.



**PROJET DE PUBLICATION :**  
**CHARACTERIZATION AND *IN VITRO* EVALUATION OF A  
VAGINAL GEL CONTAINING *LACTOBACILLUS CRISPATUS*  
FOR THE PREVENTION OF GONORRHEA**

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**Abstract**

The increasing resistance of *Neisseria gonorrhoeae* to any current antibiotic treatment and the difficulties associated with the use of prevention means such as condom urge the need for alternative methods to prevent this sexually transmitted infection. In this work, a prevention strategy based on the use of a vaginal gel containing *Lactobacilli* was assessed. A *Lactobacillus crispatus* strain was selected based on the published data on its ability to inhibit *Neisseria gonorrhoeae* and its probiotic properties were characterized. A thermo-sensitive hydrogel containing 21.5 % of poloxamer 407, 1 % of sodium alginate and 9 log<sub>10</sub> CFU by gel unit of *Lactobacillus crispatus* was developed. The gelation temperature and the rheological

characteristics of this formulation appeared suitable for a vaginal administration. *Lactobacillus crispatus* were viable in the gel for six month although a large amount of the bacteria was not culturable. The ability of *Lactobacillus crispatus* to inhibit *Neisseria gonorrhoeae* was still observed with the gel. Such system, thus, appeared promising for the prevention of gonorrhea.

### **Keywords**

Confocal scanning laser microscopy, gel, gonorrhea prevention, *Lactobacillus*, probiotics, rheology, vaginal administration.

### **Abbreviations**

ATCC, american type culture collection; BHIA, brain heart infusion agar; CDC, Center for disease control; CFU, colony forming unit; CLSM, confocal laser scanning microscopy;  $G'$ , elastic modulus;  $G''$ , viscous modulus; GC agar, chocolate agar; HIV, human immunodeficiency virus; *L. crispatus*, *Lactobacillus crispatus*; MRS, Man-Rogosa-Sharpe; *N. gonorrhoeae*, *Neisseria gonorrhoeae*; PBS, phosphate buffered saline;  $pH_i$ , initial pH; SVF, simulated vaginal fluid;  $T_{gel}$ , gelation temperature; VNC, viable but nonculturable.

## 1. INTRODUCTION

Gonorrhoea is a sexually transmitted infection, that is caused by *Neisseria gonorrhoeae* (*N. gonorrhoeae*), with 78 million new cases each year (WHO, 2016). It has become a major public health issue for many reasons. Indeed, more often this infection remains asymptomatic in women, which favors its spreading. It can induce severe damages such as infertility both in men and women, and blindness in newborns (Grodstein et al., 1993; Laga et al., 1986). It also increases the risk of contracting human immunodeficiency virus HIV (Cohen, 2004; Jarvis and Chang, 2012). There is currently an emergence of resistance to several antibiotics even to the last intention treatments (Lefebvre et al., 2018). Some untreatable strains insensitive to any known antimicrobial are now circulating. Meanwhile, neither vaccine nor new drugs are emerging on the market. Condom, sole effective prevention mean, is unfortunately not well or insufficiently used. Thus, gonorrhoea has become difficult to treat or to prevent. The main target of gonorrhoea, as all sexually transmitted infections is women, because they are more vulnerable. A strategy focused on women could allow to achieve better results in the field of prevention (Stein, 1990). Thus, recent researches are interested in woman-controlled tools for vaginal administration (Minnis and Padian, 2005; Traore et al., 2018).

Approaches based on probiotic have recently been developed for the prevention of women urogenital tract infections (Andrew and Gregor, 2017; Stapleton et al., 2011). Indeed, probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). The most important probiotic *genus* studied for urogenital infections is *Lactobacillus*. The healthy vaginal flora is typically dominated by *Lactobacillus* (L.) species, such as *L. iners*, *L. crispatus*, *L. jensenii* and *L. gasseri* (Pavlova et al., 2002; Ravel et al., 2011), but *L. crispatus* seems to largely prevail over the other *Lactobacillus* strains (Antonio et al., 1999; Vasquez et al., 2002). Moreover, *L. crispatus* has been reported to promote the stability of the normal vaginal flora (Verstraelen et al., 2009). Vaginal colonization with a human *L. crispatus* strain had been successful (Antonio and Hillier, 2003). A *L. crispatus* strain was also used with success as a vaginal suppository for healthy women with a history of recurrent urinary tract infections (Czaja et al., 2007; Stapleton et al., 2011) and for the treatment of bacterial vaginosis (Hemmerling et al., 2010). Moreover, it was shown that *L. crispatus* inhibited the infectivity of *N. gonorrhoeae* *in vitro* (Amant et al., 2002) and *ex-vivo* (Breshears et al., 2015). Therefore, in this study, *L. crispatus* was selected. Its use for a vaginal administration could be a valuable strategy for vaginal gonorrhoea prevention. As the health promoting effects of probiotics are

usually strain specific, in this work, we report on the ability of a reference vaginal *L. crispatus* strain ATCC 33197 to be used as a probiotic for the prevention of gonorrhoea. Since bacterial suspensions are liquid and can be quickly cleared after vaginal instillation, we focused on the design of a gel form intended for the administration of this *L. crispatus* strain. Indeed, hydrogels are three-dimensional polymer networks made up of water soluble polymer chains crosslinked by chemical or physical bonds. They have been studied in the fields of cell immobilization (Cellesi, 2012; Chaluvadi et al., 2012; Willaert, 2011), of tissue engineering (Maisani, 2017) and of drug delivery (Cook and Brown, 2018). They are already used and well accepted for vaginal administration, are easy to be prepared and have a low cost of production (Pilcher, 2004). They can be easily self-administered without partner knowledge. They are expected to easily cover the vaginal mucosa and ensure efficiency. Several studies have been focused on viscoelastic and temperature-sensitive hydrogels based on poloxamer 407 (Aka-Any-Grah et al., 2010; Huang et al., 2016; Liu et al., 2009). In aqueous solution, this synthetic poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer forms micelles above a certain critical micelle concentration or temperature. If the polymer concentration is high enough, these micelles organize themselves into a cubic liquid crystalline structure when the temperature is further increased above a critical temperature called gelation temperature ( $T_{gel}$ ), forming a gel-like phase (Dumortier et al., 2006). It is generally used as drug delivery systems (Karavana et al., 2012; Rençber et al., 2017) or for cell encapsulation (Gioffredi et al., 2016; Khattak et al., 2005; Wagner et al., 2014). Poloxamer 407 can be mixed with mucoadhesive polymers to improve this property. Among these mucoadhesive polymers, sodium alginate is particularly attractive. Indeed, it is a natural polymer derived from brown algae that can be used to create hydrogels by ionic cross-linking with divalent cations such as  $Ca^{2+}$ . Alginate is commonly and widely used for drug delivery, cell encapsulation and immobilization, wound dressing, enzymes immobilization (Lee and Mooney, 2012; Lin et al., 2004; Szekalska et al., 2016; Tønnesen and Karlsen, 2002). The mixtures of poloxamer 407 and alginate could be liquid at low or room temperature to allow easy administration, and, undergo gelation at body temperature (Lin et al., 2004). Previous study on this mixture also showed compatibility between poloxamer 407 and alginate (Grassi et al., 2006).

In the present study, the mixture of poloxamer 407 and sodium alginate was studied as a support for a vaginal administration of *L. crispatus* in order to obtain a temperature-sensitive gel with adequate mechanical properties and capable to inhibit *N. gonorrhoeae*. To the best of our knowledge, there is no published data dealing with the use of semisolid dosage forms (gel,

cream) for the administration of *Lactobacilli* into the vagina. The probiotic properties of the selected strain were first assessed. In a second step, the influence of *L. crispatus* on the thermogelling and viscoelastic properties of the mixture of poloxamer 407 and alginate was investigated by rheology at 25 °C, in administration conditions, and at 37 °C after dilution with simulated vaginal fluid (SVF) to mimic *in vivo* conditions after administration. The efficacy of the gel on *N. gonorrhoeae* was evaluated *in vitro* as well as the viability of the *L. crispatus* in the gel over a six-month period.

## 2. MATERIALS AND METHODS

### 2.1. Materials

*L. crispatus*, a reference strain, isolated from human vagina with collection numbers CIP 103603 (ATCC 33197; NCTC 4505; NCIMB 4505), and a reference strain *N. gonorrhoeae* with collection number CIP 107031 (ATCC 49226; CCM 4500; CCUG 26213; CDC 10.001; CNCTC 5194; DSM 9189) were purchased from Institut Pasteur (Paris). The strains were stored at -80 °C in cryovials with the appropriate broth supplemented with 20 % (v/v) glycerol.

*Streptococcus pneumoniae* ATCC 49619 and *Streptococcus pyogenes* ATCC 12344 were obtained from Institut Pasteur (Abidjan). BD-Difco™ *Lactobacilli* of Man-Rogosa-Sharpe (MRS) broth or MRS agar, brain heart infusion agar and Columbia agar base were obtained from Fisher Scientific (France). Poloxamer 407 (P) (Lutrol® F127) of pharmaceutical grade was purchased from BASF (Germany). According to the supplier, the average molecular weight was 11774 g.mol<sup>-1</sup> (9840-14600 g.mol<sup>-1</sup>). Sodium alginate (A) was purchased from Roth (France), lot N° 351172083. Its weight-average molecular weight determined by size exclusion chromatography coupled to multi-angle light scattering was 300 000 g.mol<sup>-1</sup>. Bovine serum albumin and all other reagents were supplied by Sigma-Aldrich (France) and were of analytical grade. Live/dead® bacLight™ bacterial viability kit was purchased from Fisher Scientific (France). Sterile water (resistivity 18.2 MΩ, MilliQ, Millipore, France) was used. All the antibiotic discs were from BIO-RAD (France) and the E-test from Biomérieux (France).



## **2.2. Study of *Lactobacillus crispatus***

### **2.2.1 Growth kinetic**

To determine its stationary growth phase, *L. crispatus* (1/1000 v/v) in MRS broth was incubated at 37 °C, 5 % CO<sub>2</sub>. Then, the number of colony-forming units was determined at the beginning (t<sub>0</sub>) and every three hours for 24 h, using a MRS agar plate. This test was performed in triplicate in one experiment.

### **2.2.2 Acid production and growth at different pH**

The evaluation of the acidification ability of *L. crispatus* and of the effects of low pH on its growth was achieved according to Boskey et al. (1999), with slight modifications. MRS broth was prepared and adjusted to specific initial pH (pH<sub>i</sub>) ranging from 4 to 7 with either HCl or NaOH before autoclaving. *L. crispatus* was inoculated (1 % v/v) into 10 mL of each MRS broth giving a starting concentration of about 10<sup>7</sup> cells/mL. Then, they were incubated for 24 h at 37 °C under 5 % CO<sub>2</sub>. The amount of acid produced by *L. crispatus* was indirectly determined by measuring the pH immediately after preparation (t<sub>0</sub>) and after 24 h incubation (t<sub>24h</sub>) with a pH meter (Metter Toledo equipped with an electrode Seven2GO, France). To assess the sensitivity of *L. crispatus* to the pH, the numbers of CFU/mL of each MRS broth were quantified at the beginning (t<sub>0</sub>) and after 24 h (t<sub>24h</sub>). This assay was performed in two separate experiments constituted of three replicates each.

### **2.2.3 Auto-aggregation and co-aggregation assays**

This test was conducted to appreciate the ability of *L. crispatus* to auto-aggregate and co-aggregate *N. gonorrhoeae*. It was determined according to Verdenelli et al. (2014) with slight modifications. The cultures were harvested by centrifugation (2800 g, 10 min, 4 °C), and washed twice with sterile PBS (pH7). Bacterial suspensions in PBS of *L. crispatus* and *N. gonorrhoeae* were adjusted to 0.5 McFarland. Equal volumes (1 mL) of each suspension were mixed for 20 s using a vortex, and left for 4 h at 37 °C. A droplet of the single suspensions and the mixture was then put on a glass slide immediately and after four hours. They were Gram-stained for visual observation of aggregates, defined as visible clumps of bacteria. This experiment was performed twice.

#### **2.2.4 Hemolytic activity**

To evaluate the ability of *L. crispatus* to induce hemolysis, the hemolytic activity of *L. crispatus* was determined according to Maragkoudakis et al. (2009) with slight modifications. In short, the subculture of *L. crispatus* of 16 h in MRS broth was streaked onto Columbia agar base plates supplemented with 5 % (v/v) whole sheep blood. The plates were incubated at 37 °C under 5 % CO<sub>2</sub> for 24 h and 48 h in anaerobic conditions. Then, the clear zones and the color of hemolysis around the growth colonies were observed. *Streptococcus pneumoniae* ATCC 49619 and *Streptococcus pyogenes* ATCC 12344 were used as controls for respectively  $\alpha$ - and  $\beta$ -hemolysis. This experiment was performed thrice.

#### **2.2.5 Antibiotic susceptibility profile**

*L. crispatus* susceptibility to different classes of antibiotics was evaluated by disc (6 mm diameter) method. At least two independent set of experiments were performed. As a control to the disc method, susceptibility to azithromycin, cefixime, ceftriaxone, ciprofloxacin, gentamicin, penicillin G and tetracycline was also evaluated by the E-test method according to the manufacturer's instructions. This method provides a convenient and quantitative test of antibiotic resistance. All the plates were incubated for 24 and 48 h before reading the plates. MRS agar was used in order to favor the growth of *L. crispatus*.

### **2.3. Purity control and counting of *L. crispatus***

Purity was assessed by macroscopic observation of colonies and microscopic observation after Gram staining. The serial decimal dilution method, by plating onto MRS agar medium, was used for counting total *Lactobacilli* in every suspensions and gels. Dilutions were made in PBS and after vortexing each dilution for 5 s, 100  $\mu$ L of the previous dilution were inoculated in 900  $\mu$ L of the next dilution. Then, 100  $\mu$ L of the chosen dilutions were poured on the MRS agar plates.

### **2.4. Hydrogel preparation**

A concentrated mixture of polymers was prepared. Polymer solubilization was performed by using a mixer equipped with a turbine adapted to the mixing of viscous preparations (Rayneri-turbotest, Rayneri, France) under an agitation of 1000 rpm as described by Aka-Any-Grah et

al. (2010). The alginate powder (1.250 g) was gradually added under agitation at room temperature to an ultrapure water (71,875 g). After complete dissolution of alginate, the poloxamer powder (26,875 g) was gradually added at 4 °C under the same agitation. After complete dissolution, this mixture was equilibrated 48 h at 4°C to eliminate air bubbles and was autoclaved at 121 °C, 15 min before its mixing with the *Lactobacilli* suspension. Meanwhile, frozen *L. crispatus* (-80 °C) was isolated on MRS agar for 48-72 h. Then it was subcultured twice, first for 24 h and second for 16 h (37 °C, 5 % CO<sub>2</sub>). This second subculture was centrifuged (2800 g, 10 min, 4°C) and washed twice with sterile water before being suspended in sterile water. *L. crispatus* homogenization was achieved by vortexing. *L. crispatus* suspension was enumerated by plating serial dilutions onto MRS agar. Final gels with or without *L. crispatus* were obtained by mixing respectively 1 mL of sterile water or 1 mL of the *L. crispatus* suspension to 4 g of the concentrated polymer mixture. This allowed obtaining two types of gels (P21.5A1 and P21.5A1L) whose final compositions are presented in **Table 1**. P21.5A1L contained 9 log<sub>10</sub> CFU of *L. crispatus* per gel sample (5 g).

**Table 1.** Hydrogels containing alginate (A), poloxamer (P) with or without *L. crispatus* (L)

Acronyms	Poloxamer (% w/w)	Alginate (% w/w)	<i>L. crispatus</i> (log <sub>10</sub> CFU/5 g of gel)
P21.5A1	21.5	1	-
P21.5A1L	21.5	1	9

## 2.5. Preparation of hydrogels diluted by simulated vaginal fluid

Previous studies showed that 5 mL of gels were able to properly cover the human vaginal epithelium, immediately after insertion, within the first 30 min and without sexual intercourse (Barnhart et al., 2004). The volume of ambient fluid present in the vagina at any time is approximately 0.5–0.75 mL (Owen and Katz, 1999). Therefore, to mimic the maximum dilution likely to occur in the vagina after the gel administration, 5 mL of each formulation were diluted with 0.75 mL of simulated vaginal fluid (SVF). The SVF was prepared according to Owen and Katz (1999). This SVF simulates the vaginal fluid from healthy, nonpregnant, premenopausal women and can be used to study vaginal formulations *in vitro*. P21.5A1 + SVF denoted P21.5A1 diluted with SVF and P21.5A1L + SVF the P21.5A1L one diluted with SVF. The aim of this test was to assess the influence of the dilution by SVF on the thermogelling and the rheological properties of the formulations.

## 2.6. Rheological characterization of the hydrogels

Rheological properties of the gels, at 25 °C, at 37 °C or after dilution of the gels by SVF at 37°C, were carried out on a rotational rheometer ARG2 (TA instruments, New Castle, USA) equipped with an aluminum cone/plate geometry (diameter 40 mm, angle 1° and cone truncation 28 μm) and with a solvent trap to limit evaporation during measurement. Peltier diodes, placed in the lower plate, allowed to control the temperature. The samples were equilibrated 3 min before starting the experiments. In flow measurements, the viscosity ( $\eta$ ) was recorded as a function of shear rate. The shear rate was increased gradually from 0.01 s<sup>-1</sup> to 1000 s<sup>-1</sup> over 3 min, maintained to 1000 s<sup>-1</sup> for 1 min and decreased gradually from 1000 s<sup>-1</sup> to 0.01 s<sup>-1</sup> over 3 min. In oscillatory measurements, linear viscoelastic regions, where the moduli remained constant as the shear stress increased were determined. The gelation temperature ( $T_{gel}$ ) and viscoelastic properties of the gels were assessed under a stress value belonging to their viscoelastic linear regime. A temperature sweep at a rate of 1 °C/min was performed at a frequency of 1 Hz, in a temperature range from 5 °C to 45 °C, and the elastic modulus ( $G'$ ) as well as the viscous modulus ( $G''$ ) were recorded as a function of temperature. The gelation temperature ( $T_{gel}$ ) was determined as the temperature of the crossover of  $G'$  and  $G''$ .  $G'$  and  $G''$  were also recorded during frequency sweeps from 0.01 to 50 Hz. These experiments were performed in triplicate.

## 2.7. pH measurement of the gels

Both the pH and the temperature into the gels were measured at room temperature with a pH-meter Mettler Toledo Seven2Go, suited for measurements in viscous systems. This experiment was performed in triplicate.

## 2.8. Assessment of the distribution of *L. crispatus* within the gel

The distribution and viability of *L. crispatus* within the gels were assessed by using syto 9 that stained both living and dead bacteria in green and propidium iodide that stained only dead bacteria in red. When used simultaneously, syto 9 seemed to allow the observation of living bacteria in green, and propidium iodide the observation in red of the dead ones (Moore et al., 2015). These fluorescent stains were introduced at 1/1000 (v/w) in the gels. In short, they were introduced in the 1 mL of *L. crispatus* suspension before mixing it with the gels in the

liquid state, then, they were immediately observed by microscope. This study was performed with an inverted confocal laser scanning microscope LSM 510-Meta (Carl Zeiss, Germany) using a Plan-Apochromat 63X/1.4 objective lens, equipped with an argon (488 nm excitation wavelength) and a helium neon laser (543 nm excitation wavelength). The green and the red fluorescence emissions were collected with a 505-550 nm band-pass and a 560 nm long pass emission filter respectively, under a sequential mode. The pinhole was set at 1.0 Airy unit. For each condition, single 2D images were collected as well as 3D images with an image every 0.9  $\mu\text{m}$  along the z axis to reach a thickness of 32  $\mu\text{m}$  taken arbitrarily. 12 bit numerical images were acquired with LSM 510 software version 3.2.

### **2.9. Assessment of the influence of gel state**

*L. crispatus* within the gel P21.5A1L were counted after culture on MRS agar plate at  $t_0$ ,  $t_{24\text{ h}}$  and  $t_{48\text{ h}}$  after storage at 15 °C (below gelation temperature) and at 23 °C (above gelation temperature). *L. crispatus* suspension was also analyzed in the same conditions as a control. This test was realized in triplicate.

### **2.10. Stability studies of the gel P21.5A1L**

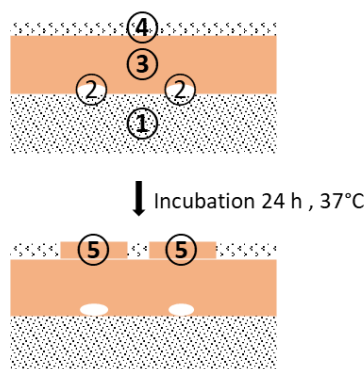
P21.5A1L was stored at two storage temperatures (4 °C and 25 °C) over six months in order to assess its stability. The gel viscosity at a shear rate of 125  $\text{s}^{-1}$  and pH were measured as previously described. Counting on MRS agar plate was performed to investigate the culturability of cells. In brief, after serial decimal dilutions, three dilutions were plated on MRS agar. Plates were incubated at 37 °C for 48 h before counting the colony forming units. Otherwise, *L. crispatus* viability was evaluated by confocal laser scanning microscopy. Briefly, the fluorescent stains were introduced at 1/1000 (v/w) in the liquid gels containing *L. crispatus*, vortexed and immediately observed by confocal microscopy as previously described in **section 2.8**.

### **2.11. Inhibition of the growth of *N. gonorrhoeae***

For the preparation of *N. gonorrhoeae* suspension, the frozen stock was isolated on Thayer-Martin medium plate and incubated for 18 h at 37 °C in a moist atmosphere enriched with 5 %  $\text{CO}_2$ . These conditions were respected because autolysis may occur during prolonged

incubation, and it may be difficult to obtain a suspension from growth on agar plates. Then, colonies were suspended in NaCl 0.85 % to a turbidity equivalent to 0.5 McFarland standard. These suspensions contained  $10^6$  *N. gonorrhoeae* cells/mL. They were mixed thoroughly using a vortex mixer to break up clumps of growth.

The inhibition of *N. gonorrhoeae* was evaluated by using a modified version of the agar overlay technique of Amant et al. (2002). **Figure 1** presents the method schematically. Brain heart infusion agars (BHIA) with different pH adjusted to pH 5, 6.5, and 7 were prepared before being autoclaved at 121 °C over 15 min. Gels or suspensions containing *L. crispatus* were inoculated onto BHIA plates (60 mm diameter), using respectively 1 µL sterile handle or 50 µL to obtain a spot. After 24 h of incubation, 4 mL of chocolate agar (containing proteose peptone, dextrose, sodium chloride, disodium phosphate, agar, hemoglobin) were poured onto the BHIA plates and allowed to solidify. Then, 500 µL of *N. gonorrhoeae* suspension were spread onto the agar overlay and incubated for 24 h. The presence of a zone of growth inhibition was considered as positive for inhibition. A control was made by the same protocol without the step of spot formation. For all experiments, the number of *L. crispatus* in the suspensions or the gels was confirmed by standard serial dilution and culture. The thickness of each agar layer was about 2 mm.



**Figure 1.** Schematic depiction of the inhibition of *N. gonorrhoeae* test: ① heart brain infusion agar, ② spots of gels or suspensions containing *L. crispatus* after incubation 24 h, 37°C, ③ chocolate agar medium, ④ spreading of *N. gonorrhoeae*, ⑤ inhibition zones.

### 3. RESULTS AND DISCUSSION

In this study, a reference strain, well identified and characterized, that originated from a human vagina and that has already shown an efficacy on *N. gonorrhoeae* (Amant et al., 2002; Breshears et al., 2015) was selected. Then, its probiotic benefits such as its acidification

ability, its ability to inhibit the growth of *N. gonorrhoeae in vitro*, and its ability to auto-aggregate or co-aggregate *N. gonorrhoeae* were confirmed before formulation development.

### 3.1. Characterization of *L. crispatus*

In this study, *L. crispatus* suspensions contained  $9 \log_{10}$  CFU/mL based on published data. Amant et al. (2002) showed the effectiveness of different dilutions of  $10^8$  *L. crispatus* on *N. gonorrhoeae* and Reid et al. (2001) showed the effectiveness of over  $10^8$  viable probiotic per day to restore and maintain a normal vaginal flora. An expert consensus document stated that the minimal number of viable cells should be  $1 \times 10^9$  CFU administered per day (Hill et al., 2014).

*L. crispatus* growth kinetic test allowed determining its stationary phase that began after 9 h of culture. Based on this result, for all the tests, cultures of 16 h of incubation were used to ensure reproducible physiological and metabolic states of the strain.

As regards *L. crispatus* acidification ability, this strain was grown in either standard growth medium (pH<sub>i</sub> 6.5) or in modified pH growth medium (4, 5, 6, 7). The use of these pH values was based on the fact that vaginal pH varies between 3.5 and 5.5 and increases up to 7 in the presence of seminal fluid (O'Hanlon et al., 2013; Owen and Katz, 1999). Therefore, the growth and the acid production of *L. crispatus* were simultaneously evaluated in MRS at pH ranging from 4 to 7 to mimic this vaginal pH. In MRS, the *Lactobacilli* had the nutrients they needed to grow, pH was the only modified parameter in order to assess its influence. The growth of *L. crispatus* was favored at pH ranging from 6 to 7 (**Table 2**). This pH range corresponded to the pH of the mixture of polymers, which was thus favorable for the mix with *L. crispatus*. Otherwise, whatever the MRS pH<sub>i</sub>, a decrease in pH values was observed after 24 h of incubation at 37 °C and 5 % CO<sub>2</sub>, mimicking the vaginal conditions. The variations of pH are given in **Table 2**. They were also higher at pH varying between 6 and 7, suggesting a correlation between *L. crispatus* viability and acid production. A reduction of pH by *L. crispatus* indicated indirectly an acidification due to lactic acid production. This result suggested thus higher lactic acid production at pH higher than 6. Both pH and lactic acid concentration of a media may inactivate pathogens individually or in combination. (O'Hanlon et al. (2013) showed that as the alkaline pH of the vagina due to seminal fluid, reduced the protective action of vaginal acidity against pathogens, the level of vaginal lactic acid was likely to reduce the transmission of vaginal acid-sensitive pathogens. As only protonated

lactic acid might have microbicidal activity, it would be better to have a pH below the pKa of lactic acid (3.90) (Tachedjian et al., 2017). In this study, the quantity of protonated lactic acid was not measured, but as the final pH in each sample was between 3,1 and 3,9, it can be supposed that lactic acid was in its protonated state favorable for its antibacterial activity. The final pH in each sample was comparable to the pH of healthy vagina dominated by *Lactobacilli* as described by O’Hanlon et al. (2013).

**Table 2.** Reduction in pH values due to *L. crispatus* acid production and growth of *L. crispatus* at different pH values as a mean of three replicates on two separate experiments.

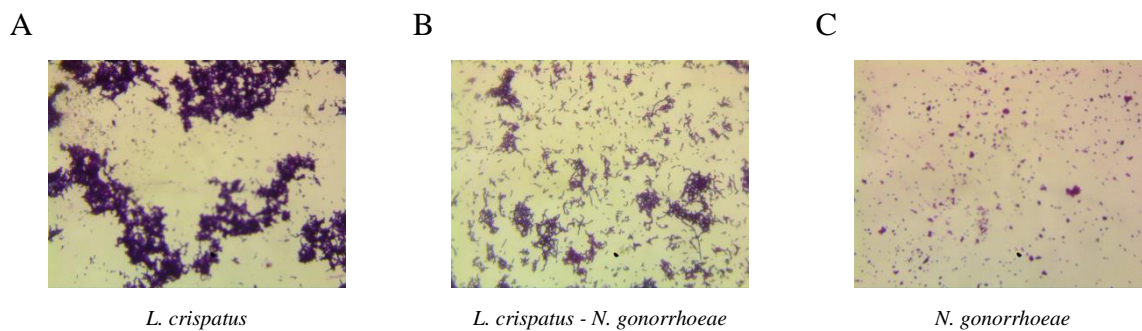
pH <sub>i</sub>	4.00 ± 0.01	5.00 ± 0.01	6.00 ± 0.01	6.50 ± 0.01	7.00 ± 0.01
Mean growth at 24 h, 37 °C, 5 % CO <sub>2</sub> (Log <sub>10</sub> CFU/mL)	1.19 ± 0.23	1.57 ± 0.41	1.85 ± 0.46	1.88 ± 0.11	2.00 ± 0.16
Mean pH reduction due to <i>L. crispatus</i> at 24 h, 37 °C, 5 % CO <sub>2</sub>	0.74 ± 0.17	1.49 ± 0.13	1.96 ± 0.02	2.24 ± 0.08	2.39 ± 0.04

Moreover, Graver and Wade (2011) showed the important role of acidification in the inhibition of *N. gonorrhoeae*. For Foschi et al. (2017), the presence of lactate seemed to be crucial for the anti-gonococcal activity, especially for pH values ranging from 4.4 to 5.3, indicating that the presence of hydrogen ions was necessary but not sufficient to inhibit *gonococci*. In our study, this evaluation of the acid production and the growth of *L. crispatus* in the pH range of 4 to 7 predicted a favorable growth in the vagina containing natural nutrients and favorable conditions for the inhibition of *N. gonorrhoeae* (see **section 3.5**). Amant et al. (2002) also showed the ability of this *Lactobacillus* strain to inhibit *N. gonorrhoeae* both at acid and neutral pH. Based on these studies, the higher reduction in pH related to higher lactic acid production was considered as more interesting.

The auto-aggregation and co-aggregation with *N. gonorrhoeae* properties of *L. crispatus* were investigated. **Figure 2** shows their organization in suspension after a 4 h-incubation time in suspension. *L. crispatus* formed large and dense visible clumps (**Fig. 2A**) whereas *N. gonorrhoeae* formed a clump similar to that observed by Anderson et al. (16) (**Fig. 2C**), with an aspect different than that of *L. crispatus* (**Fig. 2A**). Small and widely distributed aggregates were observed in the mixtures of *L. crispatus* and *N. gonorrhoeae* (**Fig. 2B**). They were arranged as microcolonies similar to the ones observed by Foschi et al. (2017). *L. crispatus* can change its organization in the presence of pathogens to co-aggregate with them. Reid et al. (1990) showed that when *Lactobacilli* formed co-aggregates and bound to pathogens, this resulted in returns to homeostasis, because co-aggregation created a hostile biochemical



micro-environment around the pathogen and prevented it from continuation of growth and domination of the niche.



**Figure 2.** Aspects of clumps in aggregation tests after 4 h of incubation (x 40).

Regarding the security aspect associated with the use of this *L. crispatus* strain, two parameters were studied, its hemolytic activity and its antimicrobial resistance. Hemolytic activity test showed that the Columbia sheep blood agar aspect around the colonies were unchanged, meaning *L. crispatus* did not exhibit hemolysis. It is a non-hemolytic bacterium. This result was in accordance with many reports which had revealed that *Lactobacilli* generally did not exhibit hemolysis (Ambalam et al., 2012; Kassaa et al., 2014; Maragkoudakis et al., 2006). The antibiotic resistance is another key factor for probiotics as the level of susceptibility to the antimicrobial agents is species-dependent. **Table 3** presents the results of disc method. Both E-test and the disc method gave similar results for the antibiotics studied by the two methods. The resistance to gentamicin and ciprofloxacin was observed both by E-test and disc diffusion methods. Results showed that *L. crispatus* was resistant to an aminoglycoside (gentamicin), quinolones (ciprofloxacin, nalidixic acid, pefloxacin) and a lincosamide (clindamycin) (**Table 3**). Several studies have reported intrinsic resistance of *Lactobacilli* to quinolones, gentamicin, and other antibiotics (Danielsen and Wind, 2003; Štšepetova et al., 2017; Temmerman et al., 2003). According to the European Food Safety Authority (EFSA) guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, bacterial strains carrying intrinsic resistance present a minimal risk for horizontal spread and thus, may be used as a food additive. Based on these *in vitro* tests, *L. crispatus* is an interesting probiotic candidate. However, further studies should be performed to search for pathogenicity genes, as well as *in vivo* studies to confirm that this *L. crispatus* strain could not induce any pathogenicity and could be regarded as a safe microorganism for a vaginal administration.

**Table 3.** Antibiotics susceptibility of *L. crispatus* by disc diffusion method as a mean of at least two repetitions, showing in gray the antibiotics to which *L. crispatus* was resistant.

Antibiotic discs (dose/disc)	Inhibition zone diameter (mm)	Antibiotic discs (dose/disc)	Inhibition zone diameter (mm)
<b>PENICILLIN CLASS</b>		<b>TETRACYCLINES CLASS</b>	
Penicillin G (10µg)	26	Doxycycline (DOX, 30 µg)	26
Ampicillin (AMP, 10 µg)	25	Minocycline (MN, 30 µg)	29
Amoxicillin (AMX, 25 µg)	24	Tetracycline (TET, 30 µg)	28
Oxacillin (OXA, 1 µg)	27	<b>MACROLIDES CLASS</b>	
Piperacillin (PIP, 75 µg)	34	Azithromycin (AZM, 15 µg)	20
Ticarcillin (TIC, 75 µg)	37	Spiramycin (SPN, 100 µg)	18
Amoxicillin-clavulanic acid (AMC, 20/10 µg)	27	Erythromycin (ERY, 15 µg)	30
Piperacillin-tazobactam (PPT, 75/10 µg)	24	<b>CHLORAMPHENICOL CLASS</b>	
Ticarcillin-clavulanic (TCC, 75/10 µg)	36	Chloramphenicol (CHL, 30 µg)	29
<b>CEPHALOSPORIN CLASS</b>		<b>LINCOSAMIDES CLASS</b>	
Cephalexin (CEF, 30 µg)	30	Lincomycin LCN, 15 µg)	13
Cefuroxime (CXM, 30 µg)	24	Clindamycin (CMN, 2 µg)	6
Cefixime (CFM, 10 µg)	28	<b>QUINOLONES CLASS</b>	
Ceftazidime (CAZ, 30 µg)	21	Pefloxacin (PEF, 5 µg)	0
Cefpodoxime (CPD, 10 µg)	19	Ciprofloxacin (CIP, 5 µg)	0
Ceftriaxone (CFO, 30 µg)	31	Nalidixic acid (NAL, 30 µg)	0
<b>AMINOGLYCOSIDES CLASS</b>		<b>ANSAMYCIN CLASS</b>	
Gentamicin (GMI, 15µg)	0	Rifampycin (RAM, 30 µg)	27
<b>CARBAPENEM CLASS</b>		<b>GLYCOPEPTIDES CLASS</b>	
Imipenem (IPM, 10 µg)	25	Vancomycin (VAN, 30 µg),	19
		Teicoplanin (TEC, 30 µg)	15

Regarding the ability of *L. crispatus* to inhibit the growth of *N. gonorrhoeae* *in vitro*, the assays were realized at three different pH (5, 6.5, 7) (Table 4). *L. crispatus* suspensions contained 8 log<sub>10</sub> cells/mL while *N. gonorrhoeae* suspension 6 log<sub>10</sub> cells/mL. The BHIA acid (pH 5) overlaid by chocolate agar (pH 7) was deleterious for *N. gonorrhoeae*. That is why there was no growth of *N. gonorrhoeae* at this condition. An inhibition zone was clearly observed at BHIA pH 6.5 and 7. Therefore, this test confirmed the inhibition of *N. gonorrhoeae* by the studied *L. crispatus* strain in a pH-dependant manner (Table 4). This result was similar to the observation of Amant et al. (2002), who demonstrated that *L. crispatus* could inhibit *N. gonorrhoeae* both in acidic and in neutral pH. Foschi et al. (2017) also showed the strict correlation between the pH levels and the anti-gonococcal effect of *Lactobacilli*. Their supernatant whose pH was below 4 induced a reduction of 100 % of *N. gonorrhoeae* viability by *L. crispatus* after 7 min of direct contact at 37 °C, 5 % CO<sub>2</sub>. The acidic environment associated to inhibitory molecules produced by *L. crispatus* may induce complete inhibition of the growth of *N. gonorrhoeae*.

**Table 4.** Inhibition of the growth of *N. gonorrhoeae in vitro* by agar overlaid method, after 24 h of incubation at 37 °C, 5 % CO<sub>2</sub>. Comparative study of *L. crispatus* suspension and of gels P21.5A1 and P21.5A1L. Four different experiments (test).

		Control = BHIA	Test 1	Test 2	Test 3	Test 4
		Agar overlaid by chocolate agar				
Suspension	Counting ( $\log_{10}$ <i>L. crispatus</i> /spot)	No spot	8.52	8.33	8.12	8.35
	Inhibition diameter at pH 5	≥ 60 mm	≥ 60 mm	≥ 60 mm	≥ 60 mm	≥ 60 mm
	Inhibition diameter at pH 6.5	No inhibition	30 mm	28 mm	30 mm	31 mm
	Inhibition diameter at pH 7	No inhibition	30 mm	20 mm	-	-
P21.5A1	Inhibition diameter at pH 5	≥ 60 mm	≥ 60 mm	≥ 60 mm	≥ 60 mm	≥ 60 mm
	Inhibition diameter at pH 6.5	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
	Inhibition diameter at pH 7	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
	Counting ( $\log_{10}$ <i>L. crispatus</i> /spot)	No spot	7.32	7.72	9.32	9.10
P21.5A1L	Inhibition diameter at pH 5	≥ 60 mm	≥ 60 mm	≥ 60 mm	≥ 60 mm	≥ 60 mm
	Inhibition diameter at pH 6.5	No inhibition	15 mm	12 mm	31 mm	30 mm
	Inhibition diameter at pH 7	No inhibition	10 mm	11 mm	-	14 mm

-non exploitable due to contamination

All these studies allowed asserting this *L. crispatus* strain may grow, produce acids, co-aggregate with *N. gonorrhoeae*, and inhibit the growth of *N. gonorrhoeae*. It could be used in order to treat or prevent gonorrhoea. Our results were in accordance with those of Foschi et al. (2017), who demonstrated that their studied *L. crispatus* strains were able to counteract *N. gonorrhoeae* viability through multiple mechanisms (acidic environment creation, lactate production, a biosurfactant production and co-aggregation), and could represent a new potential probiotic strategy for the prevention of gonorrhoea in women.

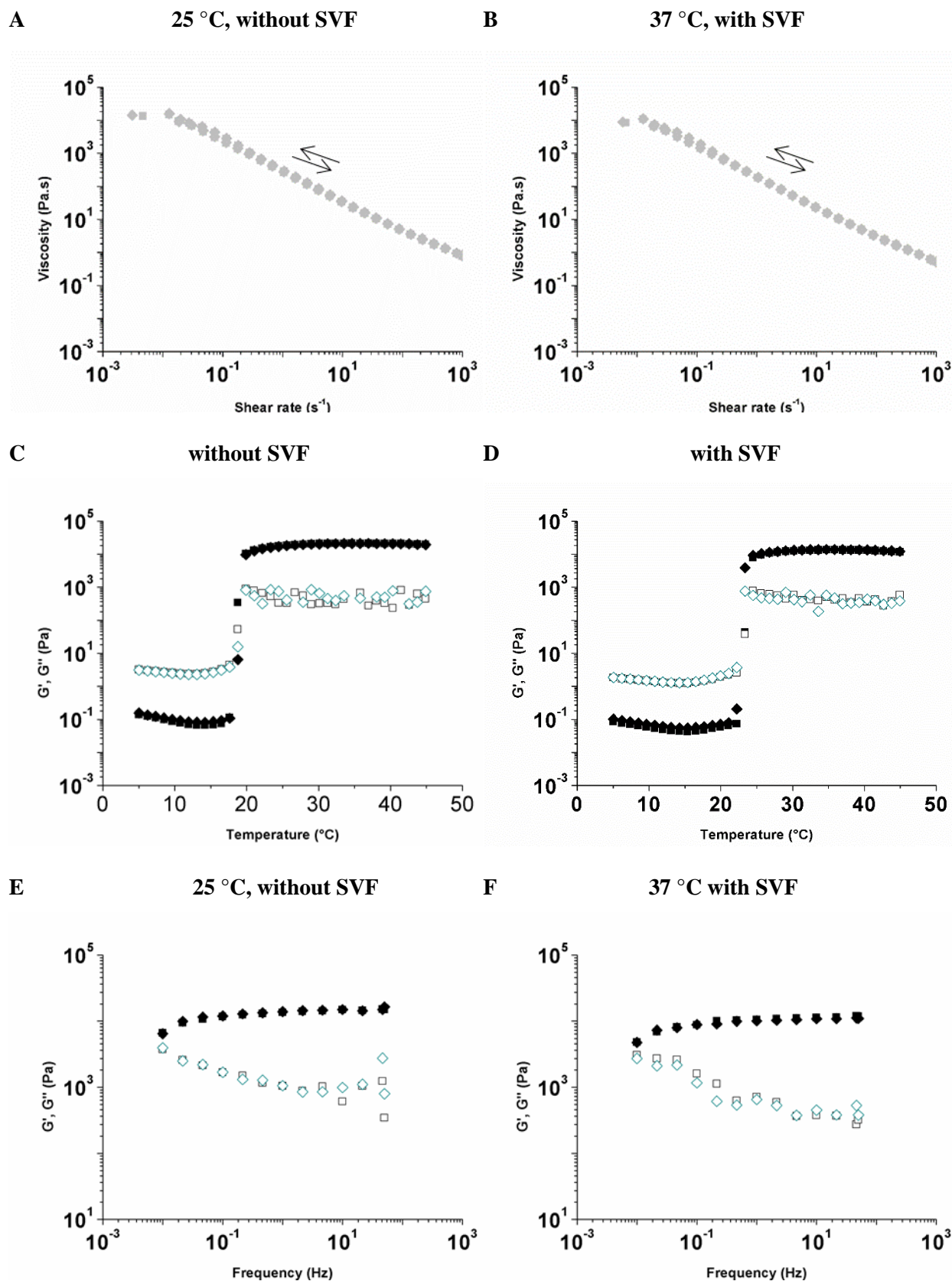
### 3.2. Preparation and rheological characterization of gels

In a second step of our study, *L. crispatus* was incorporated in an aqueous mixture of poloxamer 407 and sodium alginate in order to develop a formulation with characteristics that were suitable for a vaginal administration. In a preliminary optimization step, the concentration of poloxamer 407 (21.5 %) and sodium alginate (1 %) were selected in order to obtain a formulation that remained in gel state at 37 °C, even after dilution with SVF, with minimal polymer concentrations. Thereafter, the study focused on this gel denoted P21.5A1 and on its corresponding gel containing *L. crispatus* at 9  $\log_{10}$  CFU by gel unit (P21.5A1L).

During the process to obtain the gels containing *Lactobacilli*, the critical point was the mixture of the concentrated polymers with the suspension of *Lactobacilli*. The counting of

*Lactobacilli* of both suspensions and gels after their preparation showed that in the gel it represented 1/5 of the number in the suspension. That corresponded to the dilution ratio of *L. crispatus* suspension (1 mL) to the gel (4 g). This result suggested that the fabrication process was not deleterious for the *Lactobacilli*.

Both gels were characterized by rheology. The influence of *L. crispatus* on the flow, the gelation and the viscoelastic properties of the formulations were assessed (**Fig. 3, Table 5**). The gel without *L. crispatus* P21.5A1 showed a shear-thinning behavior (**Fig. 3A**). This behavior should allow an easy administration. This formulation was also non-thixotropic even after dilution by the simulated vaginal fluid (**Fig. 3B**). Therefore, as soon as the shear was removed, the viscosity returned to its initial viscosity, which should be favorable to maintain the gel at the administration site. P21.5A1 also had a thermogelling behavior (**Fig. 3C**). Below  $T_{gel}$ , the elastic modulus ( $G'$ ) was lower than the viscous modulus ( $G''$ ) revealing a dominant viscous behavior. Above  $T_{gel}$ ,  $G'$  was higher than  $G''$  showing a dominant elastic behavior. P21.5A1 gelation temperature was  $19 \pm 1$  °C. After dilution with SVF,  $T_{gel}$  was increased (**Fig. 3C, 3D**) whereas the viscoelastic moduli values decreased due to the diminution of the concentration of poloxamer (**Fig. 3C versus 3D and 3E versus 3F**). Aka-Any-Grah et al. (2010) also observed this phenomenon. When *L. crispatus* was introduced into the gel (P21.5A1L), the rheological properties were similar to those of P21.5A1 (**Fig. 3, Table 5**). *L. crispatus* did not significantly influence the rheological properties of this mixture of poloxamer 407 and sodium alginate even after dilution with SVF.



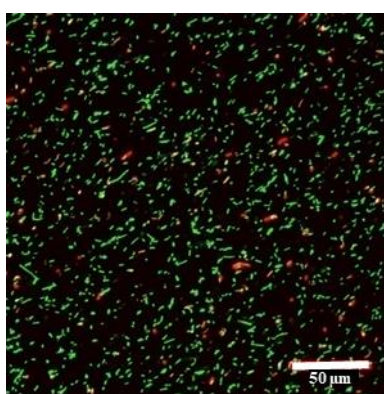
**Figure 3.** Comparative rheological properties of the gels P21.5A1 and P21.5A1L, showing flow properties (A, B), temperature sweep displaying one point data out of ten (C, D), and viscoelastic properties at 1 Hz (E, F), showing gels without dilution with SVF (A,C,E) and after dilution with SVF (B,D,F): (■)  $\eta$  for P21.5A1, (□)  $G''$  for P21.5A1, and (◆)  $\eta$  for P21.5A1L, (◇)  $G''$  for P21.5A1L

**Table 5.** Comparative study of the gel without and with *L. crispatus*. Influence of *L. crispatus* on the rheological properties of the gel containing poloxamer 407 at 21.5 % and sodium alginate at 1 % at three different conditions: 25 °C, 37 °C and 37 °C after dilution with SVF.

Samples	T <sub>gel</sub> (°C)	Viscoelastic properties			Flow properties
		Conditions	G' at 1 Hz (10 <sup>3</sup> Pa)	G'' mean at 1 Hz (10 <sup>3</sup> Pa)	
P21.5A1	19 ± 1	25°C	13.5 ± 0.4	1.0 ± 0.2	Shear-thinning Non- thixotropic
		37 °C	17.6 ± 0.5	0.5 ± 0.6	
P21.5A1 + SVF	24 ± 1	37 °C + SVF	10.5 ± 0.2	0.7 ± 0.2	
P21.5A1L	19 ± 1	25°C	13.9 ± 0.3	1.1 ± 0.5	
		37 °C	16.5 ± 0.4	0.7 ± 0.1	
P21.5A1L + SVF	23 ± 1	37 °C + SVF	10.1 ± 0.3	0.7 ± 0.2	

### 3.3. Distribution and viability of *L. crispatus* in the gel

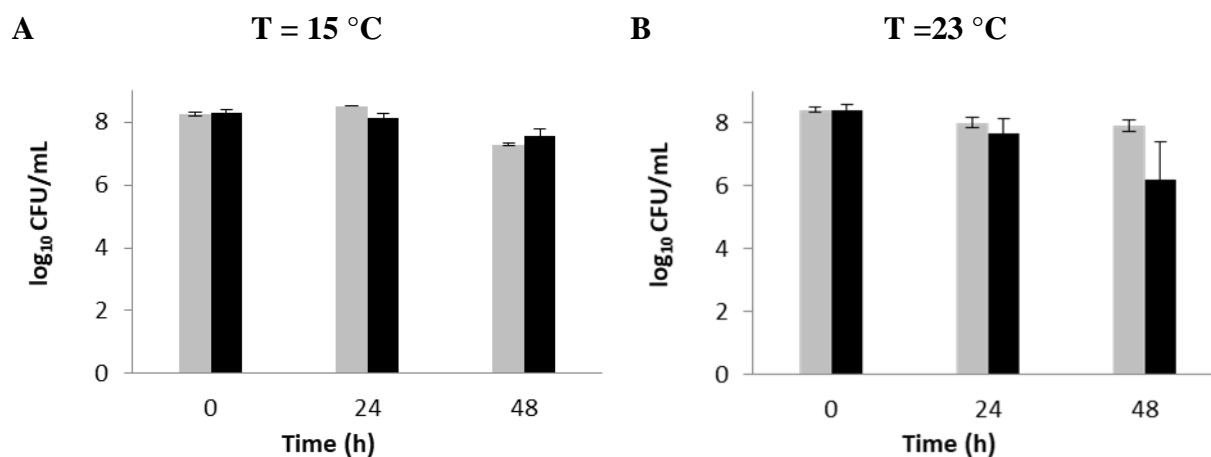
For further characterization of the gel containing *L. crispatus*, we studied the distribution of *L. crispatus* immediately after the gel preparation, by confocal fluorescence microscopy (CLSM), using both syto 9 and propidium iodide (**Fig. 4**). Almost all the *L. crispatus* cells were green stained confirming their viability after their introduction into the gel. The distribution seemed to be similar at all the observed points, from two different observed wells of the same gel. CLSM showed a homogeneous distribution of *L. crispatus* in the gel (**Fig. 4**). This distribution of *L. crispatus* was repeatable and reproducible, showing the reproducibility of the method of gel preparation. This homogeneous distribution of *L. crispatus* in the gel should be favorable to a homogeneous distribution of *L. crispatus* in the vagina.



**Figure 4.** Image representative of the observed images of P21.5A1L in gel state immediately after their preparation, by confocal microscopy, showing the majority of *L. crispatus* in green. Projection in z-axis of 32 μm of depth.

The viability of *L. crispatus* in the suspension compared to the gel as well as the influence of the gel state on the viability of *L. crispatus* was assessed (**Fig. 5**). Compared to a suspension

of *Lactobacilli*, the number of *L. crispatus* did not significantly decrease over 48 h, at 15 °C. At 23 °C, only a slight decrease was observed at 48 h. These results suggested that the formulation was not deleterious to *L. crispatus*. Below the gelation temperature (15 °C) (**Fig. 5A**), the formulation was liquid and solid-like above it (23 °C) (**Fig. 5B**). The gel state did not seem to influence the viability of *Lactobacilli* at short time.



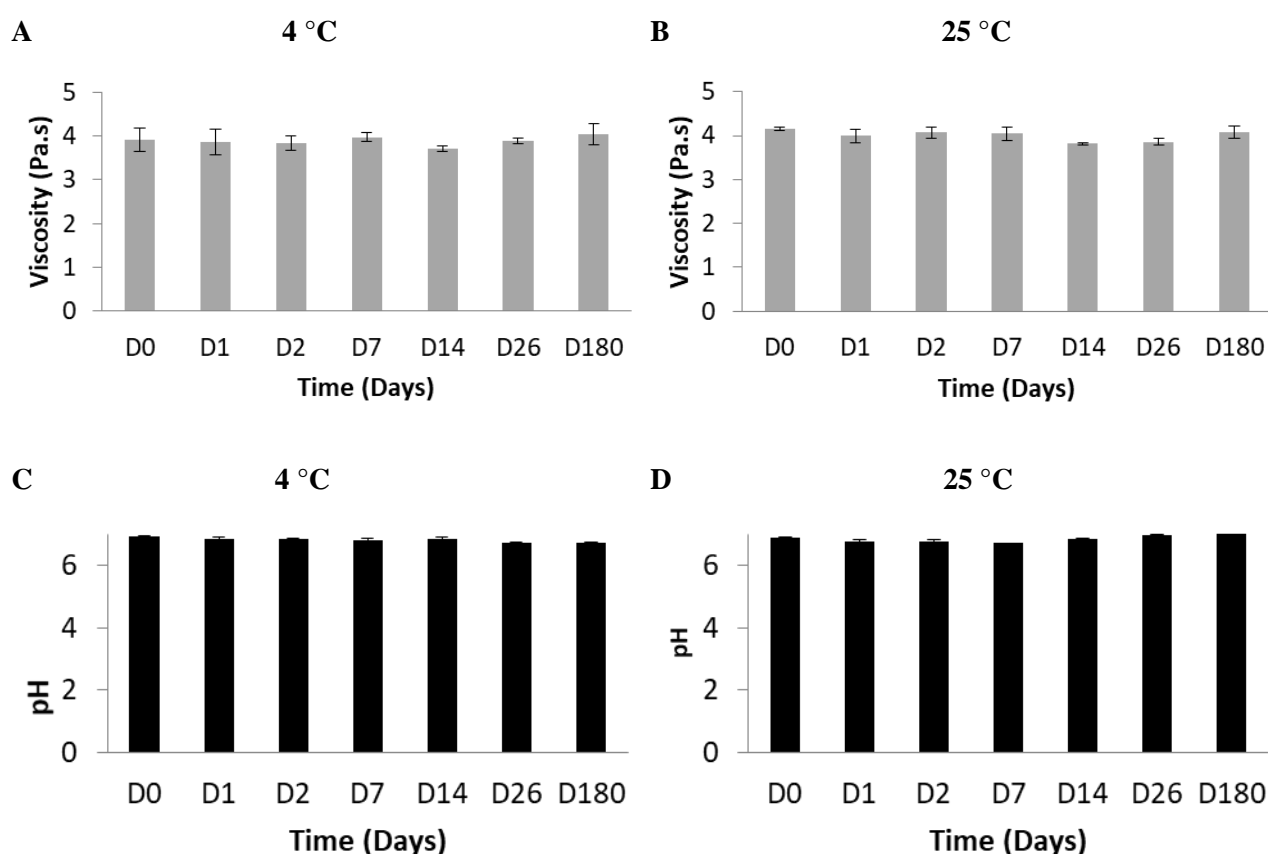
**Figure 5.** *L. crispatus* viability in a gel P21.5A1L (black bars) compared to a suspension of *L. crispatus* (gray bars) showing the results at 15 °C (below the gelation temperature) (A) and at 23 °C (above the gelation temperature) (B),  $T_{gel} = 19 \pm 1^{\circ}C$ .

#### 4. Influence of the storage conditions

The stability of the gel P21.5A1L was examined for 6 months in two different storage conditions 4 °C and 25 °C. Viscosity of the gel (**Fig. 6A, B**) as well as its pH (**Fig. 6C, D**) remained unchanged over these 6 months of storage at 4 °C (**Fig. 6A, C**) as well as at 25 °C (**Fig. 6B, D**). However, the viability and the culturability of formulated *L. crispatus* were modified (**Fig. 7, 8**). The number of *L. crispatus* assessed by counting on MRS agar plate decreased drastically after 2 days of storage at 25 °C (**Fig. 8**) and after 7 days at 4 °C (**Fig. 7**). The number of *L. crispatus* was assessed by confocal microscopy using two fluorescent stains (**Fig. 7**). Zotta et al. (2012) showed the usefulness of two stain indicators for optimal results in the evaluation of cells viability. In the present study, Syto 9 was used to stain live bacteria in green whereas propidium iodide was used to stain dead cells. Samples were analysed by two types of acquisition, the Z-stack acquisition of two different areas and the single stack of eight different areas. The coefficient of correlation between the two types of image acquisition was 0.93. Despite the observation of green-labeled bacteria on the images of the confocal

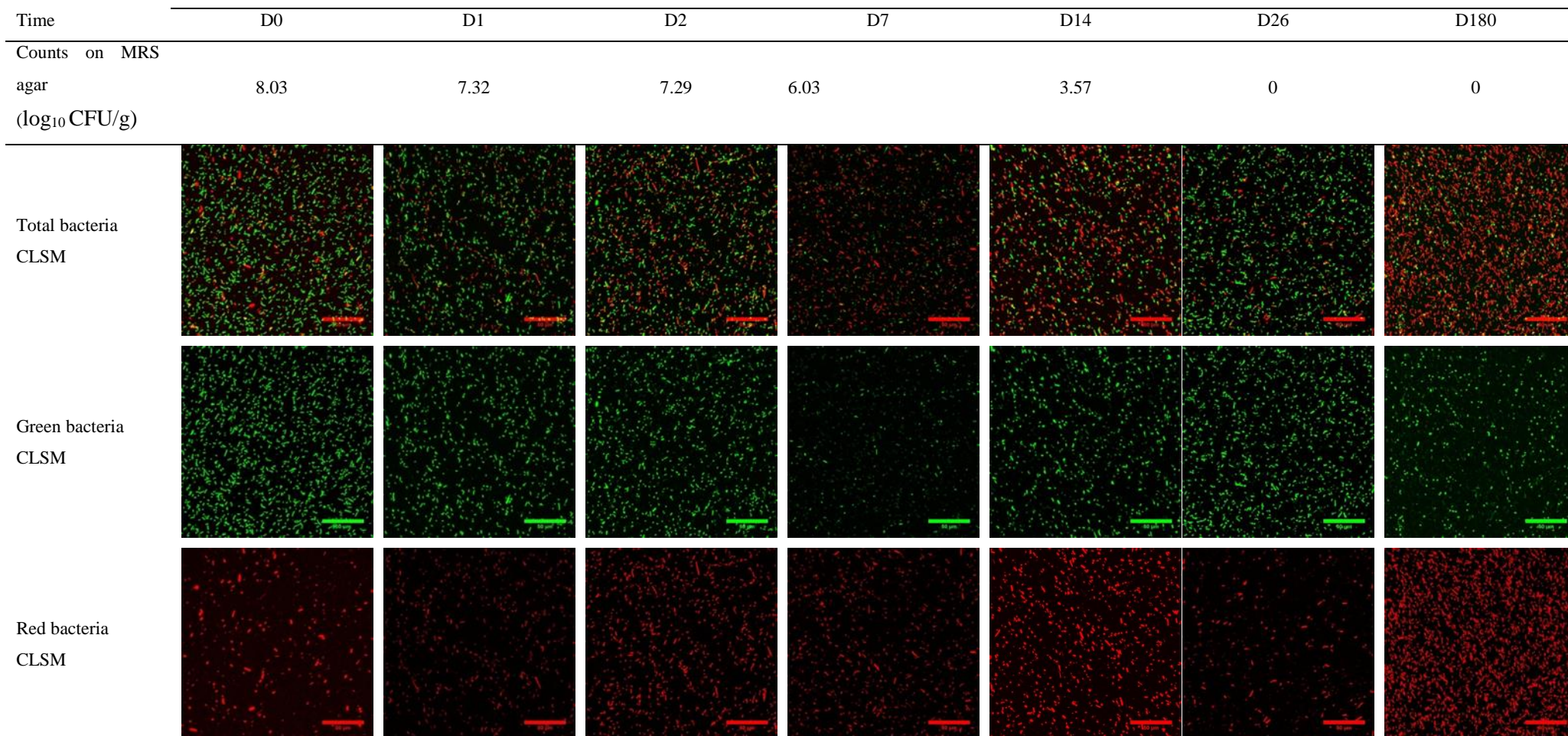
microscopy, there was no bacterial count by agar count method on MRS plate. This result suggested the development of viable but nonculturable (VNC) bacteria in the gel during storage at both 4 °C and 25 °C. This can be due to the absence of nutrient inducing a stress of *L. crispatus* in the gel. Indeed, the enumeration by agar plate method often leads to an underestimation of bacterial total number due to the formation of cells chains and clumps or to the induction of VNC state after an exposure to stress or starvation (Léonard et al., 2015; Rault et al., 2007; Zotta et al., 2009). These results are in accordance with several studies which showed that cell viability estimated by microscopy was higher than viability assessed by plate counts. VNC cells can not be cultured using standard microbiological media because they are incapable of undergoing the sustained cellular division required to form a colony on regular agar media but they often maintain a potential for metabolic activity at the single cell level (Zotta et al., 2009).

As the number of *L. crispatus* decreased in gel, further studies are needed to preserve its viability over time. The use of a lyophilization process might be an interesting strategy.

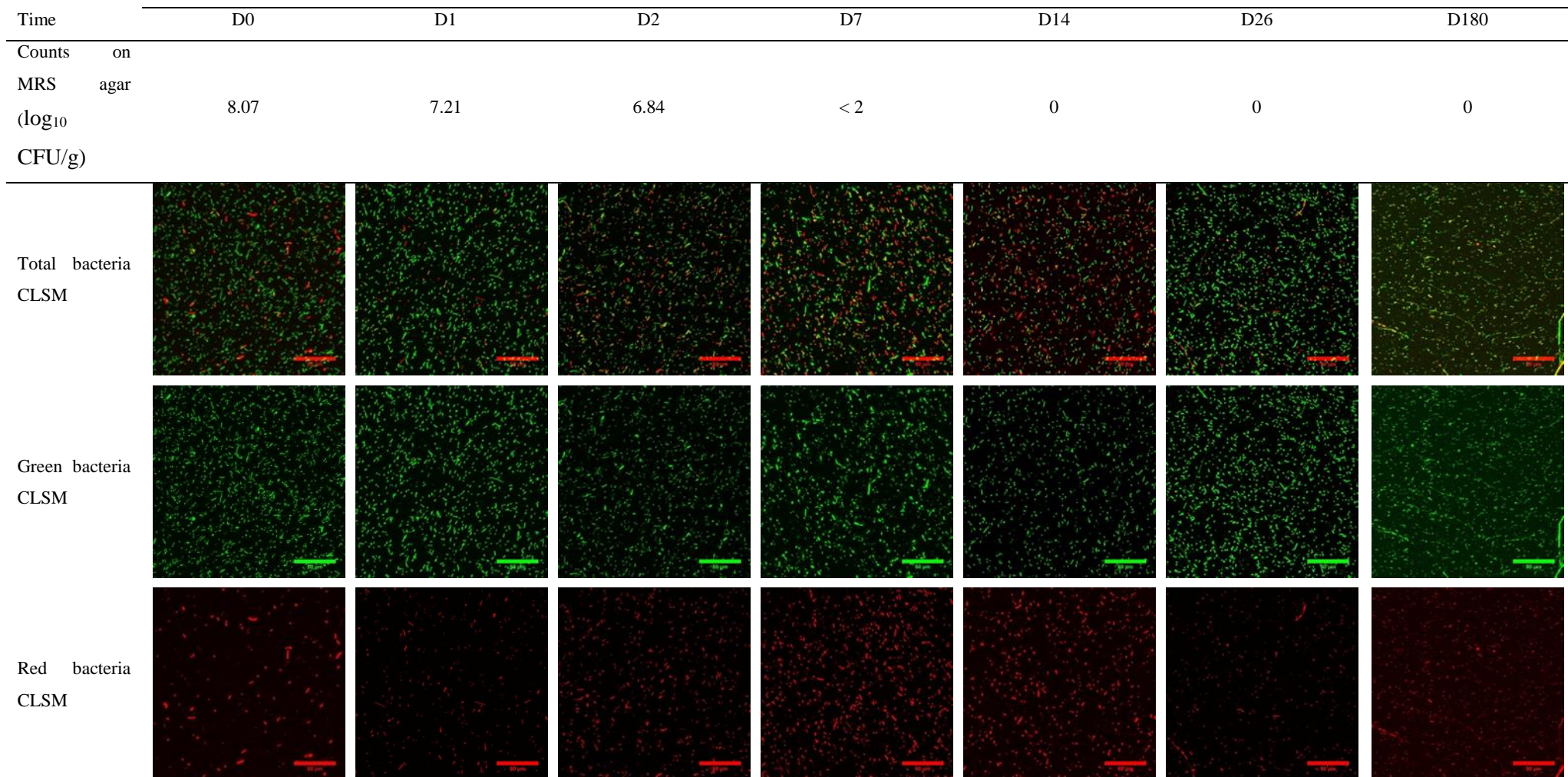


**Figure 6.** Viscosity (A, B) and pH (C, D) measurements of a gel containing poloxamer 407 at 21.5 %, sodium alginate at 1 % and *L. crispatus* at 9 log<sub>10</sub> CFU by gel unit, during its storage over six months at a storage temperature of 4 °C (A, C) or at a storage temperature of 25 °C (B, D).





**Figure 7.** Viability of *L. crispatus* containing in the gel P21.5A1L after storage at 4 °C. Observations were made immediately after preparation (D0) and at days 1, 2, 7, 14, 26, 180 (D1-D180). Counts on MRS agar plate expressed in log<sub>10</sub> CFU/g. Examples of projection of all bacteria, of green-stained bacteria and of red-stained bacteria. Scale bar corresponds to 50 μm.



**Figure 8.** Viability of *L. crispatus* containing in the gel P21.5A1L after storage at 25 °C (B). Observations were made immediately after preparation (D0) and at days 1,2, 7, 14, 26, 180 (D1-D180). Counts on MRS agar plate expressed in log<sub>10</sub> CFU/g. Examples of projection of all bacteria, of green-stained bacteria and of red-stained bacteria. Scale bar corresponds to 50 µm

## 5. Efficacy of inhibition of *N. gonorrhoeae*

P21.5A1L efficacy on *N. gonorrhoeae* was assessed. Its aim was to assess the efficacy of the gel containing different quantity of the selected *L. crispatus*. The minimal quantity of *L. crispatus* in tested gels was  $2.1 \times 10^8$  CFU/gel unit corresponding to test 1 of P21.5A1L (**Table 4**). These gels showed an efficacy depending on *L. crispatus* quantity.

The efficacy of the gel with and without *L. crispatus* was assessed *in vitro* on *N. gonorrhoeae* (**Table 4**). As observed with suspensions containing *L. crispatus*, the BHIA acid (pH 5) overlaid by GC agar (pH 7) induced no growth of *N. gonorrhoeae* due to its own acidity. Therefore, the tests on BHIA at pH 6.5 and 7 were used for comparison. The gel without *L. crispatus* P21.5A1 did not show any efficacy while the gel containing *L. crispatus* P21.5A1L inhibited the growth of pathogens (**Table 4**). *L. crispatus* was thus responsible of the inhibition by the gel at pH 6.5 and 7. *N. gonorrhoeae* optimal growth pH is pH 7. This result confirmed that *L. crispatus* inhibited *N. gonorrhoeae* but this inhibition is reinforced in more acidic environment.

At BHIA pH 6.5, the inhibition zone of the suspension containing  $8 \log_{10}$  *L. crispatus* was similar to the inhibition zone of the gel containing  $9 \log_{10}$  *L. crispatus* (**Table 4**). The inhibition of *N. gonorrhoeae* by the studied *L. crispatus* strain containing in the gel, suggested that the polymers could influence the inhibition of *N. gonorrhoeae*. The gel, due to its viscosity, could slow down the diffusion of substances that are produced by *L. crispatus* are involved in the inhibition mechanisms. Despite this lower efficacy compared to the suspension, gels remain interesting because liquid suspensions may lead to a rapid flow and clearance after vaginal administration whereas the gel is likely to have a higher residence time due to its viscosity. Otherwise, the method used to test the inhibition of *N. gonorrhoeae* only explore the influence of the environment pH and the diffusion of inhibitory molecules through the agar. Further studies by direct contact with *N. gonorrhoeae* should be performed to determine if formulated *L. crispatus* preserve all its inhibition properties on *N. gonorrhoeae*.

## 6. CONCLUSION

This study showed the probiotic potential of *L. crispatus* ATCC 33197 and its ability to inhibit *N. gonorrhoeae*. *L. crispatus* can be considered as a good candidate to be used in the development of vaginal formulations for the prevention of gonorrhea. Gels containing the poloxamer 407 at 21.5 % and alginate 1 % with or without *L. crispatus* ATCC 33197 showed

rheological properties favorable for a vaginal administration. Their process of preparation was not deleterious for the *Lactobacilli* cells. Therefore, it would be possible to prepare a gel containing *L. crispatus* 33197 as a valuable system intended to the vagina to prevent the infection by *N. gonorrhoeae*. Such a gel is promising for the prevention of gonorrhea, but *L. crispatus* viability should be optimized by lyophilization or the addition of some nutrients in the formulation.

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### **Conflict of interest**

The authors declare no conflict of interest.

### **References**

- Aka-Any-Grah, A., Bouchemal, K., Koffi, A., Agnely, F., Zhang, M., Djabourov, M., and Ponchel, G. (2010). Formulation of mucoadhesive vaginal hydrogels insensitive to dilution with vaginal fluids. *Eur. J. Pharm. Biopharm.* 76, 296–303.
- Amant, D.C.S., Valentin-Bon, I.E., and Jerse, A.E. (2002). Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. *Infect. Immun.* 70, 7169–7171.
- Ambalam, P., Ramoliya, J.M., Dave, J.M., and Vyas, B.R.M. (2012). Safety assessment of potential probiotic strains *Lactobacillus rhamnosus* 231 and *Lactobacillus rhamnosus* v92 in mouse model.. *Int. J. Bioassays* 2, 333–337.
- Anderson, M.T., Byerly, L., Apicella, M.A., and Seifert, H.S. (2016). Seminal plasma promotes *Neisseria gonorrhoeae* aggregation and biofilm formation. *J. Bacteriol.* 198, 2228–2235.
- Andrew, and Gregor, R. (2017). Intravaginal instillation of *Lactobacilli* for prevention of recurrent urinary tract infections. *Can. J. Microbiol.* 3, 339–343.

Antonio, M.A.D., and Hillier, S.L. (2003). DNA fingerprinting of *Lactobacillus crispatus* strain CTV-05 by repetitive element sequence-based PCR analysis in a pilot study of vaginal colonization. *J. Clin. Microbiol.* *41*, 1881–1887.

Antonio, M.A.D., Hawes, S.E., and Hillier, S.L. (1999). The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J. Infect. Dis.* *180*, 1950–1956.

Barnhart, K.T., Pretorius, E.S., Timbers, K., Shera, D., Shabbout, M., and Malamud, D. (2004). *In vivo* distribution of a vaginal gel: MRI evaluation of the effects of gel volume, time and simulated intercourse. *Contraception* *70*, 498–505.

Boskey, E.R., Telsch, K.M., Whaley, K.J., Moench, T.R., and Cone, R.A. (1999). Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. *Infect. Immun.* *67*, 5170–5175.

Breshears, L.M., Edwards, V.L., Ravel, J., and Peterson, M.L. (2015). *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. *BMC Microbiol.* *15*.

Cellesi, F. (2012). Thermoresponsive hydrogels for cellular delivery. *Ther. Deliv.* *3*, 1395–1407.

Chaluvadi, S., Hotchkiss Jr., A. t., Call, J. e., Luchansky, J. b., Phillips, J. g., Liu, L., and Yam, K. I. (2012). Protection of probiotic bacteria in a synbiotic matrix following aerobic storage at 4 °C. *Benef. Microbes* *3*, 175–187.

Cohen, M.S. (2004). HIV and sexually transmitted diseases: lethal synergy. *Top. HIV Med. Publ. Int. AIDS Soc. USA* *12*, 104–107.

Cook, M.T., and Brown, M.B. (2018). Polymeric gels for intravaginal drug delivery. *J. Controlled Release* *270*, 145–157.

Czaja, C.A., Stapleton, A.E., Yarova-Yarovaya, Y., and Stamm, W.E. (2007). Phase I trial of a *Lactobacillus crispatus* vaginal suppository for prevention of recurrent urinary tract infection in women. *Infect. Dis. Obstet. Gynecol.* *2007*.

Danielsen, M., and Wind, A. (2003). Susceptibility of *Lactobacillus spp.* to antimicrobial agents. *Int. J. Food Microbiol.* *82*, 1–11.

Dumortier, G., Grossiord, J.L., Agnely, F., and Chaumeil, J.C. (2006). A review of poloxamer 407 pharmaceutical and pharmacological characteristics. *Pharm. Res.* *23*, 2709–2728.

Foschi, C., Salvo, M., Cevenini, R., Parolin, C., Vitali, B., and Marangoni, A. (2017). Vaginal *Lactobacilli* reduce *Neisseria gonorrhoeae* viability through multiple strategies: an *in vitro* study. *Front. Cell. Infect. Microbiol.* *7*.

Gioffredi, E., Boffito, M., Calzone, S., Giannitelli, S.M., Rainer, A., Trombetta, M., Mozetic, P., and Chiono, V. (2016). Pluronic F127 hydrogel characterization and biofabrication in cellularized constructs for tissue engineering applications. *Procedia CIRP* *49*, 125–132.

Grassi, G., Crevatin, A., Farra, R., Guarnieri, G., Pascotto, A., Rehimers, B., Lapasin, R., and Grassi, M. (2006). Rheological properties of aqueous Pluronic-alginate systems containing liposomes. *J. Colloid Interface Sci.* *301*, 282–290.

Graver, M.A., and Wade, J.J. (2011). The role of acidification in the inhibition of *Neisseria gonorrhoeae* by vaginal *Lactobacilli* during anaerobic growth. *Ann. Clin. Microbiol. Antimicrob.* 10, 8.

Grodstein, F., Goldman, M.B., and Cramer, D.W. (1993). Relation of tubal infertility to history of sexually transmitted diseases. *Am. J. Epidemiol.* 137, 577–584.

Hemmerling, A., Harrison, W., Schroeder, A., Park, J., Korn, A., Shiboski, S., Foster-Rosales, A., and Cohen, C.R. (2010). Phase 2a study assessing colonization efficiency, safety, and acceptability of *Lactobacillus crispatus* CTV-05 in women with bacterial vaginosis. *Sex. Transm. Dis.* 37, 745–750.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514.

Huang, L., Shen, M., Li, R., Zhang, X., Sun, Y., Gao, P., Fu, H., Liu, H., He, Y., Du, Y., et al. (2016). Thermo-sensitive composite hydrogels based on poloxamer 407 and alginate and their therapeutic effect in embolization in rabbit VX2 liver tumors. *Oncotarget* 7, 73280.

Jarvis, G.A., and Chang, T.L. (2012). Modulation of HIV transmission by *Neisseria gonorrhoeae*: molecular and immunological aspects. *Curr. HIV Res.* 10, 211–217.

Karavana, S.Y., Rençbe, S., Şenyiğit, Z.A., and Baloğlu, E. (2012). A New *in-situ* gel formulation of itraconazole for vaginal administration. *Pharmacol. Amp Pharm.* 03, 417.

Kassaa, I.A., Hamze, M., Hober, D., Chihib, N.-E., and Drider, D. (2014). Identification of vaginal *Lactobacilli* with potential probiotic properties isolated from women in north Lebanon. *Microb. Ecol.* 67, 722–734.

Khattak, S.F., Bhatia, S.R., and Roberts, S.C. (2005). Pluronic F127 as a cell encapsulation material: utilization of membrane-stabilizing agents. *Tissue Eng.* 11, 974–983.

Laga, M., Plummer, F.A., Nzanze, H., Namaara, W., Brunham, R.C., Ndinya-Achola, J.O., Maitha, G., Ronald, A.R., D'Costa, L.J., and Bhullar, V.B. (1986). Epidemiology of ophthalmia neonatorum in Kenya. *Lancet Lond. Engl.* 2, 1145–1149.

Lee, K.Y., and Mooney, D. (2012). Alginate: properties and biomedical applications. *Prog Polym Sci* 37, 106–126.

Lefebvre, B., Martin, I., Demczuk, W., Deshaies, L., Michaud, S., Labbé, A.-C., Beaudoin, M.-C., and Longtin, J. (2018). Ceftriaxone-resistant *Neisseria gonorrhoeae*, Canada, 2017 (2018). *Emerging Infectious Disease journal - CDC.* 24 (2).

Léonard, L., Beji, O., Arnould, C., Noirot, E., Bonnotte, A., Gharsallaoui, A., Degraeve, P., Lherminier, J., Saurel, R., and Oulahal, N. (2015). Preservation of viability and anti-*Listeria* activity of lactic acid bacteria, *Lactococcus lactis* and *Lactobacillus paracasei*, entrapped in gelling matrices of alginate or alginate/caseinate. *Food Control* 47, 7–19.

Lin, H.-R., Sung, K.C., and Vong, W.-J. (2004). In situ gelling of alginate/pluronic solutions for ophthalmic delivery of pilocarpine. *Biomacromolecules* 5, 2358–2365.

Liu, Y., Zhu, Y., Wei, G., and Lu, W. (2009). Effect of carrageenan on poloxamer-based in situ gel for vaginal use: improved *in vitro* and *in vivo* sustained-release properties. *Eur. J. Pharm. Sci.* 37, 306–312.

Maisani, M. (2017). Conception et développement d'hydrogels pour l'ingénierie tissulaire appliquée au tissu osseux (Bordeaux).

Maragkoudakis, P.A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., and Tsakalidou, E. (2006). Probiotic potential of *Lactobacillus* strains isolated from dairy products. *Int. Dairy J.* 16, 189–199.

Maragkoudakis, P.A., Papadelli, M., Georgalaki, M., Panayotopoulou, E.G., Martinez-Gonzalez, B., Mentis, A.F., Petraki, K., Sgouras, D.N., and Tsakalidou, E. (2009). *In vitro* and *in vivo* safety evaluation of the bacteriocin producer *Streptococcus macedonicus* ACA-DC 198. *Int. J. Food Microbiol.* 133, 141–147.

Minnis, A.M., and Padian, N.S. (2005). Effectiveness of female controlled barrier methods in preventing sexually transmitted infections and HIV: current evidence and future research directions. *Sex. Transm. Infect.* 81, 193–200.

Moore, S., Kailasapathy, K., Phillips, M., and Jones, M.R. (2015). Development of a viability standard curve for microencapsulated probiotic bacteria using confocal microscopy and image analysis software. *J. Microbiol. Methods* 114, 16–22.

O'Hanlon, D.E., Moench, T.R., and Cone, R.A. (2013). Vaginal pH and microbicidal lactic acid when *Lactobacilli* dominate the microbiota. *PLoS ONE* 8, e80074.

Owen, D.H., and Katz, D.F. (1999). A vaginal fluid simulant. *Contraception* 59, 91–95.

Pavlova, S.I., Kilic, A.O., Kilic, S.S., So, J.-S., Nader-Macias, M.E., Simoes, J.A., and Tao, L. (2002). Genetic diversity of vaginal lactobacilli from women in different countries based on 16S rRNA gene sequences. *J. Appl. Microbiol.* 92, 451–459.

Pilcher, H. (2004). Starting to gel. *Nature* 430, 138–140.

Rault, A., Béal, C., Ghorbal, S., Ogier, J.-C., and Bouix, M. (2007). Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage. *Cryobiology* 55, 35–43.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., Stacey, L.M., Karlebach, shara, Gorle, R., Russell, J., Carol, O.T., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* 108, 4680–4687.

Reid, G., McGroarty, J.A., Domingue, P.A.G., Chow, A.W., Bruce, A.W., Eisen, A., and Costerton, J.W. (1990). Coaggregation of urogenital bacteria *in vitro* and *in vivo*. *Curr. Microbiol.* 20, 47–52.

Reid, G., Beuerman, D., Heinemann, C., and Bruce, A.W. (2001). Probiotic *Lactobacillus* dose required to restore and maintain a normal vaginal flora. *FEMS Immunol. Med. Microbiol.* 32, 37–41.

Rençber, S., Karavana, S.Y., Şenyiğit, Z.A., Eraç, B., Limoncu, M.H., and Baloğlu, E. (2017). Mucoadhesive in situ gel formulation for vaginal delivery of clotrimazole: formulation, preparation, and *in vitro/in vivo* evaluation. *Pharm. Dev. Technol.* 22, 551–561.

Stapleton, A.E., Au-Yeung, M., Hooton, T.M., Fredricks, D.N., Roberts, P.L., Czaja, C.A., Yarova-Yarovaya, Y., Fiedler, T., Cox, M., and Stamm, W.E. (2011). Randomized, Placebo-Controlled Phase 2 Trial of a *Lactobacillus crispatus* Probiotic Given Intravaginally for Prevention of Recurrent Urinary Tract Infection. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 52, 1212–1217.

Stein, Z.A. (1990). HIV prevention: the need for methods women can use. *Am. J. Public Health* 80, 460–462.

Štšepetova, J., Taelma, H., Smidt, I., Hütt, P., Lapp, E., Aotäht, E., and Mändar, R. (2017). Assessment of phenotypic and genotypic antibiotic susceptibility of vaginal *Lactobacillus* sp. *J. Appl. Microbiol.* *123*, 524–534.

Szekalska, M., Puciłowska, A., Szymańska, E., Ciosek, P., and Winnicka, K. (2016). Alginate: Current Use and Future Perspectives in Pharmaceutical and Biomedical Applications.

Tachedjian, G., Aldunate, M., Bradshaw, C.S., and Cone, R.A. (2017). The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health. *Res. Microbiol.* *168*, 782–792.

Temmerman, R., Pot, B., Huys, G., and Swings, J. (2003). Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int. J. Food Microbiol.* *81*, 1–10.

Tønnesen, H.H., and Karlsen, J. (2002). Alginate in drug delivery systems. *Drug Dev. Ind. Pharm.* *28*, 621–630.

Traore, Y.L., Chen, Y., and Ho, E.A. (2018). Current state of microbicide development. *Clin. Pharmacol. Ther.*

Vasquez, A., Jakobsson, T., Ahrne, S., Forsum, U., and Molin, G. (2002). Vaginal *Lactobacillus* flora of healthy Swedish women. *J Clin Microbiol* *40*, 2746–2749.

Verdenelli, M.C., Coman, M.M., Cecchini, C., Silvi, S., Orpianesi, C., and Cresci, A. (2014). Evaluation of antipathogenic activity and adherence properties of human *Lactobacillus* strains for vaginal formulations. *J. Appl. Microbiol.* *116*, 1297–1307.

Verstraelen, H., Verhelst, R., Claeys, G., De Backer, E., Temmerman, M., and Vanechoutte, M. (2009). Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol* *9*, 116.

Wagner, M.A., Marks, W.H., and Bhatia, S.K. (2014). Hydrogel Encapsulation to improve cell viability during syringe needle flow. *J. Long. Term Eff. Med. Implants* *24*.

WHO (2016). WHO | WHO guidelines for the treatment of *Neisseria gonorrhoeae*.

Willaert, R. (2011). Cell immobilization and its applications in biotechnology. pp. 313–367.

Zotta, T., Parente, E., and Ricciardi, A. (2009). Viability staining and detection of metabolic activity of sourdough lactic acid bacteria under stress conditions. *World J. Microbiol. Biotechnol.*

Zotta, T., Guidone, A., Tremonte, P., Parente, E., and Ricciardi, A. (2012). A comparison of fluorescent stains for the assessment of viability and metabolic activity of lactic acid bacteria. *World J. Microbiol. Biotechnol.* *28*, 919–927.





## **CHAPITRE VI : MANIPULATIONS COMPLEMENTAIRES**



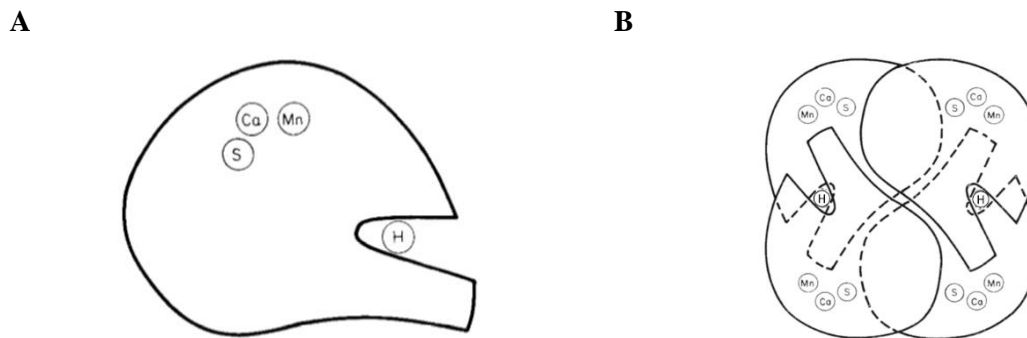
Ce chapitre vise à regrouper des travaux effectués qui n'ont pas été pris en compte dans les chapitres IV et V. Il est subdivisé en deux parties. La première partie s'intéresse aux aspects préliminaires de l'étude de l'organisation du gel par microscopie confocale après le marquage de l'alginate avec la concanavaline A. La deuxième partie concerne l'étude de l'introduction de bouillon MRS (Man, Rogosa et Sharpe) dans le gel dans le but d'améliorer la viabilité des lactobacilles en leur apportant des nutriments.

## **1. ETUDE PRELIMINAIRE DE L'ORGANISATION D'UN GEL A BASE DE POLOXAMER 407, D'ALGINATE DE SODIUM ET DE *LACTOBACILLUS CRISPATUS* PAR MICROSCOPIE CONFOCALE**

### **1.1. Introduction**

La microscopie confocale a été utilisée dans des études précédentes pour visualiser la microstructure des mélanges de protéines, de gélatine, de protéines de lactosérum et de  $\beta$ -lactoglobuline, de polysaccharides. Le contraste en fluorescence est obtenu soit par un marquage covalent avec une sonde fluorescente (polysaccharides et protéines), soit par un marquage non covalent (protéines et amidon) (Lamprecht et al., 2000; van de Velde et al., 2003). Dans le cadre de ce projet de thèse, nous avons choisi un marquage non covalent de l'alginate avec la concanavaline A (Con A) liée de façon covalente avec une sonde fluorescente, le « Texas red » (TR-Con A). Le Texas red est un dérivé chlorure de sulfonyle de la sulforhodamine 101, qui fluoresce dans le rouge (longueur d'onde d'excitation 595 nm, longueur d'onde d'émission 613 nm). La Con A est une lectine, marqueur des polysaccharides. Elle se lie aux unités glucopyranosyl et mannopyranosyl de façon multivalente (Sandoval-Altamirano et al., 2017). Dans les solutions neutres et alcalines, la Con A existe sous forme de tétramères de masse molaire environ 104 000 daltons. En revanche, dans les solutions acides (pH inférieur à 5,0), la Con A existe sous forme de dimères. La Con A possède un site de fixation du mannose ou du glucose sur chaque unimère (**Fig. 1**). Elle peut se lier à d'autres structures *via* des liaisons hydrophobes de faible affinité car elle possède un second site de fixation capable d'interagir avec des ligands hydrophobes (Edelman and Wang, 1978). Les sites de fixation des saccharides sur la Con A se trouvent au sommet de chaque sous-unité, à 20 Å de la cavité non polaire qui lie les ligands hydrophobes. De plus, la liaison du saccharide et des ligands hydrophobes ne sont ni compétitifs ni interactifs (Edelman and Wang, 1978). Dans le cas de notre étude, le pH des

formulations est compris entre 6 et 7. La concavoline A est donc sous forme de tétramères et pourrait se lier à l'alginate comme elle se fait avec les saccharides.



**Figure 1.** Représentation schématique montrant les emplacements (A) des sites de fixation pour le manganèse (Mn), le calcium (Ca), les saccharides (S) et les molécules hydrophobes (H) et la forme tétramérique de la concanavoline A (B) proposé par Edelman and Wang (1978).

La microscopie confocale a été aussi précédemment utilisée pour la visualisation des bactéries, de leur organisation et de leur viabilité. Cette étude vise à observer l'organisation de l'alginate dans la formulation mise au point qui est un mélange complexe de poloxamer 407, alginate et lactobacilles. La TR-Con A a donc été visualisée dans les polymères seuls puis dans les mélanges. Cette technique permet d'obtenir des images à deux et trois dimensions.

## 1.2. Matériel et méthodes

### 1.2.1. Matériel

*L. crispatus* (CIP 103603, ATCC 33197) provient de l'Institut Pasteur (Paris). La gélose et le bouillon MRS Difco™ proviennent de Fisher Scientific (France). Le poloxamer 407 (P) (Lutrol® F127) de masse molaire 11774 g.mol<sup>-1</sup> provient de BASF (Allemagne). L'alginate de sodium (A) de masse molaire 300 000 g.mol<sup>-1</sup> provient de Roth (France). Les marqueurs syto 9 du kit Live/dead® bacLight™ et concanavoline A TEXAS RED™ (TR-Con A) proviennent de Fisher Scientific (France). L'eau ultrapure (résistivité 18.2 MΩ, MilliQ, Millipore, France) a été utilisée.

### 1.2.2. Préparation des gels

La préparation des gels est identique à celle décrite dans le **chapitre IV paragraphe 2.3** et le **chapitre V paragraphe 2.4**.

### ***1.2.3. Marquage à la concanavaline A***

La solution mère de TR-Con A ( $5 \text{ mg.mL}^{-1}$ ) a été préparée dans du bicarbonate de sodium  $0,1 \text{ M}$  et conservée au congélateur à  $-20 \text{ }^{\circ}\text{C}$ . Avant chaque utilisation, elle a été centrifugée à  $9050 \text{ g}$  pendant  $15 \text{ mn}$  afin d'éliminer les agrégats. Le surnageant a été pipeté pour le marquage des formulations.  $20 \text{ }\mu\text{L}$  de TR-Con A ont été ajoutés à  $2 \text{ g}$  de gel sous forme liquide ( $4 \text{ }^{\circ}\text{C}$ ), mélangés au vortex et conservés à l'abri de la lumière à  $4 \text{ }^{\circ}\text{C}$  pendant  $90 \text{ minutes}$  avant d'être analysés par microscopie confocale à température ambiante.

### ***1.2.4. Double marquage concanavaline A- syto 9***

La solution mère de TR-Con A ( $5 \text{ mg.mL}^{-1}$ ) a été centrifugée à  $9050 \text{ g}$  pendant  $15 \text{ mn}$ , à température ambiante.  $50 \text{ }\mu\text{L}$  du surnageant ont été mélangés au  $4 \text{ g}$  du mélange poloxamer 407 et alginate concentré comme décrit dans le **chapitre V**, puis incubés pendant  $90 \text{ mn}$  à  $4 \text{ }^{\circ}\text{C}$  et à l'obscurité. Par ailleurs,  $5 \text{ }\mu\text{L}$  de syto 9 ont été mélangés avec  $1 \text{ mL}$  de la suspension de lactobacilles puis incorporés dans les  $4 \text{ g}$  de gel concentré marqués à la TR-Con A, immédiatement avant l'analyse au microscope (images  $20\times$ , Zoom  $2\times$ ). La composition finale du gel était P21.5A1L.

### ***1.2.5. Observation au microscope confocale***

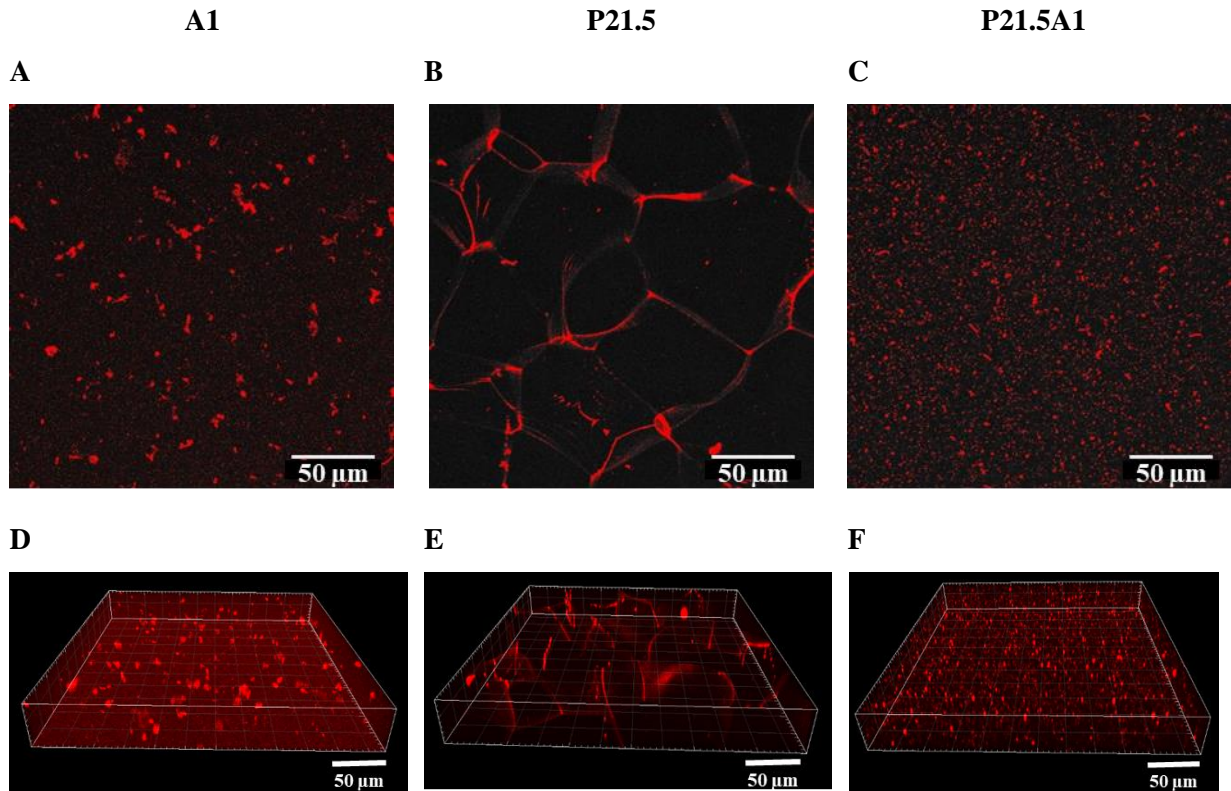
La méthode d'observation est décrite dans le **chapitre V paragraphe 2.8**.

## **1.3. Résultats et discussion**

### ***1.3.1. Marquage à la concanavaline A***

Les formulations alginate  $1 \text{ } \%$  (A1), poloxamer  $21,5 \text{ } \%$  (P21.5) et le mélange poloxamer  $21,5 \text{ } \%$ -alginate  $1 \text{ } \%$  (P21.5A1) ont été observés par microscopie confocale après marquage à la TR-Con A. Dans la solution d'alginate (**Fig. 2A**) tout comme dans le mélange avec le poloxamer 407 (**Fig. 2B**), on observe une dispersion uniforme de points rouge, révélant la répartition homogène de l'alginate dans ces formulations. En revanche, la présence de TR-Con A dans P21.5 semble délimiter des interstices d'une taille d'environ  $50 \text{ }\mu\text{m}$  qui pourraient résulter de la cristallisation du poloxamer sous forme de microdomaines. L'interaction entre le poloxamer et la TR-Con A semble complexe. Elle demanderait à être mieux comprise surtout que ces microdomaines ne sont pas observés dans le mélange (**Fig. 2C**). Une hypothèse pourrait être que la TR-Con A a plus d'affinité pour l'alginate, ce qui fait que lorsque ces

deux polymères sont associés, c'est seulement le marquage de l'alginate qui est observé. Un marquage covalent de l'alginate avec un fluorophore pourrait être intéressant pour étudier l'organisation de nos formulations

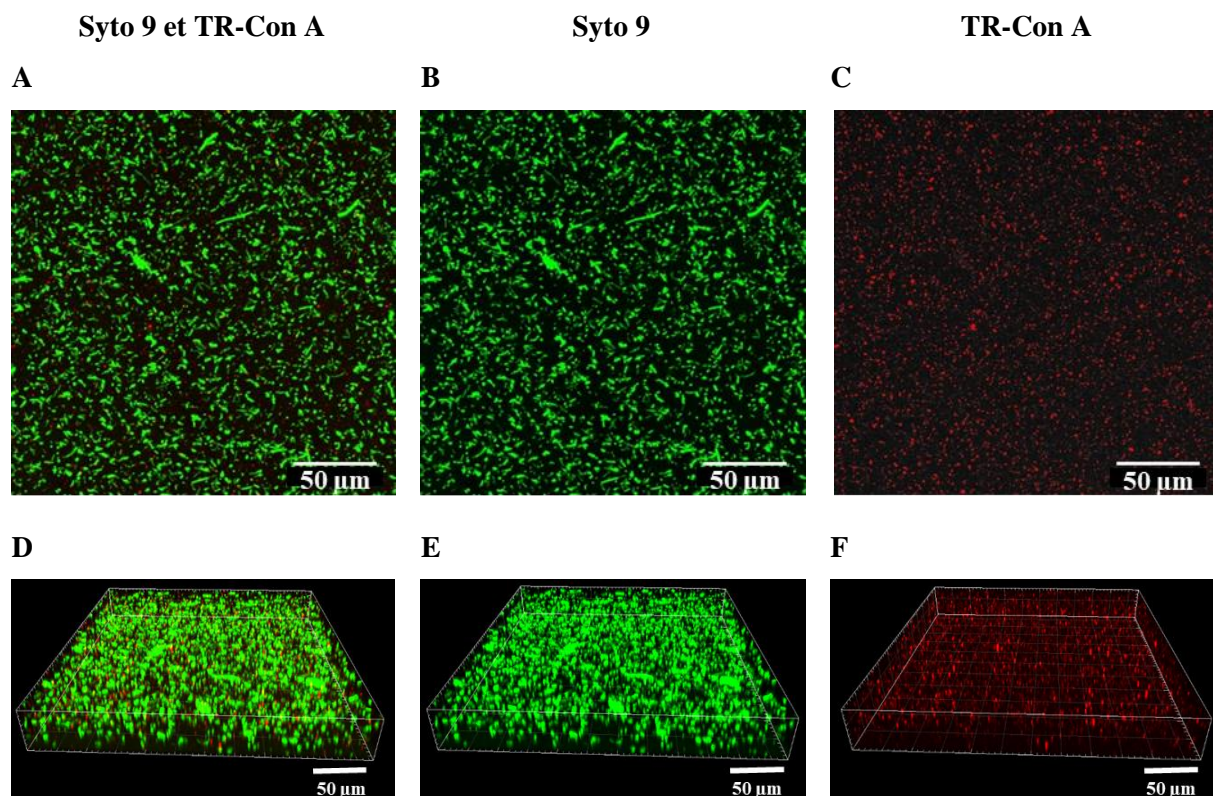


**Figure 2.** Projection (A, B, C) et image 3D (D, E, F) des formulations A1 (A, D), P21.5 (B, E), P21.5A1 (C, F) mélangées avec la TR-Con A. Observation à température ambiante, au microscope confocal, à l'objectif 20×, Zoom 2×.

### 1.3.2. Double marquage concanavine A- syto 9

Le double marquage par la-Con A et le syto 9 est généralement utilisé dans la caractérisation des biofilms afin de visualiser toutes les bactéries avec le syto 9 et les exopolysaccharides avec la Con A (Chen et al., 2007; Leccese Terraf et al., 2016). Wei et al. (2017) ont utilisé la Con A conjuguée avec l'alex 488 et le « kit live/dead baclight » respectivement pour marquer les exopolysaccharides du biofilm et les lactobacilles vaginaux. Dans le cas de cette étude, le mélange P21.5A1L a été étudié par microscopie confocale après marquage de toutes les bactéries par le syto 9 et de l'alginate par la TR-Con A (**Fig. 3**). La répartition des bactéries dans le gel semble homogène. L'alginate semble aussi être distribué dans tout le gel de façon homogène lorsqu'il est en présence de lactobacilles. La répartition de l'alginate dans le gel contenant les lactobacilles (**Fig. 3C, F**) est similaire à sa répartition dans le gel sans

lactobacilles (**Fig. 2C, F**). Ces observations sont comparables à celles faites par certains auteurs sur les exopolysaccharides de biofilms formés par *Pseudomonas fluorescens* (Baum et al., 2009) et par *Pseudomonas mandelii* (Vásquez-Ponce et al., 2017).



**Figure 3.** Double marquage Syto 9 (lactobacilles) et TR-Con A (alginate) à température ambiante. Projections (A, B, C), 3D (D, E, F), la superposition des deux canaux (A, D), le canal vert du Syto 9 marquant les lactobacilles (B, E), le canal rouge de la TR-Con A (C, F).

Des études sur les effets de l'alginate sur une bactérie Gram positif (*Streptococcus mutant*) a montré que l'alginate ne se liait pas à la surface des bactéries Gram positif contrairement aux bactéries Gram négatif (Pritchard et al., 2017). *L. crispatus* étant une bactérie Gram positif, notre hypothèse est donc que l'alginate ne se lierait pas à la surface de *L. crispatus* et resterait dispersé. Néanmoins, le gel ne subit pas une étape de lavage donc peut renfermer de la TR-Con A non liée à l'alginate. De plus, le comportement de Con A en présence de poloxamer est complexe. Des études complémentaires sont donc nécessaires.

#### 1.4. Conclusion

Ces études préliminaires de microscopie confocale suggèrent une répartition homogène de l'alginate de sodium et des lactobacilles dans tout le gel. D'autres techniques telles que la cryo-



microscopie électronique à transmission, la diffusion des neutrons aux petits angles ou la diffusion des rayons X aux petits angles, seraient utiles pour étudier l'organisation du poloxamer également dans ce mélange complexe.

## **2. INTRODUCTION DU MRS DANS LE GEL : AVANTAGES ET INCONVENIENTS**

### **2.1. Introduction**

Dans le but d'augmenter le temps de conservation et d'activité des gels, du bouillon MRS a été ajouté dans le gel afin que les lactobacilles aient directement les nutriments nécessaires à leur viabilité et leur activité et qu'une fois le gel administré dans le vagin, ils n'entrent pas en compétition avec les lactobacilles commensaux. En effet, le bouillon MRS est un bouillon nutritif utilisé pour la culture des lactobacilles et sa composition a été étudiée pour leur assurer une croissance optimale. Le bouillon MRS renferme dans sa composition 10 g de protéose peptone n° 3, 10 g d'extrait de bœuf, 5 g d'extrait de levure, 20 g de dextrose, 1 g de polysorbate 80, 2 g de citrate d'ammonium, 5 g d'acétate de sodium, 100 mg de sulfate de magnésium, 50 mg de sulfate de manganèse et 2 g de phosphate dipotassique par litre (pH  $6,5 \pm 0,2$ ).

### **2.2. Matériel et méthodes**

Le matériel et les méthodes sont les mêmes que ceux décrits dans les **chapitre IV et V**. Pour évaluer l'influence du MRS sur le gel, quatre gels ont été préparés P21.5A1, P21.5A1L d'une part et P21.5A1M, P21.5A1LM d'autre part obtenus par le même protocole que précédemment (**chapitre V**) sauf que la suspension de lactobacilles est réalisée avec du MRS au lieu de l'eau. La composition de ces formulations est donnée dans le **Tableau 1**. Des mesures de pH ainsi que des études rhéologiques, d'expulsion à travers une seringue, de viabilité et de stabilité ont été menées suivant les protocoles décrits dans les **chapitres IV et V**.

**Tableau 1.** Composition des gels étudiés contenant alginate (A), poloxamer (P), avec ou sans *L. crispatus* (L), avec (+) ou sans (-) MRS

Formulation	Poloxamer (% m/m)	Alginate (% m/m)	<i>L. crispatus</i> (log <sub>10</sub> U/5 g of gel)	MRS
P21.5A1	21,5	1	-	-
P21.5A1M	21,5	1	-	+
P21.5A1L	21,5	1	9	-
P21.5A1LM	21,5	1	9	+

## 2.3. Résultats et discussion

### 2.3.1. Influence du bouillon MRS sur la mesure du pH

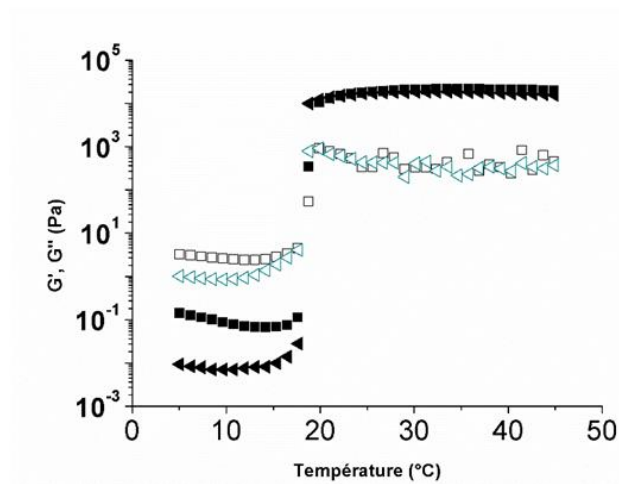
Le gel P21.5A1LM était acide ( $5,64 \pm 0,04$ ). Il était plus acide que le gel P21.5A1L ( $6,85 \pm 0,02$ ). La mesure du pH du MRS seul a montré qu'il était lui-même acide (pH 5,57), ce qui expliquerait donc la différence de pH observée pour les deux gels.

### 2.3.2. Influence du bouillon MRS sur les propriétés rhéologiques des gels

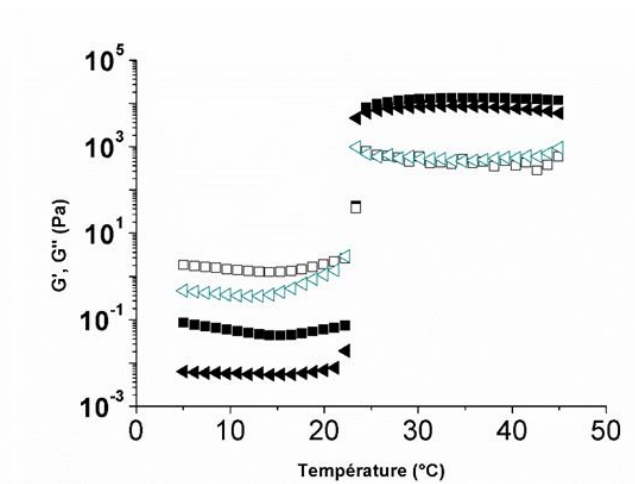
Cette étude a été menée dans le but d'évaluer l'influence du MRS sur les propriétés thermogélifiantes et viscoélastiques des gels avec ou sans lactobacilles. La viscosité, la température de gélification ( $T_{gel}$ ) ainsi que les modules élastiques  $G'$  et visqueux  $G''$  ont été déterminés pour chaque gel. Comme dans les **chapitres IV et V**, les formulations ont été analysées à 25 °C pour mimer les conditions d'administration et à 37 °C après dilution par le fluide vaginal simulé pour mimer leur comportement *in vivo*. La **Figure 4** montre l'allure des courbes obtenues comparée entre les gels sans lactobacilles P21.5A1 et P21.5A1M. La **Figure 5** reporte les résultats comparés des gels avec lactobacilles P21.5A1L et P21.5A1LM. Le **Tableau 2** rassemble les valeurs de  $T_{gel}$ ,  $G'$  et  $G''$ . Les propriétés rhéologiques (comportement thermogélifiant, rhéofluidifiant et non thixotrope, propriétés viscoélastiques) ne sont pas significativement modifiées par la présence du MRS dans les gels. La seule différence concerne les modules  $G'$  et  $G''$  pour les températures inférieures à la température de gélification. Cela peut s'expliquer par le fait que le MRS contient des sels donc sa force ionique est plus élevée que celle de l'eau. L'alginate est un polyélectrolyte chargé négativement au pH du gel. En présence de sel, la conformation est repliée alors qu'elle est plus étendue dans l'eau. Dans le MRS, les chaînes d'alginate devraient donc être plus contractées, ce qui réduirait la viscosité ainsi que les modules  $G'$  et  $G''$  comme observé avant la température de gélification (**Fig. 4 A, B**). Par ailleurs, la formulation P21.5A1LM après

dilution par le fluide vaginal simulé a une gélification plus progressive avec une température de gélification ( $20 \pm 1^\circ\text{C}$ ) différente de celle de P21.5A1L ( $23 \pm 1^\circ\text{C}$ ) (**Fig. 5B**). Cela pourrait peut-être s'expliquer par la production d'un composé par les lactobacilles lorsqu'ils sont en présence de MRS.

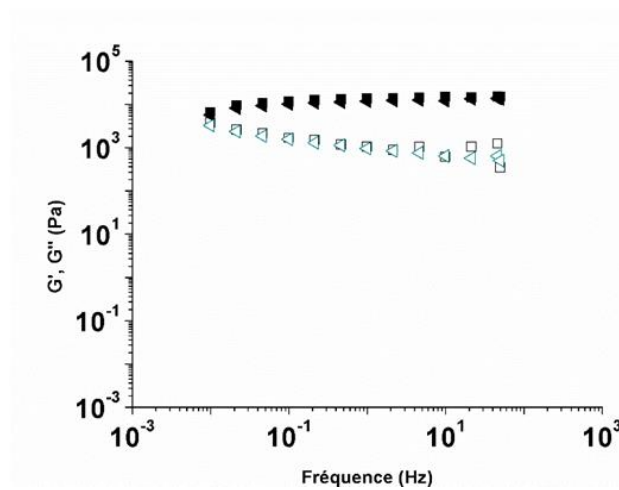
**A** Sans dilution par le SVF



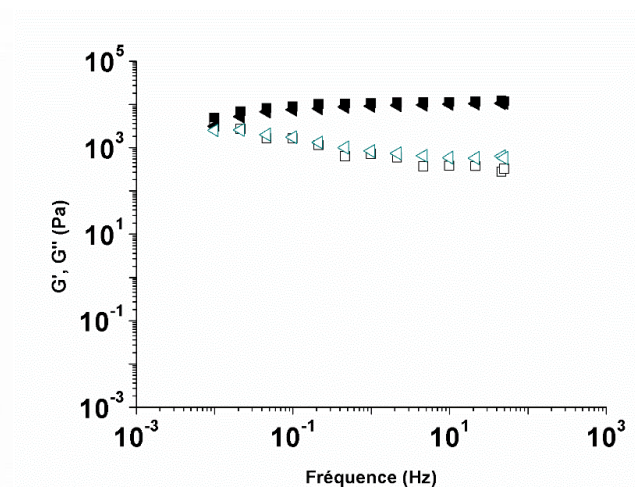
**B** Après dilution par le SVF



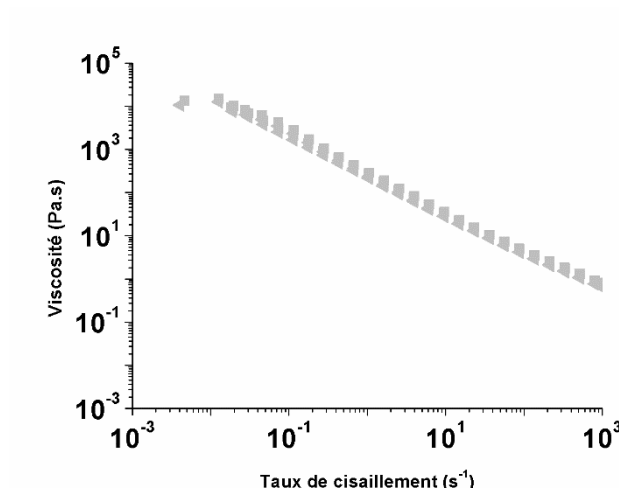
**C** 25 °C



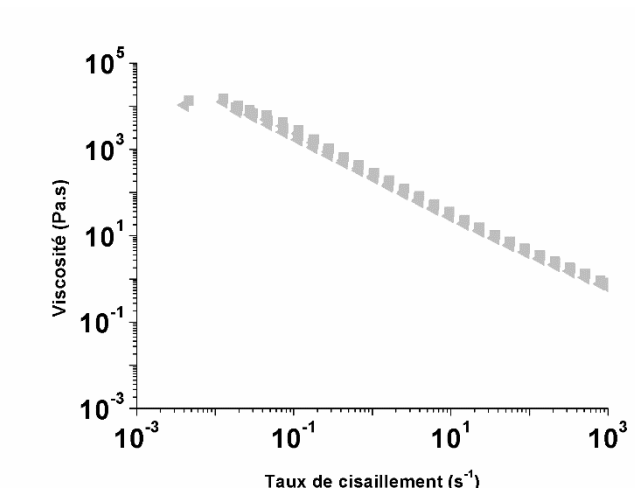
**D** 37 °C + SVF



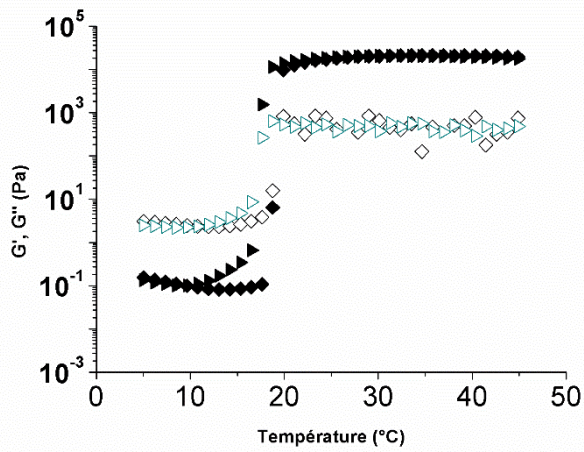
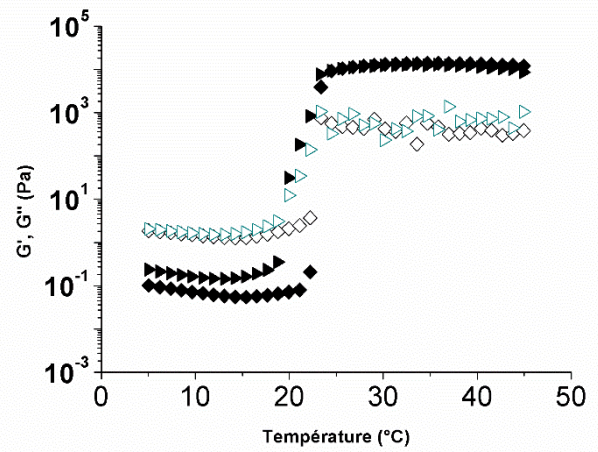
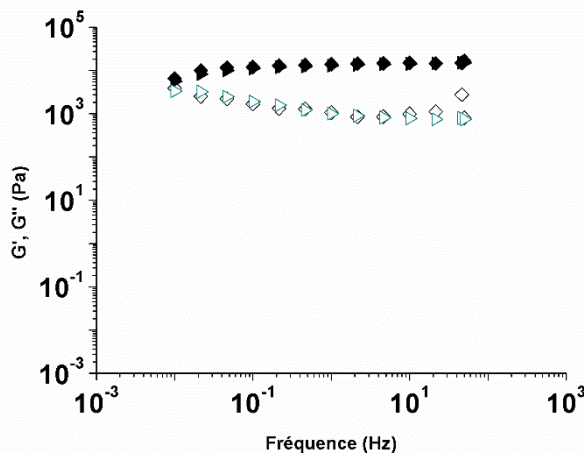
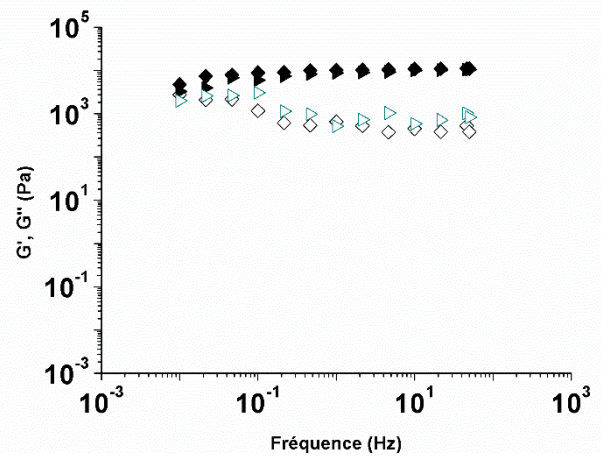
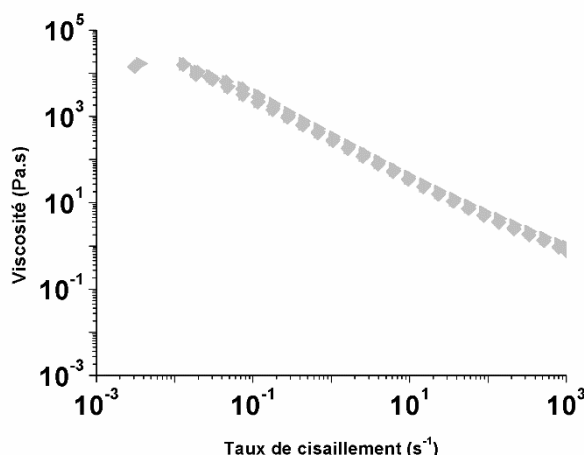
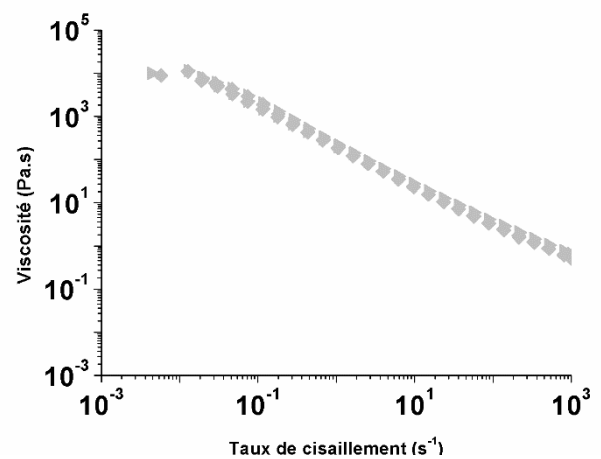
**E** 25 °C



**F** 37 °C + SVF



**Figure 4.** Propriétés rhéologiques comparées de P21.5A1 et P21.5A1M montrant le balayage en température affichant une donnée sur dix (A, B), le balayage en fréquence (C, D), et la mesure en écoulement (E, F) : (■)  $G'$  pour P21.5A1, (□)  $G''$  pour P21.5A1, (■)  $\eta$  pour P21.5A1, (◄)  $G'$  pour P21.5A1M, (◄)  $G''$  pour P21.5A1M, (◄)  $\eta$  pour P21.5A1M.

**A** Sans dilution par le SVF**B** Après dilution par le SVF**C** 25 °C**D** 37 °C + SVF**E** 25 °C**F** 37 °C + SVF

**Figure 5.** Propriétés rhéologiques de P21.5A1L et P21.5A1LM, montrant le balayage en température affichant une donnée sur dix (A, B), le balayage en fréquence (C, D), et la mesure en écoulement (E, F) : (◆)  $G'$  pour P21.5A1L, (◇)  $G''$  pour P21.5A1L, (◆)  $\eta$  pour P21.5A1L, (▶)  $G'$  pour P21.5A1LM, (▷)  $G''$  pour P21.5A1LM, (▶)  $\eta$  pour P21.5A1LM. Affichage de chaque 10 points des données de balayage en température.

**Tableau 2.** Températures de gélification et paramètres rhéologiques des gels avec ou sans MRS.

Formulation	T <sub>gel</sub> (°C)	Conditions	G' à 1 Hz (Pa)	G'' à 1 Hz (Pa)	En écoulement
P21.5A1	19 ± 1	25°C	(13.5 ± 0.4).10 <sup>3</sup>	(1.0 ± 0.2).10 <sup>3</sup>	Rhéofluidifiant Non-thixotrope
	24 ± 1	37 °C + SVF	(10.5 ± 0.2).10 <sup>3</sup>	(0.7 ± 0.2).10 <sup>3</sup>	
P21.5A1M	18 ± 1	25°C	(11.9 ± 0.4).10 <sup>3</sup>	(1.0 ± 0.1).10 <sup>3</sup>	
	23 ± 1	37 °C + SVF	(9.2 ± 0.1).10 <sup>3</sup>	(0.8 ± 0.1).10 <sup>3</sup>	
P21.5A1L	19 ± 1	25°C	(13.9 ± 0.3).10 <sup>3</sup>	(1.1 ± 0.5).10 <sup>3</sup>	
	23 ± 1	37 °C + SVF	(10.1 ± 0.3).10 <sup>3</sup>	(0.7 ± 0.2).10 <sup>3</sup>	
P21.5A1LM	17 ± 1	25°C	(13.0 ± 0.2).10 <sup>3</sup>	(1.0 ± 0.2).10 <sup>3</sup>	
	20 ± 1	37 °C + SVF	(8.9 ± 0.4).10 <sup>3</sup>	<b>(0.8 ± 0.5).10<sup>3</sup></b>	

### 2.3.3. Influence du bouillon MRS sur la capacité d'expulsion à travers un dispositif vaginal

Le **Tableau 3** présente les forces maximales (F<sub>max</sub>) ainsi que le travail (W) nécessaires pour expulser les gels à travers une seringue de 5 mL choisie comme un modèle de dispositif vaginal. F<sub>max</sub> pour les gels avec les lactobacilles est plus de trois fois plus élevée que les gels sans lactobacilles correspondants. Aussi, le travail d'expulsion des gels avec les lactobacilles est plus de six fois plus élevé que celui des gels sans lactobacilles correspondants, mais reste inférieur à 40 mJ. Le MRS n'a pas d'influence significative sur ces paramètres. Connaissant les caractéristiques de la seringue 5 mL Terumo<sup>®</sup> utilisée, ces résultats préliminaires permettront de proposer un dispositif d'administration adapté pour une application aisée par la femme elle-même et pour une meilleure répartition du gel dans le vagin.

**Tableau 3.** Evaluation de l'expulsion des gels à travers un dispositif vaginal modélisé par une seringue 5 mL.

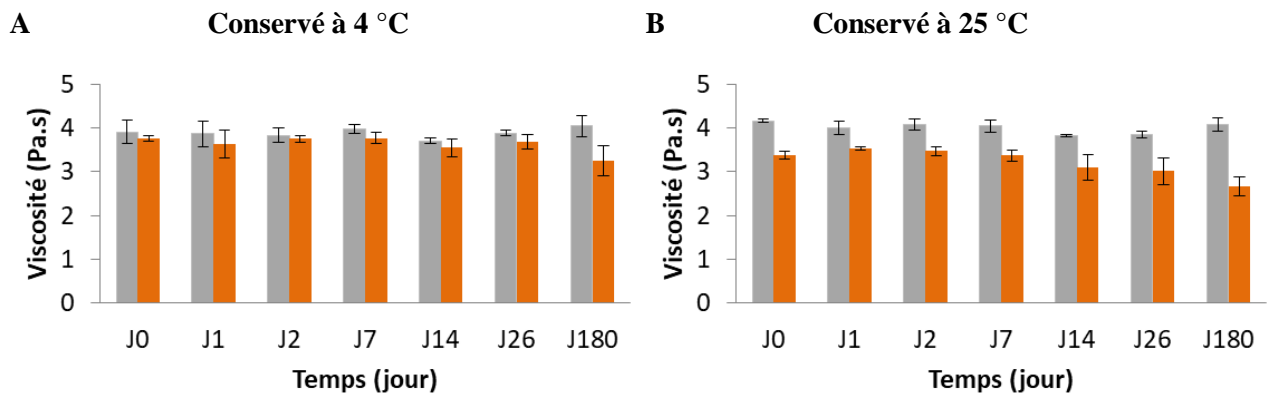
Formulation	Force maximale Fmax (N)	Travail d'expulsion (mJ)
P21.5A1	0,7 ± 0,2	6 ± 6
P21.5A1M	0,7 ± 0,2	5 ± 4
P21.5A1L	3,2 ± 0,6	37 ± 9
P21.5A1LM	2,2 ± 0,3	<b>37 13</b>

### 2.3.4. Stabilité du gel P21.5A1LM sur six mois

Pour l'étude de la stabilité des gels, plusieurs paramètres ont été suivis : la viscosité, le pH, le nombre de bactéries cultivables sur gélose MRS, ainsi que le nombre de bactéries vivantes par microscopie confocale.

## Viscosité

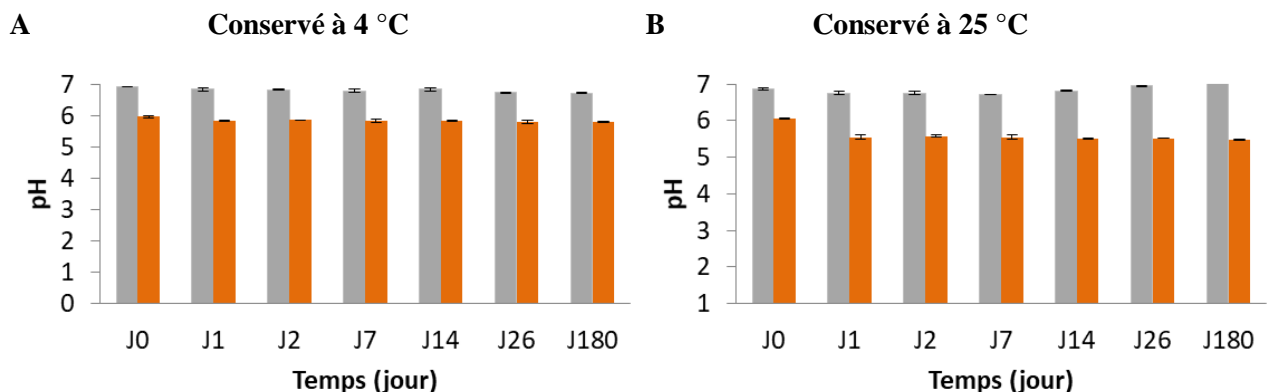
La **Figure 6** montre la viscosité pour un taux de cisaillement de  $125 \text{ s}^{-1}$  lors de l'étude de la stabilité sur 6 mois des gels avec lactobacilles. Ce taux de cisaillement, a été choisi sur la base du taux de cisaillement maximal pouvant être rencontré dans le vagin après administration qui est  $100 \text{ s}^{-1}$  (Owen et al., 2000). La viscosité de P21.5A1LM conservé à  $4^\circ\text{C}$  (**Fig. 6A orange**) semble similaire sur les 6 mois alors qu'elle décroît lorsque le gel est conservé à  $25^\circ\text{C}$  (**Fig. 6B orange**). Comparées aux viscosités de P21.5A1L (barres oranges comparées à barres grises), les viscosités des gels avec MRS ne diffèrent pas significativement lorsque les gels sont conservés à  $4^\circ\text{C}$  (**Fig. 6A**) mais sont légèrement abaissées à  $25^\circ\text{C}$  (**Fig. 6B**).



**Figure 6.** Viscosité comparée sur 6 mois de P21.5A1L (barre grise) et P21.5A1LM (barre orange).

## Mesure du pH

Le pH de P21.5A1LM est inchangé sur les six mois et toujours plus acide que P21.5A1L à  $4^\circ\text{C}$  (**Fig. 7A**). A  $25^\circ\text{C}$ , on observe un léger abaissement de pH entre J0 et J1.



**Figure 7.** Mesures de pH comparées sur 6 mois de P21.5A1L (barre grise) et P21.5A1LM (barre orange).

### ***Cultivabilité sur gélose MRS et viabilité par microscopie confocale***

La cultivabilité sur gélose MRS a été évaluée par dénombrement sur cette gélose.

La viabilité a été évaluée en utilisant deux marqueurs, le Syto 9 et l'iodure de propidium, qui, utilisés simultanément, semblent permettre d'observer les bactéries vivantes (vert) et mortes (rouge) (Moore et al., 2015).

Après l'observation par microscopie confocale des bactéries marquées en rouge ou en vert, les aires occupées par ces bactéries ont été mesurées avec Image J, respectivement aire rouge et aire verte. Ensuite le ratio des aires occupées par les lactobacilles a été calculé selon l'**Equation 1** :

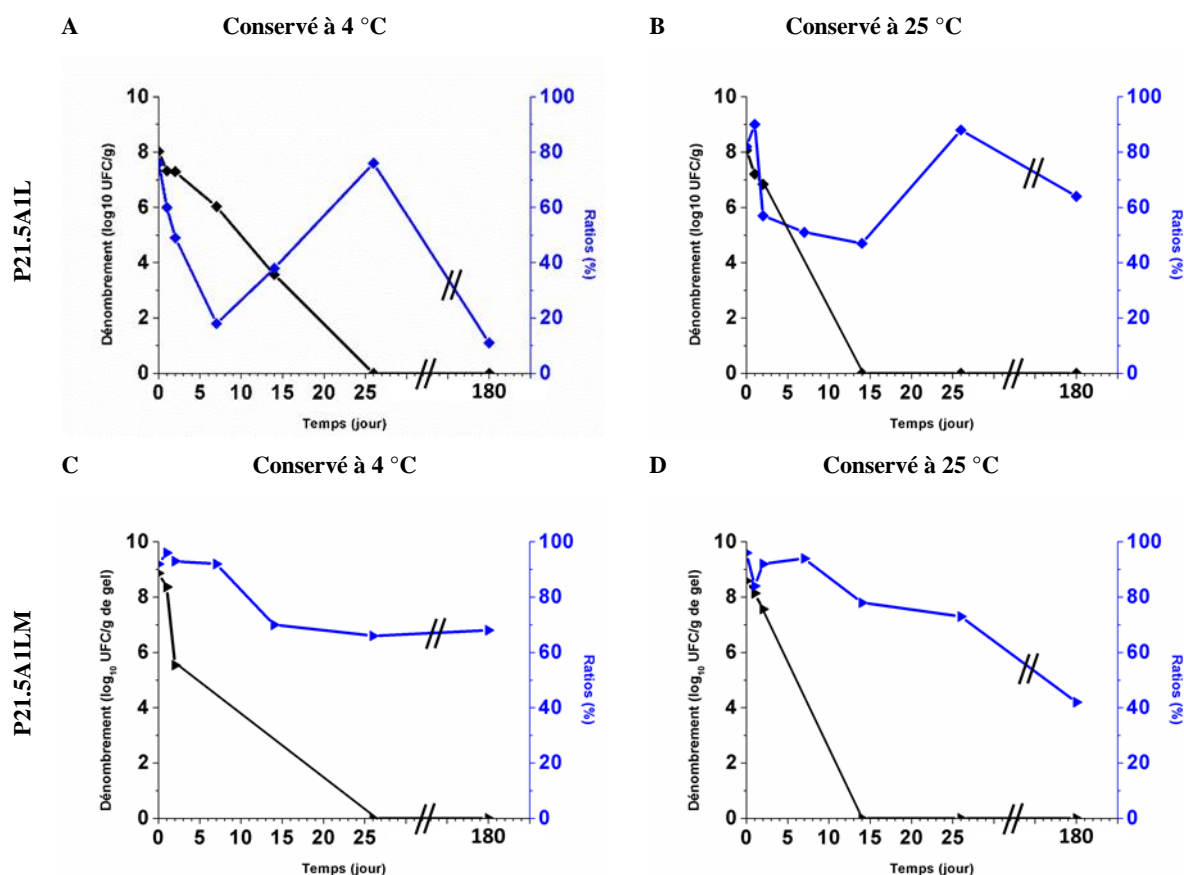
$$R = \frac{\text{Aire verte}}{\text{Aire verte} + \text{Aire rouge}} \times 100 \quad (1)$$

Ce ratio permet d'estimer la fraction des lactobacilles vivants dans le système. Les ratios (R) obtenus à chaque temps sur une période de 6 mois (180 jours) ont été reportés sur la **Figure 8**. Comme nous l'avons vu dans le **chapitre V**, le dénombrement sur gélose MRS permet de déterminer le nombre de lactobacilles actifs et capables de se multiplier pour former une colonie. La microscopie confocale permet, en revanche, de visualiser les bactéries vivantes marquées en vert et mortes marquées en rouge. Des acquisitions 2D (single) et 2D (projection en une image de plusieurs singles obtenus sur une épaisseur de 32  $\mu\text{m}$ ). Le coefficient de corrélation entre ces acquisitions 2D single et projection est de 0,93. Le nombre de bactéries cultivables sur gélose MRS a diminué fortement au cours du temps pour s'annuler à des temps courts (**Fig. 8**). De façon inattendue, la présence du MRS n'améliore pas les résultats. De façon intéressante, l'étude de la viabilité par microscopie confocale montre que les variations de R ne se font pas de la même manière que le dénombrement sur gélose. Pour des dénombrements nuls, on a observé des ratios R parfois élevés (environ 60 %). Les variations de R sont plus complexes avec une augmentation surtout dans l'eau P21.5A1L (**Fig. 8A, B**). Dans le MRS, les ratios restent plus élevés, plus longtemps pour P21.5A1LM (**Fig. 8C, D**). Il serait quand même intéressant de reproduire l'étude en faisant des temps intermédiaires. Cette étude soulève quelques interrogations. Les bactéries seraient-elles viables mais non cultivables ? Une étude de la possibilité de « résurrection » des lactobacilles par exemple par la catalase (Liu et al., 2017a, 2017b), pourrait nous permettre de l'élucider. Les bactéries vivantes se nourrissent-elles des mortes pour se multiplier et survivre ? Par ailleurs, l'utilisation du Syto 9 et de l'iodure de propidium simultanément pour marquer les bactéries pourrait être discutée. En effet, le syto 9 marque toutes les bactéries, mais en présence de l'iodure de propidium, il est remplacé par ce dernier qui a une plus grande affinité pour

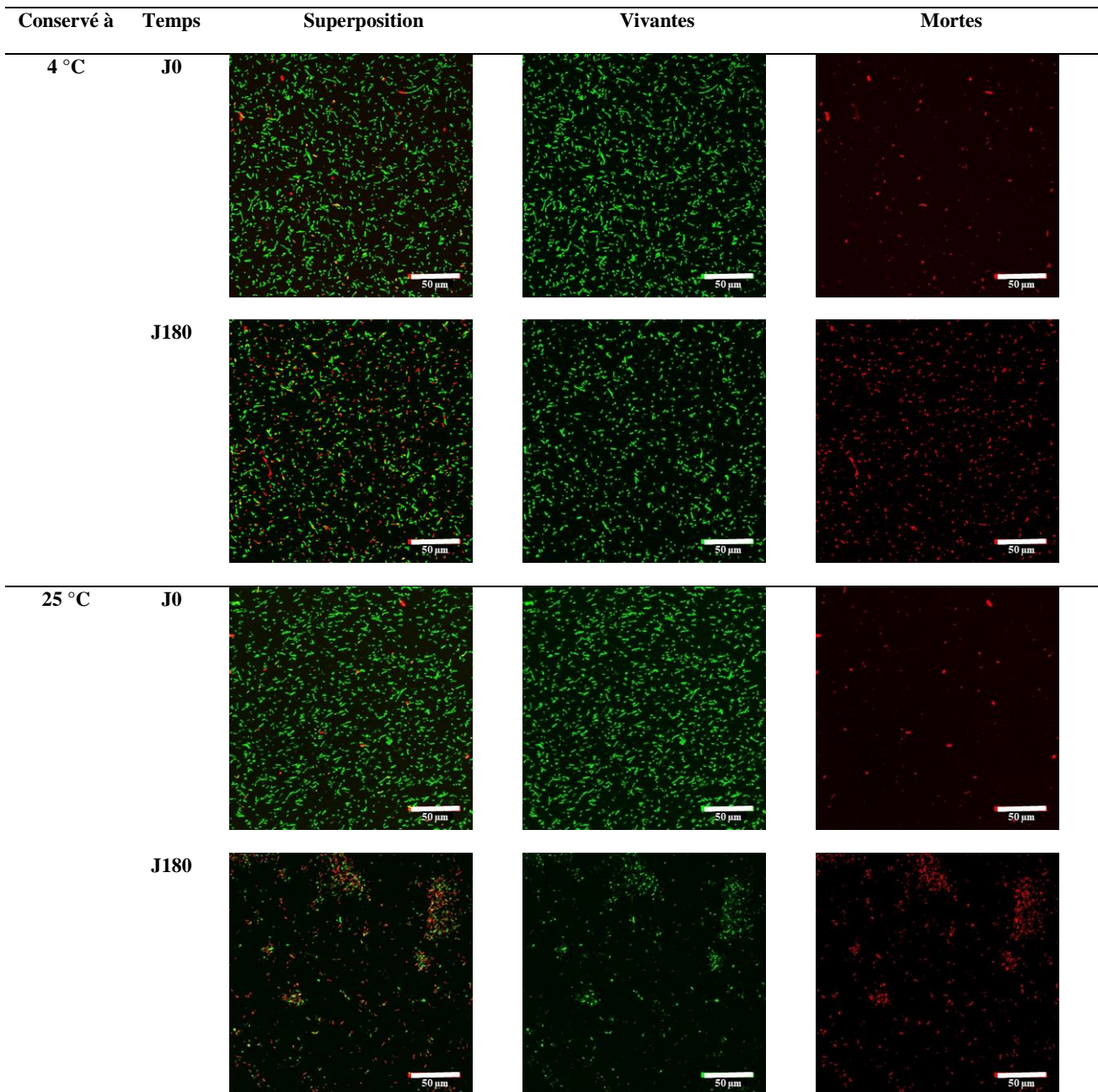


l'ADN (Stocks, 2004). Est-ce que la quantité d'iodure de propidium a été suffisante pour déplacer le syto 9 et marquer toutes les mortes en rouge au cours de notre étude ? En effet, si tel n'est pas le cas, certaines bactéries mortes resteraient marquées en vert. Certains auteurs ont parfois observé une coloration jaune au cours de ce double marquage suggérant la présence des deux marqueurs en même temps. Les images qui ont servi de base pour notre étude présentaient distinctement la couleur rouge ou verte. Ainsi, les bactéries rouges semblent mortes et les vertes vivantes. Nous n'avons pas d'interprétation pour le moment aux phénomènes observés. Il serait d'abord nécessaire de reproduire ces résultats. Ensuite, les mécanismes impliqués nécessiteraient des études approfondies.

Au bout de six mois, la répartition des lactobacilles a été conservée pour le gel P21.5A1LM conservé à 4 ° C, tandis que l'on a observé des zones plus denses ou plus pauvres en lactobacilles pour le gel conservé à 25 ° C (Fig. 9). En revanche, la répartition des lactobacilles semble plutôt homogène sur les six mois quelle que soit la température de conservation pour P21.5A1L (Fig. 7 et 8 du chapitre V), montrant que le mélange au vortex est efficace pour disperser les lactobacilles dans le gel.



**Figure 8.** Viabilité et cultivabilité comparées sur 6 mois de P21.5A1L (A, B) et P21.5A1LM (C, D) conservés à 4 °C (A, C) et à 25 °C (B, D). // rupture de la ligne du temps.



**Figure 9.** Répartition et viabilité sur six mois de *L. crispatus* dans le gel P21.5A1LM, montrant en vert les bactéries vivantes marquées par le syto 9 et en rouge les bactéries endommagées marquées par l'iodure de propidium.

### 2.3.5. Influence du MRS sur l'inhibition de la croissance de *N. gonorrhoeae*

Le **Tableau 4** reporte les résultats des tests d'inhibition de la croissance de *N. gonorrhoeae*, comparés entre les suspensions avec ou sans MRS de même qu'entre les gels avec ou sans MRS. Cette étude préliminaire, réalisée immédiatement après la préparation des systèmes, révèle que le MRS réduit l'efficacité des gels. Dans le gel P21.5A1L, les lactobacilles sont à pH 6-7 alors que dans P21.5A1LM le pH est 5-6. Le pH du milieu pourrait avoir une

influence sur la production d'acide lactique et la croissance des lactobacilles. Etudier ces deux gels au même pH pourrait permettre de mieux apprécier leur efficacité comparée. Le **Tableau 5** montre l'évolution du pH après incubation de P21.5A1L et P21.5A1LM à 37 °C pendant 24 h. La production acide semble être plus importante dans le gel avec MRS. La réduction du diamètre d'inhibition observé avec P21.5A1LM paraît alors surprenante puisqu'une production acide plus élevée correspondant à une baisse de pH plus élevée est normalement favorable à une inhibition (**Tableau 2 du chapitre V**) et un pH plus faible est favorable à l'inhibition (**Tableau 4 du chapitre v**). Ces tests doivent être répétés et des études approfondies doivent être réalisées. En effet, les lactobacilles ont un mécanisme d'action assez complexe sur les pathogènes, qui n'est pas seulement dû à la production d'acide lactique comme nous l'avons vu dans les **chapitres II et IV**. Il serait de ce fait intéressant d'explorer aussi d'autres mécanismes tels que la production de biosurfactants ou de bactériocines dans les gels.

**Tableau 4.** Inhibition de la croissance de *N. gonorrhoeae* pour les suspensions dans l'eau ou le MRS de lactobacilles et pour les gels contenant des lactobacilles dans l'eau ou le MRS.

		Contrôle= géloses superposées	Eau 1	MRS 1	Eau 2	MRS 2
Suspensions	Dénombrement (log <sub>10</sub> <i>L. crispatus</i> /spot)	Sans spot	8,12	8,10	8,35	8,27
	Diamètre inhibition à pH 5	≥ 60 mm	≥ 60 mm	≥ 60 mm	≥ 60 mm	≥ 60 mm
	Diamètre inhibition à pH 6.5	Sans inhibition	30 mm	20 mm	31 mm	20 mm
	Diamètre inhibition à pH 7	Sans inhibition	-	-	-	-
		Contrôle= géloses superposées	P21.5A1L	P21.5A1LM	P21.5A1L	P21.5A1LM
Gels	Dénombrement (log <sub>10</sub> <i>L. crispatus</i> /spot)	Sans spot	9,10	9,13	-	-
	Diamètre inhibition à pH 5	≥ 60 mm	≥ 60 mm	≥ 60 mm		
	Diamètre inhibition à pH 6.5	Sans inhibition	30 mm	6 mm		
	Diamètre inhibition à pH 7	Sans inhibition	14 mm	6 mm		

- inexploitable car contaminés

**Tableau 5.** Production acide après incubation de suspensions et gels de lactobacilles pendant 24 h à 37 °C en présence de 5 % CO<sub>2</sub> : pH<sub>i</sub>= pH initial, pH<sub>24 h</sub> = pH à 24 h.

Incubation à 37 °C, 5 % CO <sub>2</sub>	pH <sub>i</sub>	pH <sub>24 h</sub>
Suspension de-lactobacilles dans l'eau	6,70 ± 0,07	5,54 ± 0,26
Suspension de-lactobacilles dans le MRS	5,08 ± 0,03	4,08 ± 0,06
P21.5A1L	6,85 ± 0,02	6,53 ± 0,02
P21.5A1LM	5,64 ± 0,04	4,45 ± 0,04

## 2.4. Conclusion

Le MRS n'influence pas significativement les propriétés physicochimiques des gels. Néanmoins, il n'a pas eu l'effet positif espéré sur la viabilité des lactobacilles et sur l'efficacité des gels sur *N. gonorrhoeae*. La capacité du gel P21.5A1LM à inhiber le gonocoque semble diminuer alors que la production acide semble plus élevée. La présence de MRS pourrait augmenter le nombre de bactéries viables mais non cultivables. Des études complémentaires sont nécessaires pour mieux comprendre les mécanismes impliqués tels que la production de substances inhibitrices pour le gonocoque.

## Références

- Baum, M.M., Kainović, A., O'Keeffe, T., Pandita, R., McDonald, K., Wu, S., and Webster, P. (2009). Characterization of structures in biofilms formed by a *Pseudomonas fluorescens* isolated from soil. *BMC Microbiol.* 9, 103.
- Chen, M.-Y., Lee, D.-J., Tay, J.-H., and Show, K.-Y. (2007). Staining of extracellular polymeric substances and cells in bioaggregates. *Appl. Microbiol. Biotechnol.* 75, 467–474.
- Edelman, G.M., and Wang, J.L. (1978). Binding and functional properties of concanavalin A and its derivatives III. Interactions with indoleacetic acid and other hydrophobic ligands. *J. BIOLGICAI Chem.* 253, 3016–3022.
- Lamprecht, A., Schäfer, U., and Lehr, C.-M. (2000). Structural analysis of microparticles by confocal laser scanning microscopy. *AAPS PharmSciTech* 1, 10–19.
- Leccese Terraf, M.C., Juarez Tomas, maria S., Rault, L., Even, S., Le Loir, Y., and Nader-Macias, M.-E. (2016). Biofilms of vaginal *Lactobacillus reuteri* CRL 1324 and *Lactobacillus rhamnosus* CRL 1332: kinetics of formation and matrix characterization. *Arch. Microbiol.* 198, 689–700.
- Liu, J., Li, L., Li, B., Peters, B.M., Deng, Y., Xu, Z., and Shirliff, M.E. (2017a). First study on the formation and resuscitation of viable but nonculturable state and beer spoilage capability of *Lactobacillus lindneri*. *Microb. Pathog.* 107, 219–224.
- Liu, J., Li, L., Peters, B.M., Li, B., Chen, L., Deng, Y., Xu, Z., and Shirliff, M.E. (2017b). The viable but nonculturable state induction and genomic analyses of *Lactobacillus casei* BM-LC14617, a beer-spoilage bacterium. *MicrobiologyOpen* 6.
- Moore, S., Kailasapathy, K., Phillips, M., and Jones, M.R. (2015). Development of a viability standard curve for microencapsulated probiotic bacteria using confocal microscopy and image analysis software. *J. Microbiol. Methods* 114, 16–22.
- Owen, D.H., Peters, J.J., and Katz, D.F. (2000). Rheological properties of contraceptive gels. *Contraception* 62, 321–326.
- Pritchard, M.F., Powell, L.C., Khan, S., Griffiths, P.C., Mansour, O.T., Schweins, R., Beck, K., Buurma, N.J., Dempsey, C.E., Wright, C.J., et al. (2017). The antimicrobial effects of the alginate oligomer OligoG CF-5/20 are independent of direct bacterial cell membrane disruption. *Sci. Rep.* 7, 44731.

Sandoval-Altamirano, C., Sanchez, S.A., Ferreyra, N.F., and Gunther, G. (2017). Understanding the interaction of concanavalin a with mannosyl glycoliposomes: A surface plasmon resonance and fluorescence study. *Colloids Surf. B Biointerfaces* 158, 539–546.

Stocks, S.M. (2004). Mechanism and use of the commercially available viability stain, BacLight. *Cytom. Part J. Int. Soc. Anal. Cytol.* 61, 189–195.

Vásquez-Ponce, F., Higuera-Llantén, S., Pavlov, M.S., Ramírez-Orellana, R., Marshall, S.H., and Olivares-Pacheco, J. (2017). Alginate overproduction and biofilm formation by psychrotolerant *Pseudomonas mandelii* depend on temperature in Antarctic marine sediments. *Electron. J. Biotechnol.* 28, 27–34.

van de Velde, F., Weinbreck, F., Edelman, M.W., van der Linden, E., and Tromp, R.H. (2003). Visualisation of biopolymer mixtures using confocal scanning laser microscopy (CSLM) and covalent labelling techniques. *Colloids Surf. B Biointerfaces* 31, 159–168.

Wei, X.-Y., Zhang, R., Xiao, B.-B., and Liao, Q.-P. (2017). Biofilms of vaginal *Lactobacillus* in vitro test. *J. Obstet. Gynaecol.* 37, 69–73.

## **DISCUSSION GENERALE**



Le but de ce travail de thèse a été de mettre au point et de caractériser un gel à base de *Lactobacillus crispatus* destiné à une application vaginale pour la prévention de la gonococcie.

Ce travail a eu trois objectifs principaux. Le premier objectif a été d'étudier un mélange de poloxamer 407 et d'alginate de sodium pour une administration au niveau du vagin (**chapitre IV**). Etant donné que l'alginate est un polymère naturel dont la composition varie selon la source, nous avons déterminé sa masse molaire et sa composition. Les propriétés rhéologiques de l'alginate, du poloxamer 407, seuls ou mélangés ont été étudiées. Les aspects pratiques de leur administration ont été évalués par des tests d'expulsion à travers un dispositif vaginal que nous avons mimé à l'aide d'une seringue, et par l'analyse du profil textural en conditions mimant les conditions d'administration des gels. Le but était de faire de ce mélange une plateforme pour une administration vaginale. Le deuxième objectif a été de caractériser la souche de lactobacilles choisie (*L. crispatus* ATCC 33197) (**chapitre V**). Certains mécanismes d'action tels que la production d'acide, la capacité de co-agréger le gonocoque ainsi que l'inhibition *in vitro* de la croissance du gonocoque ont été étudiés. Le troisième objectif a été de caractériser de façon plus approfondie le gel contenant les lactobacilles P21.5A1L (**chapitre V et VI**).

Les questions soulevées par ce travail étaient :

- Quel pH utiliser pour ce gel probiotique ? Un pH acide pour mimer l'acidité vaginale ou plutôt proche de la neutralité favorable aux probiotiques ?
- Le mélange poloxamer 407 et alginate de sodium permet-il une gélification à la fois du poloxamer par la température vaginale et de l'alginate par les ions présents dans le fluide vaginal ?
- Quelle est l'influence des lactobacilles sur un tel mélange de polymères ?
- Quelle est l'influence des polymères sur la viabilité et l'efficacité des lactobacilles ?
- Quelle est l'influence du fluide vaginal sur les propriétés des gels ?

Dans le but de répondre aux questions qui se posaient, des mesures de pH, des études rhéologiques, des observations en microscopie confocale et des études de stabilité ont été réalisées.

## 1. CHOIX ET CARACTERISATION DE *L. CRISPATUS*

La souche de *Lactobacillus crispatus* a été sélectionnée sur la base des données publiées sur sa capacité à inhiber *Neisseria gonorrhoeae in vitro* (Amant et al., 2002) et *ex-vivo* (Breshears



et al., 2015). De plus, c'est la bactérie la plus ubiquitaire, qui domine la flore vaginale saine (Vasquez et al., 2002). Cette bactérie favorise aussi la stabilité de la flore vaginale normale (Verstraelen et al., 2009) et possède des propriétés probiotiques. Dans cette étude, la souche de référence *L. crispatus* ATCC 33197, d'origine vaginale a été utilisée pour la mise au point du gel destiné à la prévention de la gonococcie.

La flore vaginale saine est généralement composée de plusieurs espèces de lactobacilles qui interagissent ensemble. Pour mimer cette organisation naturelle, il pourrait être intéressant d'associer à *L. crispatus* d'autres souches présentant un potentiel probiotique et ayant aussi une efficacité sur le gonocoque. La flore vaginale est dominée par ces 4 espèces : *L. iners*, *L. crispatus*, *L. jensenii* et *L. gasseri* (Pavlova et al., 2002; Ravel et al., 2011). Néanmoins, *L. iners* et *L. gasseri* sont plutôt associés à des états pathologiques (Verstraelen et al., 2009). Il serait donc intéressant de s'orienter vers l'association de *L. jensenii* à *L. crispatus*. Les proportions restent à être élucidées. Spurbeck and Arvidson (2010) ont montré que *L. jensenii* inhibe l'adhésion du gonocoque aux cellules épithéliales. En mélangeant différentes espèces de lactobacilles, l'efficacité pourrait résulter de la synergie des deux souches ou bien j'une des souches pourrait inhiber l'autre (Famularo et al., 2001; Gardiner et al., 2002). Des études préliminaires devraient donc démontrer l'intérêt de cette association avant leur utilisation ensemble.

Nous avons montré que la souche de *L. crispatus* choisie pouvait se multiplier et produire des acides dans toute la gamme de pH étudiée (4-7), co-agréger avec *N. gonorrhoeae* et inhiber la croissance de *N. gonorrhoeae in vitro*. Elle a donc été utilisée dans notre formulation pour prévenir la gonococcie (**chapitre V**).

Nous avons par ailleurs vu dans le **chapitre V** qu'il existe une corrélation entre le pH et la production d'acide lactique par les lactobacilles et avons déduit que plus la variation du pH est élevée plus la production d'acide lactique est vraisemblablement élevée. Toutefois, il a été démontré que le pH et la concentration en acide lactique d'un milieu peuvent inactiver les agents pathogènes individuellement ou en combinaison. De plus, les concentrations élevées d'acide lactique protoné pourraient augmenter l'effet microbicide *in vivo* des lactobacilles (O'Hanlon et al., 2013). Le pKa de l'acide lactique étant 3,90, et le pH final dans les tubes étant compris entre 3.1 et 3.9, l'acide lactique protoné est donc la fraction la plus importante. Dans notre travail, la production d'acide lactique est estimée de façon indirecte par l'abaissement du pH. Elle devrait être mesurée de façon quantitative par des méthodes chromatographiques afin de confirmer notre analyse.

En plus des tests réalisés concernant les aspects sécuritaires de l'utilisation de *L. crispatus*, il serait intéressant d'ajouter l'utilisation de méthodes moléculaires telles que la réaction de polymérase en chaîne dans le but de déterminer les éventuels gènes de résistance de *L. crispatus*.

## 2. CHOIX ET CARACTERISATION DES POLYMERES

Dans une première approche, nous voulions développer une formulation thermosensible qui soit liquide à température ambiante pour une administration facile par la femme elle-même, suivi d'un bon étalement sur la muqueuse vaginale pour assurer une meilleure efficacité, et qui gélifie *in situ*. Nous avons donc choisi le mélange du poloxamer 407 pour obtenir la gélification avec la température corporelle, et de l'alginate de sodium pour ses propriétés mucoadhésives et sa possibilité de gélification ionique avec les ions calcium présents dans le fluide vaginal. L'association de ces deux mécanismes de gélification pourrait augmenter le temps de résidence du système. Ce mélange de polymères devait aussi maintenir la gélification après dilution par le fluide vaginal simulé. Nous avons travaillé avec des solutions d'alginate en régime semi-dilué vraisemblablement enchevêtré, de concentration variant entre 0.5 et 2 %. A 1 %, la solution d'alginate a présenté de meilleures propriétés viscoélastiques par rapport à celles à 0.5 % et était plus facile à obtenir que les solutions plus concentrées à 1,5 % ou 2 %. Cette concentration a donc été retenue. Toutefois son comportement liquide visqueux ne convient pas pour une administration vaginale lorsqu'il est utilisé seul. Les solutions de poloxamer 407 ont donc été étudiées à des concentrations variant de 16 à 21,5 % afin de déterminer la concentration optimale. Toutes les formulations de poloxamer ont présenté un comportement thermogélifiant, et un comportement solide élastique dominant, rhéofluidifiant et non-thixotrope à 25 ° C. La température de gélification de P21.5 était la plus basse ( $21 \pm 1$  ° C) mais seul P21.5 a maintenu ses propriétés thermogélifiantes après dilution par le fluide vaginal simulé à 37 °C. Ce système a donc été retenu pour le mélange avec l'alginate de sodium. L'ajout de P21.5 à A1 a amélioré l'élasticité du gel à l'état liquide. Plusieurs études ont montré que le mélange du poloxamer 407 avec l'alginate de sodium pouvait augmenter ses propriétés mécaniques (Quah et al., 2018; White et al., 2013).

Le mélange de poloxamer 407 à 21,5 % et d'alginate de sodium à 1 % (P21.5A1) a une température de gélification de  $19 \pm 1$  °C. La température ambiante dans certains pays d'Afrique subsaharienne comme la Côte d'Ivoire varie généralement entre 25 et 30 °C. Le mélange serait donc déjà sous la forme gel avant son administration. Néanmoins, son caractère rhéofluidifiant, les résultats de l'analyse du profil textural à 25 °C et de l'évaluation

de son aptitude à être expulsé d'une seringue sont propices à une administration aisée et un bon étalement. De plus, ce mélange pourrait être conservé au frais avant son administration pour être sous forme liquide. Ses propriétés rhéologiques à 37 °C après dilution par le fluide vaginal simulé sont aussi favorables pour assurer son étalement et éviter les fuites.

Dans une deuxième approche, nous voulions aussi obtenir la gélification ionique de l'alginate, mais ces essais se sont montrés infructueux même avec l'ajout de calcium dans la formulation. Toutefois, l'alginate de sodium associé à des ions calcium peut gélifier en milieu acide (Ferreira et al., 2017). Crow et Nelson (2006) ont mélangé des solutions d'alginate à du CaCO<sub>3</sub> non soluble dans l'eau à pH neutre. En ajoutant de la glucono- $\delta$ -lactone à ce mélange, ils ont dissocié l'ion calcium en abaissant le pH. Ensuite, l'ion calcium a initié la gélification de l'alginate de manière plus progressive (Crow et Nelson, 2006; Wang et al., 2008). Cette étude nous emmène à penser qu'une étape supplémentaire d'ajout de la glucono- $\delta$ -lactone dans nos formulation contenant du calcium, pourrait aider à initier la gélification interne. Cet essai pourrait être envisagé. Par ailleurs, Brus et al., (2017) ont montré que les blocs hydratés riches en M de l'alginate ont une faible capacité à se lier avec les ions de réticulation. Il serait intéressant de comparer notre alginate à un autre grade plus riche en unités guluronate, ce qui permettrait de faciliter cette gélification ionique.

Même si le mélange P21.5A1 ne remplissait pas totalement le cahier des charges initialement fixé, nous l'avons retenu pour la suite des études car nous ne savions pas quel serait l'effet des lactobacilles sur les propriétés rhéologiques.

### **3. LACTOBACILLES, POLOXAMER 407 ET ALGINATE**

Les études préliminaires sur les lactobacilles ont permis de choisir le pH optimal pour les lactobacilles compris entre 6 et 7. Le pH des gels étant similaire à ces derniers, nous n'avons pas jugé nécessaire d'ajouter un tampon dans nos formulations.

L'hydrogel optimisé P21.5A1L contient 21,5 % de poloxamer 407, 1 % d'alginate de sodium et 9 log<sub>10</sub> UFC/unité de gel de *L. crispatus*. Le processus de préparation des gels n'a pas été délétère pour les lactobacilles. La répartition de *L. crispatus* est reproductible dans le gel, montrant la reproductibilité de la méthode de préparation du gel. Cette répartition homogène de *L. crispatus* dans le gel est favorable à une distribution homogène de *L. crispatus* dans le vagin.

L'influence de *L. crispatus* sur les propriétés thermo-gélifiantes et viscoélastiques du mélange de poloxamer 407 et d'alginate a été étudiée par rhéologie à 25 °C (conditions

d'administration) et à 37 ° C après dilution avec le fluide vaginal simulé (SVF) (mimant les conditions *in vivo* après l'administration). *L. crispatus* n'influence pas de manière significative les propriétés rhéologiques du mélange de poloxamer 407 et d'alginate de sodium, même après dilution avec du SVF.

Dans cette étude, nous nous sommes intéressés à l'un des mécanismes d'action de *L. crispatus* qui est l'abaissement du pH. Nous avons pu faire la preuve du concept d'un gel probiotique *in vitro*. Néanmoins, l'inhibition du gonocoque par les gels reste moins importante que les suspensions de lactobacilles. Les gels gardent cependant tout leur intérêt car ils ont un temps de résidence plus prolongé que les suspensions après une administration vaginale.

La méthode d'étude de l'efficacité prend en compte seulement l'action liée aux molécules produites par les lactobacilles et capables de diffuser à travers le gel et la gélose (**Fig. 1 du chapitre V**). Une étude par contact direct entre le gel et le gonocoque est à envisager afin d'évaluer les autres mécanismes liés à la souche elle-même tels que la co-agrégation puis les mécanismes liés à la forme gel. Il s'agira de répondre à la question : les polymères entraînent-ils par eux-mêmes des mécanismes directs d'inhibition de *N. gonorrhoeae* ? Powell et al. (2014) ont étudié l'interaction de l'alginate de sodium composé d'unités G (> 96 %) avec la surface de *Pseudomonas aeruginosa*, une bactérie Gram négatif, en utilisant la microscopie à force atomique (AFM), la mesure du potentiel zêta et la mesure de la taille par diffusion dynamique de la lumière et des essais de mobilité. Cette étude a permis de démontrer que l'alginate était capable de se lier à la surface bactérienne, de diminuer la charge de surface, d'induire une agrégation des bactéries et d'inhiber leur mobilité. L'alginate utilisé dans notre gel ne possède pas la même composition (G = 45 %), mais pourrait présenter des avantages qu'il serait intéressant d'élucider. On peut aussi envisager de changer de grade d'alginate au profit d'un autre riche en unités guluronate. Une étude comparée permettrait de conclure sur le grade le plus approprié pour cette indication. Une étude de l'influence du mélange poloxamer-alginate sur *N. gonorrhoeae* serait aussi intéressante pour évaluer d'autres mécanismes par lequel le gel pourrait être efficace.

Dans l'étude de la viabilité des lactobacilles par microscopie confocale, les formulations ont été rendues liquides, puis mélangées au vortex avec les marqueurs fluorescents entraînant une répartition plutôt homogène des lactobacilles. Ce résultat montre que le gel pourrait retrouver facilement son homogénéité après une étape de mélange avant son utilisation, à une température inférieure à la température de gélification puisque la formulation serait liquide.

Contrairement à ce que nous envisagions, l'ajout de MRS dans le gel a présenté des inconvénients tels que (i) la baisse de l'efficacité sur le gonocoque, (ii) la baisse de la cultivabilité sur gélose MRS de *L. crispatus* des gels au cours des six mois de conservation à 4 °C et à 25 °C. Notre hypothèse est que le MRS aiderait les lactobacilles à entrer dans un état de bactéries viables mais non cultivables pour survivre longtemps. L'ajout de MRS a cependant présenté l'avantage d'une production acide plus élevée lorsque le gel P21.5A1LM a été incubé à 37 °C. Le MRS est un milieu assez complexe et la production d'acide lactique provient du métabolisme des glucides qui y sont contenus. Il serait donc intéressant de sélectionner des glucides et de les mettre à la disposition des lactobacilles dans le gel pour favoriser leur survie, leur croissance et leur activité : on parle de l'utilisation de prébiotiques. Certains prébiotiques ont déjà fait l'objet d'études pour d'autres souches de lactobacilles. Les plus étudiés sont les fructooligosaccharides et les galactooligosaccharides (Gibson et al., 2017). Toutefois, d'autres prébiotiques tels que l'inuline, le mannitol, la maltodextrine, la pectine ont montré de bonnes propriétés sur l'amélioration de la survie des lactobacilles (Yeo and Liong, 2010).

Toujours dans le but d'optimiser le gel et améliorer la viabilité des lactobacilles, une voie serait de lyophiliser les lactobacilles qui pourraient ensuite être resuspendus et incorporés dans le mélange de polymères avant administration. Le gel contenant les lactobacilles pourrait également être lyophilisé et reconstitué avec un solvant avant son administration. Ce procédé pourrait nécessiter l'utilisation de cryoprotectants dont le choix doit s'orienter vers ceux qui augmenteraient la température de gélification en restant toutefois inférieure à la température vaginale. Cette étude devrait aussi être associée à la conception d'un dispositif de mélange et d'administration. Nous pensons à un système composé de deux compartiments avec un opercule qui serait percé pour le mélange avant administration.

L'évaluation des propriétés mucoadhésives des formulations doit être envisagée ainsi que l'évaluation du temps de résidence de nos formulations dans le vagin. Les modèles animaux pour étudier le gonocoque sont les souris, les porcs, les chimpanzés (Arko, 1989; Chandler and Kraus, 1976; Lucas et al., 1971). La muqueuse vaginale du porc étant proche de celle de la femme, des études *in vivo* sur des modèles animaux de porc seraient intéressantes. Néanmoins, le gonocoque est une bactérie stricte de l'Homme. Ainsi, seuls les essais cliniques sur la femme pourront fournir la réponse définitive de l'efficacité des lactobacilles formulés en tant que probiotiques pour la prévention de la gonococcie.

Même si nous sommes encore loin d'un système utilisable en clinique, la vision que nous avons de notre système serait une formulation unidose, destinée à être administrée par les

femmes à risque présentant un antécédent d'infection vaginale et les femmes présentant naturellement un déficit de *L. crispatus* dans la flore vaginale. Cette formulation limiterait la gonococcie surtout chez les femmes qui n'ont pas la possibilité de négocier l'usage du préservatif lors des rapports sexuels avec leur partenaire. Le nombre, la fréquence et le moment d'administration, afin d'assurer la prévention de la gonococcie, sont des paramètres importants qu'il faudrait évaluer au cours d'une étude clinique.

## Références

- Amant, D.C.S., Valentin-Bon, I.E., and Jerse, A.E. (2002). Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. *Infect. Immun.* 70, 7169–7171.
- Arko, R.J. (1989). Animal models for pathogenic *Neisseria* species. *Clin. Microbiol. Rev.* 2, S56–S59.
- Breshears, L.M., Edwards, V.L., Ravel, J., and Peterson, M.L. (2015). *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. *BMC Microbiol.* 15.
- Chandler, F.W., and Kraus, S.J. (1976). Animal model of human disease. Gonorrhea. *Am. J. Pathol.* 82, 437–440.
- Famularo, G., Pieluigi, M., Coccia, R., Mastroiacovo, P., and Simone, C.D. (2001). Microecology, bacterial vaginosis and probiotics: perspectives for bacteriotherapy. *Med. Hypotheses* 56, 421–430.
- Gardiner, G.E., Heinemann, C., Bruce, A.W., Beuerman, D., and Reid, G. (2002). Persistence of *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 but not *L. rhamnosus* GG in the human vagina as demonstrated by randomly amplified polymorphic DNA. *Clin. Diagn. Lab. Immunol.* 9, 92–96.
- Gibson, G.R., Hutkins, R., Sanders, M.E., Prescott, S.L., Reimer, R.A., Salminen, S.J., Scott, K., Stanton, C., Swanson, K.S., Cani, P.D., et al. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* 14, 491–502.
- Lucas, C.T., Chandler, F., Martin, J.E., and Schmale, J.D. (1971). Transfer of gonococcal urethritis from man to chimpanzee: an animal model for gonorrhea. *JAMA* 216, 1612–1614.
- Pavlova, S.I., Kilic, A.O., Kilic, S.S., So, J.-S., Nader-Macias, M.E., Simoes, J.A., and Tao, L. (2002). Genetic diversity of vaginal *Lactobacilli* from women in different countries based on 16S rRNA gene sequences. *J. Appl. Microbiol.* 92, 451–459.
- Powell, L.C., Pritchard, M.F., Emanuel, C., Onsøyen, E., Rye, P.D., Wright, C.J., Hill, K.E., and Thomas, D.W. (2014). A nanoscale characterization of the interaction of a novel alginate oligomer with the cell surface and motility of *Pseudomonas aeruginosa*. *Am. J. Respir. Cell Mol. Biol.* 50, 483–492.
- Quah, S.P., Smith, A.J., Preston, A.N., Laughlin, S.T., and Bhatia, S.R. (2018). Large-area alginate/PEO-PPO-PEO hydrogels with thermoreversible rheology at physiological temperatures. *Polymer* 135, 171–177.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., Stacey, L.M., Karlebach, shara, Gorle, R., Russell, J., Carol, O.T., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U A* 108, 4680–4687.

Spurbeck, R.R., and Arvidson, C.G. (2010). *Lactobacillus jensenii* surface-associated proteins inhibit *Neisseria gonorrhoeae* adherence to epithelial cells. *Infect. Immun.* 78, 3103–3111.

Vasquez, A., Jakobsson, T., Ahrne, S., Forsum, U., and Molin, G. (2002). Vaginal *Lactobacillus* flora of healthy Swedish women. *J Clin Microbiol* 40, 2746–2749.

Verstraelen, H., Verhelst, R., Claeys, G., De Backer, E., Temmerman, M., and Vaneechoutte, M. (2009). Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol* 9, 116.

White, J.C., Saffer, E.M., and Bhatia, S.R. (2013). Alginate/PEO-PPO-PEO composite hydrogels with thermally-active plasticity. *Biomacromolecules* 14, 4456–4464.

Yeo, S.-K., and Liong, M.-T. (2010). Effect of prebiotics on viability and growth characteristics of probiotics in soymilk. *J. Sci. Food Agric.* 90, 267–275.

## **CONCLUSION GENERALE ET PERSPECTIVES**





La gonococcie est une infection sexuellement transmissible, causée par *Neisseria gonorrhoeae*, devenue un problème majeur de santé publique pour plusieurs raisons. En effet, cette infection reste souvent asymptomatique chez la femme, ce qui favorise sa propagation. Elle peut entraîner des complications graves, telles que l'infertilité chez l'adulte et la cécité chez le nouveau-né. Elle augmente également le risque de contracter le VIH. Il existe ni vaccin ni nouveaux médicaments émergeant sur le marché. Le préservatif, seul moyen de prévention efficace, n'est malheureusement pas bien ou insuffisamment utilisé. Actuellement une émergence de gonocoques résistants à plusieurs antibiotiques est observée, et même certaines souches non traitables car insensibles à tous les antibiotiques connus commencent à circuler. Ainsi, cette infection est devenue difficile à traiter ou à prévenir. Nous avons choisi dans le cadre de notre étude de nous intéresser aux aspects de prévention de cette infection qui nous paraissait un levier primordial pour juguler cette infection.

*Neisseria gonorrhoeae* successivement adhère aux cellules épithéliales, pénètre, se multiplie, crée une inflammation et affaiblit la barrière épithéliale. Il peut persister à l'intérieur des neutrophiles ou modifier leurs antigènes de surface pour échapper au système immunitaire. Il peut également produire certaines protéases pour éviter le développement d'une immunité à la réinfection. *Neisseria gonorrhoeae* peut modifier les sites de fixation des antibiotiques, synthétiser des enzymes pour modifier ou inactiver les antibiotiques, modifier ses protéines de porine de surface pour réduire la perméabilité des antibiotiques, utiliser une nouvelle voie métabolique pour remplacer celle inhibée par les antibiotiques, utiliser le mécanisme actif d'efflux d'antibiotiques et protéger l'enzyme cible en se liant à une autre protéine. Ces mécanismes de pathogénicité et de résistance aident le gonocoque à survivre.

Les lactobacilles, en revanche, sont des bactéries commensales prédominantes du microbiote vaginal normal. Ils participent au maintien de l'acidité vaginale et luttent naturellement contre les infections en associant différents mécanismes. En fait, ils peuvent adhérer aux cellules épithéliales, concurrencer les agents pathogènes pour les sites d'adhésion ou produire des tensioactifs pour inhiber l'adhérence des agents pathogènes, co-agréger avec des agents pathogènes pour réduire leur propagation, produire des substances antimicrobiennes telles que les acides, du peroxyde d'hydrogène et des bactériocines et stimuler le système immunitaire. L'utilisation de lactobacilles est apparue dans le domaine de la prévention des infections urogénitales. Des formulations à base de lactobacilles déjà sur le marché ou en développement ont été proposées pour le traitement des infections de la sphère urogénitale. Par conséquent, la restauration du microbiote vaginal normal par l'administration de

lactobacilles possédant des propriétés probiotiques et une activité sur *Neisseria gonorrhoeae* paraît attractive.

A l'instar du contrôle des naissances axé sur la femme qui s'avère efficace, axer la stratégie de prévention des infections sexuellement transmissibles, en particulier de la gonococcie sur la femme apparaît intéressant pour juguler cette infection. Protéger la femme, c'est aussi protéger l'homme et également protéger les nouveau-nés. Développer un moyen de prévention que la femme soit capable d'utiliser elle-même, de contrôler sans accord de son partenaire est à privilégier. Etant donné que la voie vaginale est la voie principale de transmission de cette infection, un moyen de prévention destiné à une administration vaginale nous a paru judicieux.

Dans ce projet de thèse, nous avons choisi d'administrer les lactobacilles, en particulier *Lactobacillus crispatus* ayant montré dans la littérature une efficacité contre le gonocoque, en l'incorporant dans un gel pour plusieurs raisons. En effet, il s'agit d'une forme galénique facile à préparer et à administrer, capable de tapisser la muqueuse vaginale et de réaliser un contact intime avec elle tout en augmentant le temps de résidence par rapport à une forme liquide. En particulier, les gels thermosensibles peuvent gélifier à la température corporelle après leur administration et éviter ainsi leur écoulement rapide de la lumière vaginale. Nous avons donc développé un gel à base de poloxamer 407, d'alginate de sodium et de *Lactobacillus crispatus*, puis nous l'avons caractérisé et évalué son efficacité sur le gonocoque. Le gel optimisé constitué de poloxamer 407 à 21.5 %, d'alginate de sodium à 1 % et de *lactobacillus crispatus* était (i) rhéofluidifiant, ce qui favoriserait son administration, (ii) conservait ses propriétés rhéologiques après la dilution par le fluide vaginal simulé, (iii) inhibait la croissance du gonocoque *in vitro*. Cette formulation est prometteuse pour la prévention de la gonococcie. Cette étude préliminaire a permis de faire la preuve du concept *in vitro*. Des efforts restent néanmoins encore à faire pour améliorer la viabilité des lactobacilles et la conservation de ces systèmes. L'évaluation de ce gel doit être poursuivie afin d'améliorer la compréhension de l'organisation et la structure de ce gel et ses mécanismes d'inhibition du gonocoque.

Plusieurs pistes d'optimisation de notre formulation restent donc à explorer pour compléter ce travail. L'introduction de prébiotiques dans ce gel pourrait être une stratégie intéressante pour augmenter la viabilité des lactobacilles. La lyophilisation soit des lactobacilles, soit du gel complet dans un dispositif d'administration vaginale à double compartiments adapté à cette formulation et surtout pour le mélange avant administration pourrait permettre de le conserver sur des temps plus longs. Des études de cryo-microscopie électronique à transmission, de

diffusion des neutrons aux petits angles, ou de diffusion des rayons X aux petits angles pourraient permettre d'étudier de façon plus approfondie leur organisation. Enfin, des études d'efficacité sur le gonocoque en le mettant en contact direct avec la formulation pourraient être intéressantes pour favoriser des mécanismes d'inhibition tels que la co-agrégation ou la production de substances inhibitrices par les lactobacilles, à proximité directe du gonocoque.



**Titre :** Développement d'un gel vaginal à base de poloxamer 407, d'alginate de sodium et de *Lactobacillus crispatus* pour la prévention de la gonococcie

**Mots clés :** gel, rhéologie, poloxamer 407, alginate de sodium, *Lactobacillus crispatus*, microscopie confocale, voie vaginale, gonococcie

**Résumé :** La gonococcie est une infection sexuellement transmissible due au gonocoque. Elle est devenue un problème majeur de santé publique du fait de la multirésistance aux antibiotiques, mais surtout de la résistance au traitement de dernière intention actuellement en vigueur. *Lactobacillus crispatus*, une bactérie naturelle, commensale du vagin de la femme, s'est montré efficace pour inhiber le gonocoque. Les gels, une forme galénique bien acceptée, sont déjà utilisés pour le traitement des infections génitales de la femme. Disposer d'un gel contenant *Lactobacillus crispatus*, efficace, facile à administrer par la femme elle-même paraît donc attractif pour la prévention de la gonococcie. Ainsi, nous avons conçu un gel à base d'un polymère thermogélifiant, le poloxamer 407 et d'un polymère biocompatible, l'alginate de sodium.

Dans un premier temps, une étude physico-chimique du mélange de polymères a permis de retenir les concentrations optimisées. Dans un second temps, la souche de *Lactobacillus* choisie a été caractérisée et introduite dans le mélange de polymères. Les propriétés physicochimiques dont les caractéristiques rhéologiques, l'expulsion d'un dispositif d'administration, la stabilité, la microstructure ainsi que l'efficacité *in vitro* du gel obtenu ont été étudiés. Une répartition homogène de *Lactobacillus crispatus* a été observée dans le gel. Ce système est facilement administrable et possède des propriétés rhéologiques favorables à son étalement et son maintien dans la lumière vaginale. Ce gel a permis d'inhiber la croissance du gonocoque *in vitro*.

**Title:** Development of a gel containing poloxamer 407, sodium alginate and *Lactobacillus crispatus* for the prevention of gonorrhoea

**Keywords :** gel, rheology, poloxamer 407, sodium alginate, *Lactobacillus crispatus*, confocal microscopy, vaginal administration, gonorrhoea

**Abstract:** Gonorrhoea is a sexually transmitted infection caused by *Neisseria gonorrhoeae*. It has become a major public health issue due to multidrug resistance, especially resistance to the current last-intention treatment. *Lactobacillus crispatus*, a natural bacterium, commensal to the woman's vagina, has been shown to inhibit *Neisseria gonorrhoeae*. Gels, a well-accepted dosage form, are already used for the treatment of woman's genital infections. Having a gel containing *Lactobacillus crispatus*, that is effective, easy to administer by the woman herself, would be ideal for the prevention of gonorrhoea. Thus, we designed a gel based on a thermogelling polymer, poloxamer 407, and a biocompatible polymer, sodium alginate.

First, a physicochemical study of the polymers mixtures allowed to select the optimized concentrations. Second, the selected *Lactobacillus* strain was characterized and introduced into the optimized polymer mixture. Physicochemical properties including rheological characteristics, expulsion from a device, stability, microstructure as well as *in vitro* gel efficacy were studied. A homogeneous distribution of *Lactobacillus crispatus* was observed in the gel. It was easily administered and its rheological properties were suitable for its spreading and its long residence time in the vaginal lumen. This gel showed an inhibition of gonococcal growth *in vitro*.