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Innovative therapeutic strategies for the treatment of vaginal infections

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Resumo

A bacteriose vaginal (BV) e a candidose vulvovaginal (VVC) são as infeções vaginais mais prevalentes e afetam um elevado número de mulheres, apresentando um impacto negativo significativo na sua autoestima e estando associadas a um enorme desconforto e alterações nas atividades diárias, especialmente na vida sexual.

A VVC é causada sobretudo por *Candida albicans*, que é considerada um agente patogénico oportunista, mas outras espécies de *Candida* como C. *glabrata*, *C. krusei*, *C. tropicalis* e *C. parapsilosis* também podem ser considerados agentes etiológicos. Os sintomas mais comuns associados a VVC são irritação vaginal, ardor, prurido, edemaciação e alteração do fluído que se apresenta com aspeto de queijo coalhado. O tratamento de primeira linha da VVC é feito com azóis, mais frequentemente dose única de fluconazol (150 mg) ou itraconazol (200mg em duas tomas num dia), ou aplicação de produtos tópicos como creme de clotrimazol e comprimido vaginal de clotrimazol 500mg, durante um curto período. Porém, 5-8% das mulheres em idade reprodutiva são afetadas por recorrências clínicas definidas em termos de frequência como 3 ou mais episódios num período de 12 meses (VVC crónica), quer por falta de resposta à terapêutica (VVC persistente) seja por serem devidas a espécies de *Candida* não *albicans* ou *Candida albicans* resistentes aos azóis, quer por reinfecção.

Embora algumas estejam assintomáticas, a maioria das mulheres com BV experiencia um corrimento abundante, pouco viscoso e com mau odor. Esta condição clínica é considerada uma disbiose, em que ocorre a substituição da flora vaginal saprófita saudável, dominada por *Lactobacillus* spp, por biofilmes polimicrobianos densos, estruturados, principalmente constituídos por *Gardnerella vaginalis*. As recomendações internacionais indicam como tratamento de primeira linha a administração oral ou vaginal de metronidazol, clindamicina ou tinidazol. Contudo, o tratamento com estes antibióticos está associado a elevados níveis de falha terapêutica e de recorrência, o que pode estar relacionado com resistência aos antibióticos, a incapacidade de erradicar os biofilmes polimicrobianos e/ou posterior falha no restabelecimento do pH vaginal acídico e uma flora comensal dominada por lactobacilos.

Foi objetivo deste trabalho desenvolver e caracterizar abordagens terapêuticas inovadoras alternativas ou complementares das existentes para o tratamento de infeções vaginais. Especificamente, o nosso objetivo foi desenvolver dois produtos seguros e eficazes com potencial para serem utilizados terapeuticamente e comercializados e que são: um gel bioadesivo de bicarbonato de sódio para o tratamento da VVC e uma folha vaginal com óleo essencial de *Thymbra capitata* para o tratamento da BV.

No desenvolvimento dos géis de bicarbonato de sódio, o potencial anti *Candida* foi estudado através de um método de referência internacional (CLSI M27 ”Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts”) e de um protocolo adaptado a partir de *Challenge Test* da farmacopeia europeia 10.0 (monografia 5.1.3). Também avaliámos a

performance tecnológica das formulações considerando parâmetros que mimetizam a condição patológica. O pH, osmolalidade, viscosidade, firmeza e adesividade foram determinados diretamente nas formulações não diluídas a temperatura ambiente e após diluição das formulações em simulante de fluido vaginal a 37 ºC. O perfil de bioadesão foi determinado usando um modelo *ex vivo* vaginal de suíno. A formulação C (5% de bicarbonato de sódio, 1% de carbómero e 94% de água) foi a mais eficaz a inibir o crescimento de *Candida albicans*. Para além disso, a formulação C apresentou características organolépticas que estão de acordo com as preferências das utilizadoras e perfil tecnológico adequado, fazendo prever uma adequada *performance in vivo*.

As folhas vaginais desenhadas para o tratamento da BV contêm gelatina, como principal macromolécula, associada a polímeros bioadesivos como quitosano, álcool polivinílico e hidroxipropilmetilcelulose, para promover um contato íntimo e prologado dos produtos com o epitélio. As folhas contêm ainda plastificantes (glicerina e propilenoglicol) que lhes conferem flexibilidade. A inclusão do tampão de acido lático/lactato de sódio nas formulações representa um mecanismo adicional para promover o ambiente acídico favorável ao restabelecimento da flora saprófita saudável, contribuindo para a eficácia do tratamento e prevenindo recorrências. As folhas vaginais foram usadas como sistema de veiculação de fármaco para o óleo essencial de *Thymbra capitata*, que atua diretamente no controle das bactérias que causam a BV, enquanto simultaneamente e de imediato pretendem reduzir os sintomas associados ao corrimento vaginal abundante e ao seu odor desagradável, dando conforto às utilizadoras. A folha vaginal D.O apresentou a capacidade de tampão mais elevada e a melhor capacidade para absorver o simulante de fluído vaginal, de entre as folhas vaginais com óleo estudadas, apresentando ainda um dos melhores perfis de bioadesão. Adicionalmente, D.O apresenta flexibilidade (isto é, resiliência) e resistência (isto é, dureza), de modo a permitir que seja facilmente enrolada a volta de um dedo ou sobre si mesma e manuseada sem quebrar. Numa perspetiva de segurança, a citotoxicidade foi avaliada em duas linhas celulares e num modelo de epitélio vaginal humano reconstituído e o potencial para causar irritação vaginal também foi avaliado através do ensaio "Hen´s Egg Test-Chorioallantoic Membrane Assay" (HET-CAM). A substância ativa (óleo essencial de *Thymbra capitata*) a 0.32 µL/mL no modelo celular evidenciou toxicidade relevante, contudo no modelo tecidular tridimensional mostrou-se biocompatível. A incorporação de óleo essencial de *Thymbra capitata* (1% m/m) na folha base D reduziu a viabilidade em modelo celular (em ambas as linhas) e no modelo tecidular. A viabilidade após contacto com a folha D.O em modelo celular foi muito inferior quando comparada com o placebo universal, porém é necessário considerar que *in vivo* a folha vaginal vai gradualmente ser diluída no fluido vaginal e é proposta para tratamentos de curta duração. A folha vaginal D.O diluída a 10% m/v foi biocompatível no modelo tecidular.

Em conclusão: foram desenvolvidos dois produtos para o tratamento de infeções vaginais inovadores e com potencial para serem transferidos da academia para a indústria farmacêutica. O gel de bicarbonato de sódio e a folha vaginal com óleo essencial de *Thymbra capitata* foram desenvolvidos para responder aos requisitos clínicos e simultaneamente às preferências das utilizadoras, permitindo ultrapassar desvantagens associadas a formas

farmacêuticas convencionais. Embora os resultados obtidos sejam promissores ao nível da previsível aceitabilidade, *performance* tecnológica, eficácia e segurança, são necessários estudos mais completos numa fase pré-clínica e obviamente a prova definitiva nos ensaios clínicos.

Palavras-chave

Infeção vaginal; candidose vulvovaginal; bacteriose vaginal; tratamento; tecnologia farmacêutica; gel bioadesivo; bicarbonato de sódio; óleo de rícino; folha vaginal; óleo essencial de *Thymbra capitata*

Resumo alargado

As infeções vaginais apresentam repercussões significativas na vida das mulheres, sendo causadoras de ansiedade, desconforto e sentimentos negativos com impacto desfavorável na sua autoestima, levando a alterações das rotinas quotidianas e inclusivamente da vida sexual.

A bacteriose vaginal (BV) e a candidose vulvovaginal (VVC) são as infeções vaginais mais prevalentes e afetam um elevado número de mulheres em todo o mundo. As infeções vaginais estão associadas a alterações na flora vaginal normal saudável e substituição da mesma por microrganismos capazes de proliferar e causar infeção.

A VVC é causada sobretudo por *Candida albicans*, que é considerada um agente patogénico oportunista, mas outras espécies de *Candida* como C. *glabrata*, *C. krusei*, *C. tropicalis* e *C. parapsilosis* também podem ser consideradas agentes etiológicos.

Os sintomas mais comuns associados a VVC são irritação vaginal, ardor, prurido, edemaciação alteração do fluído que se apresenta com aspeto de queijo coalhado.

O tratamento de primeira linha da VVC é feito com azóis, mais frequentemente dose única de fluconazol (150 mg) ou itraconazol (200mg em duas tomas num dia), ou aplicação de produtos tópicos como creme de clotrimazol e comprimido vaginal de clotrimazol 500mg, durante um curto período. Algumas mulheres são afetadas por recorrência definidas em termos de frequência como 3 ou mais episódios num período de 12 meses (VVC crónica), quer por falta de resposta à terapêutica (VVC persistente) seja por serem devidas a espécies de *Candida* não *albicans* ou *Candida albicans* resistentes aos azóis, quer por reinfecção.

Na BV, a flora vaginal saprófita saudável, dominada por *Lactobacillus* spp, é substituída por biofilmes polimicrobianos densos, estruturados, principalmente constituídos por *Gardnerella vaginalis*.

Embora algumas mulheres sejam assintomáticas, a maioria das mulheres com BV experiencia um corrimento abundante, pouco viscoso e com odor desagradável a peixe podre.

As recomendações internacionais indicam como tratamento de primeira linha a administração oral ou vaginal de metronidazol, clindamicina ou tinidazol. Contudo, o tratamento *standard* está associado a elevados níveis de falha terapêutica devido ao aumento das resistências aos antibióticos e incapacidade de penetrar e erradicar os biofilmes polimicrobianos e elevados níveis de recorrência por falha no restabelecimento do ambiente vaginal saudável em termos de flora comensal dominada por lactobacilos e pH vaginal acídico.

Desta forma, torna-se emergente dar resposta efetiva a estas infeções vaginais, sobretudo nos casos em que os tratamentos *standard* não se revelam eficazes, fazendo com que estas patologias se tornem recorrentes, o que acarreta um enorme impacto na saúde de um elevado número de mulheres em todo o mundo.

O objetivo desta investigação foi desenvolver novas abordagens terapêuticas para o tratamento das duas infeções vaginais mais comuns, alternativas ou complementares aos tratamentos atualmente disponíveis. Especificamente, o objetivo foi desenvolver dois produtos

seguros e eficazes com potencial para serem utilizados terapeuticamente uma vez comercializados e que são: um gel bioadesivo de bicarbonato de sódio para o tratamento da VVC e uma folha vaginal com óleo essencial de *Thymbra capitata* para o tratamento da BV. Para além disso, foi objetivo desta investigação caracterizar os protótipos desenvolvidos quanto ao perfil tecnológico considerando condições que mimetizam o ambiente vaginal *in vivo* característico da patologia em questão e *performance* preditiva; quanto ao perfil de segurança dos protótipos mais promissores usando metodologias complementares; e quanto à eficácia *in vitro* com base na capacidade para inibir o crescimento de alguns microrganismos causadores destas infeções.

O desenho racional destas formulações teve por base a seleção de substâncias ativas alternativas aos tratamentos convencionais com potencial para inibir o crescimento dos microrganismos causadores da infeção e excipientes considerados seguros para a administração vaginal. Estas formulações foram desenhadas de modo a serem adaptadas aos objetivos específicos do tratamento de cada patologia, de modo a promover o sucesso terapêutico, mas também para irem de encontro às preferências das utilizadoras, para favorecer a adesão à terapêutica.

Foram desenvolvidas 8 formulações de géis à base de bicarbonato de sódio. O potencial anti *Candida* foi estudado através de um método de referência internacional (CLSI M27 ”Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts”) e de um protocolo adaptado a partir de *Challenge Test* da farmacopeia europeia 10.0 (monografia 5.1.3), sendo que a formulação C (5% de bicarbonato de sódio, 1% de carbómero e 94% de água) foi a mais eficaz a inibir o crescimento de *Candida albicans*, sendo também eficaz contra outras estirpes de *Candida*. Adicionalmente, a formulação C apresentou características organolépticas que estão de acordo com as preferências das utilizadoras. Sendo um gel pouco viscoso e de baixa firmeza, o espalhamento e revestimento do epitélio encontram-se facilitados e, graças às suas características bioadesivas (avaliadas em modelo *ex vivo* vaginal de suíno), é promovido um contacto íntimo e prolongado com o mesmo. Apesar do gel C apresentar um pH e osmolalidade superiores aos valores fisiológicos reportados para o fluído vaginal de mulheres saudáveis em idade fértil, os ensaios de segurança realizados nesta investigação indicam que a formulação é biocompatível na linha celular testada (não tóxico para todas as concentrações testadas até 50% (m/v) e classificado como não irritante severo no ensaio HET-CAM. Após diluição em simulante de fluído vaginal (5g de gel em 0.825mL de simulante), o pH e osmolalidade resultantes tornam-se mais próximos dos valores fisiológicos, embora ainda superiores aos limites definidos para lubrificantes, de acordo com as recomendações atuais. Porém deve ser considerado que este produto se destina a um tratamento durante um curto período e que, uma vez que o gel C apresenta um potencial anti-*Candida* muito potente, poderá ser reduzida a quantidade de gel a aplicar para 1g, tornando o pH e osmolalidade mais próximos dos fisiológicos, e simultaneamente reduzindo a possibilidade de escorrimento através do canal vaginal após aplicação.

As folhas vaginais desenhadas para o tratamento da BV foram usadas como sistema de veiculação de fármaco para o óleo essencial de *Thymbra capitata*, que atua diretamente no

controle das bactérias que causam a BV, enquanto simultaneamente e de imediato absorvem o excesso de fluído com odor desagradável, prevendo-se que ofereçam conforto às utilizadoras. No desenho racional das folhas vaginais foi incluída a gelatina associada a polímeros bioadesivos (quitosano, álcool polivinílico e hidroxipropilmetilcelulose) e plastificantes (glicerina e propilenoglicol) que lhes conferem flexibilidade. A inclusão do tampão de ácido lático nas formulações representa um mecanismo complementar, contribuindo para o restabelecimento do ambiente vaginal saudável. A folha vaginal D.O apresentou a capacidade tampão mais elevada, o que está de acordo com o esperado, já que foi incluído nesta formulação uma solução tampão com maior força. Para além disso, a folha vaginal D.O apresentou a melhor capacidade para absorver o simulante de fluído vaginal entre as folhas vaginais com óleo, podendo contribuir para um alívio imediato dos sintomas ao absorver o fluído de mau odor e gradualmente se transformar num gel. A folha vaginal D.O apresentou um dos melhores perfis de bioadesão avaliado em modelo de suíno *ex-vivo*. Adicionalmente, D.O apresenta flexibilidade (isto é, resiliência) e resistência (isto é, dureza), de modo a permitir que seja facilmente enrolada a volta de um dedo ou sobre si mesma e manuseada sem quebrar, o que permite uma aplicação sem desconforto. A folha vaginal D.O na concentração 0.32 µL/mL de óleo essencial de *T. capitata* conseguiu reduzir significativamente a carga bacteriana em todas as espécies de *Gardnerella* testadas, sendo menos eficaz em *G. piotti*. Numa perspetiva de segurança, a citotoxicidade do protótipo mais promissor foi avaliada em duas linhas celulares (HEC-1A e HeLa) e em modelo de epitélio vaginal humano reconstituído (SkinEthic™) e o potencial para causar irritação vaginal também foi avaliado através do ensaio "Hen´s Egg Test-Chorioallantoic Membrane Assay". A substância ativa (óleo essencial de *Thymbra capitata*) na concentração 0.32 µL/mL no modelo celular evidenciou toxicidade relevante, contudo no modelo tecidular tridimensional mostrou-se biocompatível. Isto revela que este modelo é mais robusto e provavelmente mais próximo do que acontece *in vivo*. A incorporação de óleo essencial de *Thymbra capitata* (1%m/m) na folha base reduziu a viabilidade em modelo celular (em ambas as linhas) e no modelo tecidular. A viabilidade após contacto com a folha D.O em modelo celular foi muito inferior quando comparada com o placebo universal, porém é necessário considerar que *in vivo* a folha vaginal vai gradualmente ser diluída no fluido vaginal e é proposta para tratamentos de curta duração. A folha vaginal D.O diluída a 10% m/v foi biocompatível no modelo tecidular.

Em conclusão: foram desenvolvidos dois produtos para o tratamento de infeções vaginais com potencial para serem transferidos da academia para a indústria farmacêutica. Embora os resultados obtidos para o gel C de bicarbonato de sódio e da folha vaginal com óleo de *Thymbra capitata* (D.O) sejam promissores ao nível da previsível aceitabilidade, *performance* tecnológica, eficácia e segurança, são necessários estudos mais completos numa fase pré-clínica e obviamente a prova definitiva nos ensaios clínicos. Uma vez comprovados os dados de segurança e eficácia e atestada uma relação risco/benefício favorável, estes produtos poderão ser disponibilizados no mercado farmacêutico e constituir uma resposta efetiva para o tratamento das duas infeções vaginais mais comuns, particularmente nos casos de falha terapêutica da primeira linha de tratamento e recorrência, representando assim uma melhoria significativa na qualidade de vida e saúde sexual da mulher.

Abstract

Bacterial vaginosis (BV) and vulvovaginal candidosis (VVC) are the most prevalent vaginal infections, both affecting many women, with negative impact on their self-esteem and being associated with huge discomfort and changes in daily routines, especially the sexual life.

VVC is predominantly caused by *Candida albicans*, which is considered an opportunistic pathogen, but other *Candida species* such as *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* can also be etiological agents. The most common symptoms associated with VVC are vaginal irritation, vulvar burning, pruritus, swelling and discharge with vaginal fluid usually described as "cottage cheese-like". For the treatment VVC, standard treatment is presently based on azoles (single dose of fluconazole 150mg or Itraconazole 200 mg twice daily for one day or short-course topical formulations, such as clotrimazole cream or clotrimazole vaginal tablet 500 mg). Nevertheless, 5–8% of women of reproductive age are affected by recurrent episodes of vulvovaginal candidosis (cVVC), defined by three or more episodes occurring in a 12 months period, as a result of resistance to azoles, particularly in Non *albicans Candida* species cases, or re-infection.

Although some women are asymptomatic, most women with of BV, experiences an unpleasant thin vaginal malodorous discharge. This clinical condition is considered to be a dysbiosis, that is the replacement of saprophytic vaginal flora, dominated by *Lactobacillus* spp. by dense, structured and polymicrobial biofilm primarily constituted by *Gardnerella vaginalis*. For the treatment of BV, international guidelines recommend the administration of metronidazole, clindamycin or tinidazole orally or intravaginally as the standard treatment. However, the treatment with these antibiotics is associated with high levels of failure and recurrence rates. These may be associated with antibiotic resistance, the inability to eradicate the polymicrobial biofilms, and failure to re-establish acidic pH and the *Lactobacillus*-dominated commensal flora.

The aim of this work was to develop and characterize new therapeutic approaches for the treatment of vaginal infections. Specifically, we aimed to design two effective and safe products with potential to be transferred to the pharmaceutical industry: a bioadhesive sodium bicarbonate gel for the treatment of VVC and a vaginal sheet with *Thymbra capitata* essential oil (EO) for the treatment of BV.

In the development of sodium bicarbonate gels, the anti-*Candida* potential was studied through both the standard guideline (CLSI M27 "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts") and a protocol adapted from the Challenge Test of the European Pharmacopoeia 10.0 (monograph 5.1.3). We also assessed the technological performance of formulations considering pathological parameters (volume of fluid was adapted to this specific condition). The pH, osmolality, viscosity, firmness and adhesiveness were determined directly on plain formulations at room temperature and after diluting in vaginal fluid simulant at 37 ºC. The bioadhesion profile was determined using vaginal ex vivo porcine model. Formulation C (5% sodium bicarbonate, 1% carbomer and 94% water) was the most effective to inhibit *C. albicans*' growth. Additionally, formulation C presented organoleptic characteristics that are in accordance with the users' preferences and adequate technological characteristics, predicting adequate in vivo performance.

Vaginal sheets were developed containing gelatin, as main macromolecule, associated with bioadhesive polymers such as chitosan, polyvinyl alcohol (PVA) and hydroxypropyl methylcellulose (HPMC) to promote an intimate and prolonged contact of the product formulation with the epithelium. Plasticisers (glycerine or propylene glycol) were included to confer flexibility to formulations. The inclusion of lactic acid (LA) buffer in these formulations represents an additional mechanism to help re-establish the healthy saprophytic flora, contributing for treatment efficacy and preventing recurrence. Vaginal sheets were used as drug delivery system for *Thymbra capitata* EO, acting directly on causing bacteria while simultaneously and immediately relieving abundant vaginal discharge and its unpleasant odour, providing comfort to users. Among those studied vaginal sheets containing oil vaginal sheet D.O presented the higher buffer capacity and ability to absorb vaginal fluid simulant (VFS), and one of the best bioadhesive profiles. Besides, D.O presented flexibility (resilience) and resistance (hardness) that allow it to be easily rolled around finger and handled without breaking. Under a safety perspective, cytotoxicity was evaluated in two cell lines and in a Reconstructed Human Vaginal Epithelium model and the potential to cause vaginal irritation was also studied using *Hen's Egg Test-Chorioallantoic Membrane Assay* (HET-CAM) assay. The active substance (*T. capitata* EO) at 0.32 µL/mL in cellular model showed toxicity but in the tissue model it was biocompatible. The addition of *T. capitata* EO (1% w/w sheet) to base formulation D, as a coating, reduced the viability in the cellular model (both lines) and in tissue model. Comparing D.O with universal placebo, the viability in the tissue model was much lower, however, it must be considered that in vivo vaginal sheet will be gradually diluted on vaginal fluid and that

it is designed to be used for short periods. Vaginal sheet D.O diluted at 10% w/v was biocompatible in model tissue.

Concluding: two products with potential to be transferred from academy to industrial pharmacy were designed for the treatment of two common vaginal infections. A sodium bicarbonate gel and a vaginal sheet with *T. capitata* EO were developed to respond to the clinical requirements but also to women's preferences, overcoming disadvantages associated with conventional dosage forms. Although the present results are promising in terms of predictable acceptability, *in vivo* technological performance, efficacy and safety, more complete studies are needed in a pre-clinical phase and finally clinical assays will for confirm their usefulness.

Keywords

Vaginal infections; Vulvovaginal Candidosis; Bacterial Vaginosis; Treatment; Pharmaceutical Technology; Bioadhesive gel; Sodium Bicarbonate; Castor oil; Vaginal sheet; *Thymbra capitata* essential oil

Thesis overview

The work presented in this thesis aimed to find solutions to a health problem that affects a massive number of women, having huge impact in their life routines and self-esteem: vaginal infections. The main objective of this work was, therefore, to develop and characterize (in terms of sensorial features, technological attributes, laboratory efficacy and safety) innovative approaches for the treatment of vaginal infections, not only exploring other antimicrobial substances, but also investigating drug delivery systems that increase the efficacy of treatment and simultaneously responding to users' preferences in order to overcome drawbacks associated with conventional dosage forms.

The present work is part of a more comprehensive and continuous investigation that has been carried out by our research group over the years, which aims to better understand the vaginal environment mainly in the context of clinical disturbances, particularly those that are difficult to ameliorate or revert successfully.

This thesis is divided into three chapters and a concise section of concluding remarks.

Chapter 1 is an introductory contextualization that provides theoretical concepts applied to the experimental work, focusing topics such as the etiopathology and physiopathology of the two most prevalent vaginal infections (vulvovaginal candidosis and bacterial vaginosis), the signs and symptoms associated with them, the standard treatments recommended by international clinical guidelines and also promising alternative or complementary strategies of treatment that can be particularly useful when the first line of treatment does not achieve therapeutic success. The high prevalence of these clinical conditions, the tremendous impact of their symptoms and the growing recurrence of these pathologies that are described in this chapter was the problem question that justified the development of this research, which we propose to contribute to by generating scientific knowledge to provide an answer. The therapeutic approaches presented in the following chapters that comprise alternative antimicrobial substances and innovation on pharmaceutical technology perspective are developed based on the information described in this chapter.

Chapter 2 comprises the development and characterization of sodium bicarbonate gels as a new promising strategy for the treatment of VVC.

Chapter 3 describes the initial stages of development of vaginal sheet as dosage form and the application of this drug delivery system for *Thymbra capitata* essential oil for the treatment of BV.

Finally, the conclusion discusses the main outcomes of the present research as concluding remarks and future perspectives.

Most of the data presented in this thesis has been previously published in Peer-Reviewed International Scientific Journals and some of them are submitted to patent creation.

List of publications

Publications in Peer-Reviewed International Scientific Journals included in this thesis

- **Mariana Tomás**, Ana Palmeira-de-Oliveira, Sérgio Simões, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, "*Bacterial vaginosis: standard treatments and alternative strategies*". International Journal of Pharmaceutics. 2020, doi: https://doi.org/ 10.1016/j.ijpharm.2020.119659

- **Mariana Tomás,** Joana Rolo, Carlos Gaspar, Ana Palmeira-de-Oliveira, Sérgio Simões, David F. Katz, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, "*Sodium bicarbonate gels: a new promising strategy for the treatment of vulvovaginal candidosis*". European Journal of Pharmaceutical Sciences. 2020, doi: https://doi.org/10.1016/j.ejps.2020.105621

- Rita Machado, **Mariana Tomás**, Ana Palmeira-de-Oliveira, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, "*The vaginal sheet: an innovative form of vaginal film for the treatment of vaginal infections*". Drug Development and Industrial Pharmacy. 2020, doi: 10.1080/03639045.2019.1711386

- Lúcia G. V. Sousa, Joana Castro, Carlos Cavaleiro, Lígia Salgueiro, **Mariana Tomás**, Rita Palmeira-de-Oliveira, José Martinez-de-Oliveira, Nuno Cerca, *"Synergistic effects of Carvacrol, α-Terpinene, γ-Terpinene, ρ-Cymene and Linalool against Gardnerella species"*. Scientific reports. 2022, doi: 10.1038/s41598-022-08217-w

- **Mariana Tomás**, Lúcia G. V. Sousa, Ana Sofia Oliveira, Carolina Gomes, Ana Palmeira-de-Oliveira, Carlos Cavaleiro, Lígia Salgueiro, Nuno Cerca, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, "*Vaginal sheet with Thymbra capitata essential oil designed for the treatment of bacterial vaginosis"*. (*Submitted manuscriopt*)

Other publications in Peer-Reviewed International Scientific Journals published during the PhD Course

- Izamara Gomes Maocha, Josué Carvalho, Jéssica Lopes-Nunes, Tiago Rosado, Eugénia Gallardo, **Mariana Tomás**, Ana Palmeira-de-Oliveira, Rita Palmeira-de-Oliveira, José Martinez-de-Oliveira, Maria Paula Cabral Campello, António Paulo, Carla Cruz, "*Drug formulations for localized treatment of Human Papillomavirus-induced lesions"*. Journal of Pharmaceutical Sciences. 2022, doi: 10.1016/j.xphs.2022.02.004

- Rita Palmeira-de-Oliveira, Ana Sofia Oliveira, Joana Rolo, **Mariana Tomás**, Ana Palmeira-de-Oliveira, Sérgio Simões, José Martinez-de-Oliveira, *"Women's preferences and acceptance for different drug delivery routes and products"*. Advanced drug delivery reviews. 2022, doi: 10.1016/j.addr.2022.114133

- Mariana Tomás, Ana Sofia Agonia, Lígia Borges, Ana Palmeira-de-Oliveira, Rita Palmeira-de-Oliveira, *"Isothiazolinones Quantification in Shampoo Matrices: A Matter of Method Optimization or Stability Driven by Interactions?"*. Cosmetics. 2020, doi: 10.3390/cosmetics7010004

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Oral communications

- Mariana Tomás, Ana Palmeira-de-Oliveira, José Martinez-de-Oliveira, Lígia Salgueiro, Carlos Cavaleiro, Nuno Cerca, Rita Palmeira-de-Oliveira, *"Vaginal sheet as a drug delivery system for Thymbra capitata essential oil to treat bacterial vaginosis",* XV ANNUAL CICS-UBI SYMPOSIUM, Covilhã, Portugal (2020)

Poster communications

- **Mariana Tomás**, Joana Rolo, Carlos Gaspar, Ana Palmeira-de-Oliveira, Sérgio Simões, David F Katz, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, *"Sodium bicarbonate gel for the treatment of vulvovaginal candidosis:in vitro efficacy and safety"*, XV ANNUAL CICS-UBI SYMPOSIUM, Covilhã, Portugal (2020)

- Izamara Gomes-Maocha, Josué Carvalho, Jéssica Lopes-Nunes, **Mariana Tomás**, Ana Sofia Agonia, Rita Palmeira-de-Oliveira, Ana Palmeira-de-Oliveira, José Martinez-de-Oliveira, Carla Cruz, *"Development of drug-loaded gelformulations for intravaginal administration of anti-HPV drugs"*, XV ANNUAL CICS-UBI SYMPOSIUM, Covilhã, Portugal (2020)

- Jéssica Lopes-Nunes, **Mariana Tomás**, Ana Sofia Agonia, José Moutinho, Eugenia Gallardo, Rita Palmeira-de-Oliveira, Ana Palmeira-de-Oliveira, José Martinez-Oliveira, Maria P.C. Campello, António Paulo, Carla Cruz, *"AS1411-gold nanoparticles as cervical cancer drug delivery system"*, XV ANNUAL CICS-UBI SYMPOSIUM, Covilhã, Portugal (2020)

- Rita Palmeira-de-Oliveira, Carlos Gaspar, Francisca Oliveira, **Mariana Tomás**, Ana Palmeira-de-Oliveira, José Martinez-de-Oliveira, Fátima Nader-Macías, David F Katz, Sérgio Simões, *"Microscopic and physico-chemical characterization of vaginal fluids obtained from pregnant and non-pregnant women"*, XV ANNUAL CICS-UBI SYMPOSIUM, Covilhã, Portugal (2020)

- **Mariana Tomás**, Joana Rolo, Carlos Gaspar, Ana Palmeira-de-Oliveira, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, *"New strategy to treat postmenopausal vulvovaginal candidosis"*, III International Congress in Health Sciences Research towards innovation andentrepreneurship: Trends in Aging and Cancer, Covilhã, Portugal (2019)

- **Mariana Tomás**, Joana Rolo, Ana Palmeira-de-Oliveira, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, *"Influence of formulation factors in the anti candida activity of sodium bicarbonate gels",* XIV CICS-UBI Annual Symposium, Covilhã, Portugal (2019)

Patent application:

Rita Palmeira-de-Oliveira, José Martinez-de-Oliveira, Ana Palmeira-de-Oliveira, Mariana Tomás, Nuno Cerca, Lúcia Sousa, Aliona Rosca, Joana Castro, Carlos Cavaleiro, Lígia Couto, *"Folha vaginal para o tratamento e prevenção de vaginose bacteriana"*, (2021) patent pending process 20211000045617.

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List of abbreviations

ABC	Absolute Buffering Capacity
AmB	Amphotericin B
ASS	Anhydrous sodium sulphate
AUC	Area under the curve
BA	Boric acid
BASHH	British Association for Sexual Health and HIV
CLIN	Clindamycin
cVVC	Chronic vulvovaginal candidosis
DMSO	Dimethyl sulfoxide
EO	Essential oil
F_{max}	Maximum force
GC/FID	Gas chromatography with flame ionization detection
HET-CAM	Hen's Egg Test-Chorioallantoic Membrane Assay
HPMC	Hydroxypropylmethylcellulose
ICH	Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IUSTI	European International Union against sexually transmitted infections
IS	Irritation score
LA	Lactic acid
MET	Metronidazole
MIC	Minimal inhibitory concentration
mVFS	Modified Vaginal Fluid Simulant
NC	Negative control
NAC	Non-*albicans Candida*
PBS	Phosphate buffer solution
PVA	Polyvinyl alcohol
PC	Positive control
RBC	Relevant Buffer Capacity
RVVC	Recurrent vulvovaginal candidosis
SDS	Sodium dodecyl sulfate
TI	Tinidazole
WHO	World Health Organisation

Chapter 1
Introductory contextualization: vulvovaginal candidosis and bacterial vaginosis

The content of this chapter is partially published in:

- **Mariana Tomás**, Ana Palmeira-de-Oliveira, Sérgio Simões, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, "*Bacterial vaginosis: standard treatments and alternative strategies*". International Journal of Pharmaceutics. 2020, doi: https://doi.org/10.1016/j.ijpharm.2020.119659

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1. Introduction

The healthy vaginal microbiota is constituted mainly by Gram-positive bacilli of the genus *Lactobacillus (L.)* [1–3]. The vaginal microbiota is unique. The dominant species are *Lactobacillus* and the pH varies according to the ethnic groups [1]. *Lactobacillus crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii* are the commonest species.

Lactobacillus spp. are credited of being essential to maintain the health of the vagina due to the production of antimicrobial compounds (hydrogen peroxide, lactic acid (LA), bacteriocin-like substances) and their capability to adhere to the vaginal epithelium (competitive exclusion of other bacteria), and the ability to competitively use available nutrients. The production of LA is responsible for and so directly correlated with the low vaginal pH (pH 3.5-4.5). Hydrogen peroxide is an oxidizing agent toxic to catalase-negative bacteria such as most anaerobic microorganisms [3–7]. The H_2O_2-producing *Lactobacilli* spp. also contribute to the microbiota homeostasis stimulating the antimicrobial secretions from epithelial cells and increasing the activity of already synthesized factors (muramidase and lactoferrin) [6,7]. However, recently, the antimicrobial role of hydrogen peroxide has been questioned, since oxygen is required to produce hydrogen peroxide in large amounts to achieve the antimicrobial activity and the cervicovaginal environment is microaerobic (hypoxic) and considering that hydrogen peroxide is inactivated by the reducing capabilities of cervicovaginal fluid and semen [8].

An alteration of the physiological vaginal microbiota with depletion of *Lactobacillus* spp. allows the overgrowth of microorganisms responsible for dysbiosis [9].
BV and VVC are the most prevalent vaginal infections.

1.2. Vulvovaginal candidosis

1.2.1. Physiopathology and clinical diagnosis

VVC is one of the most prevalent vaginal infections with a profound negative impact on quality-of-life [10]. It is estimated that between 29% and 49% of women will have at least one lifetime episode of VVC [11]. Although acute uncomplicated VVC is the most frequent, about 5% of women develop recurrent disease [12,13]. RVVC is defined by three or more episodes occurring in a 12 month period [13–16].

The most common symptoms associated with VVC are vaginal irritation, vulvar burning, pruritus, swelling and discharge. The vaginal fluid is usually described as “cottage cheese-like”, ranging from homogeneously thick to floccular, with none, milky, or fermentation odour) and presenting invariably normal pH (3.5-4.5) [11,17–20].

VVC is predominantly caused *by Candida albicans*, but other *Candida* species such as *Candida glabrata*, *Candida krusei*, *Candida tropicalis* and *Candida parapsilosis* can also be etiological agents. *C. albicans* is considered an opportunistic pathogen, due to the existence of a prolonged colonization state before active infection. In fact, *C. albicans* can be present in the vaginal milieu and may remain in the vagina between clinical episodes, even after treatment [11,17–20]. In recent times, however, there has been a notable change in the etiologic agent of

candidosis with non-*albicans Candida* (NAC) species gaining importance, with some studies reporting 10% to 45% of VVC cases, influenced by the widespread and indiscriminate use of antifungals, some of them available as over-the-counter medications. Treatment failure is common in NAC-VVC, since some of these species are intrinsically resistant (*C. krusei*) or show low susceptibilities to commonly used antifungal agents, such as fluconazole. Thus, definitive laboratory procedures to identify *Candida* isolates from suspected VVC cases to a species level should be performed in clinic to ensure the selection of effective antifungal agents [21].

1.2.2. Standard treatment

The standard treatment for VVC is presently based on azoles [13–15,17] [22]. For acute uncomplicated VVC, international guidelines recommend treatment with a single dose of fluconazole 150mg or Itraconazole 200 mg twice daily for one day or short-course topical formulations, such as clotrimazole cream or Clotrimazole vaginal tablet 500 mg as single dose [15].

The treatment of CVV with azoles results in a cure rate of 80–90% [12,13,15,23].

However, in recent years, there has been an increase in therapeutic failure and recurrence.

Clinically recurrent cVVC that affects 5–8% of women of reproductive age is a therapeutic challenge due to the paucity of alternative drugs [14,20].

International guidelines recommend that the treatment of RVVC should include maintenance therapy, commonly six months of weekly oral fluconazole [10,13,15]. Indeed, azole-resistance is a growing problem difficult to manage, resulting from years of indiscriminate drug administration and unnecessary exposure. The acquired resistance of *Candida* spp. can result from frequent and/or prolonged azole exposure that enhances efflux pumps capacity resulting in drug extrusion from the yeast which is one of the main mechanisms involved in resistance development [23–29].

Recently, the British Association for Sexual Health and HIV (BASHH) published guidelines that consider sensitivity testing for fluconazole to decide the treatment for RVVC and suggested treatment with nystatin pessaries or boric acid pessaries for azole resistant *Candida spp*. [12].

Non-*albicans Candida* species such as *C. glabrata* and *C. krusei* present greater resistance to standard therapy with azole derivatives (mainly fluconazole) than *C. albicans*, and are responsible for up to 33% of RVVC cases [22,24].

As VVC affects many women and has a high influence on their self-esteem and life quality, it is urgent to develop new alternative treatments with high cure rates and low recurrence rates, especially for RVVC.

1.2.3 Alternative treatments

The first line standard treatments are becoming ineffective since there is an increasing resistance to azoles. This is the result of this being, for years, the exclusive therapeutic option,

sometimes used inappropriately and indiscriminate, allowing *Candida* spp. to develop resistance mechanisms (such as drug efflux pumps or changes in drug target structure) and the growing incidence of NAC (intrinsically more resistant to azoles) and the biofilm organization that harms and prevents the action of drugs and promotes the survival of these yeasts [18,22–24,30].

Azole agents were introduced some decades ago and since then there has been a paucity of new drugs in the market. Due to the increasing resistance of VVC-causing agents to azoles, the treatment of cVVC becomes a challenge that needs emergent solutions. So, recently the British Association for Sexual Health and HIV actualized its guidelines and included the treatment with nystatin or boric acid when *Candida* species are resistant to the first line standard treatment. However, there were other promising strategies that have been explored in some studies, such as the administration of probiotics to promote the normal vaginal flora (not consensual), the use of other drug classes (which are not approved for antifungal purposes), amphotericin B, natural compounds, and incorporation of excipients which have an intrinsic antimicrobial activity to increase the product efficacy (such as chitosan) or other formulation's strategies including nanotechnological products.

Boric acid does represent an alternative treatment for VVC caused by *C. albicans,* particularly when conventional treatment fails, such as in RVVC caused by other species and/or azole resistance [22,31]. Boric acid is fungistatic or fungicidal depending on concentration and the inhibition of oxidative metabolism seems to be its key antifungal mechanism [32]. Moreover, boric acid can inhibit some virulence factors of *C. albicans* such as germ-tube formation due to a disruption of apical cytoskeletal elements of growing hyphae [33]. Importantly, boric acid maintenance regimens of 300 to 600 mg used twice weekly intravaginal appeared to be well tolerated and effective [34]. Guaschino et al evaluated the efficacy of a topical long-term treatment with boric acid versus an oral long-term treatment with itraconazole and concluded that both treatments presented similar antifungal efficacy and ability to control the symptoms, but that the efficacy of boric acid ends with the suspension of the therapy [35]. Sobel et al reported that 600 mg daily vaginal capsule, for 2 to 3 weeks, presented therapeutic success in 64% (group 1) and 71% (group 2) of symptomatic women with vaginitis caused by *C. glabrata* [36]. In their study, Ray et al reported that women with *C. glabrata* VVC exhibited higher therapeutic cure with boric acid vaginal suppositories given for 14 days compared with single-dose oral 150-mg fluconazole (63.6% vs. 28.6%, $p<0.01$) [37].

Fan and Liu concluded that the microbiological cure rate of women with complicated VVC treated with nystatin was 85.4% (examination on days 7–14) and 83.4% (follow-up at 30-35 days) and both *C. albicans* and non-*albicans Candida* species were susceptible to nystatin, *in vitro* [38].

A study involving cervical-vaginal samples isolated from women reported the anti-*Candida* activity of nystatin (MIC50 of 4.0 μg/mL and MIC90 8.0 μg/mL for *C. albicans and C. glabrata* [39]. Choukri et al confirmed the nystatin can be used to treat VVC caused by non-*albicans Candida* species, since nystatin MIC90 was 4 mg/L for non-*albicans Candida* and *Candida albicans* species tested, on the other hand MIC90 of azole agents (econazole,

miconazole, clotrimazole) was 0.06 mg/L for *C. albicans* isolates and ranged from 0.5 to 8 mg/L, from 1 to 4 mg/L and from 0.12 to 4 mg/L, for econazole, miconazole, clotrimazole, respectively [40]. Fan et al compared the efficacy of vaginal nystatin suppositories for 14 days each month with a weekly single oral dose of fluconazole for the treatment of RVVC. At the end of maintenance therapy period, the microbiological cure rates were 80.7% for nystatin group and 72.7% for the fluconazole group. Considering the microbiological cure rates of RVVC caused by *C. glabrata*, fluconazole was less effective than nystatin (12.5% versus 64.3%). Moreover, nystatin was effective in 55% of women with RVVC caused by fluconazole-resistant *Candida* [41]. Importantly, *in vivo* (rat model), nystatin improves the immune response against *Candida albicans* and simultaneously protects the ultrastructure of the vaginal epithelium [42].

Some studies reported the potential anti-*Candida* effect of amphotericin B. Majdabadi et all defined the MIC values of amphotericin B (0.42 μg/mL) and of fluconazole (40.51 μg/mL) using *Candida* species isolated from women with cVVC [43]. The ability of amphotericin B to destroy *Candida* biofilms is not yet completely defined and this effect seems to be related with the species, strains, and stage of biofilm maturation [44–47].

Although not consensual, some studies suggest the benefits of probiotics for the prevention and treatment of VVC. Lactobacilli adhere to the vaginal epithelial cells and then hinder the adhesion of *Candida* spp. to vaginal epithelium by direct competition, exhibiting a direct fungicidal effect. Moreover, after adhering, probiotics produce lactic acid, hydrogen peroxide and bacteriocins which allow the reestablishment of healthy vaginal microenvironment and inhibit pathogen or microorganism colonizer's growth, promoting an healthy microbiome dominated by *Lactobacillus* spp. [18,24,48–55].

Several studies suggested that chitosan shall be used as excipient in order to potentiate VVC treatment [56–62]. Chitosan, a natural bioadhesive polymer, can promote the efficacy of treatment not only by increasing the residence time and prolonging the contact of drugs in the vaginal milieu, but also by its intrinsic antifungal activity [61]. Nanotechnology developed chitosan-containing liposomes and also chitosan nanocapsules were proposed as delivery systems for tioconazole and econazole and showed potent antifungal activity against *C. albicans* [56] [57]. Lo et al reported the synergic anti-*Candida* effect of chitosan and fluconazole against *C. albicans* and *C. tropicalis* [60]. Palmeira-de-Oliveira et al also studied chitosan as promising vehicle for the incorporation of active, having itself an intrinsic anti-*Candida* activity [58]. This activity seems to be related with the interaction of ionic reaction between its amino free groups and ionic charges at the yeast cell surface, explaining different actions on different *Candida* species [62].

Previous work has suggested the use of other drug classes (which are not approved for antifungal purposes) such as selective serotonin reuptake inhibitors antidepressants (sertraline and fluoxetine), lidocaine and nitro glycerine due to their anti-*Candida* activity [63–67] which are valuable in difficult to treat cases mainly those resulting from yeast resistance.

Natural compounds have been used since ancient times for the treatment of pathologies, namely vaginal infections [68]. The anti-*Candida* effect of some EOs such as oregano oil (*Origanum* oil), Thyme oil (*Thymus* spp. oil), tea tree oil (*Melaleuca alternifolia* oil), mint oil

(*Mentha piperita*, *Mentha spicata* and *Mentha cervina*), castor oil (*Ricinus communis* oil), winter savory oil (*Satureja montana*), and clove oil (*Syzygium aromaticum oil*) has been reported. Their high hydrophobicity changes the permeability of cellular membranes, resulting in a cascade of events that culminate with cellular death. Since EOs are mixtures of components and there is some chemical variability depending on the growing region, part of the plant from which it was obtained, season of year and extraction method employed, it becomes difficult to identify which chemical compound are responsible for antifungal activity or even if this activity is necessarily due to the interaction of several compounds [22,69,70].

Bona et al analysed the effect of 12 EOs against *Candida albicans* and demonstrated that the most potent were oregano, winter savory and mint oils. Oregano and winter savory oils inhibited the growth of 100% of the tested strains, while mint oil was effective on 80% of tested strains [70]. MIC of oregano EO was lower than 1% v/v in 64% of the isolates and MIC of winter savory was lower than 1% v/v in 31% of the isolates

Ellah et al develop coated polyethylene glycol vaginal suppositories containing EO of cumin seeds that exhibited good anti-*Candida* activity *in vitro* while being biocompatible with vaginal tissues of rabbits. In a clinical study, the treatment allowed to relieve symptoms such as itching, discharge and dyspareunia and achieved negative cultures in 70% of participants [71].

Tea tree EO can inhibit *C. albicans* and also presented antibiofilm efficacy, attributed mainly to its major component terpinen-4-ol [72]. Vaginal suppositories containing tea tree oil exhibited fungicidal effect against several strains of *Candida* spp. and, importantly, as a selective effect, strains of probiotics remained viable and were only affected by concentration ≥4% v/v of tea tree oil [73]. Besides, Mertas et al suggested the combination of tea tree oil with the standard treatment fluconazole to overcome the fluconazole-resistant of *C. albicans*. Fluconazole-resistant *C. albicans* strains were exposed to sublethal concentrations of tea tree oil which allowed to enhance fluconazole susceptibility against these strains (fluconazole MIC decreased from an average of 244.0 μg/mL to 38.46 μg/mL, and the fluconazole minimal fungicidal concentrations decreased from an average of 254.67 μg/mL to 66.62 μg/mL) [74].

Moreover, Rosato et al showed that the combination of *Pelargonium graveolens* oil and Amphotericin B allowed the reduction of Amphotericin B dose necessary to inhibit *Candida* species though a synergic antifungal effect and so consequently minimizing the side-effects of treatment [75].

Zakeri et al studied the effectiveness of vaginal cream of *Achillea millefolium* extract comparing with vaginal cream clotrimazole 1% for the treatment of VVC and concluded that, although *Achillea millefolium* extract cream presented some efficacy, standard treatment was more efficient to relive the symptoms and achieve an higher microbiological cure rate (negative cultures in 77% patients of control group and 53% in patient of experimental group (p<0.05)) [76].

In a randomized, placebo-controlled trial, 2% dill (*Anethum graveolens*) vaginal suppositories were as effective as 100mg clotrimazole vaginal tablets in relieving symptoms of VVC and achieve microbiological cure (no significative differences were observed between groups) [77].

Romero-Cerecero et al compared a daily administration of a vaginal suppository containing 7% standardized extract of *Ageratina pichinchensis* and 100mg clotrimazole (control) for 6 days to treat VVC and found that, 2 weeks after concluding the treatment, there was no statistical difference in the cure rates of the two groups (86.6% of women in the control group and 81.2% in experimental group) [78].

Capoci et al studied the antifungal action of a propolis extract solution against *Candida albicans* isolated from patients with VVC and reported that a concentration of 546.87 μg/mL caused the death of 75.8% of the isolates and additionally presented antibiofilm effect [79].

Medical grade honey can be an alternative strategy for the treatment of RVVC due to its anti-Candida activity and also due to the ability to eradicate biofilms, while simultaneously presenting benefits to the vaginal environment due to the anti-inflammatory and antioxidative properties [80].

Promising results of *Salvia officinalis* in vaginal tablet formulation use, alone or in combination with clotrimazole gave symptoms relief and high rates of microbiological cure (seven days after the end of treatment 80.6% in clotrimazole group, 97.2% in *S. officinalis* group and 94.3% in *S. officinalis* and clotrimazole groups, respectively, exhibited negative microbiologic culture test) [81].

In vitro, curcumin showed its ability to inhibit the growth of *Candida* species (MIC values 125–1000 μg/ml), being more potent against *Candida glabrata*. These results were confirmed *in vivo* (rat model), by curcumin reduced *Candida* burden. The mechanism of action seems to be related with the attachment to ergosterol of yeast membrane [82]. Abouali et al in a clinical study reported that although curcumin-based vaginal 10% cream was as effective as clotrimazole vaginal 1% cream to relieve the symptoms of VVC, it is significantly less effective that clotrimazole cream to achieve microbiological cure (p=0.002) [83].

Sodium bicarbonate appears to have anti-*Candida* activity at certain concentrations, despite having no action on biofilms [84–86].

1.2.4. Advances in the field of pharmaceutical technology

Several strategies have been proposed to improve the *in vivo* performance of products, to add therapeutic advantages and also to guarantee better technological characteristics that promote patients' compliance [87,88].

The conventional semi-solid dosage forms for vaginal administration (creams, gels) to treat vaginal infections presented the disadvantage of low retention time. So, to overcome this disadvantage, the inclusion of bioadhesive excipients has been proposed [87].

Bassi et Kaur developed carboxymethyl derivative of fenugreek gum which possess film forming properties and bioadhesive potential. The carboxymethyl derivative of fenugreek gum was used to develop a film containing nystatin, which allows 100% drug release over 5h. This film was non-toxic, compact (being discreet) and presented with good antifungal properties in vivo [89].

Gupta et al developed bioadhesive vaginal tablets containing clotrimazole loaded microspheres, using bioadhesive polymers such as hydroxypropyl methylcellulose, sodium carboxymethylcellulose and Carbopol. This drug delivery system (DDS) allowed a controlled intravaginal drug release, providing long-term therapeutic activity of clotrimazole at the site of infection, promoting the efficacy of treatment [90].

Also nanosystems (such as liposomes, nanoparticles and micelles), resulting from the application of nanotechnology in pharmaceutical industry, can be explored to overcome solubility problems of some drugs, improve local drug distribution over epithelium and prolong their retention [87,88].

Calvo et al designed a chitosan nanocapsule based delivery system containing tioconazole and econazole, overcoming the problems related with the low water solubility that affect their therapeutic efficiency. The encapsulation efficiency was high (more than 87%), and the drug delivery system was stable over two months and presented potent antifungal activity against *Candida albicans* while showing to be non toxic for the vaginal epithelium [57].

Albertini et al used spray-congealing technology to produce mucoadhesive microparticles for the vaginal delivery of econazole nitrate. The incorporation of chitosan, sodium carboxymethylcellulose and poloxamers promoted the bioadhesion of the microparticles to mucosal tissue. Furthermore, poloxamers/Gelucire®-based microparticles presented a potent inhibition of *C. albicans* growth, suggesting that when included into capsules, can keep their effectiveness with reduced frequency of administration when compared to conventional vaginal semi solid products[91].

Carbone et al developed solid lipid nanoparticles loaded with clotrimazole and alphalipolic acid in order to obtain a synergistic treatment against *Candida albicans*, adding to the antifungal action of clotrimazole the ability of alphalipolic acid to reduce the generation of reactive oxygen species [92].

Itraconazole-Loaded Polycaprolactone-Nanoparticles were developed to increase drug delivery and reduce itraconazole toxicity. These nanoparticles improved anti-*Candida* activity by increasing the contact of the drug with the epithelium and the ability to reduce the inflammation caused by the infection [93].

Clotrimazole-loaded nanoemulsion can offer higher deposition of drug on mucosae and stronger antifungal efficacy compared to commercially conventional semisolid formulations [94]

The local administration of nystatin is limited by poor water solubility and spontaneous easy aggregation. To overcome this limitation, Song et al produced an exopolysaccharide/nystatin nanoemulsion by ultrasonic method for the treatment of VVC. The nanoemulsion demonstrated high stability and slow drug release, exhibiting a potent anti-*Candida* activity (MIC 0.125 μg/ml) [95].

Antifungal vaginal sponges loaded with butoconazole nitrate have been prepared by lyophilization of bioadhesive polymers (chitosan, HPMC and carbopol 934), using calcium chloride as crosslinking agent and sodium carboxymethylcellulose as viscosity modifier. The

sponge prepared with chitosan:HPMC (1: 1) was the most effective to inhibit *C. albicans*, with longer residence time than conventional dosage forms [96].

A novel multifunctional carrier Ketoconazole/β-cyclodextrin co-ground mixture into chitosan/gellan gum gel-flakes was developed to treat *Candida* infection. The drug has poor water-solubility and low penetration capacity, being less effective in deep-seated *Candida* infection. This technological strategy allows reducing dosage regimes due to sustained release of drug, being as effective as the commercial available cream, and to reach deep-seated infections [97].

Clotrimazole was incorporated into a new hybrid mucoadhesive formulation of dextran and alginate nanofiber which presented higher and faster antifungal properties comparing to clotrimazole films and also two-fold adhesiveness to the mouse tissue [98].

Thermogels which responds to the variation of temperature by changing their viscosity, were also explored to improve the treatment of VVC. A vaginal delivery system of amphotericin B (AmB) nanosuspension-loaded thermogel, was formulated with Poloxamer P407/P188 to confer a proper sol–gel transition. The gelation temperature of the most promising formulation was around 31 ºC, forming an *in situ* gel due to the higher temperature of thebody. This drug delivery system provided a sustained release for 12 h and anti-*Candida* activity [99]. Deshkar and Palve developed thermosensitive cyclodextrin-based *in situ* gel of voriconazole, using Poloxamer 407 and Poloxamer 188. This drug delivery system presented an excellent mucoadhesion and sustained drug release for more than 8h [100].

1.3. Bacterial vaginosis

1.3.1. Physiopathology and clinical diagnosis

Bacterial vaginosis (BV) is one of the most common vaginal infections in reproductive aged women [5,101–104]. It is characterized by the replacement of beneficial lactobacilli by an overgrowth of anaerobic bacteria [102,105]. The etiopathogenesis of BV is not yet fully understood due to the great diversity and complexity of microorganisms involved. BV involves the presence of a biofilm, which is a structured group of bacteria that have synergistic interactions, adhered to a biological tissue. The dense, structured and polymicrobial biofilm is primarily constituted by *Gardnerella vaginalis* clusters. Its great ability to strongly adhere to vaginal epithelial cells represents a key event for the establishment of BV, facilitating the adherence and growth of other BV-associated anaerobes, such as *Atopobium vaginae*, *Mobiluncus mulieris*, *Prevotella bivia*, *Fusobacterium nucleatum* and *Peptoniphilus sp.* [3,103,104,106,107].

Biofilms present a sophisticated internal architecture, consisting of an extracellular matrix (which constitutes a physical barrier against antimicrobial molecules and human immune responses and increases tolerance of unfavourable conditions) containing channels that facilitate the circulation of nutrients [102,104,108]. Modifications in microbiota may influence the drug release. Biofilm environment-related factors (pH, pCO_2, or pO_2) may affect the drug

release from formulation, modify the solubility of drug and even impair the efficacy of the substances [104].

The diagnosis of BV is based either on the Nugent score or Amsel criteria. To define Nugent score time, resources, and expertise are required while Amsel criteria allow for a syndromic point-of-care diagnosis, and so in routine clinical practice these method is commonly preferred by clinicians [109].

In the so called Amsel approach any combination of three of its four diagnostic criteria (abundant creamy, grey-white adherent vaginal discharge, rotten fish odor - spontaneous or alkali induced -, pH > 4.5, microscopic detected presence of clue cells (exfoliated vaginal epithelial cells covered by *G. vaginalis*) leads to the diagnosis [17,104–106,109,110].

The semi-quantitative microscopic evaluation of Gram-stained vaginal smears is the basis of Nugent scoring. The score is defined based on the combination of the following morphotypes: lactobacilli, *G. vaginalis* or bacteroides (small gram-variable rods or gram-negative rods), and curved gram variable rods. BV is diagnosed in samples that present a replacement of lactobacilli by anaerobic microorganisms (score ≥ 7.0) while the healthy vaginal samples are characterized by abundant lactobacilli (Nugent score ≤ 3). Cases scoring between these two limits (Nugent score 4-6) are considered to be intermediate flora [111].

Approximately 50% of women with BV are asymptomatic [106,109]. These group of women may correspond to the IV group of Ravel classification that had lower proportions of lactic acid bacteria and higher proportions of strictly anaerobic organisms, including *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia*, and *Mobiluncus*. Communities not dominated by species of *Lactobacillus* (group IV) have higher median pH (5.3 ± 0.6), but these vaginal bacterial communities are common and appear normal and healthy in black and Hispanic women. So, these normal group of women may be misdiagnosed, since lactobacilli are not the dominant flora and the pH is above 4.5 [1].

Over 50% of women with BV experience an unpleasant thin vaginal malodorous discharge, which is associated with a significant negative impact on self-esteem, sexual relationships, and quality of life [105].

The predominant bacterial species associated with BV produce malodorous volatile amines and are associated with increased vaginal fluid transudation and squamous epithelial cell exfoliation. The pH increases to values above 4.5 (in some cases up to 7) by a significant reduction of the number of protective lactobacilli and consequently by the decrease in the production of LA. The alkalization of vaginal pH allows potential pathogens to invade or expand their colonization of the vagina [17,104,109].

Women with BV have higher frequency and concentration of bacteria producing mucin-degrading enzymes so significantly lowering the viscosity of vaginal fluid, explaining why this is thin in consistency [17,112].

BV is associated with reproductive and obstetric complications, like increased risk of acquiring pelvic inflammatory disease, or having spontaneous abortion, preterm delivery, low birth weight newborns, and postpartum endometritis [105,106].

Vaginal biofilms play a key role not only in BV pathogenesis, but also in its treatment failure, with recurrences (chronicity). The biofilm's structure prevents from antimicrobial penetration in the matrix and the contact with the microorganisms. Simultaneously, an increase in resistance to antibiotics is observed, resulting in treatment failures [102,105,106,108,113].

Other treatment options for BV include alternative antimicrobial agents or agents that modify the pathological vaginal environment while formulation strategies have also been proposed to improve the efficacy of vaginally applied products. The main goals of these treatments are either to destroy biofilms, to eradicate pathogens and/or to re-establish the healthy commensal flora.

1.3.2. Standard antimicrobial treatment

International guidelines propose treating women diagnosed with BV with antibiotics (metronidazole (MET), clindamycin (CLIN) or alternatively tinidazole (TI)) orally or intravaginally (table 1) [15,114–117].

Table 1: International guidelines for treatment of BV

Guidelines for the Management of Sexually Transmitted Infections [114]	Standard regimens	MET, 400 or 500mg orally, twice daily for 7 days
	Alternative regimens	MET, 2g orally, single dose, OR CLIN cream (2%), 5g intravaginally for 7 days MET gel (0.75%), 5g twice daily, intravaginally for 5 days CLIN, 300mg orally, twice daily for 7 days.
UK National Guideline for the management of Bacterial Vaginosis [115]	Standard regimens	MET 400 mg orally, twice daily for 5-7 days, OR MET 2 g orally, single dose, OR Intravaginal MET gel (0.75%), once daily for 5 days, OR CLIN cream (2%) intravaginally, once daily for 7 days.
	Alternative regimens	TI 2 g orally, single dose, OR CLIN 300 mg orally, twice daily for 7 days.
Sexually Transmitted Diseases Treatment Guidelines [116]	Standard regimens	MET 500 mg orally, twice a day for 7 days, OR MET gel (0.75%) 5g intravaginally, once daily for 5 days, OR CLIN cream (2%) 5g intravaginally, once daily for 7 days.
	Alternative regimens	TI 2 g orally, once daily for 2 days, OR TI 1 g orally, once daily for 5 days, OR CLIN 300 mg orally, twice daily for 7 days, OR CLIN ovules 100 mg intravaginally, once daily for 3 days.
Australian STI Management guidelines for use in primary care [117]	Standard regimens	MET 400 mg orally, twice daily for 7 days, OR MET (0.75%) gel intravaginally, 5 g once daily for 5 days, OR CLIN (2%) vaginal cream intravaginally, 5 g once daily for 7 days, OR CLIN 300 mg orally, twice daily for 7 days.
	Alternative regimens	MET 2 g orally, single dose, OR TI 2 g orally, single dose.

European International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge (2018) [15]	Standard regimens	MET 400–500 mg orally twice daily for 5–7 days MET gel (0.75%), intravaginally, once daily for 5 days CLIN cream (2%), intravaginally, once daily for 7 days
	Alternative regimens	MET 2 g orally, single dose TI 2 g orally, single dose TI 1 g orally, for 5 days CLIN 300 mg orally twice daily, for 7 days Dequalinium chloride 10 mg vaginal tablet one daily, for 6 days

In the oldest guideline the standard regimen was limited to MET, while in the most recent guidelines intravaginal administration was added to standard regimen. MET remains the most commonly used antimicrobial agent for the treatment of BV [104,118]. MET was superior to CLIN for *Prevotella* spp., *Bacteroides* spp., *Peptoniphilus* spp., *Anaerococcus tetradius* and *Finegoldia magna*. CLIN had greater activity against *Atopobium vaginae*, *G. vaginalis* and *Mobiluncus* spp. compared to the nitroimidazoles. Moreover, nitroimidazoles have no *in vitro* activity against lactobacilli while CLIN has activity against these beneficial microorganisms [118].

A systematic review of trials concluded that CLIN and MET, using either oral or vaginal formulations, showed identical efficacy to treat BV at two weeks and one month follow up and also that CLIN tended to cause a lower rate of adverse events than MET [119].

Tinidazole is a second-generation nitroimidazole with a longer half-life than metronidazole (12–14 h and 6–7 h, respectively) and with a better adverse effect profile [104].

Different studies suggest lack of efficacy of traditional antimicrobial treatments and high recurrence rates of BV due to their inability to eradicate the vaginal biofilm completely and/or to their negative impacts of antibiotics on healthy vaginal microflora [109].

Recurrence rates of 30% and 76% within 4 weeks and 6 months, respectively, after treatment with standard antimicrobials, respectively, had been described [5,120].
Furthermore, a recent study concludes that metronidazole (MET) can't disintegrate the existing *G. vaginalis* biofilms and the viability remained fully intact after 40 h of action in concentration of 0.1 mg/mL and higher [121].

Single dose therapies have lower cure rates than prolonged treatment [15].

In the "European (IUSTI/WHO) International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge" dequalinium chloride was introduced as an alternative regimen to antibiotics [15].

1.3.3 Alternative treatments

Several studies reported the potential of alternative treatments (table 2).

Table 2 Alternative treatments for BV

Group	Examples	Activity
Other antimicrobials	Rifaximin	Inhibit the growth of various BV-related bacteria (*Atopobium vaginae, Prevotella, Megasphaera, Mobiluncus* and *Sneathia*)
	Secnidazole	Antimicrobial activity against BV-associated bacteria
Antimicrobial peptides	Retrocyclin	Inhibit the activity of vaginolysin and decrease the biofilm formation
	Subtilosin	Inhibit the growth of *G. vaginalis* by forming pores in the cell membrane
Antiseptics	Dequalinium chloride Povidone iodide Hydrogen peroxide Chlorhexidine Octenidine	Large spectrum antibacterial activity, acting by non-specifically disrupting cell membrane
Surfactants	Cocoamphopropionate	Large spectrum antibacterial properties
Natural Compounds	*Prangos ferulacea* *Myrtus communis* *Berberis vulgaris* *Zataria multiflora* *Thymus vulgaris* *Eugenia caryophyllus* *C. officinalis* *Tribulus terrestres* *Myrtus commuis* *Foeniculum vulgare* *Tamarindus indica* *Thymbra capitata* *Artemisia princeps Pamp.*	Several pharmacological activities depending on the plant: inhibition of the growth of *G. vaginalis*, antibiofilm activity or alleviating the symptoms (such as vaginal itching, burning sensation, odor, dysuria, and dyspareunia)
Acidifying agents	Vitamin C Lactic acid Polycarbophil Boric acid	Lower pH below 4.5, re-establishing the normal vaginal pH
Probiotics	*L. reuteri* *L. rhamnosus* *L. plantarum* *L. crispatus* *L. fermentum* *L. gasseri* *L. brevis* *L. acidophilus*	Re-establish saprophytic vaginal flora
Prebiotics	Fructooligosaccharides Sugar alcohols Galacto-oligosaccharides Lactulose Raffinose	Promote the selective growth of beneficial microorganisms
Other substances	Nifuratel	Antimicrobial activity on *G. vaginalis* and *A. vaginae*
	Benzoyl peroxide	Effective against *G. vaginalis*
	DNase	Inhibit the *G. vaginalis growth,* acting on the biofilm extracellular DNA that is essential to maintain the structural integrity

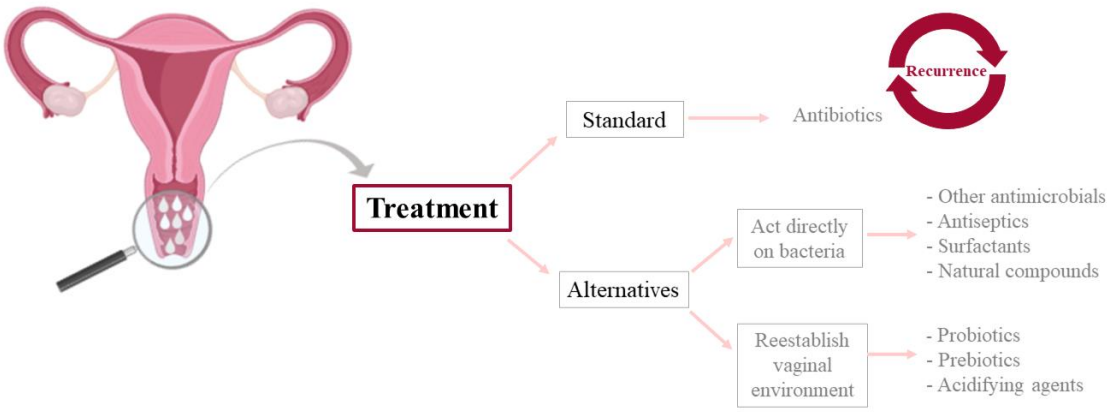

Figure 1. Summary of standard and alternative treatments for BV

1.3.3.1. Other antimicrobials

1.3.3.1.1. Rifaximin

Rifaximin, a rifamycin derivative, has been tried as vaginal tablets with 25 mg for 5 days and achieved a cure rate of 48% 7–10 days after the end of therapy, significantly higher (p=0.04) than that of placebo (19.0%) [122]. Rifaximin can reduce the growth of various BV-related bacteria (*Atopobium vaginae, Prevotella, Megasphaera, Mobiluncus* and *Sneathia*) being *A. vaginae* and *Sneathia* the most susceptible [123]. Rifaximin treatment was associated with an increase in the *Lactobacillus* spp./BV-related bacteria ratio, as well as with an increase in LA concentration and a decrease of metabolites typically produced by BV-related bacteria [124].

1.3.3.1.2. Secnidazole

Secnidazole is a next-generation 5-nitroimidazole and differs from MET and TI in the groups attached to the ring nitrogen adjacent to the nitro group. Secnidazole is characterized by potent *in vitro* antimicrobial activity against BV-associated bacteria, as well as prolonged elimination (half-life), which allow a single dose treatment unlike other nitroimidazoles that require daily dosing. A single 2-g oral dose of secnidazole results in plasma concentrations of secnidazole that are maintained above the MIC of BV-associated pathogens for longer than 72 h [125]. Secnidazole has similar *in vitro* activity against the range of BV related microorganisms compared to MET or TI and it has no harmful activity on lactobacilli (MIC90>128µg/ml) [118,125]. After a single oral dose 2 g of secnidazole the cure rates achieved in BV in two different studies sere similar: 58.9% (n=125), compared to 24.6% of placebo (n=64) and 67.7% (n=52) compared to 17.7% of placebo (n=52) [126,127].

1.3.3.2. Antimicrobial peptides: Retrocyclin and subtilosin

The potential of antimicrobial peptides to treat BV has been studied.

Retrocyclins are cyclic antimicrobial peptides with broad spectrum activity [121]. In an in vitro study, synthetic retrocyclin (RC-101) inhibited biofilm formation but not planktonic growth of *G. vaginalis*. RC-101 also inhibited the activity of vaginolysin (toxin produced by *G. vaginalis*) and decreased the biofilm formation, so it is a promising candidate agent for BV prevention [128].

Subtilosin is a cyclic anionic peptide produced by *Bacillus subtilis* and *Bacillus amyloliquifaciens*. In non-clinical studies, subtilosin-containing hydrogels were shown to inhibit the growth of *G. vaginalis* while not significantly inhibiting *Lactobacillus* spp. Subtilosin promoted the death of *G. vaginalis* by forming pores in the cell membrane, resulting in an efflux of ATP [121,129].

1.3.3.3. Antiseptics

Antiseptics (including dequalinium chloride, povidone iodide, hydrogen peroxide, chlorhexidine, octenidine) have been used to treat vaginal infections during several decades, since they have large spectrum antibacterial activity, acting by non-specifically disrupting cell membrane [130].

The vaginal administration of dequalinium chloride is a convenient option for empiric therapy of different vaginal infections since it is an antiseptic agent with a broad bactericidal and fungicidal activity and the systemic absorption is very low [131]. Weissenbacher et al. showed in a randomised trial (n=321) that dequalinium chloride vaginal tablets have equivalent efficacy to CLIN vaginal cream in the treatment of BV. Cure rates with dequalinium chloride 7 days after the end of treatment (81.5%) were similar to CLIN (78.4%) [132].

The vaginal administration of a chlorhexidine sustained-release gel is also an effective and well-tolerated treatment of BV. In a randomised trial, four weeks after the treatment, the cure rate in the group treated with chlorhexidine 0.5% vaginal gel was 93% comparing to 74% in the group treated with metronidazole vaginal tablets 500 mg [133].

Octenidine and phenoxyethanol as aqueous solution spray proved to be as effective as the standard therapy with MET in BV treatment [134]. Less promising results were achieved by Swidsinski et al in a clinical trial (n=44) using octenidine as it showed to be initially highly effective in BV treatment, but the efficacy of repeated and prolonged treatment led to bacterial resistance [135].

1.3.3.4. Surfactants

Surfactants are characterised by having an hydrophilic portion and an hydrophobic portion which give them antibacterial properties [121].

In a controlled randomized clinical trial, the administration of pessary containing an amphoteric surfactant (cocoamphopropionate) was found to reduce biofilms after MET

treatment but did not prevent the recurrence. In the group who received pessary containing the amphoteric surfactant after MET therapy, 86.7% of women were considered healthy, according to Nugent classification, at visit 4 [136]. Furthermore, *in vitro*, the application of sodium cocoamphoacetate led to the disintegration of existing *G. vaginalis* biofilms and it was able to increase the effect of MET [121]. The emulsifier lecithin had little effect in preventing biofilm formation and no effect in disrupting pre-existing *G. vaginalis* biofilms, *in vitro* [121].

1.3.3.5. Natural Compounds

Traditionally, plants have been used for the treatment of vaginal infections [137].

Natural compounds such as *Prangos ferulacea, Myrtus communis, Berberis vulgaris* and *Zataria multiflora,* can be used in combination with MET improving the efficacy of antimicrobial therapy and reducing the recurrence of BV [138–142].

Plant-derived therapies can also be combined with probiotics to treat BV. In a recent observational study, the administration of vaginal capsules containing *Thymus vulgaris*, *Eugenia caryophyllus, Lactobacillus fermentum* LF10 and *Lactobacillus plantarum* LP02 improved the signals and symptoms and reestablished flora in 80.0% of cases with BV. This indicates a selective action of these plant extracts and probiotics as they are active against BV-related bacteria but not against lactobacilli [143].

Likewise, in a randomized trial (n=80), Pazhohideh et al. proved that *Calendula officinalis* extract-based cream was an effective alternative to standard antimicrobial treatment. One week after the intervention, all women in both groups (group treated with *C. officinalis* extract-based cream or group treated with MET vaginal cream) were free of symptoms, such as vaginal itching, burning sensation, odor, dysuria, and dyspareunia [144].

Boselli et al. demonstrated that suppositories and cream with *Triticum vulgare* extract are effective to treat BV (therapeutic success of 98.8%) [145].

Additionally, Baery et al. in their trial (n=127) reported that suppositories containing a mixture of *Tribulus terrestres*, *Myrtus commuis*, *Foeniculum vulgare* and *Tamarindus indica* are as effective as MET suppositories to relieve BV symptoms. Fourteen days after intervention, in the group treated with herbal suppositories 14.06% of women presented discharge odour, 3.1% presented cervical inflammation and 12.5% presented pelvic pain, comparing to 7.9%, 9.5% and 11.5%, respectively, in the group treated with MET [146].

Furthermore, *Thymbra capitata* EO and its main compound carvacrol exhibited potent antibacterial activity (*in vitro)* against *G. vaginalis* and showing antibiofilm action. Lactobacilli demonstrated a significantly higher tolerance to the *Thymbra capitata* EO than *G. vaginalis* (MIC of lactobacilli (1.25–2.50 μL/mL) was near tenfold higher than *G. vaginalis*) [147]. Similarly, *Artemisia princeps Pamp*. EO and its constituents eucalyptol and α-terpineol were found to inhibit the growth of *G. vaginalis* [148]. Moreover, thymol (present in thyme EO) has demonstrated *in vitro* an inhibitory effect on newly formed and mature *G. vaginalis* biofilms [121,149]. Thymol is a small hydrophobic molecule able to interact with the lipid bilayer of membranes, promoting the loss of integrity and the leakage of cellular material. It also impairs

the mechanisms of the initial phase of attachment to the substrate, acting in the initial stages of biofilm organization [149].

1.3.3.6. Acidifying agents

The physiologic pH of the vagina is defined as acidic (generally less than 4.5) during the reproductive years. Acidification can be also a strategy to treat BV, because most BV-causing bacteria cannot grow at a pH below 4.5 and the acidic environment facilitates the growth of *Lactobacillus* species [109,150]. The low pH strongly disturbs biofilm formation and integrity of *G. vaginalis* biofilms even the already established ones [121].

The failure to achieve vaginal acidification after antibiotic therapy can be associated with BV recurrence [151].

Some vaginal acidifiers such as vitamin C, LA, polycarbophil or boric acid (BA) were incorporated in the form of gels, suppositories or tablets, although there is controversy regarding the efficacy in the treatment and prevention of BV [5,109,150].

Women with BV were treated with an aqueous gel formulated at pH 3.9, with potent buffering capacity. The product, containing Carbopol 974, was originally a microbicide/spermicide that has completed phase I safety for contraceptive and sexually transmitted diseases prevention trials in which it was verified a significant decrease in BV prevalence after its use, so the potential to treat BV was subsequently investigated. This gel applied vaginally once a day for 6 days appears to be a well-tolerated and moderately effective to treat BV. The cure rate was 70% (n=7) at 2–3 days after treatment and 40% (n=4) one month after treatment [152].

BA has been used for years to treat vaginal infections, since it is a potent microbicide, promotes the acidification of vaginal fluid and has little absorption from the vagina [153,154]. In a trial involving 53 women diagnosed with BV, two formulations containing BA achieved clinical cure rates of BV about 50%. Each formulation also contained EDTA, which enhance the antimicrobial activity of BA and provide superior antibiofilm potency against *G. vaginalis* biofilm while sparing protective lactobacilli [155]. The potential of BA to treat BV was also confirmed by Reichman et al. which hypothesized that BA might remove bacterial mucus or biofilm facilitating direct contact of antimicrobials with pathological bacteria. Women with recurrent BV were treated with oral nitroimidazole during 7 days, followed by 21 days of intravaginal BA (600 mg/day). The cure rates after nitroimidazole and BA therapy were 88% and 92%, 7 and 12 weeks, respectively, after starting treatment [156].

Polycarbophil is a weak polyacid with buffer capacity near its pKa (4.3) so it can re-establish the acid pH of the vagina. A mucoadhesive vaginal gel constituted by polycarbophil and carbopol (a carbomer) proved to be an effective treatment for BV (cure rates after 6 week of treatment were 93% in the intervention group and 6% in the placebo group) [157].

A single study enrolling 142 women was designed to use 250 mg ascorbic acid vaginal tablets on 6 days per month for 6 months after successful treatment of BV with MET or CLIN. This

strategy decreased by half the risk of recurrence (recurrence rate for placebo was 32.4% and for vitamin C group was 16.2% (p = 0.024)) [158].

Since the LA production by lactobacilli is a mechanism that prevents the growth of pathogenic microorganisms it is expected that its administration can normalize vaginal dysbiosis [5]. In a trial involving 123 women, Bahamondes et al. verified that a soap containing LA used for external intimate hygiene can reduce BV recurrence after treatment with oral MET. The rate of recurrence of BV was 19.0%, 24.2% and 7.1%, 30, 60 and 90 days after starting treatments, respectively [159]. Decena et al. in a *in vivo* trial (n=90 women) concluded that the treatment with LA vaginal gel combined and MET administered orally had lower rate of recurrences and promoted the lactobacilli colonization, comparing to MET alone. After a week of treatment, only 3% of women in the LA group, 4% in the MET group, and 0% in the combination group were positive for clue cells. Moreover, within the period from day 15 to day 56, 6.7% of those given LA gel, 14.3% in the MET arm, and 3.6% in the combination arm, were observed to have recurrent vaginal discharge [160].

By contrast, other studies indicate that acidifying agents are ineffective in treating BV. Holley et al., in a trial (n=44), reported that vaginal acidification with an acetic acid gel (pH 3.9 to 4.1) was ineffective to treat BV [161]. Furthermore, Simoes et al. proved that acid-buffering gel was significantly less effective than high-dose MET gel for the treatment of symptomatic BV. At the first follow-up visit (7-12 days after treatment), 88% of the women in the MET group were cured compared with only 23% in the acid-buffered gel group (P<0.001) [150]. Additionally, Boeke and colleagues demonstrated in a randomized clinical trial that the cure rates two weeks after the treatment with LA suppositories were 49%, comparing to 89% in the group treated with MET and 47% in the placebo group [162].

1.3.3.7 Probiotics

The administration of probiotics has been used for the maintenance and reestablishment of an healthy vaginal microbiome [17,163,164].

Lactobacillus strains commonly used as probiotics are *L. reuteri* RC-14, *L. fermentum*, *L. gasseri*, *L. rhamnosus* GR-1, *L. brevis*, *L. acidophilus*, *L. crispatus*, and *L. plantarum* administrated orally or intravaginally [2,163].

While vaginal application is understood as a direct strategy for rebalancing the local microbiota, oral administration represents an indirect route for the protective microorganisms to benefit the vaginal environment. For the later, probiotics shall resist the conditions of the gastrointestinal tract (frequently achieved through formulation strategies) and they are believed to either stabilize the intestinal environment concerning protective microorganisms, or the ability to reach the vagina after rectal excretion. Oral capsules have been used as dosage forms to administer vaginal probiotic strains in clinical trials with positive results in the treatment of BV [165–167].

Lactobacilli exhibited phenotypic surface properties (aggregation, adhesion and biofilm formation) that were specific for each strain. Terraf et al. studied the phenotypic and genetic characteristics of lactobacilli isolated from human vagina and concluded that *L. reuteri* CRL

1324 and *L. reuteri* CRL 1327 showed high auto-aggregation, adhesion to mucin and biofilm formation ability. The genes of these strains encoded three adhesion proteins [168].

Furthermore, Gregorio et al. reported that co-aggregation (a process in which different micro-organisms become attached to one another via specific molecules) is a strain specific phenomenon. When lactobacilli form co-aggregates with pathogens, homeostasis could be restored, allowing the elimination of pathogens, partially explaining the efficacy of probiotics to treat vaginal infections, such as BV [169].In their study Hutt et al. aimed to characterize and compare the probiotic potential between different *Lactobacillus* species. Most of *L. crispatus* (89%) and *L. jensenii* (86%) strains produced hydrogen peroxide. The best LA producers were *L. gasseri* (18.2±2.2 mg/mL) followed by *L. crispatus* (15.6±2.8 mg/mL) and *L. jensenii* (11.6±2.6 mg/mL). Additionally, they found that there was no significant difference in antagonistic activity against *G. vaginalis* between strains of these three *Lactobacillus* species [170]. Castro et al. showed that preadhering *Lactobacillus crispatus* to HeLa epithelial cells reduced the cytotoxicity of *G. vaginalis*, reducing the expression of vaginolysin and sialidase transcripts 2.58-fold and 1.89-fold, respectively [171].

Santos et al. evaluated the *in vitro* probiotic potential of 23 lactobacilli isolated from the vaginal ecosystem of healthy women. Most strains showed high co-aggregation rates with *G. vaginalis* and *L. fermentum* 137 showing the best co-aggregation ability (97.5%). This feature reflects the ability of *Lactobacillus* spp. To surround *G. vaginalis* and prevent it from adhering to the epithelium. Additionally, *Lactobacillus* spp. Can form biofilms, which promote colonization and prevent pathogenic microorganisms from adhering to the epithelium, by competing directly with them. According to this research, this characteristic is particularly relevant in *L.gasseri* 202 and *L. fermentum* 137, which were classified as “very strong” and “strong” biofilm producers, respectively. Lactobacilli presents excellent ability to adhere to monolayers of HeLa cells. Among them *L.fermentum 197* presents the highest potential to colonize the epithelium [172].

Probiotics may be incorporated into solid dosage forms (capsules, tablets) or even into vaginal rings. These are low moisture content dosage forms, being the former an important requirement for lactobacilli viability maintenance throughout storage [173]. Other factors to take into account in the development of the formulation are the dosage (amount of bacteria per unit) and the maintenance of viability during the storage period that can be analysed by stability studies [4]. In fact, the effectiveness of probiotic products depends on the number of viable cells per administration. The incorporation of the probiotic in a formulation ensuring an effective and sustainable release through the matrix and the maintenance of viability over the storage time may be a challenge [174]. Tomás et al. concluded that the encapsulation (by extrusion-ionic gelation) and freeze-drying in the presence of lyoprotectors and subsequent storage in refrigeration conditions favoured the maintenance of vaginal *L. reuteri* CRL 1324 viability and efficacy [175].

There is still some controversy regarding the use of probiotics as main or adjuvant therapy (in combination with antimicrobial agents) for the treatment of vaginal infections. A recent meta-analysis concluded that the currently limited evidence does not support that MET

combined with probiotic supplementation is more effective than MET alone for the treatment of BV [176]. However, two other meta-analyses conclude that although the evidence is limited and weak, probiotics show a beneficial effect as alternatives or co-treatments of BV [48,177].

Several clinical studies are presented in table 3.

Further studies are needed to confirm the efficacy and safety of this therapeutic strategy, using an appropriate sample size and experimental design.

Table 3 Clinical studies about the efficacy of probiotics in BV treatment

Article	Route	Dosage form	Posology	Species	Principal findings
Effects of BV-Associated Bacteria and Sexual Intercourse on Vaginal Colonization with the Probiotic *Lactobacillus crispatus* CTV-05	Vaginal	Suppository	Women diagnosed with BV were treated with MET gel for 5 days. 24 to 72 h after antimicrobial a part of these initiated administration of LACTIN-V once daily for 5 consecutive days. Subsequently administration was repeated on days 12 and 19.	*L. crispatus* CTV-05	44 % of BV cases were colonized with CTV-05 at 28 days, and cases not colonized had higher median concentrations of BV-associated bacteria [178].
Effectiveness of Lactobacillus-containing vaginal tablets in the treatment of symptomatic bacterial vaginosis	Vaginal	Tablet	Patients with BV received either one *Lactobacillus*-containing tablet or placebo daily for 7 days	*L. brevis* (CD2), *L. salivarius* (FV2) and *L. plantarum* (FV9)	Two weeks after completion of therapy, treatment was successful in 61% of *Lactobacillus*-treated patients as compared with 19% of those in the placebo group ($p < 0.05$) [179].
Restoring vaginal microbiota: biological control of bacterial vaginosis. A prospective case–control study using Lactobacillus rhamnosus BMX 54 as adjuvant treatment against bacterial vaginosis	Vaginal	Tablet	Patients selected were divided in Group A (MET 500 mg orally twice a day for 7 days) and Group B (undergoing the same standard antibiotic regimen followed by vaginal tablets containing *Lactobacillus rhamnosus* BMX 54 once a day for 10 days, twice a week for 15 days, and once every 5 days for 7 months as maintenance therapy.)	*L. rhamnosus* BMX 54	Probiotic supplementation seems to be useful in hindering bacteria growth after antibiotic therapy (Vaginal flora was significantly replaced and pH decreased) [9].
Effectiveness of the Two microorganisms *Lactobacillus fermentum* LF15 and *Lactobacillus plantarum* LP01, Formulated in Slow-release Vaginal Tablets,	Vaginal	Slow release tablet	Once a day for 7 consecutive nights, followed by 1 tablet (400 million live cells per dose) every 3 nights for a further 3-week application (acute phase) and, finally, 1 tablet per week to maintain a	*L. fermentum* LF15, *L. plantarum* LP01	*L. fermentum* LF15 showed the strongest in vitro inhibitory activity towards *G. vaginalis* ATCC 10231 after both 24 and 48 h. *L. fermentum* LF15 and *L. plantarum* LP01, significantly reduced the Nugent

in Women Affected by Bacterial Vaginosis - A Pilot Study			long-term vaginal colonization against possible recurrences		score below 7 after 28 days in 91.7% of patients (P<0.001) [180].
Observational prospective study on *Lactobacillus plantarum* P 17630 in the prevention of vaginal infections, during and after systemic antibiotic therapy or in women with recurrent vaginal or genitourinary infections	Vaginal	Capsule	One vaginal capsule per day for 6 days, then a capsule per week for 16weeks were administrated after systemic antibiotic therapy	*L. plantarum* P 17630	This observational study suggested that *Lactobacillus plantarum* may reduce the frequency of genitourinary infection after antibiotics treatment for bacterial respiratory tract infections and in women with recurrent vaginal infection or bacterial genitourinary infections after standard treatment for the conditions, however results were not statistically significant [181].
Efficacy and safety of vaginally administered lyophilized *Lactobacillus crispatus* IP 174178 in the prevention of bacterial vaginosis recurrence	Vaginal	Capsule	After oral metronidazole treatment (1 g/day for 7 days) patients were randomised to receive vaginal capsules of probiotic or placebo once a day, for 14 days over the first two menstrual cycles and another 14 days of the same treatment for the following two menstrual cycles	*L. crispatus* IP174178	During the treatment period, 41% of patients in the placebo group had at least one recurrence versus 20.5% in the probiotic supplementation group (P = 0.0497). The time to recurrence was longer in the probiotic supplementation group (3.75±0.16 months) vs. the placebo group (2.93±0.18 months) (P = 0.0298) [182].
Vaginal colonisation by probiotic lactobacilli and clinical outcome in women conventionally treated for bacterial vaginosis and yeast infection	Vaginal	Capsule	Trial I: Women with BV were treated with clindamycin and metronidazole followed by probiotic capsules, for 5 consecutive days after each antibiotic treatment. Trial II: women with BV receiving clindamycin and metronidazole treatment together with a prolonged administration of probiotic capsules (10 consecutive days after each antibiotic treatment followed by weekly administration of capsules for next four months)	*L. rhamnosus* DSM 14870 and *L. gasseri* DSM 14869	Cure rates for BV was 50% and 67% in 6-months for trial I and trial II, respectively [183].
Augmentation of antimicrobial metronidazole therapy of	Oral	Capsule	Women with BV were treated with oral metronidazole (500 mg) twice daily	*L. rhamnosus* GR-1 and	88% were cured in the antibiotic/probiotic group compared to 40% in the

bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial			from days 1 to 7, and randomized to receive oral probiotic or placebo twice daily from days 1 to 30	*Lactobacillus reuteri* RC-14	antibiotic/placebo group ($p< 0.001$) [167].
Improved cure of bacterial vaginosis with single dose of tinidazole (2g), *Lactobacillus rhamnosus* GR-1, and *Lactobacillus reuteri* RC-14: a randomized, double-blind, placebo-controlled trial	Oral	Capsule	After receive a single dose of TI (2g) patients were divided into 2 groups: placebo group and probiotic group. Two capsules were administered every morning for 4 weeks.	*L. rhamnosus* GR-1 and *L. reuteri* RC-14	After 4 weeks, the probiotic group had a significantly higher cure rate (87.5%) of BV than the placebo group (50.0%) [166].
Supplementation of standard antibiotic therapy with oral probiotics for bacterial vaginosis and aerobic vaginitis: a randomised, double-blind, placebo controlled trial	Oral	Capsule	One probiotic or placebo capsule was administered together with metronidazole twice daily for 10 days. During follow up, patients took one capsule daily for 10 days perimenstrually.	*L. fermentum* 57A, L. *plantarum* 57B, *L. gasseri* 57C	Probiotic use reduced and maintained low vaginal pH and Nugent score and lengthened the time to clinical relapse [165].

1.3.3.8. Prebiotics

The administration of prebiotics (substances that promote the selective growth of beneficial microorganisms) is a strategy that allows reestablishment of a healthy vaginal flora [184–186].

Fructooligosaccharides, sugar alcohols, galacto-oligosaccharides, lactulose, and raffinose are examples of prebiotics that selectively promote the growth of *Lactobacillus* spp. [185,186].

Rousseau et al. showed the *in vitro* capacity of oligosaccharides to selectively promote the growth of vaginal lactobacilli and so their potential to be used as substrate by protective bacteria while the commonest vaginal pathogens can not metabolize them [184].

Posteriorly, Coste et al. concluded that a prebiotic vaginal gel containing alpha-glucooligosaccharides was able to improve the recovery of normal vaginal flora and to reduce the pH in women previously treated for BV with oral metronidazole. After 16 days of treatment, all subjects who received the prebiotic gel still showed normal Nugent scores (versus 24% in the placebo group) [187].

Collins et al. in a research aimed to evaluate the prebiotic potential of lactitol, lactulose, raffinose and oligofructose in monocultures of commensal *Lactobacillus crispatus, L. vaginalis,*

L. gasseri, *L. johnsonii*, *L. jensenii,* and *L. iners* in addition to BV associated organisms showed that lactulose promoted commensal *Lactobacillus* spp. growth (including the strongly health-associated *L. crispatus*) and resulted in decrease of pH partially through LA production, while antagonizing BV-related microorganisms. Although lactulose did not improve the viability of *L. iners,* it has been hypothesized that it was fermented by them to produce acidic metabolites. Lactitol was also fermented by vaginal lactobacilli and increased the proportional abundance of *Lactobacillus* spp. but did not re-establish an acidic pH. Although raffinose was fermented by several *Lactobacillus* strains, it did not directly stimulate lactobacilli and also antagonize *L. iners* [186].

Additionally, another study concluded that raffinose can be used by *Trichomonas vaginalis,* the vaginal parasite responsible for trichomoniasis. Therefore, raffinose does not appear to be promising as prebiotic [188]. Furthermore, oligofructose did not increase the proportion of lactobacilli, and it antagonized *L. iners* AB-1 in culture, so it also does not appear to be promising as prebiotic [186].

Lactoferrin, an iron-binding glycoprotein prebiotic with bacteriostatic and bactericidal properties, protect against infection by binding and regulating the iron needed for bacterial proliferation and by immunological processes. In an open prospective randomized trial (n=60) the administration of lactoferrin vaginal pessaries significantly decreased the occurrence of bacteria associated with BV and increased the occurrence of *Lactobacillus* species. The Nugent score reduced below the threshold of 7 in 92.8% of women treated with lactoferrin 200 mg [189]. The prebiotic potential of lactoferrin was confirmed by Russo et al. in a clinical trial (n=48) upon a probiotic mixture (*Lactobacillus acidophilus* GLA-14 and *Lactobacillus rhamnosus* HN001) with lactoferrin after MET oral therapy improve symptoms of BV, comparing to the group that received MET oral therapy and placebo [190]. These findings are supported by the research of Katsufumi Otsuki and Noriaki Imai, although, in this case, the study was carried out considering a very small number of patients (n=6) with refractory BV [191].

The efficacy of a gel containing 2% red clover extract, 10% inulin, and 10% fructo-oligosaccharide combined with oral MET was compared with MET alone. The cure rate on the group treated with MET oral and prebiotic gel was 76% and in the control group (receiving MET oral and placebo gel) was 30%, 10 days after the end of treatment. This data shows this probiotic mixture is promising in the treatment of BV [192].

1.3.3.9. Other substances

There are molecules used in the treatment of other pathologies whose potential in the treatment of BV has been investigated, such as nifuratel and benzoyl peroxide. Nifuratel is used to treat wide spectrum vaginal infections and it is active against BV related bacteria without affecting lactobacilli [193,194]. *In vitro*, nifuratel was more active on *G. vaginalis* and *A. vaginae* than metronidazole (MICs ranging from <0.125 to 4 μg/ml and from <0.125 to 1 μg/ml, respectively) [194]. It is reported that benzoyl peroxide encapsulated in a Polycarbophil/Carbopol 934P hydrogel was effective against *G. vaginalis* and was well tolerated

by *Lactobacillus* spp [195]. Additionally, Algburi et al. demonstrated that benzoyl peroxide can have a quorum sensing-mediated biofilm controlling effect [196].

Additional promising therapeutic agents for the treatment of BV include DNase which act on the biofilm extracellular DNA that is essential to maintain the structural integrity. *In vitro*, purified bovine pancreas DNase inhibited *de novo* biofilm formation and also liberated *G. vaginalis* from existing biofilms into the supernatant fraction without killing them, so the biofilm density decreased and the effect of antimicrobials was potentially enhanced [197].

1.3.4. Advances in the field of pharmaceutical technology to treat BV

Advances in the field of pharmaceutical technology aim to improve the effectiveness of conventional treatments as well as overcome some disadvantages associated with vaginal administration of drugs.

1.3.4.1. Formulation strategies

Formulation strategies can contribute to improve the efficacy of conventional treatments by modifying the release of active substance, promoting synergistic effects with excipients, improving direct contact with pathogens, and enhancing drug retention and the adhesion to the vaginal tract epithelium.

Local treatment of vaginal infections usually results in higher local drug concentrations with fewer side effects than the systemic therapy with the same drug by oral administration [17]. Treatment of infections is one of the main reasons to administrate products through the vaginal route [103,198]. However, leakage is one of the major disadvantages of vaginal route and it is associated with discomfort [198]. The anatomical and physiological characteristics of the vagina such as extensive surface area, the S shape of the vaginal canal, the hormonal changes during the menstrual cycle and the surface coating with vaginal fluid contribute to reduce the retention time and promote leakage. The inclusion of bioadhesive polymers in the development of dosage forms or delivery systems can overcome this limitation, resulting in a prolonged *in situ* residence [17,103].

Bioadhesion means the attachment to biological tissue. Mucoadhesion is defined as a particular type of bioadhesion used whenever the biological tissue is mucosal membrane. Bioadhesive/mucoadhesive polymers used in vaginal dosage forms promote the intimate contact between the formulation and the vaginal epithelium. These include polyacrylates (carbomers and polycarbophil), cellulose derivatives (hydroxypropylmethyl cellulose, hydroxyethyl cellulose, hydroxypropyl cellulose), chitosan, sodium alginate, hyaluronic acid, pectin and natural gums [17,104].

Besides the mucoadhesive properties, chitosan presents antimicrobial activity including against BV related bacteria [199]. Furthermore, it can promote disruption of *P. aeruginosa* biofilms that is not typically associated with BV but is an opportunistic pathogen occasionally isolated from vaginal smears, and often difficult to treat with conventional antibiotic therapy [200]. Moreover, chitosan-coated liposomes containing resveratrol were studied as a potential

treatment of BV. This strategy overcomes the limitation of solubility of resveratrol and allows a synergistic action of both molecules [201].

Polyacrylates, such as polycarbophil and carbomers, are particularly advantageous as excipients incorporated into formulations for the treatment of BV because as they are acid, they promote the correction of vaginal pH [17].

Different formulation strategies were also developed to improve the efficacy of traditional MET therapy.

The introduction of mucoadhesive polymers in MET gel to prolong the retention time has been studied. The combination of hydroxyethyl cellulose with chitosan or modified chitosan (5-methyl-pyrrolidinone-chitosan) is more mucoadhesive than the carbomer-based metronidazole commercial gel. The combination with modified chitosan is more mucoadhesive than the combination with chitosan since the amine groups are enclosed into a heterocyclic ring in 5-methyl-pyrrolidinone-chitosan determining less availability to link hydroxyethyl cellulose and consequently it presents increased ability to establish hydrogen bond with mucin chains [202].

Moreover, *in situ* gel formulations ware also developed. Ibrahim and colleagues developed poloxamer-based formulations (pluronic F-127 alone and in combination with pluronic F-68) containing MET. The final thermogelling system showed better mucoadhesion and higher cure rates than the marketed preparation used to establish a comparison. The viscosity of the final thermogelling system increases as the temperature increases, and it allows a free flow which facilitates the application of the required dose accurately and would result in good spreading and coating of the vagina. At 37 ºC the viscosity of the final thermogelling system is higher than the marketed product, avoiding leakage. Besides, Pluronic formulations might serve as a rate-controlling barrier, allowing the sustained release of MET [203]. Similarly, other study reports that a thermo-sensitive gel containing MET achieved higher cure rates than the commercial product (80% versus 47% in the conventional gel (p=0.034)). Since the formulation is liquid before the administration it can be applied in an accurate and reproducible quantity and spread more easily. Besides, the bioadhesiveness of pluronics improves the intimacy of contact and increases the residence time. The *in situ* gel preparation promotes sustained-release of MET [204].

Furthermore, the design of pH-sensitive hydrogels is interesting since it would control any early change due to relapse of the infection, by controlling the drug release. pH-sensitive hydrogels are designed to undergo a volume phase transition (collapse or swelling) so in BV treatment an increase in pH will stimulate the release of the drug from the formulation [104].

The development of bigels is a strategy that also allows controlling the release of MET. Bigels are prepared by mixing hydrogel (aqueous solvent) and organogel (apolar solvent) in defined proportions. Singh et al. formulated a bigel containing carbopol 934 hydrogel as the external phase and sorbitan monostearate-sesame oil organogel as the dispersed phase. When the portion of organogel increases, MET is released in a controlled mode, on the other hand, when the proportion of the aqueous layer is higher the rate of release increases [205].

Liposomes have been developed to allow sustained release of MET [206–210].

Furthermore, liposomes improve the drug delivery to bacteria by promoting contact with the bacterial membrane, while avoiding permeation to the systemic circulation, reducing systemic side effects [206].

Pavelic and colleagues designed liposomes with egg phosphatidylcholine and egg phosphatidylglycerol–sodium containing MET. To achieve adequate viscosity and to improve stability, liposomes were incorporated in a bioadhesive Carbopol gel. After 24 h of incubation in vaginal fluid simulant at 37 ºC, more than 50% of the originally entrapped metronidazole was still retained in the gel [210]. Similarly, Patel and Patel developed MET liposomes containing soy lecithin and cholesterol incorporated in Carbopol gel. They reported that 50% of the drug was released within 12 h, in comparison to 80% of drug released from a control MET gel [209]. Studies are concordant and show that the incorporation of MET liposomes in Carbopol gel keeps the original particle size distribution and allows the prolonged release of MET by the gel which may be useful in the treatment of BV [209,210].

Vanic et al. reports the liposomes *in situ* formation during the disintegration, dissolution or erosion of tablets. This delivery system combines the ability of liposomes to encapsulate and protect MET and the increased stability associated with dry compressed formulations [206].

In a preliminary study, Andersen et al. developed MET liposomes containing mucoadhesive polymers (pectin and chitosan) by the one-pot preparation method to increase the retention time on the mucosal surface. Both pectosomes and chitosomes were found to entrap more metronidazole than conventional plain liposomes [211].

As described above the incorporation of MET into liposomes prevent the systemic absorption of drug. In vaginal infections the deeper epithelial layers are frequently affected. Therefore, the ability of the drug delivery system to reach these deep epithelial layers may be vital for therapeutic success. Deformable liposomes contain phospholipids and substances responsible for increased deformability and malleability of bilayers, so they have improved penetration capacity, able to delivery MET more efficiently. Single chain surfactants act as edge activators, making the bilayer less rigid and more fluid, able to adapt the conformation allowing the liposomes to reach deeper layers. Surfactants used included sodium deoxycholate, Tween 80, Span 60 and Span 80. The safety of these formulations must be carefully evaluated because the surfactants may be toxic to the vaginal epithelium [207,208].

1.3.4.2. Dosage forms

Looking beyond traditional vaginal dosage forms such as tablets, capsules, suppositories, semi-solids (gels, creams) and liquid preparations new delivery systems such as rings and films have been developed [17].

Films are polymeric thin strips that dissolve or disperse in contact with vaginal fluids, forming a gel which spread and may adhere to the vaginal epithelium. Films are easy to apply and do not require an applicator. They are thin and solid so they do not increase significantly the volume of vaginal fluids, therefore the leakage and discomfort are reduced [17,103,212,213].

Hani and colleagues developed bioadhesive vaginal films that allow a prolonged release of MET using bioadhesive polymers such as chitosan and cellulose derivatives. [214]

Furthermore, Dubaria and Mashru designed vaginal films of CLIN using hydroxypropyl cellulose, hydroxyethyl cellulose, xanthan gum and carrageen as bioadhesive polymers. Some of these films present desirable aesthetic and physicodynamic properties and with their use it is expected an improvement of compliance and the efficacy of BV treatment. [213]

Substances to treat BV may be loaded in vaginal delivery devices, such as intravaginal rings, achieving controlled release of substances and reducing the frequency of application. These devices allow the controlled release of substances, reducing the frequency of application.

Verstraete et al. reported that intravaginal ring of thermoplastic polyurethanes promotes LA release over a period of 28 days and metronidazole release over 4-7 days [215]. In another research, intravaginal ring poly(e-caprolactone) matrices promote a rapid release of MET (35–55% of MET over 24 h) followed by a gradual release (80% of the drug content occurred over the following 12 days) when immersed in mVFS pH 4.2 [216]. Verstraelen et al. designed an intravaginal ring matrix consisting of a mixture of ethylene vinyl acetate and methacrylic acid-methyl methacrylate copolymer to load DL-lactic acid. This device is less rigid than the usual rings, promoting patients' comfort although the expulsion risk is increased. More studies are needed to confirm if the amount of acid released is sufficient to obtain the desired prebiotic effect and if the L/D-lactic acid ratio is the more effective [217].

Moreover, vaginal rings can also load probiotics. Each silicone intravaginal ring was loaded with 10 tablets coated with poly(D,L-lactide) to form pods of *Lactobacillus gasseri* ATCC 33323. The device release between 1.1-14×107 cells per day for up to 21 days [173].

1.4. Justification and aim of research

The standard treatment of BV with antibiotics is associated with high levels of therapeutical failure. These can be explained by the incapacity to eradicate the BV multispecies biofilms, associated with the increasing resistance to antibiotics and the inability to penetrate in these structures, but also by failures in the reestablishment of physiologic vaginal environment (such as saprophytic flora and vaginal pH) after the antibiotic therapy.

Similarly, in the treatment of VVC an increasing resistance to azoles was perceived as the result of years of their exclusive therapeutic use, sometimes inappropriate and indiscriminate, that allowed *Candida* spp. to develop resistance mechanisms. Moreover, the biofilm organization and the growing incidence of Non-*albicans Candida* that are intrinsically more resistant to azoles can also explain the recurrence of the disease.

Vaginal infections are a high prevalent clinical condition that affects women at some point in their lives who will experience symptoms that cause discomfort and changes in everyday life with negative emotional impact. Therefore, it is emergent to develop alternative therapeutic approaches that can replace or be combined with the actual standard therapies to promote the efficacy of treatment and avoid recurrence.

In the present research we aimed to explore alternative strategies for the treatment of VVC and BV that comprise alternative antimicrobial substances and innovation under a

pharmaceutical technology perspective. Specifically, we aimed to develop and characterize a bioadhesive sodium bicarbonate gel for the treatment of VVC and a vaginal sheet with *Thymbra capitata* EO for the treatment of BV.

Chapter 2

Sodium bicarbonate gels: a new promising strategy for the treatment of vulvovaginal candidosis

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2.1. Introduction

The antifungal potential of sodium bicarbonate (in applications other than vaginal fungal infections) has been reported. Shao et al reported the antifungal activity of sodium bicarbonate against *C. albicans* isolates using the broth microdilution method [84]. Sousa et al reported that a 5% sodium bicarbonate proved to be an effective disinfecting solution for dental prostheses, reducing *Candida albicans'* adherence to acrylic resins, and reduction of 66% in the number of CFU/mL, was confirmed, comparing to the control [85]. Betscher-Bru et al reported the antifungal action of sodium bicarbonate against several agents that cause superficial cutaneous infections, including *C. albicans* [86]. Lastauskiene et al studied the potential of sodium bicarbonate to treat cutaneous fungal infections. They concluded that exposition of the yeast cells to MICs of sodium bicarbonate (5%) resulted in cell death mostly by the necrotic pathway while at lower concentrations sodium bicarbonate stimulated yeast to hyphae switching. These factors should be taken in consideration in treatments, since an accidental use of an insufficient concentration (lower than MIC) can result in exacerbation of VVC [218]. Likewise, Matare et al, testing isolates obtained from oral swabs of patients presenting with thrush has demonstrated that sodium bicarbonate in low concentrations (0mM to 30mM) induced tube germinative formation while at higher values, up to 50 μM, a gradual decline in the percentages of cells with visible germ tubes was evident [219]. Faria-Gonçalves et al in a previous study of our research group reported pH-independent fungistatic action of sodium bicarbonate (MIC value against *C. albicans* 12.5 mg/ml (pH 8.97)) and that *C. albicans*'s virulence is attenuated in its presence (reduction of 93% in the cells producing hyphae in the presence of at least 2 times the MIC, when compared with cells not exposed to sodium bicarbonate). Moreover, in this study the antibiofilm activity of sodium bicarbonate was also reported (50% reduction in biofilm mass, when *C. albicans* cells were exposed to 15 times the MIC) [220]. On the other hand, Vanetini-Mioso et al evaluated the effectiveness of several hygiene clinical protocols in reducing the microbial viability of biofilms collected from dentures and concluded that a 5% sodium bicarbonate solution cannot inhibit the *C. albicans* growth (results are similar to control in which the dentures were cleaned with water) [221]. Overall, sodium bicarbonate appears to have anti-*Candida* activity at certain concentrations, despite having no action on biofilms.

The use of sodium bicarbonate either in a bath or in vaginal irrigation for the treatment of cytolytic vaginosis was reported. [222–224] . Cytolytic vaginosis is often misdiagnosed as VVC, since these pathologies share many symptoms. The observation of the microscopic characteristics of vaginal discharge is vital for a correct diagnosis [222,225]. Being a physiologic component of the pH buffer solution, sodium bicarbonate is used as alkalinizing agent for the treatment of metabolic acidosis, diabetic ketoacidosis, pyrosis and dyspepsia, for the correction of electrolyte imbalances and also for the prevention and treatment of acute kidney injury [226–230].

Some studies report the anti-*Candida* activity of castor oil. Castor oil has been used in oral cleansers (such as denture cleanser solutions and dentifrices), due to their *in vitro* capacity to control the growth of potential oral pathogenic microorganisms, including *Candida albicans*

[231–238]. Suubaar et al reported that methanolic and aqueous extracts of the leaves of *R. communis* presented a MIC of 12.5 mg/mL for *C. albicans* isolates [236].

In the present work, we aimed to study the potential of sodium bicarbonate, when incorporated in gel, to treat VVC and we characterized the technical and biological performance of trial products. We also aimed to study the influence of formulations factors on anti-*Candida* potential and *in vitro* performance, specifically the influence of excipients, such as the polymer and castor oil.

2.2. Materials and Methods

2.2.1. Materials

For the preparation of gel formulations the following excipients were used: carbomer (Carbopol 940) (Lubrizol, United States of America); HPMC (Methocel K100 – viscosity 100000 cP, 2% aqueous solution) (Dow, United States of America); sodium bicarbonate (purity level >99.7%) (Sigma, Germany); castor oil (E.J. Campos, Portugal); Polysorbate 80 (Sigma, Germany) and MilliQ water (obtained in house through a Merck Milli-Q® Reference equipment). For the preparation of the vaginal simulant the following excipients were used: sodium chloride (JT Baker, United States of America); potassium hydroxide (VWR Prolabo, France); calcium hydroxide (Acros Organics, United States of America); Bovine Serum Albumin (Sigma, Germany); lactic acid (Sigma, Germany); acetic acid (Fischer Scientific, United States of America); glycerol (Acofarma, Spain); urea (VWR Prolabo, France); glucose (VWR Prolabo, France) and porcine gastric mucin type II (Sigma, Germany). Reagents used in the cellular toxicity assay included Dulbecco's Modified Eagle Medium F12 (DMEM F12, Gibco); 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT, Alfa Aesar); fetal bovine serum (FBS, Sigma-Aldrich); penicillin and streptomycin (Sigma, Sigma-Aldrich); phosphate buffer solution (PBS, VWR Chemicals); sodium dodecyl sulfate (SDS, VWR Chemicals) and 2-Propanol (Honeywell).

2.2.2. Preparation of formulations

We prepared 8 formulations containing sodium bicarbonate. The base formulations (A, B, C, D) contained sodium bicarbonate, water and gelling polymer (table 4).

Table 4. Composition of formulations and placebos presented as % w/w (Note: formulations E-H are composed by formulations A-D as aqueous phase; hydrochloric acid was added to adjust the pH of formulation B1)

Composition	**Formulation code**										
(%w/w)	**A**	**B**	**B1**	**C**	**D**	**Placebo HPMC**	**Placebo Carbomer**	**E**	**F**	**G**	**H**
$NaHCO_3$	1	1	1	5	5						
HPMC		1	1		1	1					

Carbomer	1			1			1						
Water	98	98	98	94	94	99	99						
HCl 5M			Enough to adjust pH as A										
A								93					
B									93				
C										93			
D											93		
Polysorbate 80								2	2	2	2		
Castor oil								5	5	5	5		

Sodium bicarbonate was dissolved in water and then the polymer (carbomer or HPMC) was gradually dispersed using a helical stirrer (Heidolph RZR 2041, Heidolph Instruments GmbH & Co., Germany) at 500 rpm.

Formulations E, F, G and H additionally contained castor oil and tween 80 (table 4). The respective base formulations were vigorously mixed with castor oil and polysorbate 80 at 750 rpm during 15 min (Heidolph RZR 2041, Heidolph Instruments GmbH & Co., Germany).

2.2.3. Preparation of mVFS

The mVFS was prepared as described by Owen and Katz [239], with the addition of mucin, as follows: sodium chloride 3.51 g (JT Baker, United States of America); potassium hydroxide (KOH) 1.4 g (VWR Prolabo, France); calcium hydroxide (Ca(OH)2) 0.22 g (Acros Organics, United States of America); Bovine Serum Albumin 0.018 g (Sigma, Germany); lactic acid 2.00 g (Sigma, Germany); acetic acid 1.00 g (Fischer Scientific, United States of America); glycerol 0.16 g (Acofarma, Spain); urea 0.4 g (VWR Prolabo, France); glucose 5.00 g (VWR Prolabo, France) and 15.00 g porcine gastric mucin type II (Sigma, Germany) were added to a milliQ water slightly volume less than 1L and stirred mechanically until complete dissolution. The pH of the mixture was then adjusted to 4.2 using hydrochloric acid (HCl), and the final volume was adjusted to 1 L.

Mucin (1.5% w/v) was added to enable the preparation to simulate the bioadhesion properties of vaginal fluid [17,240–242].

2.2.4. Sensorial Characteristics

Sensorial characteristics studied were: general aspect (liquid, solid, semi-solid); homogeneity (homogeneous, with sediment, undissolved particles); colour (colour tone, colourless, transparent, opaque, translucent); odour (odourless, characteristics of odour, intensity of odour) and feel to touch when spread over the back of the hand (soft, with particles, greasy, leaves residue). The visual and sensory analysis was always performed by the same

operator, 24 h after preparation of the formulation. Observations were recorded and photographs were taken.

2.2.5. pH

pH measurements were performed at room temperature (25 °C) directly on the plain (undiluted) formulations and at vaginal physiologic temperature (37 °C) after diluting in mVFS. The amount corresponding to the daily dose envisaged in marketed antifungal vaginal products (5 g) was diluted in 0.825 mL of mVFS. This procedure was established to simulate the pH of the formulations when put in contact with vaginal fluid, since 0.75 mL is the estimated mean volume of fluid present in the vagina at any moment [17,243–245]. We assumed that there is an increase of 10% in that volume through accumulation of the discharge in VVC. Additionally for Gel C, additional pH measurements were performed considering less amount of gel applied to the vagina. We also evaluated the pH of sodium bicarbonate solutions described for the treatment of cytolytic vaginosis. Measurements were performed, in triplicate (Thermoscientific Orion Star A211 pH meter, *ThermoFisher Scientific*, USA).

For formulations B1, Placebo HPMC, Placebo Carbomer, the pH was only determined in the plain (undiluted) formulations since these were used only as controls for comparison assays of anti-*Candida* activity.

2.2.6. Osmolality

The osmolality was determined in triplicate using a freezing point osmometer (Osmomat 3000, Gonotec, Germany), as previously described [240] [246] [247], on a 50 mg aliquot.

Osmolality measurements were performed directly on plain formulations and after diluting in mVFS, as described above in the pH section. Additionally for Gel C, additional osmolality measurements were performed considering less amount of gel applied to the vagina. We also evaluated the osmolality of sodium bicarbonate solutions described for the treatment of cytolytic vaginosis.

Measurement standardization was performed using three standards: distilled water (zero point), NaCl 300 mOsm/Kg and NaCl 850 mOsm/Kg.
Measurements were performed in triplicate, in three independent samples from the same batch.

2.2.7. Viscosity

Viscosity was assessed using a cone-plate rheometer (Brookfield DV-3 T, Brookfield, USA), using 0.5 mL of the formulation [240]. Viscosity measurements were performed at room temperature (25 ± 0.2 °C) and at vaginal physiologic temperature (37 ± 0.2 °C), for plain formulations and after diluting in mVFS respectively. Formulations dilution to physiologic conditions was performed as described on the pH section.

The cone spindle used in the rheometer was the CPA-52Z (Brookfield, USA) had 3° and 1.2 cm, cone angle and radius, respectively.

The viscosity was determined using shear rates of 0.4 1/s for formulations A and E and 500 1/s for formulations B, C, D, F, G and H (torque 10–100%). The elapsed time of each determination was set so that the cone completed at least 4 rotations in each reading. Tests were performed in triplicate.

All formulations were left to rest for 1 min between measurements [248].

2.2.8. Texture: Firmness and Adhesiveness

Texture analysis included adhesiveness (N.mm) and firmness (N) determinations using a texturometer (TAXT Plus, Stable Micro Systems, United Kingdom). These two parameters were determined in the same run, using a cylindrical probe with a diameter of 10 mm (P10) in compression mode and "return to start test". Measurements were performed at room temperature (25 ± 1 ºC) in plain formulations and at 37 ºC after diluting formulations in mVFS. Formulations were placed in a plastic container with a base area of 9.62 cm^2 (10 g of plain formulation or 10 g of formulation diluted in 1.65 mL of mVFS). The pre-test speed, test speed and post-speed were 3 mm/s. The maximum positive force (N) to penetrate the formulation for 5 mm was registered and characterized the formulation firmness. Adhesiveness represents the negative area under the curve (AUC) of the Force (N) vs Distance (mm) curve. Measurements were performed, in triplicate at three different locations (where the probe was applied) for each formulation.

2.2.9. Bioadhesion

The formulation strength of adhesion to the porcine vaginal tissue was assessed using a texturometer (TAXT Plus, Stable Micro Systems, United Kingdom). This method is a mechanical approach to bioadhesion, since it is based on the evaluation of tensile strength of the interfacial layer formed between the formulation and the vaginal epithelium [249]. Epithelium was excised from porcine vaginal tubes (obtained from 6 animals approximately aged 6 months, kindly provided from a local slaughterhouse). The vaginal tubes were cut longitudinally, washed with physiological saline solution, wrapped in aluminium foil, and preserved in an air-tight bag at −20 °C.

For the experiment, vaginal tissue was thawed at room temperature, washed with physiological saline solution *and* cleaned with sterile gauze.

The porcine vaginal tissue was fixed using a mucoadhesion rig (A-MUC) which was placed on the equipment's base. Immediately before measurement, the tissue was hydrated with 50 µL of mVFS (containing mucin, since it is the protein most likely to be responsible for bioadhesion). Double-sided adhesive tape was used to attach a small piece of cellulose acetate membrane to the probe. Formulations were directly weighted on the cellulose acetate membrane attached to the probe, then 30 mg of formulation was adsorbed on the probe surface. The whole system (mucoadhesion rig with the tissue and the probe with the formulation) was kept at 37 ± 1 °C in an oven.

The texturometer's software was used in *bioadhesion mode*. The pre-test speed was 0.5 mm/s with a trigger force of 0.02942 N to allow for sensitive detection of the tissue. The test

speed and the post-test speed were 0.1 mm/s [240,250]. The contact/hold time was 180 seconds, and the force applied was 2.5 N [240,251].
In total six porcine vaginal tubes were used. Each vaginal tube was divided into small portions. The 8 formulations and the control were tested on adjacent portions of epithelium from the same vaginal tube.

The measurement output parameters maximum force of detachment (Fmax), debonding distance (Ddist) and work of adhesion (Wad) were used to characterize the bioadhesive potential of the formulations. These parameters were compared to the control, consisting of a cellulose acetate membrane fixed in the double-sided adhesive tape.

2.2.10. Evaluation of anti-*Candida* activity

2.2.10.1. Micromethod protocol of the standard guideline CLSI M27 "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts"

In order to evaluate the *in vitro* anti-*Candida* activity, we used the broth microdilution method, according to the international protocol M27 – A2 of CLSI "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts" [252]. For these studies we used a collection *Candida albicans* strain (ATCC 10231) and clinical strains of *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* obtained from vulvovaginal human isolates belonging to the CICS-UBI microbiology laboratory strains collection. The highest concentration studied was 20%, since the viscosity of gels did not allow testing at higher concentrations, due to limitations related to pipetting.

The microorganism suspension was prepared by inoculating isolated colonies of each specie in saline solution, to a final concentration of 10^6 CFU/mL estimated using optical density measurements (McFarland Densitometer DEN-1, *Biosan Medical-Biological Research & Technology*, *Latvia*). The suspensions were then diluted in RPMI medium to a final concentration of 1000 CFU/mL, according to the ML27 protocol.
In a multiwell plate, serial dilutions of formulation were incubated with the suspensions in RPMI medium, at 37 ºC for 24 h (Binder incubator, *GmbH*, Germany).

The relative viability of *Candida spp.* in multiple concentrations of each formulation was calculated by comparing the absorbance at 600 nm with the control (strain tested and medium) (Microplate reader Bio-Rad Xmark spectrophotometer, *Bio-Rad Laboratories*, USA).
Additionally, MIC values were read visually after 24 h of incubation at 37 ºC by macroscopic observation of the turbidity in the culture medium. MIC value was defined as the lowest concentration of formulation that inhibited visible yeast growth (absence of turbidity).

All determinations were performed in duplicate, and the concordant results of two independent experiments were considered.

2.2.10.2. Anti-*Candida* activity: Protocol adapted from Challenge Test of European Pharmacopoeia 10.0

The ability of the gels to control the proliferation of *Candida albicans* was studied through a protocol based on the Challenge Test (monograph 5.1.3. Efficacy of antimicrobial preservation) of the European Pharmacopoeia (10th Edition), used to evaluate the preservative capacity of products. Briefly, formulations were contaminated with a suspension of *Candida albicans* ($1x10^4$ CFU/mL) and incubated at 37 ºC during 48 h (Binder incubator, GmbH, Germany).

After 24 and 48 h, the CFUs counts were performed by plating 1 mL of serial dilutions (in water) of the contaminated formulations in Sabouraud Dextrose Agar and incubated at 37 ºC for 24 h.

The log (CFU/mL) after 24 and 48 h of incubation of formulation with the microorganism was calculated using the average value of two concordant independent assays (with a standard deviation less than 1 log).

2.2.11. Cellular Toxicity

2.2.11.1 Epithelial Cells

The HeLa cell line was obtained from the American Type Culture Collection (ATCC-LGC Promochem, *Teddington*, United Kingdom). This cell line is derived from human uterine cervical adenocarcinoma. These cells were cultured in DMEM-F12 medium supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% FBS and is further referred to as DMEM complete medium (passages 54-70).

2.2.11.2 Test Product Preparation

The cytotoxicity of the most promising formulation (formulation C) was tested. The formulation was diluted to 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78% (w/v), in complete culture medium. A negative control (NC) (cells only with culture media), solvent control (cells with 50% diluted culture media) and positive controls (PC) SDS 5% (w/v) were included.

2.2.11.3. Cytotoxicity test (MTT Assay)

The MTT reduction assay was performed as previously described and according to ISO/EN 10993-5 for *in vitro* evaluation of medical devices [253–256]. Cells were seeded onto 96-well plates (100,000 cells/mL) with complete culture media. Cells were left to adhere for 24 h at 37 C, under 5% CO2 atmosphere. After obtaining a half-confluent culture, 100 µL of the test formulation was added and left in contact for 24 h. After this period, cells were washed with PBS and incubated for 4 h with a 0.5 mg/mL solution of MTT reagent prepared in culture medium. Subsequently to formazan crystals formation, extraction was accomplished with 100 µL of 2-propanol for 15 min, through mild agitation on an orbital shaker, protected from light. Absorbances were then measured at 570 nm, using a microplate spectrophotometer (GloMax,

Promega). In addition, cells were observed and photographed before and after the application of the MTT reagent solution to visualize the formation of the formazan crystals, using an inverted microscope (VWR, model IT415PH) (data not shown). The NC consisted of cells without any treatment (only culture media in assay), which was considered as the 100% viability reference for products toxicity calculation.

2.2.12. Vaginal irritation - Hen's Egg Test-Chorioallantoic Membrane Assay (HET-CAM)

2.2.12.1. Eggs and incubation conditions

The HET-CAM assay was performed on fresh fertile White Leghorn chicken eggs that were clean and weighed (45-65 g). Upon arrival at the lab, eggs were checked for damage. Damaged eggs were discarded, and undamaged eggs were incubated at 37.8 ± 0.3 ºC in a relative humidity of 58 ±2% and under automatic rotation for 8 days (Corti AF-50 and Copele 30652, Spain). On the eighth day, eggs were observed using a LED light to confirm embryo formation. Non-embryonated and non-viable (dead) eggs were discarded. Viable eggs were incubated for one further day under the same conditions of temperature and humidity, but without rotation.

2.2.12.2. HET-CAM assay

Previous studies reported the suitability of the HET-CAM assay for vaginal irritation testing of medicines, cosmetics, hygiene products or medical devices in preclinical safety assessments [257]. The HET-CAM assay (according to the ICCVAM – Recommended Test Method (NIH Publication No. 10-7553 – 2010) was used to test the potential vaginal severe irritation of gel C since it allow the identification of severe irritants [258]. The test does not provide differentiation amongst mild irritants and non-irritants. At day 9, eggs were taken out of the incubator and placed on an appropriate support. The shell was opened using a scalpel and tweezers and the internal membrane was exposed and then hydrated with NaCl 0.9% (w/v) for a maximum of 30 min. The solution was aspirated and the membrane was carefully peeled off, without damaging the blood vessels. 0.3 mL of gel C and control solvents was applied to the chorioallantoic membrane (n=3 eggs per sample). NaCl 0.9% (w/v) was used as negative control and NaOH 0.1N and SDS 1% were used as positive controls.

The irritant effects of gel C and controls were evaluated by observation of three endpoints: haemorrhage (vessel bleeding), lysis (vessels disintegration) and coagulation (intra and extra-vascular protein denaturation) at predetermined time intervals (0.5, 2, and 5 min). These observations allowed calculation of the irritation score (IS (A)) as the sum of the scores obtained at each time point (table 5). The irritancy classification is defined as non-severe irritant (IS 0-9) or severe irritant (>9 to 21). Photographs were taken at each time points (1 egg per sample).

Table 5. Irritation score calculation according to the endpoint at each time point

Endpoint	Score at time point		
	0.5 min	2 min	5 min
Lysis	5	3	1
Hemorrhage	7	5	3
Coagulation	9	7	5

2.2.13. Screening stability tests

For screening of stability, gel C was submitted to centrifugation and temperature cycling tests. In the first test, gel C was centrifugated (Heraeus Multifuge 1S-R centrifuge, *Thermo eletric corporation*, UK) at 3000 rpm for 30 min. The appearance of gel (homogeneity, color, transparency, apparent viscosity) was evaluated before and after centrifugation [259]. Temperature cycling tests were performed since they can be more severe than continuous storage, and can reveal possible changes more quickly. [260–262]. In the cycling test, the gel was submitted to alternating heating and refrigerating conditions: 40 ± 2 ºC (Binder incubator, *GmbH*, Germany for 24 h, and then 4 ± 2 ºC for 24 h, for a total period of 4 weeks. The sensorial characteristics, pH, viscosity and osmolality were evaluated before and after the test. Gel C was stored at room temperature (25 ºC) for 24 h before these parameters were evaluated.

2.2.14. Data Processing and Statistical Analysis

Data were analysed to produce arithmetic means and standard deviations. Analysis of variance (using the Bonferroni post-hoc test) was performed to determine the significance of the difference between sets of data ($p < 0.05$). A two-way ANOVA (Dunnett's multiple comparisons test) was performed to compare formulations determinations with the control, at a 95% level of significance. The correlation between parameters was calculated using Pearson Correlation, whenever the data had a normal distribution. If the data did not pass the normality test (Shapiro Wilk or Kolmogorov-Smirnov, $p<0.05$), the correlation between parameters was determined by the Spearman rank based non-parametric test.

All analyses were conducted using GraphPad Prism (version 6.01 for Windows, GraphPad Software, San Diego, USA).

2.3. Results and discussion

2.3.1. Sensorial Characteristics

Patient acceptability has a significant influence on treatment compliance. Acceptability is multifactorial and is strongly dependent upon a product's sensory properties, such as appearance, odour and texture [103,198,263,264] Acceptability and the user meaning of sensory properties of vaginal dosage forms may vary as based on intended use, geographical region, culture, previous experiences and sexual practices [103,263].

In the design of the formulations here studied, women's preferences and acceptability were considered. Previous studies showed that women have positive preferences for vaginal products presented as semisolids (gels, creams or ointments), odourless and colourless [103,198]. Gels were the dosage form we selected regarding women's preferences, since gels are

comfortable, easy to apply and have the ability to spread onto the surface of vaginal epithelium [103,198,265,266].

The sensory properties of the developed formulations were in accordance with this description (table 6).

Table 6. Sensorial characteristics of formulations: general aspect, homogeneity, colour, odour and feel to touch

Formulation	General aspect	Homogeneity	Colour	Odour	Feel to touch
A	Semi solid. Very viscous, consistent gel.	Homogeneous	Colourless, transparent	Odourless	Soft, not particulated
B	Semi solid. Fluid gel.	Homogeneous	Colourless, transparent	Odourless	Soft, not particulated
C	Semi solid. Low consistency gel, very fluid.	Homogeneous	Slightly whitish, transparent	Odourless	Soft, not particulated
D	Semi solid. Fluid gel.	Homogeneous	Colourless, transparent	Odourless	Soft, not particulated
E	Semi solid Very viscous, consistent gel	Homogeneous	White, opaque	Slight castor oil characteristic odour	Soft, not particulated
F	Semi solid. Low consistency gel, very fluid.	Homogeneous. After several hours of rest, phase separation begins to occur which is reversible after shaking.	Pearl white, translucent	Slight castor oil characteristic odour	Soft, not particulated
G	Semi solid. Low consistency gel, very fluid.	Homogeneous. After several hours of rest, phase separation begins to occur which is reversible after shaking.	Pearl white, translucent	Slight castor oil characteristic odour	Soft, not particulated
H	Semi solid. Fluid gel.	Homogeneous. After several hours of rest, phase separation begins to occur which is reversible after shaking.	Pearl white, translucent	Slight castor oil characteristic odour	Soft, not particulated

2.3.2. pH

This property was determined directly on the plain formulations and on formulations diluted in mVFS (pH of 4.22) at 37 ºC ± 0.5, used as the standard during measurements simulating *in vivo* conditions in the vagina (table 7).

Table 7. pH and osmolality studies of formulations included in this study. Results are presented as mean ± standard deviation (n=3). Measurements were performed on plain formulations and after being diluted in mVFS (5g+ 0.825 mL mVFS)

	Direct pH at room temperature 25 ºC ± 0.5	**pH of formulations diluted in VSF at 37º C ± 0.5**	**Direct osmolality**	**Osmolality of formulations diluted in VSF**
	pH ± S.D.	**pH ± S.D.**	**Osmolality ± S.D. (mΩ/Kg)**	**Osmolality ± S.D. (mΩ/Kg)**
A	7.10 ± 0.03	6.25 ± 0.07 $^{\delta}$	55 ± 2	85 ± 3$^{\delta}$
B	8.83 ± 0.08	8.80 ± 0.04	6 ± 2	41 ± 1$^{\delta}$
B1	7.06 ± 0.07	ND	ND	ND
C	8.55 ± 0.03	8.41 ± 0.06 $^{\delta}$	845 ± 7	761 ± 6$^{\delta}$
D	9.20 ± 0.05	9.08 ± 0.02 $^{\delta}$	1016 ± 1	917 ± 7$^{\delta}$
E	7.27 ± 0.07	6.19 ± 0.04 $^{\delta}$	47 ± 2	84 ± 3$^{\delta}$
F	8.90 ± 0.08	8.08 ± 0.05 $^{\delta}$	13 ± 3	48 ± 1$^{\delta}$
G	8.95 ± 0.05	8.81 ± 0.02 $^{\delta}$	861 ± 7	784 ± 2$^{\delta}$
H	9.32 ± 0.04	9.24 ± 0.04	1069 ± 2	949 ± 7$^{\delta}$
Carbomer placebo	2.81 ± 0.06	ND	ND	ND
HPMC placebo	7.42 ± 0.02	ND	ND	ND

ND: not determined

$^{\delta}$ represents statistically difference between dilutions with mVFS and the undiluted formulation (two-way ANOVA, Bonferroni Post-test, $p < 0.05$).

Both the dilution and the increase from room temperature (e.g. during product storage) to body temperature (37 ºC) may change the characteristics and properties of the formulations. Therefore, these conditions should be included in technical evaluations related to the *in vivo* performance of products [240,245].

As expected, an increase in sodium bicarbonate concentration led to an increase in the pH of the gel (formulation A with 1% of sodium bicarbonate (pH 7.10) and formulation C with 5% of sodium bicarbonate (pH 8.55); formulation B with 1% of sodium bicarbonate (pH 8.83) and formulation D with 5% of sodium bicarbonate (pH 9.20)). This is due to the sodium bicarbonate dissociation, as described by the *Henderson-Hasselbalch* equation [267].

The type of polymer influenced the pH of the formulation. Gels that contained carbomer as the gelling polymer had lower pH values than gels that contained HPMC. Results for carbomer based formulations (A, C, E, G) were statistically different from HPMC based formulations (B, D, F, H) (two-way ANOVA, Bonferroni Post-hoc test, $p<0.05$). These results can be explained by the acidic nature of carbomers which are polymers formed from repeating units of acrylic acid crosslinked with allyl sucrose or allyl pentaerythritol, while HPMC is a non-

ionic cellulose derived polymer. The colloidal acidic dispersion of the polymer was neutralized by the addition of sodium bicarbonate [268–270].

The addition of castor oil to gels resulted in increased values of pH, which were significant different between gels A and E and between gels C and G (two-way ANOVA, Bonferroni Post-hoc test, $p<0.05$).

After diluting formulations in mVFS the pH decreased slightly. Machado et al determined the pH of some commercial antifungal vaginal products directly on the product and after dilution with mVFS (5g of gel + 0.75mL mVFS), prepared as described by Owen and Katz [239]. The pH values of commercial antifungals were significantly lower compared to the formulations herein prepared (Dermofix® pH=2.70, Gino-Canesten® pH=5.89, Gino Travogen® pH=3.56, Gyno-Pevaryl® pH=2.74, Lomexin® pH=3.57 and Sertopic® pH=2.71). After dilution in mVFS the pH values became closer to physiological vaginal pH, ranging from 4.02 to 4.22 [240]. The pH of the sodium bicarbonate formulations was higher than those of most commercially available products, due to the basic nature of the active substance.

Ideally, the pH of vaginal formulations should be within the vaginal pH range. This is normally considered to be 3.5–4.5, but may vary according to hormonal stimulation, menstrual cycle phase, presence or absence of infections. [1–3,17,104,271,272]**.** In menopausal women pH values up to 6.7 have been reported [273]. Furthermore, vaginal pH and flora differ significantly by ethnicity. Ravel et al reported that pH was higher in Hispanic women (pH 5.0 ± 0.59), followed by black women (pH 4.7 ± 1.04), Asian women (pH 4.4 ± 0.59) and white women (pH 4.2 ± 0.3). These values are related to the composition profile of the vaginal microbiome associated with these different groups [1]. It is well known that increased vaginal pH is associated with the growth of potential pathogenic bacteria [3,17,101,109,113,274].

Although sodium bicarbonate confers a basic pH to preparations, which is higher than healthy vaginal pH, it has been used for vaginal administration, particularly as an alkalizing agent in vaginal douching for the treatment of cytolytic vaginosis, an underdiagnosed pathology that mimics VVC [222–224,275,276]. Baths with sodium bicarbonate solutions (30 to 60 g of sodium bicarbonate to approximately 1L of water) two to three times per week and then once or twice a week) and vaginal irrigations (15 to 30 to 0.5L of water) two to three times per week and then once or twice a week have traditionally been used to treat cytolytic vaginosis [222,224]. These preparations contain 3-6% sodium bicarbonate, similar concentrations to the prepared gels here. The pH of 3% sodium bicarbonate solution was 8.18 and the pH of 6% sodium bicarbonate solution was 8.51. Therefore, the use of preparations based on sodium bicarbonate with this pH range is currently used in gynaecology.

We also note that in the testing here the reference amount of product was the recommended dose of benchmark products (sold with 5g product applicators). However, the application of 5g of product in the vaginal cavity is known to be excessive and can promote leakage [277]. Therefore, smaller applied amounts of bicarbonate gel can be envisaged (for example 1g as used for hormonal replacement and up to 3g which have been proposed for lubricants). These would experience higher dilution in vaginal fluids and the resultant pH would be decreased. For gel C pH measurements were also performed for the proportion 1g of

gel+0.825 mL of mVFS and for 3g of gel + 0.825 mL of mVFS. The pH of mixtures decreased to 8.08 and 8.36, respectively. Further, unlike use of lubricants, which are not recommended if product pH exceeds 7, our products are not intended for chronic or recurrent use. They are for a time-limited treatment (marketed products are generally used as daily administration, for 7 days). [278].

Clearly, the evaluation of high pH effects on vaginal milieu in prolonged treatments shall not be underestimated and further studies shall be designed if this purpose is considered, for example for prevention of relapses.

2.3.3. Osmolality

As expected, gels with higher concentration of sodium bicarbonate (higher amount of solute) present higher values of osmolality: formulation C presented higher osmolality than formulation A and formulation D presented higher osmolality than formulation B (table 7). The addition of castor oil to the base formulations did not significantly affect osmolality values, as expected.

As described by Machado et al, a complete osmolality assessment should not only comprise the direct measurement but also the dilution in the mVFS, since this methodology has the capacity to early predict the *in vivo* formulation behaviour [240]. The dilution of formulations in mVFS resulted in a decrease of osmolality of hyperosmotic formulations and in an increase of osmolality of hypoosmotic formulations, since mVFS is isosmolar (262 mOsmol/kg). As described for pH determinations the amount of formulation applied in the vagina obviously impacts the final osmolality after dilution with the vaginal fluids. Therefore, for gel C we also performed osmolality measurements considering the proportion of 3f of gel + 0.825mL of mVFS and 1f of gel + 0.825mL of mVFS. In this case the osmolality of the mixtures decreased to 707 and 580 mOsm/Kg respectively, becoming closer to the physiologic values.

The osmolality of the mVFS (262 mOsmol/Kg) was similar to that described in the literature for mVFS prepared as originally described by Owen and Katz without mucin [240,245]. This value was in accordance with those reported to the biological female vaginal secretions (260–290 mOsmol/Kg) [278]. Ayehunie et al reported a slightly higher osmolality value for vaginal fluids obtained from healthy women of reproductive age when collected with a menstrual cup (370 ± 40 mOsm/Kg) [279].

In this study, the osmolality of all formulations was lower than 1200 mOsmol/Kg – the limit value considered in the advisory note about lubricants for male and female condoms of The World Health Organization (WHO in collaboration with the United Nations Population Fund and Family Health International. In the same advisory note, it was reported that values of 380 mOsmol/kg or lower as desirable (hypo and isosmolal), [278]. Osmolality has been used as safety parameter for vaginal semi-solids (especially lubricants), since hyperosmolar lubricants have been associated to vaginal toxicity [247,279–281]. Machado et al evaluated the osmolality of vaginal antifungal products. The osmolality of the products tested was very different, ranging from 43 ± 2 mOsmol/Kg (Gino Travogen) to 1446 ± 20 mOsmol/Kg (Lomexin) [240].

Only formulations A, B, E and F had osmolality lower than the desirable value referred by the advisory note (380 mOsm/kg). Formulations A, B, E and F were hypoosmotic and formulations C, D, G and H were hyperosmotic with osmolality results below 900 mOsm/Kg (table 7). The impact of these hyperosmotic formulations on the vaginal epithelium, even if the treatment is time-limited, must be evaluated.

The osmolality of sodium bicarbonate solutions 3 and 6% currently used to treat cytolytic vaginosis was 616 and 1191 mOsmol/kg, respectively.

2.3.4. Viscosity

The viscosity of formulations was determined on plain formulations at 25 ºC and after dilution in mVFS at 37 ºC. Product viscosity may change after its application due to the effect of dilution in vaginal fluids, and environmental temperature, self-cleansing action of the vagina and even women's movements [240,243,245,282]. All gels exhibited shear thinning behaviour, in which the viscosity decreased with increasing applied shear rate (rotational speed in the rheometer, data not shown). Neutralization of the carbomer polymer (with alkaline agents such as sodium bicarbonate) produces different viscosities depending on the final pH of the preparation. Gel C had a higher pH than gel A, and a resulting lower viscosity [268].

Leakage out from the vaginal canal is one of the disadvantages of semi-solid drug-dosage forms [103,198]. The rheological properties influence the efficacy of semi-solid products. [240,244,249,282–284] More viscous formulations are less likely to leak through the vaginal canal, presenting longer retention time in the vagina. However, less viscous formulations flow better through the applicator, spread more easily and better coat the epithelium. Formulations A and E were the most viscous and are the most likely to have longer retention times in the vagina (figure 2 – part 2). In contrast, formulations F and G were the less viscous and therefore more likely to leak out from the vaginal canal (figure 2 – part 1).

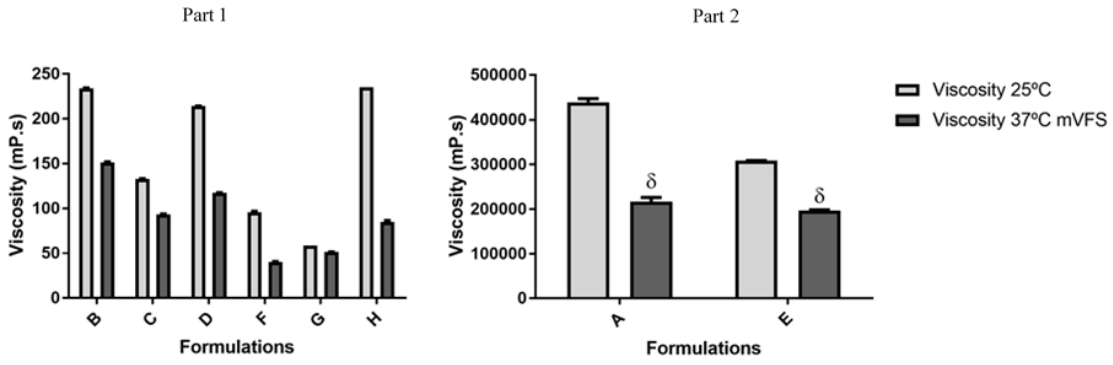

Figure 2. Determination of viscosity directly at room temperature and after diluting in mVFS at 37 ºC. Part 1: Formulations A and E, determined using shear rates of 0.4 1/s; Part 2: Formulations B, C, D, F, G, H, determined using shear rates of 500 1/s. Individual columns and vertical bars represent mean and SD values, respectively (n=3).$^{\delta}$ represents statistically difference between dilutions with mVFS and the undiluted formulation (two-way ANOVA, Bonferroni post-test, $p < 0.05$).

2.3.5. Texture: firmness and adhesiveness

The textural properties (firmness and adhesiveness) were determined by mechanical measurements (figure 3).

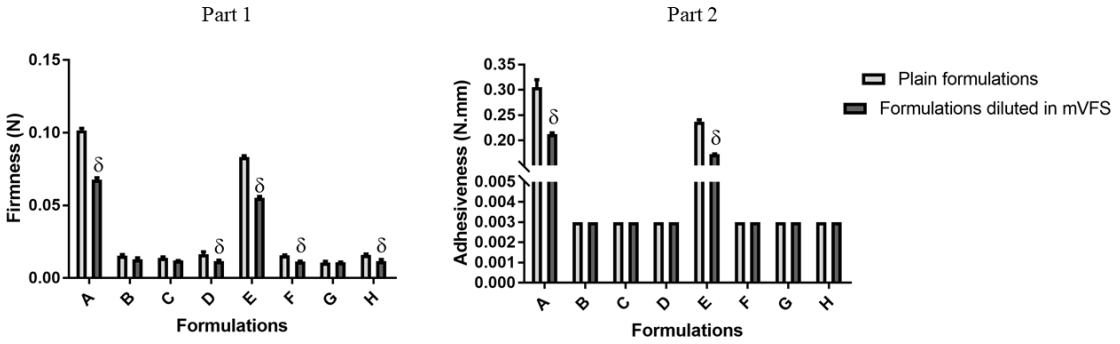

Figure 3. Determination of firmness (N) adhesiveness (N.mm) directly at room temperature and after diluting in mVFS at 37 ºC. Individual columns and vertical bars represent mean and SD values, respectively (n=3)
δ represents statistically difference between dilutions with mVFS and the undiluted formulation (two-way ANOVA, Bonferroni post-test, $p < 0.05$).

Formulations A and E exhibited the highest values of firmness and adhesiveness.

After dilution in mVFS the adhesiveness of formulations A and E decreased ($p<0.05$). For other formulations there were only slight variations in adhesiveness and the firmness decreased (statistically significant for A, D, E, F, H, $p <0.05$) except for G where it was unchanged.

These measurements of firmness complement the rheological measurements, in that firmness also relates to the ability to spread over the epithelium. Less firm products spread faster and more easily on a surface [240].

Firmness results were compared with those for viscosity. There was a strong correlation at 25 ºC in plain formulations (Spearman nonparametric test, CI95%, r=0.8251), as well as at 37 ºC after diluting formulations in mVFS (Spearman nonparametric test, CI95%, r=0.7618). Formulations A and E exhibited the highest values of firmness and viscosity, indicating these formulations are more likely to be retained in the vagina, but have diminished and/or delayed ability to spread and cover the entire epithelium over time. Machado et al studied the textural behaviour of vaginal of a set of vaginal antifungal products, reporting that they have medium adhesiveness (0.400–0.600 N.mm, approximately) and medium to high firmness (0.070–0.300 N, approximately) [240]. The formulations developed in our study exhibited lower firmness and adhesiveness values than those for commercial antifungal products. Only the textural profile of formulations A and E was comparable to those of commercial products. Those products contain a number of excipients such as, liquid paraffin, cetyl palmitate, propylene glycol and cetostearyl alcohol, that influenced their textural profiles [240]. Unlike commercial products, the prepared formulations here are very simple with few added excipients.

2.3.6. Bioadhesion

We tested bioadhesion as the ability of a formulation to adhere to the porcine vaginal tissue. The test principle is based on applying a force that allows the formulation to create initial contact with vaginal tissue and then determine the force required to break the adhesive stresses (such as chemical bonds) resulting from that contact. The determination of a mucoadhesive profile using texture analyser could be influenced by instrument parameters (such as the probe and the base selected) and test conditions (contact time, contact force, test speed, post-test speed) and experimental design (biological surface). Such variations in the method make it difficult to directly compare studies [240,244,249,285]. Due to ethical and practical problems of using human tissues, the use of *ex vivo* vaginal tissues has been proposed. The porcine vaginal tissue is particularly similar to the human in terms of anatomy, physiology and histology [240,243,249,286].

The peak force (adhesiveness), the work of adhesion and the debonding distance were measured to determine the bioadhesive profile of formulations (table 8).

Table 8. Bioadhesive parameters (work of adhesion (N.mm), peak force-adhesiveness (N) and debounding distance (mm)) determined for the formulations. S.D. =Standard deviation (n = 3). $^{\alpha}$ represents statistically difference from the control, One-way ANOVA, Dunnett's Multiple Comparison Test, $p<0.05$.

	Peak Force (Adhesiveness) ± S.D.	**Work of Adhesion ± S.D.**	**Debonding Distance ± S.D.**
	N	**N.mm**	**mm**
A	0.053 ± 0.024	0.049 ± 0.020	2.522 ± 0.424
B	0.093 ± 0.044 $^{\alpha}$	0.056 ± 0.018	2.588 ± 0.420 $^{\alpha}$
C	0.095 ± 0.040 $^{\alpha}$	0.079 ± 0.026 $^{\alpha}$	2.352 ± 0.271
D	0.115 ± 0.032 $^{\alpha}$	0.089 ± 0.027 $^{\alpha}$	2.505 ± 0.220
E	0.088 ± 0.054	0.057 ± 0.035	2.367 ± 0.373
F	0.072 ± 0.056	0.036 ± 0.016	2.168 ± 0.642
G	0.065 ± 0.053	0.047 ± 0.037	2.480 ± 0.120
H	0.093 ± 0.034 $^{\alpha}$	0.065 ± 0.017 $^{\alpha}$	2.177 ± 0.350
Control	0.025 ± 0.008	0.020 ± 0.006	1.930 ± 0.456

It has been suggested that the work of adhesion is the most appropriate metric for evaluating the mucoadhesive ability of formulations [285]. Furthermore, in our study, there was a strong positive correlation between peak positive force-adhesiveness and work of adhesion (Pearson, IC95%, $r=0.8248$, $p<0.05$), indicating that both parameters change in the same way to represent the bioadhesive profile. So, herein, work of adhesion will be considered as bioadhesion.

The bioadhesion in formulations C, D and H was different from the control, performed without any formulation (One-way ANOVA Dunnett's Multiple Comparison Test, $p < 0.05$).

Formulations C and D were the most bioadhesive, while formulations F and G were the least bioadhesive.

There was no statistically significant difference in bioadhesion profile regarding the bioadhesive polymer used (two-way ANOVA Bonferroni post-test (IC95%). It has been previously reported that carbomer alone shows better bioadhesive properties than cellulose derivatives (such as HPMC), since carbomer has higher concentration of carboxylic groups, making it able to adhere strongly to mucosal surface [287].

The bioadhesion of formulations offers the possibility of creating an intimate and prolonged contact with the vaginal epithelium, promoting efficacy and improving patient compliance by reducing the frequency of administration [17,242,243,249].

Bioadhesive properties of formulations can be promoted by the use of bioadhesive polymeric excipients, such as polyacrylic acid derivatives (carbomer and polycarbophil), cellulose derivatives, chitosan, hyaluronic acid, alginate and carrageenan [242,244,249,265,288,289]. The rational design of formulations requires an adequate selection of mucoadhesive polymers. Carbomer is a safe and stable polymer, widely used in pharmaceutical industry, including vaginal products. [240,265,268,288–290]. Carbomer has a high ability to strongly adhere to the vaginal epithelium since it has a high density of carboxylic groups on the surface of the polymer, which are able to form hydrogen bonds with mucin [287,291]. HPMC is a nonionic bioadhesive polymer, presenting high swellability characteristics. It is nontoxic, non-irritant and stable at vaginal pH. The bioadhesive properties are related to the long chain structure, with large number of hydroxyl groups able to form hydrogen bonds with mucins [268,289,292].

Machado et al compared the bioadhesion with the pure textural parameters of vaginal commercial products. They found a moderate to strong uphill positive linear correlation with adhesiveness (Pearson, CI95%, r = 0.6233), suggesting that, in practice, bioadhesion could be predicted by adhesiveness in earlier stages of product development [240]. However, in the study here, the bioadhesion was compared to adhesiveness (at 37 ºC after diluting in mVFS) and the results did not have significant correlation (Pearson, CI95%, r=-0.2482).

2.3.7. Anti-*Candida* activity

2.3.7.1. Potential to inhibit the *Candida albicans*' growth

Using the micromethod protocol of the standard guideline CLSI M27 "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts", we concluded that formulation C had the most potent anti-*Candida* activity, since it exhibited the lowest MIC value (MIC=2.50%) (figure 4) [252]. Formulations B, B1, F and H were the less effective in inhibiting *C. albicans'* growth.

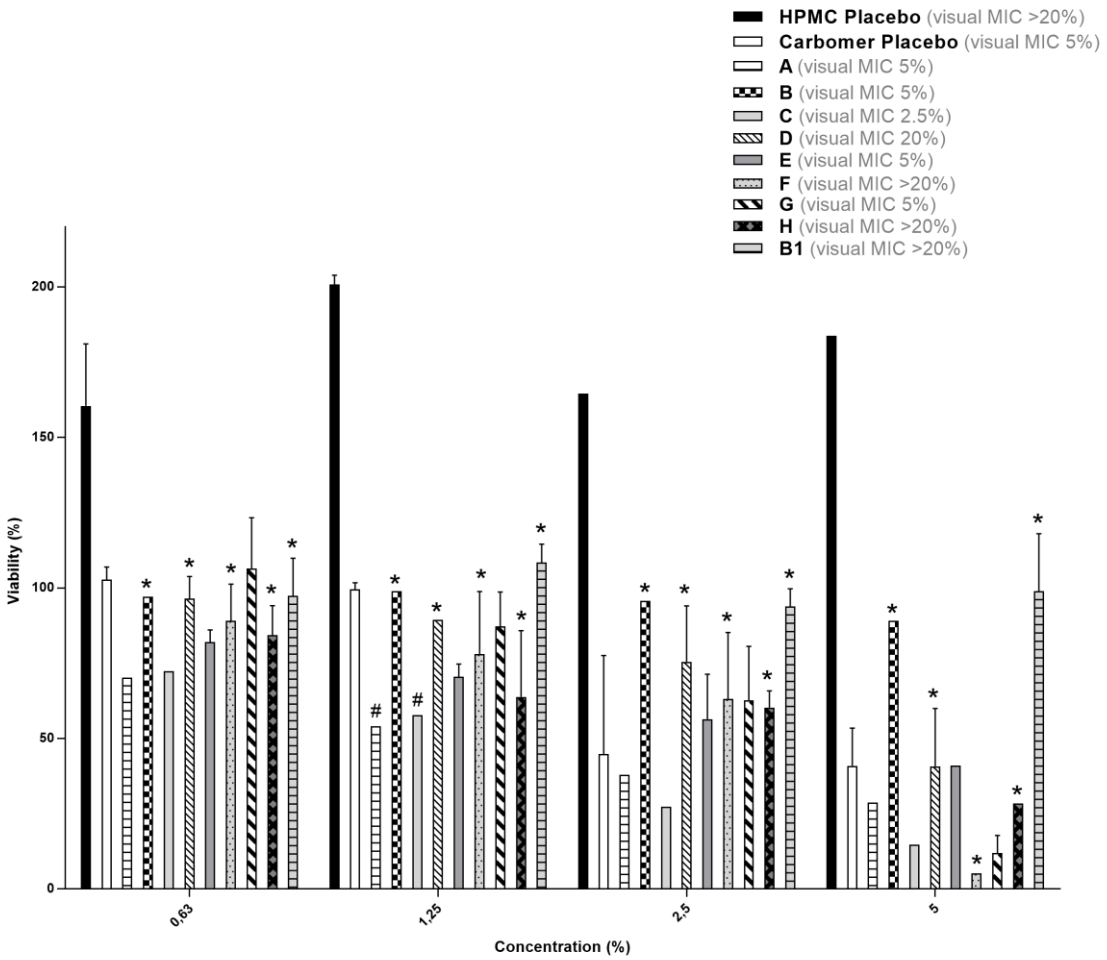

Figure 4. Viability of *Candida albicans* at different concentration of formulations evaluated with the micromethod protocol of the standard guideline CLSI M27 "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts" (mean and SD values).
Represents statistically difference from the control Carbomer Placebo (two-way ANOVA, Bonferroni Post-test, $p < 0.05$)
* Represents statistically different from the control HPMC Placebo (two-way ANOVA, Bonferroni Post-test, $p < 0.05$)

MIC determination is subject to possible operator visualization errors and to possible interference with turbidity generated by the formulation. In order to avoid these errors turbidity was compared with the negative control (formulation base and pure medium). To confirm the visual MIC results, the viability of the microorganism was determined after 24 h of incubation with the formulations by reading the absorbance at 600nm (figure 4).

According to the micro method protocol of the standard guideline CLSI M27, which considers the cut-off for susceptibility of the standard fluconazole as 50% viability, B and B1 were the less potent formulations (figure 4). For formulations F and H (visual MIC determined for concentrations >20%, figure 4), viability results were below 50% when the gel was tested at 5% concentration. This difference may be due to the opacity of these formulations related to their composition in castor oil.

Results from the protocol adapted from the Challenge Test of European Pharmacopoeia 10.0 showed that all formulations were resistant to contamination with *Candida albicans* since

they inhibited its growth (figure 5) [293]. The effect was more evident after 48 h of incubation, except for formulation G which had lower ability to inhibit growth at 48 h than at 24 h incubation.

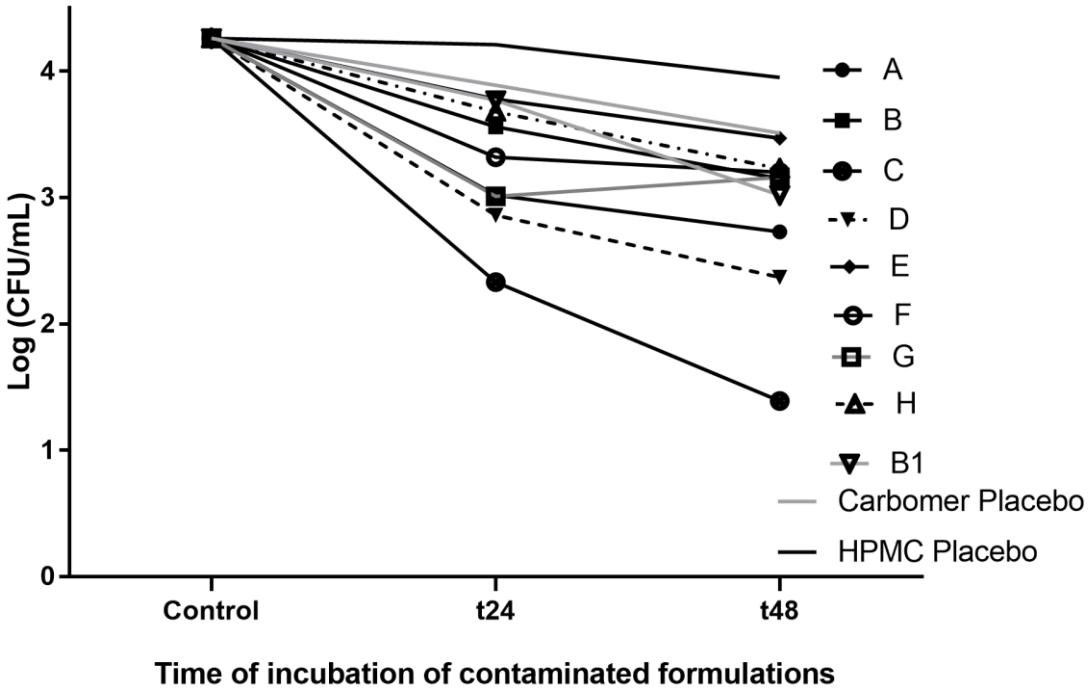

Figure 5. Variation of the Log (CFU/mL) with the time of incubation of formulation and *C. albicans* evaluated with a protocol based on the Challenge Test of European Farmacopoeia.

The international protocol M27 – A2 of CLSI is a reference method for testing antifungal susceptibility. On the other hand, the protocol adapted from the Challenge Test of European Pharmacopoeia 10.0 evaluates the capacity of a formulation to resist contamination. Both protocols were used to evaluate anti-*Candida* effects of our formulations. Results of both methods were globally concordant. Formulation C (5% sodium bicarbonate gellified with carbomer) was the most potent among those evaluated.

At both concentrations (1 and 5% sodium bicarbonate), gels inhibited the growth of *Candida albicans*, supporting the results of previous studies [84,85,218,219]. Gels containing 5% sodium bicarbonate inhibited *Candida albicans* growth more effectively than gels containing 1% sodium bicarbonate, comparing formulations with the same polymer. A higher sodium bicarbonate concentration promotes the anti-*Candida* activity of the testing formulations. It is important to note that low concentrations of sodium bicarbonate can stimulate germination at least in some conditions [218].

The base formulations without oil showed higher anti-*Candida* activity than the formulations with oil, comparing formulations with the similar concentration of sodium bicarbonate and the same polymer. It seems like the addition of oil may hamper the release of sodium bicarbonate from the matrix. Although several studies reported the anti-*Candida*

potential of oral cleasers containing *Ricinus communis* aqueous extracts and oil, in this study, the incorporation of castor oil in formulations did not improve the ability to inhibit *Candida albicans* growth. [232,234,237,238].

Comparing the formulations with the same sodium bicarbonate concentration but different polymers, we found those with carbomer were more effective than those with HPMC. The higher anti-*Candida* activity of gels containing carbomer comparing to that of gels containing HPMC does not seem to be related with their lower pH. Formulation B1 had the same excipients as B, but the final pH was adjusted to the same pH as formulation A. Formulation A inhibited more effectively *Candida albicans* growth than B1, according to both methods, indicating that the effect was determined by the polymer and not by the pH.

The anti-*Candida* activity of placebos was evaluated. According to the micromethod protocol of standard guideline CLSI M27, placebo HMPC promoted *C. albicans*' growth, since the viability was higher than that of the positive control in all concentrations tested. However, according to the protocol adapted from the Challenge Test, after 24h incubation, placebo HMPC had no effect on *C. albicans*' growth, although after 48 h of incubation there was a slight inhibition in growth.

According to both methods, carbomer appeared to be a more suitable excipient for use in anti-*Candida* products, exhibiting an intrinsic anti-*Candida* activity. The properties of the molecule or even the structure of polymeric network may interfere with some cellular component of *Candida albicans*. Andersen et al used carbomer-containing liposomes loaded with metronidazole as control to evaluate the anti-Candida activity of Chitosan-containing liposomes with metronidazole. For the Carbopol-containing liposomes with MET no inhibition of C. albicans growth was observed [56]. Those data do not reinforce a possible anti-Candida activity of the polymer, as we found in our study.

According to the protocol adapted from the Challenge Test, formulation E was the least potent inhibiting *Candida albicans* growth. On the other hand, the MIC for formulation E was 5%, indicating that formulation E was one of the most potent formulations. Furthermore, when the viability of *Candida albicans* was measured formulation E seemed to have an intermediate potential. The high viscosity of the formulation may have contributed to these variations between methods, especially in the visual assessment of MIC.

2.3.7.2. Anti-*Candida* activity of gel C against other etiological agents of VVC

Since gel C was the most promising formulation in inhibiting *Candida albicans*' growth its potential was also tested against other etiological agents of CVV. Gel C proved to have similar efficacy against all species tested, since the visual MIC was 2.5% for all species. Furthermore, the viability profiles per concentration of gel C were similar for all tested species (figure 6).

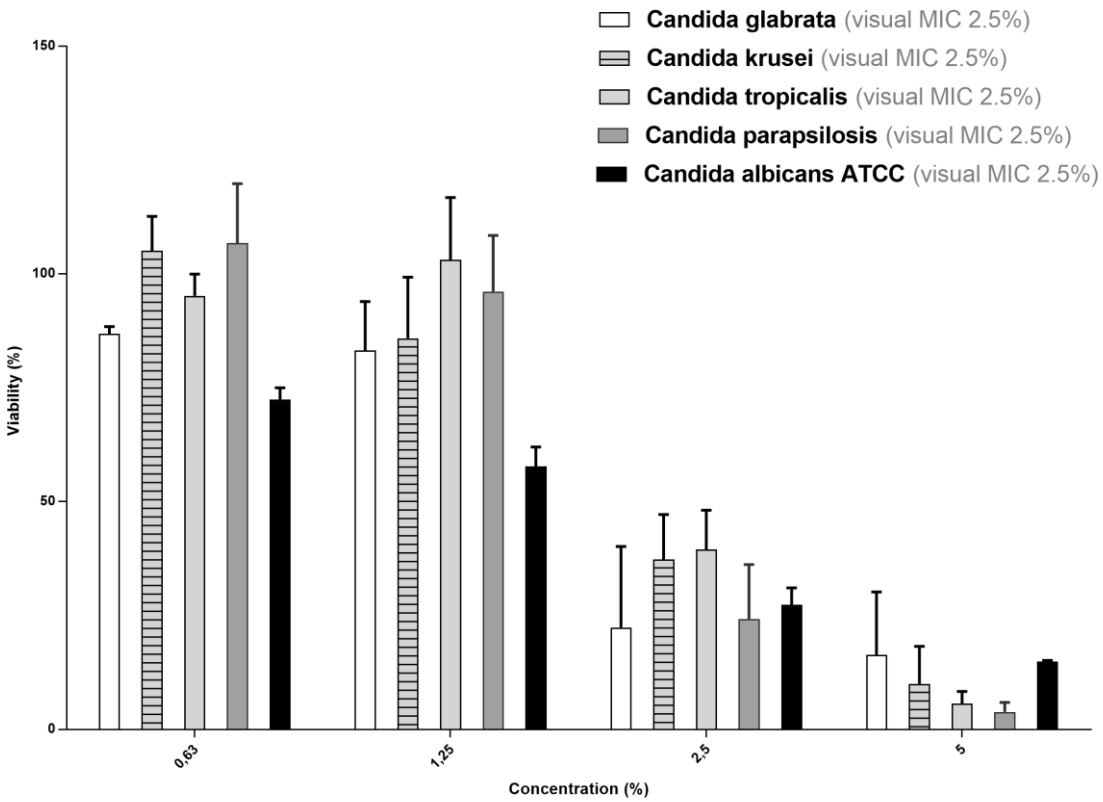

Figure 6. Viability of *Candida albicans* ATCC, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis* at different concentration of gel C evaluated with the micromethod protocol of the standard guideline CLSI M27 "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts" (mean and SD values).

2.3.8.Cellular toxicity

Formulation C was biocompatible with the tested cell line (figure 7). The rational design of all formulations here selected with their excipients and active substances are predictably biocompatible with vaginal epithelium.

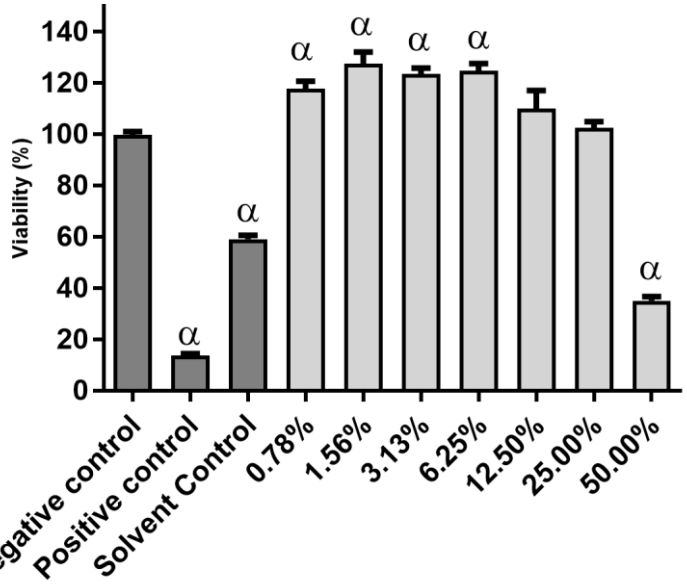

Figure 7. Cellular viability profile (MTT assay) for formulation C at dilutions ranging from 0.78% to 50% (w/v). Cell viability is represented as percentage of the control treated only with culture media. Results are presented as the mean and bars represent standard deviations from 3 independent experiments. α represents statistical difference from negative control.

Only at the concentration 50%, did formulation C exhibit toxic effect on the cells, representing a viability of 30% compared to the negative control. For concentrations equal or less than 25% there was no toxic effect. At the concentrations 0.78%, 1.56%, 3.15% e 6.25%, formulation C slightly increased growth of cervical epithelial cells. Importantly, at the minimum concentration demonstrating anti-*Candida* effect (MIC = 2.5%), formulation C was not toxic for the Hela Cell line. As expected, solvent control presented some viability reduction, since the culture medium was diluted at 50%. The viability of solvent control was significantly higher than the viability of the concentration 50% ($p<0.05$, one-way ANOVA (Dunnett's Multiple Comparisons Test) indicating that at the formulation concentration 50% there was some toxicity associated with the formulation.

2.3.9. Vaginal irritation - Hen's Egg Test-Chorioallantoic Membrane Assay (HET-CAM)

Gel C was defined as a non-severe irritant according to this methodology (table 9). The only endpoint observed was lysis (slight and limited) (figure 8).

Table 9. Irritation Score and classification of samples. Results are presented as mean values ± standard deviation (SD), n = 3

		IS (mean ± standard deviation)	**Classification**
Controls	NaCl 0.9% (w/v)	0 ± 0	Non-severe irritant
	NaOH (0.1N)	20 ± 1	Severe irritant
	SDS 1% (w/v)	10 ± 0	Severe irritant
Tested product	Gel C	4 ± 1	Non-severe irritant

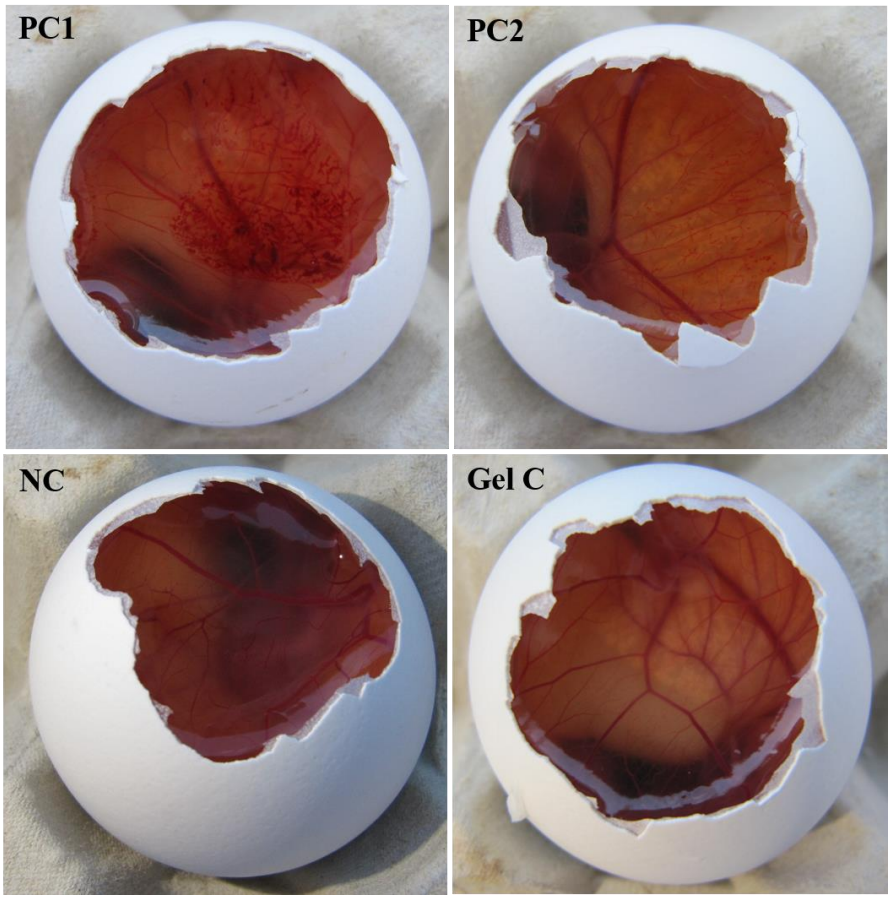

Figure 8. Irritation potential. Representative photographs for observation of the different endpoints at certain timepoints for negative control, positive controls and gel C. PC1: Positive control (NaOH 0.1N) at timepoint 5min with observations of lysis, haemorrhage and coagulation (egg 2). PC2: Positive control (SDS 1%) at 5 min with observations of lysis and hemorrhage (egg 1). NC: Negative control (NaCl 0,9%) at 5 min with none of the endpoints being observed (egg 3). Gel C at 2min. Lysis can be observed at this timepoint (egg 1).

For eggs 1 and 3, lysis was observed at timepoint 2 min and for egg 2 it was observed lysis at timepoint 0.5 min. Thus, the irritation score was 4 ± 1. This result shows that the pH and osmolality deviations from the ideal ranges considering vaginal application of gel C did not produce a severe irritation potential. The result can be explained by the selection of components with low irritating potential. Gel C contains carbomer 940 as polymer which is widely used and considered safe for vaginal administration and sodium bicarbonate, which is very biocompatible, being a physiologic component of the pH buffer solution in the body.

Palmeira-de-Oliveira et al, evaluated the vaginal irritation potential of commercial vaginal semisolids intended either as therapeutics for different pathological conditions or as lubricants for sexual or postmenopausal discomfort. Among the studied products, Ginix® exhibited the highest irritation score (3.7), followed by Ginix plus ®, KY Jelly®, Vidermina® and Geliofil® (irritation score of 3). Among the antifungals tested, Gyno Pevaryl® presented the higher value of irritation score (2.3) [257]. Thus, we can conclude that gel C, when tested using the same methodology, exhibited a potential for vaginal irritation similar to that of some commercial vaginal products.

2.3.10. Screening stability tests

Gel C maintained the same appearance after the centrifugation test. Further, no visible changes in the gel structure were observed: there was no syneresis.

In the temperature cycling test, no changes in appearance, color, odor and texture were detected. For the gel characteristics pH, osmolality and viscosity only small increases were detected, compared to the initial determinations (table 10).

Table 10. Evaluation of sensorial characteristics, pH, osmolality and viscosity after 4 weeks of cycling test (t4), comparing to the initial evaluation (t0)

	t0	**t4**
Sensorial Characteristics	Homogeneous fluid gel, slightly whitish, transparent, odorless, soft, not particulated	All characteristics were maintained
pH	8.62 ± 0.02	8.80 ± 0.01
Osmolality (mΩ/kg)	838 ± 2	849 ± 1
Viscosity (mPa.s) Shear rate 500 1/s	129.26 ± 0.09	130.29 ± 0.09

Overall, Gel C appeared to be stable in the context of the multiple characteristics evaluated here.

2.4. Conclusion

The results of this study showed that sodium bicarbonate gels are a possible and promising alternative strategy for treating VVC. Amongst a set of prototypes with differing compositions, formulation C (5% sodium bicarbonate, 1% carbomer and 94% water) was the most effective in inhibiting *C. albicans'* growth and it was also effective against other species of *Candida*. Additionally, formulation C exhibited sensorial characteristics that are in accordance with current knowledge of users' preferences. Formulation C exhibited low viscosity and low firmness, suggesting good spreading and coating of the epithelium. Reduced volume would help mitigate leakage. The mucoadhesive characteristics of the formulation contribute to its efficacy by promoting an intimate and prolonged contact with the epithelium Although the pH of formulation C was significantly higher than the healthy physiologic vaginal pH, we do not expect gel application to significantly alter vaginal pH since gel use is proposed as treatment for a short period of time. Additionally, gel C was hyperosmolar, which results from the dissociation of sodium bicarbonate, but its osmolality is still within that measured for marketed products for antifungal vaginal short-course treatment. The high pH and osmolality values of gel C compared to the reference values in standard guidelines for lubricant safety (which are products of recurrent use, continuous and not limited in time), are explained by the dissociation of the therapeutic agent that is essential for inhibition of *Candida*'s growth. However, based on the potent anti-*Candida* activity of the gel. we can propose application of a relatively low amount of product. The dilution of a lower amount of gel C (1 g, for example) in vaginal fluid results in pH and osmolality values closer to the physiologic ones. Despite not having an ideal pH and osmolality values for vaginal administration, we found that, gel C appears to be biocompatible. In fact, it demonstrated low potential for damage or irritation of vaginal epithelium in the tests performed thus far. Overall, although further safety studies are clearly needed, gel C is

promising as an alternative or complementary treatment to standard antifungal therapies for VVC.

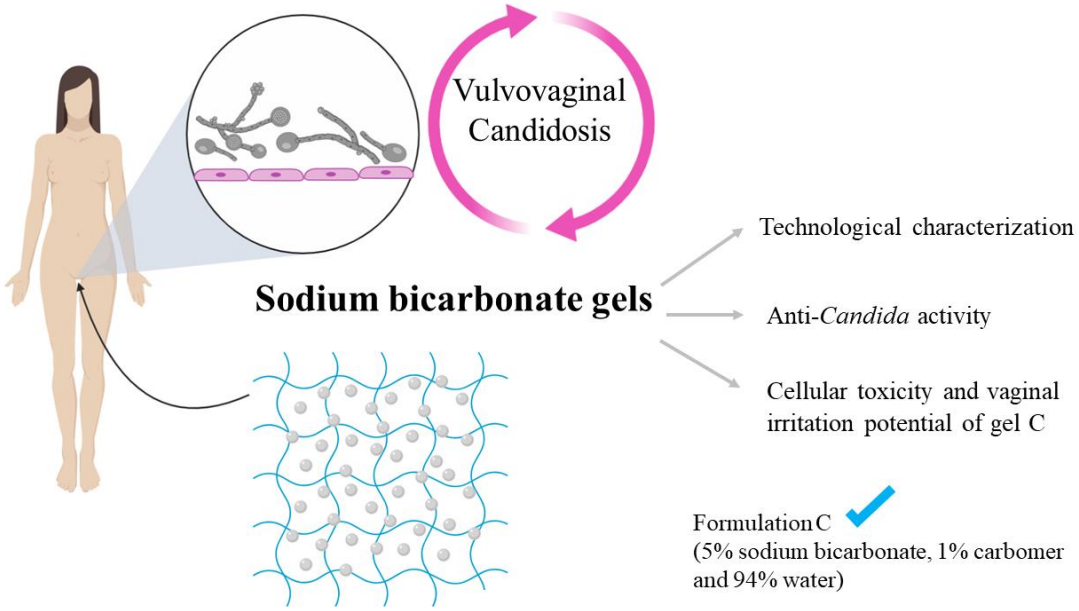

Figure 9. Graphical summary of chapter 2

Chapter 3

Vaginal sheets with *Thymbra capitata* essential oil designed for the treatment of bacterial vaginosis

The content of this chapter is partially published in:

- Rita Machado**, Mariana Tomás**, Ana Palmeira-de-Oliveira, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, "*The vaginal sheet: an innovative form of vaginal film for the treatment of vaginal infections*". Drug Development and Industrial Pharmacy. 2020, doi: 10.1080/03639045.2019.1711386

- Lúcia G. V. Sousa, Joana Castro, Carlos Cavaleiro, Lígia Salgueiro, **Mariana Tomás**, Rita Palmeira Oliveira, José Martinez Oliveira, Nuno Cerca, *"Synergistic effects of Carvacrol, α-Terpinene, γ-Terpinene, ρ-Cymene and Linalool against Gardnerella species"*. Scientific reports. 2022, doi: 10.1038/s41598-022-08217-w

Part of the content of this chapter will be published in:

- **Mariana Tomás**, Lúcia G. V. Sousa, Ana Sofia Oliveira, Carolina Gomes, Ana Palmeira-de-Oliveira, Carlos Cavaleiro, Lígia Salgueiro, Nuno Cerca, José Martinez-de-Oliveira, Rita Palmeira-de-Oliveira, "*Vaginal sheet with Thymbra capitata essential oil designed for the treatment of bacterial vaginosis". Submitted*

- Rita Palmeira-de-Oliveira, José Martinez-de-Oliveira, Ana Palmeira-de-Oliveira, **Mariana Tomás**, Nuno Cerca, Lúcia Sousa, Aliona Rosca, Joana Castro, Carlos Cavaleiro, Lígia Couto, "Folha vaginal para o tratamento e prevenção de vaginose bacteriana", (2021) patent pending process 20211000045617

3.1. Introduction

Alternative treatments for BV have been studied in view of reducing recurrences after standard antimicrobial treatments [102,294]. Natural compounds with antimicrobial activity against BV-related bacteria and substances that aim to re-establish natural saprophytic flora (probiotics, prebiotics and acidifying agents) have been proposed as alternative strategies to treat BV. *Thymbra capitata* EO and its main component carvacrol exhibited potent antibacterial activity against *G. vaginalis* planktonic cells. Additionally, *T. capitata* EO presented significant antibiofilm activity (more evident than the one found with isolated carvacrol), suggesting that this effect is not only dependent on the major component carvacrol but also on a synergic action of major and minor components. This antimicrobial effect is attributed to the hydrophobicity caused damage on cellular membrane leading to cell destruction. Importantly, *T. capitata* EO presented a selective antibacterial action, since it is significant less effective against lactobacilli, presenting MIC (1.25–2.50 µL/mL) and MLC values (1.25–2.50 µL/mL) near tenfold higher than those of *G. vaginalis* [147].

BV is caused by a replacement of saprophytic flora by anaerobe bacteria which act together in synergistic mechanism to form dense and structured biofilms. Polymicrobial biofilms, mainly constituted by *G. vaginalis* clusters that strongly adhere to vaginal epithelium associated with other bacteria such as *Atopobium vaginae*, *Mobiluncus* spp. and *Prevotella* spp., allow to organize a sophisticated internal architecture that avoid drug to penetrate them and to be effective [3,104,106,107,147,295,296]. The interaction between *G. vaginalis* that dominates the polymicrobial biofilms and other co-infecting bacteria (namely *Atopobium vaginae* and *Prevotella bivia*) is extremely complex and may involves the modulation of virulence factors and other biological processes which allow for an intimate cooperation and exceptional organization that represents a challenge for treatment [297–300]. Although *Gardnerella* species have been detected as the most common bacteria present in cases of BV, the role of this pathogen in the infection is still controversial, since *Gardnerella* colonization does not always cause clinical episodes of infection [301]. Recently, a study suggested that what has been previously referred to as *Gardnerella vaginalis*, in fact, includes 13 different species of the genus *Gardnerella*, of which 4 have been described: *G. vaginalis*, *G. piotii*, *G. leopoldii*, and *G. swidsinskii* [302]. Actually, it is not totally clear what the role of each species of *Gardnerella* in the initialization of infection and if a specific *Gardnerella* species has higher virulence potential [302].

In previous studies, our research group has proposed a formulation approach specifically designed for bacterial vaginosis in view of absorbing the excessive amount of vaginal fluid that characterizes this pathology as a swelling-hygroscopic formulation, immediately contributing to the relief of one of the symptoms that causes discomfort. The vaginal sheet is a variation of vaginal films but unlike vaginal films it is supposed not to dissolve immediately to release the drug. Furthermore, the sheet is bigger and thicker and must present the ability to absorb vaginal fluids without losing the essential structure at short time.

In this study we aimed to further explore and optimize this vehicle to promote the efficacy of incorporation of *Thymbra capitata* EO, while preventing its volatilization, providing

a suitable vaginal product for the treatment of BV. We aimed to develop and characterize vaginal sheets with *Thymbra capitata* EO regarding technological characterization, predictable *in vivo* performance, *in vitro* efficacy against *Gardnerella* spp. and safety.

3.2. Methods and materials

3.2.1. Materials

For the preparation of vaginal sheets the following excipients were used: glycerin (Acofarma, Spain); gelatin (Acofarma, Spain); lactic acid 90% (VWR, France); sodium lactate 50% (VWR, France); sodium sulphate anhydrous (Labchem, United States of America); lactose (Fagron, Spain); chitosan low molecular weight with 50-190 KDa (Sigma-Aldrich, Germany); Hydroxypropyl Methylcellulose (Methocel K100, viscosity 100000 cP, 2% aqueous solution) (Dow, United States of America); propylene glycol (Labchem, USA); Polyvinyl alcohol (PVA) 115000 (VWR, France) and MilliQ water (obtained in house through a Merck Milli-Q® Reference equipment).

Aerial parts of *T. capitata* plant were collected at the flowering stage in Lagoa, Algarve (south of Portugal). The oils were isolated by hydro distillation for 3 h, using a Clevenger-type apparatus according to the European Pharmacopoeia [303]. The oils were preserved in a sealed vial at 4 ºC.

For the preparation of the vaginal simulant the following excipients were used: sodium chloride (JT Baker, United States of America); potassium hydroxide (VWR, France); calcium hydroxide (Acros Organics, United States of America); Bovine Serum Albumin (Sigma, Germany); lactic acid (Sigma, Germany); acetic acid (Fischer Scientific, United States of America); glycerol (Acofarma, Spain); urea (VWR, France); glucose (VWR, France) and porcine gastric mucin type II (Sigma, Germany).

For the preparation of universal placebo hydroxyethylcellulose (Ashland, United States of America), sodium chloride (Honeywell fluka, United States of America) and sorbic acid 99% (Alfa Aesar, United States of America) were used.

Other reagents included: Dulbecco's Modified Eagle Medium F12 (DMEM F12) (Gibco, United States of America); RPMI medium (Biowest, United States of America); sodium bicarbonate (Sigma, Germany); 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) (Alfa Aesar, Germany); Fetal bovine serum (FBS) (Sigma, Germany); penicillin and streptomycin (Sigma, Germany); phosphate buffer solution (PBS) (VWR, France); sodium dodecyl sulfate (SDS) (VWR, France); dimethyl sulfoxide (DMSO) (Fisher Chemical, United Kingdom); trypsin (Fischer Scientific, United States of America); 2-propanol (Honeywell, United States of America); sodium chloride (JT Baker, United States of America) and sodium hydroxide (VWR, France), carvacrol (Sigma-Aldrich, United States of America), ρ-cymene (Sigma-Aldrich, United States of America) and linalool (Sigma-Aldrich, United States of America).

3.2.2. Rational design of vaginal sheets with *T. capitata* essential oil

Basic formulations of vaginal sheets, presented as a variation of vaginal films, with potential to incorporate actives for the treatment of vaginal infections, were previously developed by our research group [304]. The study developed in this thesis represents the continuation of this line of investigation by improving the ability of vaginal sheets to target specific pathological changes of BV (excessive fluids, pH changes), while delivering *T. capitata* EO as active substance. The outcomes of the initial stage of development of basic formulations were applied in the rational design of vaginal sheets with *Thymbra capitata* oil [304]. The proportion of polymer:plasticizer was maintained, although the concentration of gelatin was reduced to incorporate bioadhesive polymers that were selected for this work. The previously optimized method of production was applied. This is based on freezing vaginal sheets at -80 ºC overnight before the freeze-drying process, of 24 h duration, using a Scanvac CoolSafe™ freeze drier (temperature reached -110 ºC; pressure 0.019hPa) after which the obtained vaginal sheets were further coated with a rigorous amount of *T. capitata* EO. Throughout the development process, petri dishes were used as molds (4.5 cm diameter), for practical reasons although the envisaged final dimension of the product could be adapted to the physiological cavity (we previously proposed 7x2.4 cm, as desired dimension for that purpose). The rational design of formulations considered the selection of excipients approved for vaginal administration and the previous experience of the research group in the development of vaginal sheets. For the development of these vaginal sheets, PVA and HPMC were further added to chitosan and gelatin as bioadhesive polymers and a pH buffer solution was used to promote restoration of vaginal pH upon administration. As already stated, the *T. capitata* EO was added as active agent after the freeze-drying process, using a coating procedure.

Gelatin was used as the main macromolecule in all formulations since it is biocompatible, non-toxic, widely used in a variety of pharmaceutical formulations. It presents hygroscopic properties which allow to absorb the abundant vaginal fluid [265,268,304].

Chitosan was specifically selected to promote efficacy. Chitosan is a cationic polyamine with a high charge density at pH<6.5, that adheres to negatively charged surfaces, which can explain the excellent bioadhesive properties of this polysaccharide [249,265,268,305]. This biocompatible polymer can interact with epithelial tight junctions decreasing the epithelial barrier (in a reversible and mild process) but without causing epithelial damage [17,242]. The antimicrobial activity of chitosan, namely against agents that cause vaginal infections, has been studied. This antimicrobial activity can be explained by the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes that causes the disruption of microbial membrane and lead to leakage of intracellular constituents. Furthermore, chitosan can contribute to the disruption of BV-related biofilms, and also improve the treatment with metronidazole when used as excipient to formulate liposomes [17,56,58,199,200,202,294,306,307].

Polyvinyl alcohol (PVA) was also selected as a polymer to be included in these vaginal sheets. It is derived from polyvinyl acetate and is commonly used in rapidly dissolving vaginal films [268,308–314]. This polymer is biocompatible and it is particular suitable for vaginal

administration, due to its pH and osmolarity in solution, that are similar to those of the vaginal fluid [268,313].

Hydroxypropylmethylcellulose (HPMC) is a non-ionic, semi-synthetic cellulose derived polymer. It is water soluble and presents high swelling properties, while being non-irritant and stable at vaginal pH. HPMC also presents bioadhesive properties due to the long chain structure with hydroxyl groups which can interact with mucins, forming hydrogen bonds [268,289,292]. HPMC is commonly used as polymeric excipient in vaginal films and it allows to obtain flexible films [212,292,318,309–311,313–317].

Powdered active substances can also be included in vaginal sheets. Lactose (inert powder) was added as a placebo powder to study changes that a powder can induce in vaginal sheets physical properties. Anhydrous sodium sulphate (ASS) was studied due to its hygroscopic properties which can potentiate the ability to absorb vaginal fluid, if needed.

Glycerine and propylene glycol were used as plasticizers. Both are commonly used in vaginal products, including films, and are considered safe, although at high concentrations they can confer high osmolality to formulations which leads to toxicity [240,247,268,290,319–321]. Preliminary studies in the development of vaginal sheets as dosage form showed that glycerine may promote a better buffer capacity than propylene glycol. Moreover, propylene glycol showed higher potential to cause toxicity [322]. So, glycerine was preferentially used as plasticizer.

Lactic acid/sodium lactate (LA) pH buffer solution was the main solvent in gel preparation. LA was selected since lactic acid is the physiologic component produced by lactobacilli which allows the low pH that is the typical indicator of healthy vaginal fluid. BV is characterized by an increase of the vaginal fluid pH for values above 4.5 [17,104–106,109,110]. So, acidifying agents can be considered an alternative strategy to treat BV, because most BV-causing bacteria cannot grow at a pH below 4.5 and the acidic environment facilitates the growth of *Lactobacillus* species [109,121,150,294]. Some studies suggest that lactic acid, used as acidifying agent, can normalize vaginal dysbiosis, promoting the lactobacilli colonization and simultaneous inhibiting pathogenic bacteria's growth [5,159,160,294]. We incorporated in formulations the LA buffer solution, to further promote the reestablishment of physiologic vaginal pH after administration. The buffer solution is expected to be more effective in normalizing the pH than lactic acid alone. Two different strengths of buffer were tested.

We developed 7 prototypes of base formulations of vaginal sheets including variations of these excipients that were submitted to the following characterization assays.

3.2.3. Preparation of vaginal sheets with *Thymbra capitata* essential oil

To prepare vaginal sheets, homogeneous gels containing buffer solution, gelatin, polymers (HPMC, PA and chitosan) and plasticizers (glycerine and propylene glycol) were prepared according to table 11. For the preparation of gels, lactic acid/sodium lactate buffer was first heated to 50ºC (in a water bath) and then the plasticizers were added. Gelatin was dissolved in the mixture, and all the remaining constituents were incorporated. To prepare

formulation D, chitosan was first dissolved in lactic acid/sodium lactate buffer, that is, before the addition of plasticizer and gelatin. To prepare formulations E and F, PVA was first dissolved in buffer by heating in a water bath at 90 ºC, for 60 min. Mechanical stirring with a helical stirrer (Heidolph RZR 2041) at low speeds of rotation was performed to obtain homogeneous gels. Gels were centrifugated at 800rpm, for 5 min, to remove entrapped air bubbles. After centrifugation, 5 g of each gel were poured into standard small plastic Petri dishes (5.5 cm diameter). Formulations were allowed to cool down and were frozen at -80 ºC, overnight. The plates were then freeze-dried, during 24 h, using a Scanvac CoolSafe™ freeze drier (temperature reached -110 ºC; pressure 0.019 hPa).

T. capitata EO was added to one surface of the vaginal sheet as a coating to a final concentration of 1% w/w (weight of *T. capitata* EO/weight of vaginal sheet). For this purpose, *T. capitata* EO was spread with a spatula on the upper surface of base formulations. *Thymbra capitata* EO was mainly composed by carvacrol (73.9-80%).

The dimension of 7x2.4 cm was previously proposed for vaginal sheets, corresponding to the mean dimension of the human vagina [304]. Throughout this study, formulations were prepared and tested using the shape of the mold selected for freeze-drying (circular shape, 5.5 cm diameter), resulting in smaller portions of vaginal sheets (for laboratorial scale purposes).

Table 11. Qualitative and quantitative composition (% w/w) of gels used for vaginal sheets preparation.

	Water	LA (90% v/v)	Sodium lactate (50% v/v)	Gelatin	HPMC	PVA	Glycerine	Propylene glycol	ASS	Lactose	Chitosan
A	70.42	0.81	2.52	15.0			11.25				
B	67.54	0.79	2.42	15.0			11.25		3.0		
C	67.54	0.79	2.42	15.0			11.25			3.0	
D	63.65	1.56	4.79	13.5			15.0				1.5
E	81.82	0.95	2.93	4.0	1.0	2.4	6.0	0.9			
F	70.45	0.82	2.53	10.0		1.2	15.0				
G	70.65	0.82	2.53	10.0	1.0		15.0				

LA – lactic acid/sodium lactate buffer solution; HPMC Hydroxypropylmethylcellulose; PVA - Polyvinyl alcohol; ASS - Anhydrous sodium sulphate

3.2.4. Freeze drying efficiency

Conversion of gels into vaginal sheets was achieved through freeze dying. This process aims to eliminate water from the formulation. Freeze drying efficiency was calculated according to the follow expression, assuming that all weight loss was due to water removal:

$$\text{Freeze drying efficiency (\%)} = \frac{initial\ weight\ of\ gel - weight\ of\ sheet\ after\ freeze-drying}{weight\ of\ water\ in\ formulation} \times 100$$

3.2.5. Preparation of mVFS

The Modified Vaginal Fluid Simulant (mVFS) was prepared as described by Owen and Katz with the addition of mucin, as follows: sodium chloride 3.51 g; potassium hydroxide (KOH) 1.4 g; calcium hydroxide ($Ca(OH)_2$) 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; glucose 5.00 g and 15.00 g porcine gastric mucin type II were added to a milliQ water slightly volume less than 1L and stirred mechanically until complete dissolution [239]. The pH of the mixture was then adjusted to 5 using sodium hydroxide, to mimic the pH characteristic of BV (pathological condition), and the final volume was adjusted to 1 L.

Mucin was added to this preparation to simulate the bioadhesion properties of vaginal fluid [17,241,242].

3.2.6. Sensorial characteristics

The sensorial characteristics studied were colour, transparency (transparent, opaque, translucent); odour (odourless, characteristic odour, intensity of odour) and feel to touch (soft, hard, flexible, very flexible). The visual and sensory analysis was always performed by the same operator, 24 h after preparation of the formulation. Observations were recorded and photographs were taken.

3.2.7. Thickness

Thickness was measured using a digital micrometer IP54 (Vogel, VWR) on three different points (one at the center and two at side edges locations) of two independent vaginal sheets of each formulation.

3.2.8. Folding endurance

Folding endurance was measured by repeatedly folding the vaginal sheet at the same place (by horizontal and longitudinal folding), until broken. If the vaginal sheet was folded for 300 times without breaking, it was considered to pass this test [250,323].

3.2.9. Absorption efficiency of vaginal fluid simulant

The evaluation of the absorption efficiency of vaginal fluid simulant was performed using portions of vaginal sheets 1.75x0.6 cm (25% reduction of the proposed final size, maintaining the same proportion). For contact with vaginal fluid simulant not only the pH of the solution was adapted to the characteristics of this infection (see section 3.2.5) but also the volume of solution was increased in relation to physiologic conditions. It is generally considered that 0.75 mL are present in the vagina, in each moment, in normal (physiologic) conditions [239]. For BV, excessive amounts of vaginal fluids are present as a typical symptom, but the exact volume of these fluids is not specifically described in the literature. For the purpose of this work, it was considered that an excess of 50% of fluid characterizes BV. Therefore, 1.125mL of

fluid with pH 5.0 was considered to reproduce BV vaginal fluid. For contact purposes with the vaginal sheet, the volume of fluid was reduced to 25% to keep the proportion determined by the reduction of the size of vaginal sheet. So, 0.281mL of mVFS (25% of the vaginal fluid present in the vagina, at each moment, in BV), corresponding to 0.2834 g, based on density, was added to the upper side of the sheets (corresponding to 25% of the standard size). At certain timepoints (10 min, 30 min, 1 h, 3 h, 5 h, 7 h, 11 h, 24 h), the vaginal sheet portions were cleaned to remove the unabsorbed mVFS that remained on the surface and were weighed (n=3). Independent samples were tested for each timepoint.

The absorption efficiency of vaginal fluid simulant was calculated according to the follow equation:

$$\text{Absorption efficiency (\%)} = \frac{weight\ at\ timepoint\ X(tx) - initial\ weight(t0)}{weight\ (mVFS\ added)} \times 100$$

3.2.10. pH and buffer capacity

The vaginal sheet was dissolved at 37ºC in a 1:20 ratio (sheet weight:solvent volume). The solvents used were 0.9% w/v NaCl (since it exhibits low buffering capacity) and mVFS pH5 (pH of BV vaginal fluids).

The initial pH of the obtained dilutions was measured, then, 20 µL of 1N NaOH aqueous solution (corresponding to 0.02 meq NaOH) were added until the pH was equal to or greater than 9. Control assays were performed only with dissolution media (0.9% w/v NaCl or mVFS pH 5). The Absolute Buffering Capacity (ABC), NaOH meq necessary to rise the pH one unit, and the Relevant Buffer Capacity (RBC), NaOH meq necessary to obtain pH higher than 5, were calculated [247].

3.2.11. Texture analysis: Hardness and resilience

The evaluation of hardness and resilience was performed using a TA.XT Plus Texture Analyser (Stable Micro Systems, United Kingdom), as described by Rita Machado et al [304]. Both tests were performed on *return to start mode*.

Hardness was determined by the Fmax exerted by the 2 mm needle shape probe (P2N, Stable Micro Systems, United Kingdom) on the formulation sample placed on a heavy duty platform using the following test conditions: a test speed of 3 mm/s, a penetration distance of 0.2 mm and a trigger force of 0.05 N. Resilience, which is the ability of a sample to return to its shape after a force has been exerted on it, was measured using a P2 (2 mm) flat probe and a ring platform to avoid movements of the formulation. Test speed was 3 mm/s, distance was 2 mm and trigger force was 0.05 N.

For each formulation, hardness and resilience were measured at three different points in two independent vaginal sheets (n=6), and the mean and standard deviation of these six measurements were calculated.

3.2.12. Bioadhesion

The bioadhesion of vaginal sheets to the porcine vaginal tissue was measured using texturometer TAXT Plus (Stable Micro Systems, United Kingdom).

The tissue was excised from vaginal tubes from approximately 6 months' old animals, kindly provided from a local slaughterhouse. The vaginal tubes were cut longitudinally, washed with a sodium chloride solution (0.9% w/w) wrapped in aluminium foil, and preserved at −20 °C (figure 10).

Immediately before the experiment, vaginal tissue was thawed at room temperature and washed with sodium chloride solution.

The vaginal sheets were cut into circular portions with 10 mm diameter (corresponding to the diameter of the probe base). On the upper side of each circular portion 53 µL of mVFS were added and were left in contact for 20 min immediately before the test. The volume of 53 µL was defined proportionally, considering the area of the vaginal sheet circular portions and the increased volume of vaginal fluid present in the vagina at every moment in this pathology (see section 3.2.9 for details on the rational) [17,239,304]. A double-sided adhesive tape was used to attach the hydrated sheet portions to the probe. The vaginal epithelium samples were fixed on the equipment base using a mucoadhesion rig (A-MUC) and were hydrated with 20 µL of mVFS immediately before the beginning of the determination. The whole system was kept at 37 ± 1 °C by means of an oven (Stable Micro Systems, United Kingdom). Figure 10 illustrates the general steps for the procedure preparation.

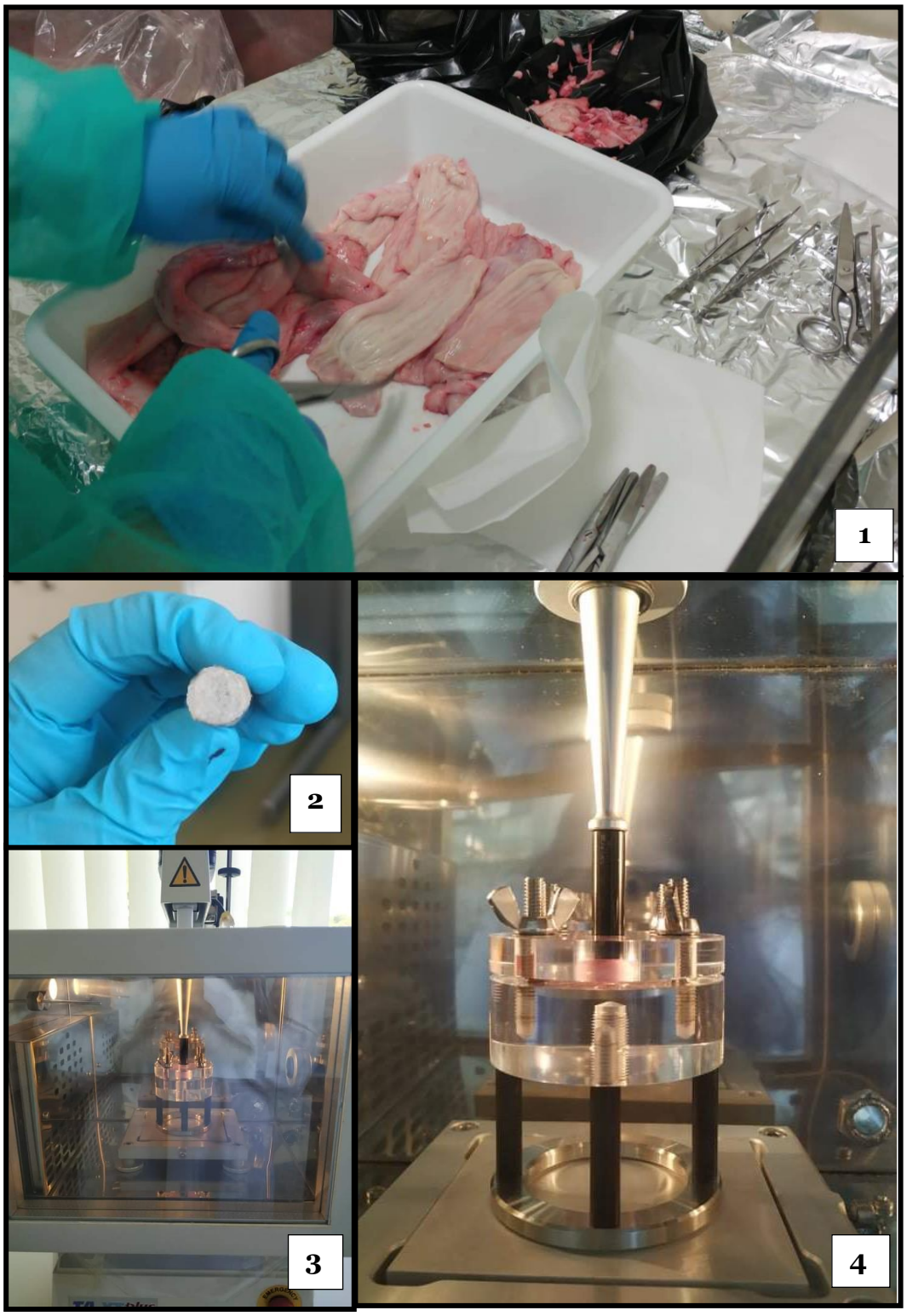

Figure 10. Illustration of the method for evaluation of bioadhesive profile of vaginal sheets on a texturometer using *ex-vivo* porcine vaginal tissue. 1- Preparation of vaginal porcine epithelium; 2- A double sided adhesive tape allowed for circular portions of vaginal sheets attachment; 3- The whole system was maintained at 37°C by means of an oven; 4- Porcine vaginal tissue was fixed using a mucoadhesion rig (A-MUC), avoiding its movement when the probe moves and allowing intimate contact between the formulation and the epithelium.

The software was used in *bioadhesion mode*. The pre-test speed was 0.5 mm/s with a trigger force of 0.02942 N. The test speed and the post-test speed were 0.1 mm/s. The contact/hold time was 180 seconds, and the force applied was 2.5 N [304].

In total, 12 porcine vaginal tubes were used. Each vaginal tube was divided into small portions. Each formulation was tested on 6 portions of different vaginal tubes (n=6).

Bioadhesive profiles of vaginal sheets were expressed as work of adhesion (N.mm) and were compared to the control, consisting of a cellulose acetate membrane fixed in the double-sided adhesive tape and treated with the same procedure as samples.

3.2.13. Stability studies

Vaginal sheets were wrapped in aluminium foil and stored at room temperature (20-25 ºC), under refrigerated conditions (5 ±3 ºC) and accelerated conditions (40±2 ºC) during 3 months. The storage temperatures were selected based on *International* Council for *Harmonisation* of Technical Requirements for Pharmaceuticals for Human Use (ICH Q1A (R2)). Humidity was not controlled. We studied the changes in sensorial characteristics, gravimetry (variation of weight), pH after the dilution on mVFS and textural properties. Observations were recorded and photographs were taken. Results were compared to the initial characterization (t0).

3.2.13.1. Quantification of *Thymbra capitata* EO components when incorporated in vaginal sheets

The quantification of carvacrol in vaginal sheet D.O was performed immediately (t0) after the incorporation of *T. capitata* EO 1% w EO/w of sheet and after storage for 3 months (t3) at room temperature (20-25 ºC), 5 ºC (±3 ºC) and 40 ºC (±2 ºC). Furthermore, sheets were prepared incorporating a mixture of pure compounds, carvacrol and linalool (80:20) at 1% w/w of sheet. These sheets, stored in the same conditions, were used to support the validation of the quantification procedures of carvacrol and linalool in the vaginal sheets. The incorporation rate of *T. capitata* EO into vaginal sheets was calculated immediately after the incorporation (t0) of *T. capitata* EO 1% w/w considering two independent batches (n=1 for batch 1 and n=3 for batch 2). Carvacrol content was used for assessment of the incorporation rate of the *T. capitata* EO in the formulation and for assessment of its stability during storage. Carvacrol in the vaginal sheets was quantified by gas chromatography equipped with a flame ionization detector (GC-FID) after a suitable liquid-liquid extraction.

Briefly, vaginal sheets (unitary samples of 2 g) were roughly divided and submitted to extraction with 3 x 20 mL of a mixture of n-pentane / diethyl oxide (93:7) during 2 h, under continuous shaking. The extractive solutions were decanted and combined. The vaginal sheet remaining's were then added of 20 mL deionized water and 20 mL of n-pentane / diethyl oxide (93:7) and kept under continuous shaking with during 2 h. After centrifugation (6000 rpm x 3 min) the organic liquid phase was recovered and joined to the previous solution. The volume of the extractive solution was reduced to less of 18 mL by distillation under reduced pressure (640

mbar) at room temperature; 10 mg of camphor were added as internal standard [1.0 mL of a camphor solution at 10.0 mg.mL-1 in n-pentane : diethyl oxide (93:7)] and the final volume adjusted to 20 mL with the solvent mixture.

Each sample was analyzed by gas chromatography in a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector (FID) and a SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 µm) column. Oven temperature was programmed to raise from 70 °C to 190 °C (at a rate of 12°C.min-1); injector temperature was set 250 °C; helium was used as mobile phase with a flow adjusted to maintain a linear velocity of 30 cm.s-1; volume sample was 1µL injected in splitless mode; FID temperature was set at 250 °C.

Quantification of carvacrol was made by the internal standard method referring to the GC peaks' raw areas (carvacrol and camphor peaks) in samples and in an standard solution containing carvacrol at 0.7 mg.ml-1 and camphor at 0.5 mg.mL-1 (as internal standard).

Limit of Quantification for carvacrol was estimated as 7000 µg per gram of sample.

3.2.14. Cellular Toxicity

3.2.14.1. Epithelial Cells

The HeLa cell line and the HEC-1A cell line were obtained from the American Type Culture Collection (ATCC-LGC Promochem, Teddington, United Kingdom). HeLa cell line is derived from human uterine cervical adenocarcinoma and HEC-1A cell line is derived from human endometrial adenocarcinoma. HeLa cell line was cultured in DMEM-F12 medium supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% FBS and is further referred to as DMEM complete medium (passages 39-49). The uterine HEC-1A cells were cultured in RPMI 1640 supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% FBS, further referred to as RPMI complete medium (Passages 50-55).

3.2.14.2. Samples tested

Cytotoxicity testing was performed for base formulation D, formulation D with *Thymbra capitata* EO, *Thymbra capitata* EO and mixtures of isolated components of *Thymbra capitata* EO which presented potent antimicrobial activity against *G. vaginalis* biofilms (A - 0.08 µL/mL Carvacrol + 0.32 µL/mL Linalool; B - 0.02 µL/mL Carvacrol + 1.25 µL/mL Linalool and C- 0.04 µL/mL Carvacrol + 2.5 µL/mLp-cymeno + 1.25 µL/mL Linalool [324]), in order to study the biocompatibility of products. Sheets were diluted to 10%, 5%, 2.5%, 1.25%, 0.63%, 0.31% (w/v) in complete culture medium containing 0.5% (v/v) of DMSO to ensure proper solubility of formulations. The dissolution of sheets at the higher concentration (10% w/v) was performed during 4 h at 37 ºC and the solutions were then vortexed during 3 min, then serial dilutions were performed. *Thymbra capitata* EO was diluted to 0.25%, 0.125%, 0.063%, 0.031%, 0.015%, 0.007% (v/v) in complete culture medium containing 0.125% (v/v) of DMSO and vortexed during 3 min. The mixtures of isolated components of *T. capitata* EO were

prepared in culture medium. The resulted emulsion was homogeneous, stable and was visually inspected for phase separation (that did not occur throughout the study timeframe).

3.2.14.3. Evaluation of MTT conversion due to non-cellular mechanisms

Chemicals absorbing light in the same range as MTT formazan and compounds able to directly reduce MTT may interfere with the cell viability measurements [325]. Some components of plant extracts have reducing activity that converts MTT [326,327].

Before performing cytotoxicity tests, we checked for possible interference due to the conversion of MTT by components of *Thymbra capitata* EO, adapting the proposed methodology referred in Test Guideline No. 439 In vitro skin irritation [325]. To identify direct MTT reducers, *Thymbra capitata* EO was added to a freshly prepared MTT solution (1 mg/mL in incomplete culture medium) at several concentrations. To further eliminate this interference, we prepared one plate containing dead fixed cells (killed by absolute ethanol in contact with cells during 7 min at -20 ºC) for each independent test. These plates containing dead fixed cells were submitted to the same procedure of cytotoxicity test (contact with tested samples at the same concentrations during the 24 h, washing step, incubation with MTT solution and extraction).

3.2.14.4. Cytotoxicity test (MTT Assay)

The MTT reduction assay was performed as previously described and according to ISO/EN 10993-5 for *in vitro* evaluation of medical devices [256]. Cells were seeded onto 96-well plates (100,000 cells/mL) with complete culture media. Cells were left to adhere for 24 h at 37 ºC, under 5% CO2 atmosphere. After obtaining a half-confluent culture, 100 µL of tested samples were added and left in contact for 24 h. After this period, cells were washed with PBS and incubated for 3 h with 50 µL a 1 mg/mL solution of MTT reagent prepared in incomplete culture medium. Subsequently to formazan crystals formation, extraction was accomplished with 100 µL of 2-propanol for 15 min, through mild agitation on an orbital shaker, protected from light. Absorbances were then measured at 570 nm, using a microplate spectrophotometer (Biorad xMark, EUA). The negative control consisted of cells without any treatment (only culture media in assay), which was considered as the 100% viability reference for products toxicity calculation. Positive control (SDS 2%), which causes extreme decrease in viability, was included. Solvent controls (SC): 0.5% DMSO in culture media for the assays of D and D.O cytotoxicity evaluation and 0.125% DMSO in culture media for the assays of EO cytotoxicity evaluation were also included to evaluate the contribution of solvents used in the extraction to the global effect in viability.

3.2.15. Vaginal irritation – SkinEthic™ *Reconstructed Human Vaginal Epithelium* model

For safety characterization, vagina irritation was further studied in a commercial 3D model (SkinEthic™ HVE / Human Vaginal Epithelium, provided by Episkin France), using the MTT test. This model is based on the ability of the cell line A431 (derived from vulvar epidermoid carcinoma) to form a pluri-stratified epithelium with strong histologic resemblance to the *in vivo* vaginal mucosa, after cultivation on an inert polycarbonate filter at the air liquid interface in a chemically defined medium.

3.2.15.1. Pre incubation

Upon reception, the inserts containing the tissues were visually inspected for integrity and individually transferred (using sterile forceps, under aseptic conditions) to a well of 6-well plates (VWR, Portugal) pre-filled with 1 mL of Maintenance Medium (provided by the Episkin together with the tissues). The tissues were then, pre-incubated overnight, at 37°C, ≥ 90% humidity, 5% CO_2 (Binder APT.lineTM C150E2, USA).

3.2.15.2. Samples

We tested the vaginal irritation caused by *Thymbra capitata* oil at 0.32 µL/mL, diluted on sesame oil), mix-A (0.08 µL/mL Carvacrol + 0.32 µL/mL Linalool diluted on sesame oil) which we previously showed to have antimicrobial potential against several species of *Gardnerella* spp [324]; sesame oil (as solvent control); vaginal sheet D diluted on PBS 10% w/v; vaginal sheet D.O diluted on PBS 10% w/v; vaginal sheet D.O (undiluted, tested in direct contact with tissue) and universal placebo, used as comparator/reference. Universal Placebo was prepared according to Tien et al. by dissolving of 2.7 g of hydroxyethyl-cellulose (2000cP) in 96.3 g of water containing 0.85 g of sodium chloride and 0.1g of sorbic acid. The final pH was adjusted to 4.4 by adding sodium hydroxide, and the gel was stored at 2–8 ºC [328].

Sesame oil (Ph Eur. Grade, Sigma) was used as solvent for oily components, since this is the oily vehicle recommended for skin irritation testing according to ISO 10993-23:2021. phosphate buffer saline without Ca^{2+} and Mg^{2+} (DPBS, VWR) and Sodium Lauryl Sulfate 1% w/v, were used as negative and positive controls, respectively.

3.2.15.3. Sample application

After pre-incubation, tissues were then transferred to new 24-well plates (one plate per condition) containing 300 µL of Maintenance Medium, and then 30 µL of the liquid/semi solid test substances or controls were gently dispersed over the entire tissue surface. Vaginal sheet D.O was further tested as solid sample, undiluted, and in this case the application procedure was different. Circular portions of vaginal sheet D.O with the same diameter of tissues was cut and 10 µL of PBS was applied on the surface of tissue to hydrate it immediately before the application of vaginal sheet's portions over the surface. After the application of samples, tissues were incubated for 24 h at 37 °C, ≥90% humidity, 5% CO_2.

3.2.15.4. MTT assay

After 24 h of incubation at 37°C, ≥ 90% humidity, 5% CO_2, tissue integrity was assessed by the MTT assay, as previously described [253,279]. Briefly, the tissues were washed with PBS and gently dried. Then, the tissues were transferred to a 24-well plate containing 300 µL per well of a 0.5 mg/mL MTT (Alfa Aeser) solution (in PBS, VWR) and incubated for 3 h, at 37 °C, ≥ 90% humidity, 5% CO_2, protected from light. After this period, the tissues were transferred to a single 24-well plate containing 750 µL of isopropyl alcohol and more 750 µL were further added at the top of each tissue to allow for full extraction of formazan for ≥2 h, in a sealed plastic bag, under agitation in a plate stirrer Absorbances were then measured at 570 nm, using a microplate spectrophotometer (Promega GloMax® Explorer System, USA). The negative control consisted of tissues treated with PBS which was considered as the 100% viability reference for products toxicity calculation. Wells with isopropyl alcohol were used for background deduction applied to all absorbance values.

3.2.16. Vaginal irritation - Hen's Egg Test-Chorioallantoic Membrane Assay (HET-CAM)

3.2.16.1. Eggs and incubation conditions

The HET-CAM assay was performed on fresh fertile White Leghorn chicken eggs that were clean and weighed (45-65 g). Upon arrival at the lab, eggs were checked for damage. Damaged eggs were discarded, and undamaged eggs were incubated at 37.8 ± 0.3 ºC in a relative humidity of 58 ± 2% and under automatic rotation for 8 days (Corti AF-50 and Copele 30652, Spain). On the eighth day, eggs were observed using a LED light to confirm embryo formation. Non-embryonated and non-viable (dead) eggs were discarded. Viable eggs were incubated for one further day under the same conditions of temperature and humidity, but without rotation.

3.2.16.2. Samples tested

Base formulation D and formulation D with oil have been ground to fine particles, as suggested on ICCVAM-Recommended Test Method Protocol: Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) Test Method for solid test substances: 0.3g were directly applied onto the CAM, ensuring that at least 50% of the CAM surface area is covered. Additionally, the same formulations were diluted on NaCl 0.9% (w/v) to a final concentration of 10% (w/v). Saline solution was selected to dissolve vaginal sheets since it was considered as non-severe irritant. The resultant viscous gels were thermostated at 37 ºC before being applied.

3.2.16.3. HET-CAM assay

Previous studies performed by our research group reported the potential of the HET-CAM assay for vaginal irritation testing of medicines, cosmetics, hygiene products or medical devices in preclinical safety assessments [257].

The HET-CAM assay (according to the ICCVAM – Recommended Test Method (NIH Publication No. 10-7553 – 2010) was used to test the potential vaginal severe irritation of samples, since it allows the identification of severe irritants as an *in vitro* alternative to the *in vivo* Draize rabbit eye test [258]. At day 9, eggs were taken out of the incubator and placed on an appropriate support. The shell was carefully opened using a scalpel and tweezers and the internal membrane was exposed and then hydrated with NaCl 0.9% (w/v) for a maximum of 30 min. The solution was aspirated, and the membrane was carefully peeled off, without damaging the blood vessels. 0.3 mL of base formulation D and formulation D with oil diluted on saline solution (10% w/v) and control solvents and 0.3g of base formulation D and formulation D with oil finely divided were applied to the CAM (n=3 eggs per sample). NaCl 0.9% (w/v) was used as negative control and NaOH 0.1N and SDS 1% were used as positive controls.

The irritant effects of samples and controls were evaluated by observation of three endpoints: haemorrhage (vessel bleeding), lysis (vessels disintegration) and coagulation (intra and extra-vascular protein denaturation) at predetermined time intervals (0.5, 2, and 5 min). These observations allowed calculation of the irritation score (IS (A)) as the sum of the scores obtained at each time point (table 5). The irritancy classification is defined as non-severe irritant (IS 0-9) or severe irritant (>9 to 21). This score does not provide differentiation amongst mild irritants and non-irritants. Photographs were taken at each time points (1 egg per sample).

3.2.17. Evaluation of vaginal sheet D.O efficacy against Gardnerella species biofilms

3.2.17.1. Bacterial growth conditions

Gardnerella vaginalis UM137, *G. piotii* UM035, *G. leopoldii* UGent 09.48 and *G. swidsinskii* GS 9838-1 were grown on Columbia Base Agar medium (Liofilchem, Roseto degli Abruzz, Italy) supplemented with 5% (v/v) of defibrinated horse blood (Oxoid Ltd, Hampshire, UK) for 48 h [296]. For each experiment, the bacterial species were grown in Brain Heart Infusion (BHI, Liofilchem) supplemented (sBHI) with 2% (w/v) gelatin (Liofilchem), 0.1% (w/v) starch (Panreac, Barcelona, Spain) and 0.5% (w/v) yeast extract (Liofilchem) and incubated for 24 h at 37 °C and 10% CO_2 (Panasonic MCO-18AC, Bracknell, UK).

3.2.17.2. Activity of dissolved vaginal sheets on Gardnerella species biofilm

For biofilm formation, 24 h bacterial inoculums of *Gardnerella vaginalis* UM137, *G. piotii* UM035, *G. leopoldii* UGent 09.48 and *G. swidsinskii* GS 9838-1 were adjusted to a concentration of 10^7 CFU/mL, determined by flow cytometry, as previously described [299].

Then, 1 mL of each suspension was dispensed on 24 well-plates (Orange Scientific, Braine-l'Alleud, Belgium) and incubated for 24 h at 37°C and 10% CO_2.

The vaginal sheet D.O was dissolved (at 10% w/v concentration), using sBHI medium containing 0.5% (v/v) of DMSO to ensure proper solubility of formulations. Required concentrations of the dissolved vaginal sheets were prepared in sBHI medium. After 24 h, the medium from the biofilms was removed and 1 ml of the correspondent vaginal sheet suspension was added to the biofilm and the plates were incubated for further 24 h at the same conditions. A negative control was performed where the medium was replaced by fresh sBHI. Another control was included where the effect of DMSO was verified by replacing the medium at 24 h by sBHI with 0.5% of DMSO. The effect of the vaginal sheet D without EO was also assessed using the same method applied to the EO containing vaginal sheet. Vaginal sheet D was tested at concentration 0.03 g/mL that corresponds to the concentration of base formulation at the higher concentration of D.O tested (*Thymbra capitata* EO 0.32 µL/mL).

At 48 h of biofilm formation, the medium was removed and the biofilm was washed once with NaCl 0.9% (w/v). Then, 1 mL of sBHI was added to each well and the biofilm was detached from the plates. 100 µL of each condition was diluted in NaCl 0.9% (w/v) and serial dilutions were performed. Lastly, 10 µL of each dilution were plated on CBA plates and incubated for 72 h at 37 °C and 10% CO_2. Colony Forming Units (CFU) counts were performed and results expressed as log CFU/mL. The experiments were repeated at least three times with technical duplicates.

3.3. Results and discussion

3.3.1. Freeze drying efficiency

Results shown in table 12 show that the freeze-drying process is efficient at removing water from formulations, with freeze drying efficiency ranging from 93.8% ± 3.0 (D) to 98.5% ± 0.5 (A). As a result, vaginal sheets contain only residual amounts of water so preventing microbial contamination.

Table 12. Freeze drying efficiency of vaginal sheets

Base formulation	Freeze drying efficiency (%)
A	98.5 ± 0.8
B	94.7 ± 1.9
C	97.9 ± 0.6
D	93.8 ± 3.0
E	96.4 ± 0.4
F	94.4 ± 1.2
G	96.5 ± 3.0

3.3.2. Sensorial characteristics

Sensorial characteristics of vaginal sheets are presented in table 13 and figure 11. Physical characteristics and sensorial perception of vaginal products influence the acceptability and the adherence of users [103]. In general, women prefer vaginal products to be colorless (or light-colored) and odorless. Natural ingredients tend to be valued by some [103,329,330].

Table 13. Sensorial characteristics of vaginal sheets

Formulation	Colour	Transparency	Odour	Feel to touch
A	Very light yellow	Transparent	Odourless	Hard, flexible, surface smooth
A.O	Light yellow	Transparent	*Thymbra capitata* essential oil odour	Flexible, surface smooth
B	Yellowish white	Translucent	Odourless	Flexible, surface smooth
B.O	Very light yellow	Translucent	*Thymbra capitata* essential oil odour	Hard, flexible, surface smooth
C	Very light yellow	Transparent	Odourless	Flexible, surface smooth
C.O	Light yellow	Transparent	*Thymbra capitata* essential oil odour	Hard, flexible, surface smooth
D	Light yellow	Transparent	Slight shellfish odour	Flexible, surface smooth
D.O	Light yellow	Transparent	*Thymbra capitata* essential oil odour; slight shellfish odour	Very flexible, surface smooth
E	White	Opaque	Odourless	Soft, flexible, surface smooth
E.O	White	Opaque	*Thymbra capitata* essential oil odour	Soft, Very flexible, surface smooth
F	Very light yellow	Translucent	Odourless	Soft, flexible, surface smooth
F.O	Very light yellow	Translucent	*Thymbra capitata* essential oil odour	Soft, Very flexible, surface smooth
G	Pearl White	Opaque	Odourless	Soft, flexible, surface smooth
G.O	Pearl White	Opaque	*Thymbra capitata* essential oil odour	Soft, very flexible, surface smooth

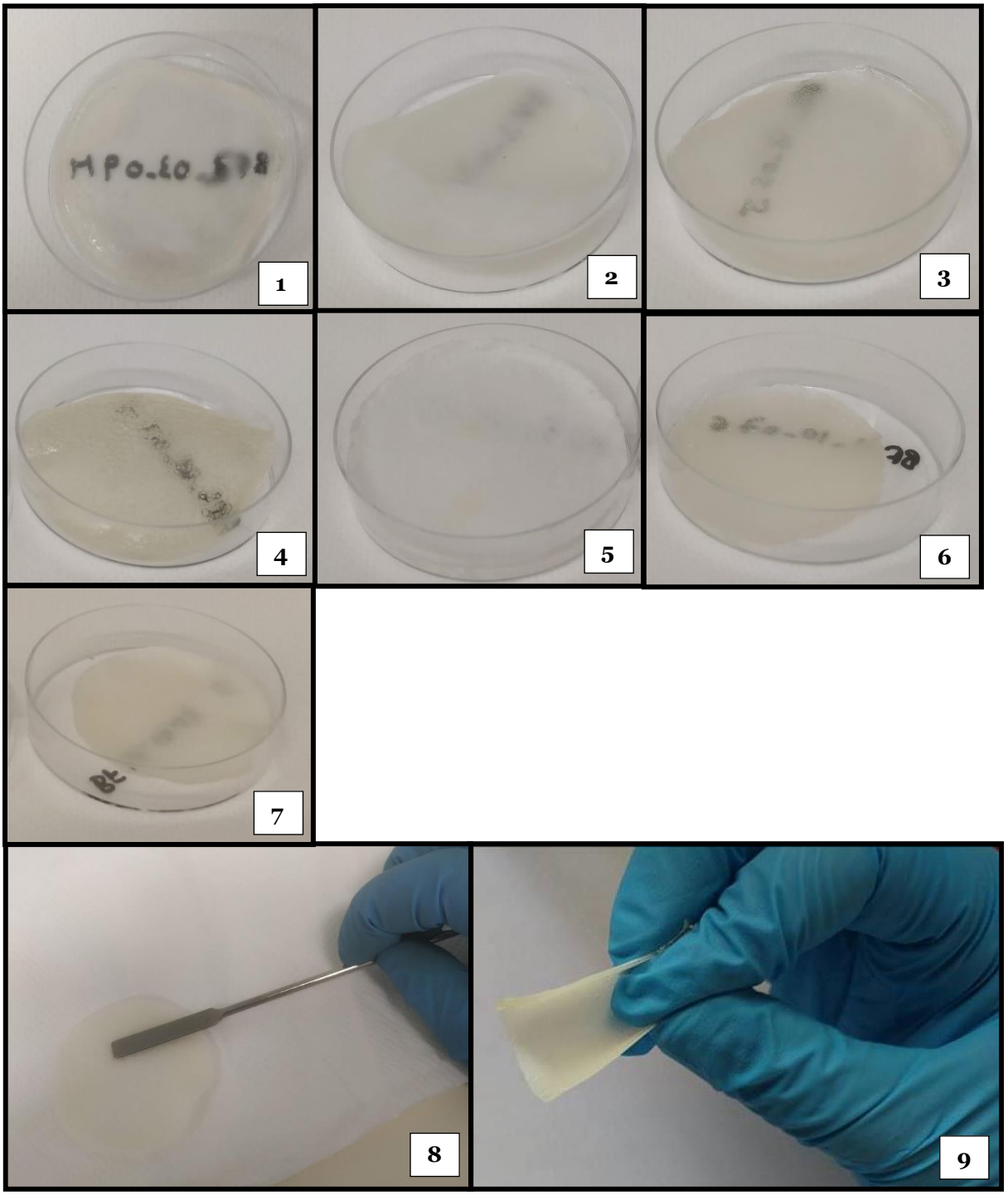

Figure 11. General aspect of vaginal sheets and how to handle and apply. Legend: 1 – vaginal sheet A; 2 – vaginal sheet B; 3 – vaginal sheet C; 4 – vaginal sheet D; 5 – vaginal sheet E; 6 – vaginal sheet F; 7 – vaginal sheet G; 8 – Method of application of *T. capitata* essential oil on the surface of vaginal sheets after the freeze-drying process using a spatula; 9 – Application of vaginal sheet to be inserted into the vagina wrapped around the finger.

Previous studies reported the acceptability of vaginal films for the prevention of HIV transmission. Fan et al reported that women most frequently preferred vaginal films to be smooth and thin, translucent and square size (5.08 cm x 5.08 cm). The majority of women preferred odorless, colorless and flavorless films, maintaining the natural characteristics of fluid. Women also preferred translucent or opaque films, since transparent films are associated with a low level of visual discernibility [329]. Guthrie el al reported that 33.3% of women within their study preferred the size of film 7.62 x 2.54 cm and women related the size with the ease of insertion, efficacy and leakage. Films with texture could improve insertion by providing better grip on the film. On the other hand, approximately 50% of participants preferred the film with one smooth and one textured side, indicating that the texture could improve insertion by providing better grip [330].

The optimal final size of vaginal sheets (7x2.4 cm) was defined considering the mean dimension of the human vagina to allow a complete coverage. The light colors of vaginal sheets do not predicable promote significative changes in the natural color of vaginal fluid. The odor of vaginal sheets of *Thymbra capitata* EO can be an advantage for the product, since it can attenuate the characteristic unpleasant odor of BV vaginal fluid. Furthermore, the fact that the EO is a natural active substance may even improve acceptability among women that are more prone to use natural products [198,331,332].

3.3.3. Thickness

The thickness of vaginal sheets ranged from 0.813 ± 0.046 mm (base formulation D) to 1.111 ± 0.176 mm (base formulation B) – table 14. This variation between formulations can be explained by the polymers used and respective concentrations. Vaginal sheets are thicker than vaginal films, since the freeze-drying process allows to maintain the poured thickness originated in the network structure of gels. Vaginal sheets presented homogeneous thickness. The addition of oil to the base formulations did not change the structure of vaginal sheets (thickness was similar).

Table 14. Thickness (mm), folding endurance and pH after diluting vaginal sheets on mVFS pH 5 (1:10 w/w)

	Thickness (mm) (n=6)	**Folding endurance (n=2)**	**pH dilution with mVFS pH5 1:10 w/w pH + S.D (n=3)**
A	0.935 ± 0.081	✓	4.72 ± 0.01
A.O	0.925 ± 0.025	✓	4.48 ± 0.02
B	1.111 ± 0.176	× (287 times)	4.63 ± 0.03
B.O	1.086 ± 0.072	✓	4.47 ± 0.03
C	0.815 ± 0.039	✓	4.61 ± 0.01
C.O	0.821 ± 0.018	✓	4.45 ± 0.01
D	0.813 ± 0.046	✓	4.67 ± 0.01
D.O	0.820 ± 0.023	✓	4.52 ± 0.01
E	0.921 ± 0.041	× (174 times)	4.43 ± 0.01
E.O	0.906 ± 0.012	× (144 times)	4.38 ± 0.01
F	1.027 ± 0.023	✓	4.52 ± 0.01
F.O	1.005 ± 0.009	✓	4.40 ± 0.01

G	0.920 ± 0.024	✓	4.55 ± 0.01
G.O	0.924 ± 0.010	× (295 times)	4.40 ± 0.02

3.3.4. Folding endurance

Vaginal sheets are designed to be inserted rolled around the finger, so it must have flexibility and simultaneously resistance to allow this application. We evaluated these characteristics using folding endurance test (table 14). Formulation B was hard and not very flexible, so it was broken at the final stage of the test. After the incorporation of the oil, it became more flexible and already passed the test. Although formulation E and formulation E.O were very flexible, they were very soft, not having enough resistance to pass this test. Similarly, G.O was very soft, being fragile to pass this test. The other formulations presented adequate characteristics for the intended use. This flexibility and low thickness are advantageous characteristics of the formulation when compared with classic solid dosage forms such as ovules (vaginal suppositories), since they are able to adapt to the vaginal cavity, causing less discomfort [316].

3.3.5. Absorption efficiency of mVFS

BV is characterized by an abundant creamy, grey-white vaginal discharge with rotten fish odor [17,104–106,109,110]. Vaginal sheets can gradually absorb mVFS and dissolve on it, ultimately becoming a bioadhesive gel that adheres to the epithelium (table 15).

Table 15. Absorption efficiency (%) of mVFS of vaginal sheets during 24 h. Results are presented as mean values ± standard deviation (SD), n = 3. Bold values represent the maximum absorption (swelling) timepoint from which preparations start to lose their structure.

	10 min	**30 min**	**1 h**	**3 h**	**5 h**	**7 h**	**11 h**	**24 h**
A	21 ± 3	35 ± 1	54 ± 0	55 ± 2	59 ± 3	**63 ± 0**	59 ± 8	57 ± 1
A.O	29 ± 2	42 ± 2	42 ± 2	**53 ± 6**	46 ± 3	47 ± 5	47 ± 2	33 ± 1
B	29 ± 4	44 ± 4	54 ± 0	62 ± 3	66 ± 0	67 ± 7	**84 ± 14**	55 ± 1
B.O	28 ± 4	39 ± 3	45 ± 2	53 ± 2	**60 ± 2**	54 ± 4	45 ± 1	32 ± 0
C	22 ± 3	42 ± 7	39 ± 1	56 ± 3	53 ± 0	55 ± 7	**59 ± 14**	50 ± 1
C.O	29 ± 0	41 ± 2	44 ± 0	50 ± 6	50 ± 2	**53 ± 4**	52 ± 4	34 ± 1
D	25 ± 5	45 ± 5	47 ± 2	59 ± 12	63 ± 5	69 ± 2	**70 ± 1**	61 ± 0
D.O	40 ± 4	42 ± 8	49 ± 5	48 ± 1	56 ± 3	**63 ± 0**	45 ± 6	32 ± 0
E	**42 ± 9**	39 ± 5	32 ± 3	29 ± 10	10 ± 0	-	-	-
E.O	46 ± 7	**47 ± 2**	42 ± 5	35 ± 8	6 ± 2	-	-	-
F	20 ± 15	37 ± 7	40 ± 11	49 ± 4	**58 ± 0**	47 ± 1	47 ± 6	23 ± 0
F.O	36 ± 4	41 ± 3	53 ± 1	**56 ± 2**	55 ± 2	55 ± 3	55 ± 3	37 ± 5
G	31 ± 1	40 ± 11	44 ± 6	**47 ± 2**	47 ± 4	46 ± 10	47 ± 5	26 ± 11
G.O	38 ± 3	48 ± 2	48 ± 8	**52 ± 5**	51 ± 8	51 ± 4	47 ± 3	38 ± 3

This dosage form is particularly interesting for the treatment of BV, since it can contribute to relieve one of the most important symptom which is associated with a significant negative impact on self-esteem, sexual relationships, and quality of life [304]. Comparing with gels, vaginal sheets (similarly to films) avoid leakage and messiness [212,304,313]. Initially, vaginal sheets presented a swelling behaviour, absorbing the mVFS (with pH and volume adapted to the pathological condition). After that, and depending on their composition, vaginal sheets begin to present a viscous gel, resulting from the dissolution of some components into mVFS, that is, they begin to lose their structure. Among base formulations, formulation B presented the higher absorption ability (84% ± 14 after 11 h). Among sheets with oil, D.O exhibited the higher absorption ability (63% ± 0 after 7 h). Vaginal sheet D.O exhibited a typical swelling behaviour until 7h, and only after this timepoint, it begins to dissolve on mVFS and gradually forms a gel. The fluid absorption profile of vaginal sheet D.O can be particularly relevant in the context of a possible clinical use, since, similarly to almost all vaginal products, it is intend to be applied at bedtime. Therefore, it could absorb the excess of vaginal fluid during the night and then be partially expelled and partially becoming a bioadhesive gel during the daytime (avoiding leakage). After 24h, vaginal sheet D.O did not totally lose its structure. On the other hand, formulation E.O presented the lower absorption efficiency of mVFS (47% ± 2 after 30 min) and the entire sheet was dissolved in mVFS, with total loss of the initial structure and formation of a gel, after 7 h.

3.3.6. pH and buffer capacity

We used the mVFS with pH 5 to simulate this pathologic fluid. After the dilution of vaginal sheets in mVFS (1:10 w/w), the resultant pH is closer to the healthy vaginal fluid pH (table 17), suggesting that vaginal sheets with LA buffer solution can normalize the mVFS pH presenting a complementary action against BV-related bacteria.

The addition of *T. capitata* EO to the base formulations caused a slight decrease of pH (significantly different for all formulations, $p<0.05$, Tukey's Multiple Comparison Test). We studied the buffer capacity of formulations (figure 12).

We included two solvents for the dilution of vaginal sheets: mVFS and saline solution (since it has lower buffer capacity) [240,247,304]. The mean value of RBC and ABC for each vaginal sheet considering the solvent (mVFS or saline solution) was significantly different (Two-way ANOVA, $p<0.05$), highlighting some contributions from the solvent to the final buffering capacity.

However, the ABC of all vaginal sheets with the exception of B.O and F.O was significantly different from controls (Dunnett's multiple comparisons test, $p<0.05$). We can conclude that the acid-buffering capacity was attributed to vaginal sheets, although there were small contributions of the solvents in which they were dissolved.

D.O exhibited the higher ABC and RBC, which was in line with our expectations since this formulation contains the highest strength buffer. This ability to reduce the vaginal pH represents a therapeutic advantage for the treatment of BV.

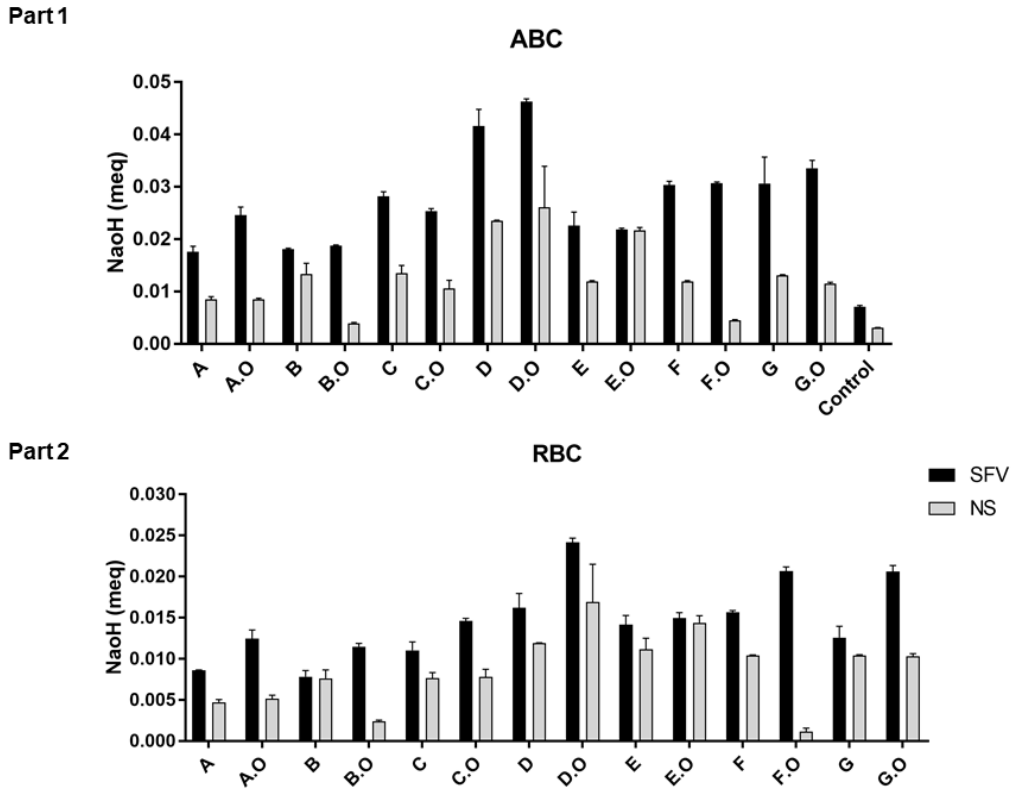

Figure 12. Determination of absolute buffer capacity (part 1) and relevant buffer capacity (part 2) of base formulations and sheets with oil. Bars represent the mean of 3 determinations and lines the standard deviation. NS = Normal saline; mVFS = vaginal fluid simulant;

3.3.7. Texture analysis: Hardness and resilience

As demonstrated in the preliminary study of development of vaginal sheets, hardness and resilience of formulations were dependent on the concentration of polymer and plasticizer and/or their respective proportion. Vaginal sheet C is harder than B and both formulations differ only on the powder included, so the inclusion of lactose gives greater hardness than SSA (figure 13). Furthermore, no significant differences were observed between formulation A and B meaning that the inclusion of the powder in B did not significantly influence the hardness of the formulation (Dunnetts multiple comparisons test, $p<0.05$)

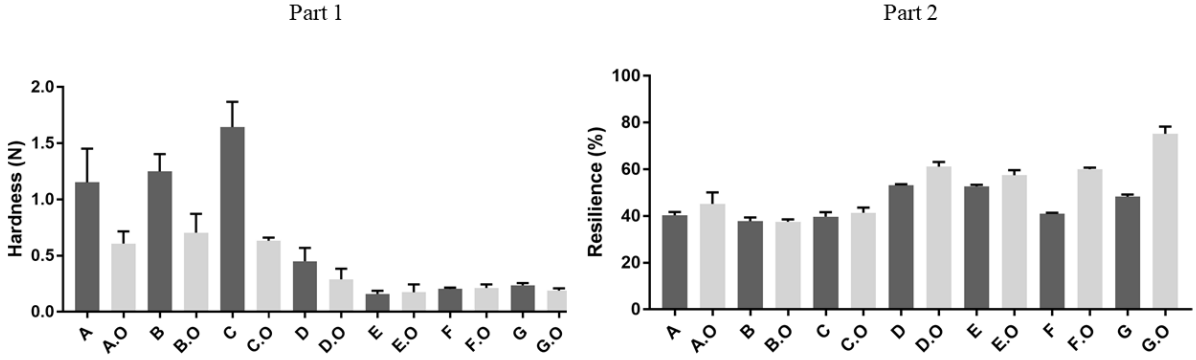

Figure 13. Study of textural properties of vaginal sheets: hardness (N) – part 1 and resilience (%) – part 2

The addition of oil to the hardest base formulations (A, B, C) results in a decrease of hardness (statistically significant $p<0.05$, Tukey's multiple comparisons test). On the other hand, formulations E, F and G were the less hard. These are the compositions with the lowest content of gelatin, despite containing other polymers such as HPMC (1%w/w gel) for E and G and PVA (2.4% and 1.2% w/v gel for E and F, respectively). The incorporation of *T. capitata* EO in base formulations caused an increase in resilience (statistically significative for A, D, E, F and G, $p<0.05$, Tukey's multiple comparison test). Among vaginal sheets with oil, G.O was the most resilient, followed by vaginal sheets D.O, E.O and F.O. This analysis of textural properties was in accordance with the results of folding endurance and the subjective evaluation of sensorial properties. As previously described, the resistance and the flexibility of vaginal sheets influence ease of insertion and the perception of users [304].

3.3.8. Bioadhesion

The assessment of bioadhesion was based on applying a force that allows the vaginal sheet to create initial contact with porcine vaginal tissue and then the force required to break the adhesive stresses (such as chemical bonds) resulting from that contact. Bioadhesive polymers, such as polyacrylic acid derivatives (carbomer and polycarbophil), cellulose derivatives (hydroxypropyl methylcellulose, carbomers and polycarbophil), chitosan, hyaluronic acid, alginate and others can be included in vaginal formulations in order to improve their bioadhesive properties, prolonging the residence time and avoiding leakage associated mainly to semi-solid products [242,244,249,265,268,288].

The rational design of vaginal sheets included the selection of bioadhesive polymers that promote an intimate and prolonged contact of sheets with epithelium, while absorbing and gradually dissolving on vaginal fluid. The resultant gel, after dilution of vaginal sheets in vaginal fluid will also present these bioadhesive properties.

Among base formulations, D was the most bioadhesive (figure 14) and among vaginal sheets with oil, formulation E.O was the most bioadhesive, followed by D.O. These results are in accordance with the rational design of these formulations, since D and E include bioadhesive polymers: chitosan and HPMC. The addition of *T. capitata* EO to base formulations promoted changes in the interactions between the vaginal sheets and the vaginal epithelium, so the bioadhesive profile was altered (increased or decreased).

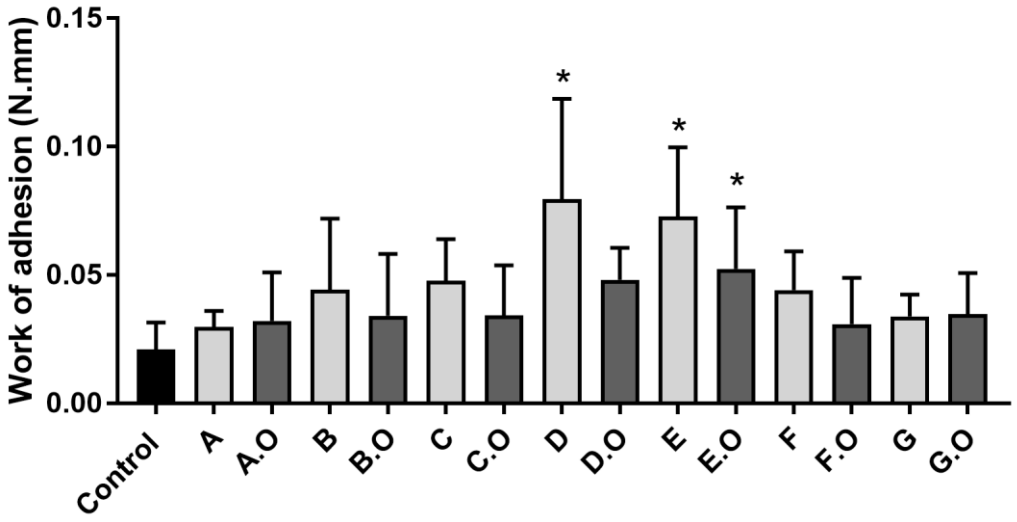

Figure 14. Evaluation of bioadesive profiles of vaginal sheets (work of adhesion (N.mm)). Individual columns and vertical bars represent mean and SD values, respectively (n=6)

3.3.9. Stability studies

At room temperature there was no significant change regarding vaginal sheets weight indicating that no significant gain (absorption) of water was observed throughout the study (table 16).

Table 16. Gravimetry (weight variation %) after storage for 3 months comparing to to

Formulation	Variation of weight (%)		
	TA	5 ºC	40 ºC
A	-1.60	6.16	-1.12
A.O	-1.90	3.90	-5.88
B	1.35	5.88	-4.92
B.O	-1.18	4.19	-3.26
C	1.37	6.08	-3.51
C.O	-0.51	3.24	-7.97
D	1.29	8.12	-4.88
D.O	0.30	4.28	-7.81
E	2.98	7.83	-1.35
E.O	-0.86	5.93	-5.09
F	2.55	8.15	-2.07
F.O	0.60	5.97	-6.56
G	0.24	9.96	-4.66
G.O	0.47	6.23	-9.26

At 5 ºC, vaginal sheets slightly increased in weight, which can be explained by the fact that they are freeze-dried and can therefore absorb some moisture from the cabinet. At 40 ºC, vaginal sheets decreased in weight, which can be explained by the evaporation of residual water and eventually of *Thymbra capitata* EO.

For vaginal sheets stored at room temperature and 5 ºC, no changes in color, odor, texture, hardness or malleability were detected, with the exception of vaginal sheet E.O which

presented yellow spots after storage under both conditions. Vaginal sheet E.O is the only composition containing propyleneglycol together with glycerin as plasticizer and additionally contains a mixture of gelatin, HPMC and PVA as polymers so its composition is very different from the other vaginal sheets. It presented high and fast absorption capacity and completely disintegrated into a gel in that test. It is possible that some absorption of the EO occurs during the coating procedure which can impair a homogeneous distribution of this active substance on the surface, leading to areas that have a greater amount of *Thymbra capitata* EO (this fact is only noticeable after storage).

Base formulations stored at 40 ºC were slightly yellow, harder and less flexible, comparing to t0 (after production). Vaginal sheets with EO were found to be more yellow (dark yellow) after storage indicating that some oxidation may occur and vaginal sheets C and C.O turned brown possible due to the degradation (caramelization) of lactose. So, the addition of antioxidant to these formulations might be considered in the future. Nevertheless, oxidation of essential oils has been shown to be highly dependent on temperature and to vary with the specific composition of the essential oil [333]. So, although accelerated stability was performed at 40 ºC in view of predicting longer term storage stability at room temperature (according to Arrhenius equation), it is possible that the extent of oxidation observed at 40 ºC do not correlate to oxidation at room temperature.

Vaginal sheets with *Thymbra capitata* EO stored at 40 ºC during 3 months, still presented the *Thymbra capitata* EO typical odor but less intense comparing to t0, possibly because some of the volatile components of oil may have volatilized [334]. Furthermore, in D and D.O sheets the characteristic chitosan odor was altered. Moreover, vaginal sheets with oil were harder and less flexible, making it difficult to separate from the aluminium foil.

There was no significant variation in pH after dilution in mVFS for vaginal sheets stored at room temperature (table 17). At this condition, all formulations presented pH variations from timepoint 0 below 0.2 pH units (that is a variation accepted for duplicate measurements). Similar observations were obtained for vaginal sheets stored at 5 ºC (variation values were below 0.24). On the contrary, vaginal sheets stored at 40 ºC exhibited an overall pH increase (more than 0.2 pH units) for all formulations except for base formulations A and D. Formulation D.O exhibited an increase of 0.27 in pH from t0 (much lower than those observed for other vaginal sheets that ranged from 0.34-0.57). These results show that formulation D.O is the most stable regarding pH at accelerated conditions that may predict a more stable behaviour for prolonged periods of storage at room temperature. However, this parameter shall be closely monitored throughout long-term (confirmation) stability studies.

In short, all vaginal sheets were stable at room temperature for three months and this packaging was adequate. These results highlight the importance of choosing a sealed package for the vaginal sheets (since they are produced though freeze-drying process and they have been coated with EO) and the temperature conditions, being greatly affected by higher temperatures. Data from accelerated conditions point to higher stability of formulation D and D.O compared to other formulations, despite the occurrence of oxidation reactions in essential oil that is

probably associated with the higher temperature (40 ºC) that makes it difficult to extrapolate for long term stability periods at room temperature.

Table 17. pH after diluting vaginal sheets on mVFS pH 5 (1:10 w/w) after storage for 3 months. Results are presented as mean values ± standard deviation (SD), n = 3

	pH dilution with mVFS pH5 1:10 w/w pH + S.D (n=3) to	pH dilution with mVFS pH5 1:10 w/w pH + S.D (n=3) t3 TA	pH dilution with mVFS pH5 1:10 w/w pH + S.D (n=3) t3 40 ºC	pH dilution with mVFS pH5 1:10 w/w pH + S.D (n=3) t3 5 ºC
A	4.72 ± 0.01	4.78 ± 0.03	4.91 ± 0.02	4.79 ± 0.02
A.O	4.48 ± 0.02	4.62 ± 0.02	4.98 ± 0.01	4.64 ± 0.03
B	4.63 ± 0.03	4.69 ± 0.03	4.97 ± 0.03	4.71 ± 0.01
B.O	4.47 ± 0.03	4.53 ± 0.03	4.89 ± 0.02	4.60 ± 0.01
C	4.61 ± 0.01	4.64 ± 0.01	5.04 ± 0.02	4.69 ± 0.03
C.O	4.45 ± 0.01	4.51 ± 0.01	4.99 ± 0.03	4.61 ± 0.02
D	4.67 ± 0.01	4.71 ± 0.01	4.81 ± 0.02	4.72 ± 0.02
D.O	4.52 ± 0.01	4.69 ± 0.02	4.79 ± 0.03	4.72 ± 0.01
E	4.43 ± 0.01	4.54 ± 0.01	4.97 ± 0.01	4.64 ± 0.01
E.O	4.38 ± 0.01	4.49 ± 0.01	4.91 ± 0.01	4.56 ± 0.02
F	4.52 ± 0.01	4.61 ± 0.02	5.02 ± 0.02	4.64 ± 0.02
F.O	4.40 ± 0.01	4.52 ± 0.01	4.92 ± 0.03	4.58 ± 0.01
G	4.55 ± 0.01	4.61 ± 0.01	5.01 ± 0.01	4.65 ± 0.02
G.O	4.40 ± 0.02	4.58 ± 0.02	4.97 ± 0.03	4.64 ± 0.02

3.3.9.1. Quantification of *Thymbra capitata* EO components when incorporated in vaginal sheets

Since vaginal sheet D.O presented the most promising technological characteristics (higher buffer capacity, higher absorption capacity, adequate textural properties) and one of the most favorable bioadhesive profiles, it was the prototype selected for the following characterization steps: quantification of active, safety and efficacy studies.

The incorporation rate of the *Thymbra capitata* EO into vaginal sheet D.O was 96.4% ± 2.9 (mean value of 4 quantifications) considering the vaginal sheet as an unitary dosage form (i.e. considering the vaginal sheet as a whole). In the optimization process of the incorporation rate determination, we also tested the quantification using portions of vaginal sheet. However, results were not reproductible, indicating existent variations between different portions of the preparation. These preliminary results highlighted the importance of considering the vaginal sheet as an unitary dosage form, since the methodology of adding *T. capitata* EO to vaginal sheets, despite having the aim to allow an homogeneous distribution over the entire surface, only guarantees that each unit presents the final concentration of 1% wEO/w sheet.

Carvacrol concentration significantly decayed in the vaginal sheet D prepared by incorporation of the artificial mixture of carvacrol + linalool, independently of the storage conditions. Vaginal sheets prepared by incorporation of the *Thymbra capitata* EO are apparently more stable during storage (regarding carvacrol concentration decay) than those prepared by incorporation of the artificial mixture of carvacrol + linalool through coating. These

results may indicate that other components (minor components) of *Thymbra capitata* EO can contribute to increasing stability, preventing volatilization.

In vaginal sheet D.O prepared by incorporating *Thymbra capitata* EO through coating (% w/w) the decrease of carvacrol concentration after storage at 5 °C for 3 months is negligible (-1.91%). On the other hand, the storage at 40 ºC significantly caused the decrease of carvacrol concentration, proving that high temperature caused the degradation or volatilization of volatile compounds as observed through loss of weight in previously shown stability data. Therefore, we propose that for this prototype a sealed primary package shall be selected, and the products shall be stored at room temperature or 5 °C, since the decrease of carvacrol after 3 months of storage is low. These results indicate that this vaginal sheet with *T. capitata* EO is stable over time and that the three-dimensional network conferred by the freeze-drying of gel effectively helped retaining the volatile compounds of EO, preventing their volatilization.

Incorporation of the EO before the freeze-drying process was not considered since it would be expected that extensive volatilization of this active would occur throughout the process.

Table 18. Evaluation of *Thymbra capitata* EO content (represented by carvacrol component) after 3 months of stability at 40 ˚C, 5 ˚C and room temperature (20-25 ˚C) in vaginal sheet D (preferred composition).

	Conditions of storage during 3 months	**Decrease of concentration of carvacrol after storage (%)**
Vaginal sheet D **Mixture Carvacrol + Linalool (0.8+0.2% w/w)**	5 °C	-41,44%
Vaginal sheet D **Mixture Carvacrol + Linalool (0.8+0.2% w/w)**	Room temperature (20-25 ºC)	-82,72%
Vaginal sheet D **Mixture Carvacrol + Linalool (0.8+0.2% w/w)**	40 °C	-93,45%
D.O ***Thymbra capitata* EO (1% w/w)**	5 °C	-1,91%
D.O ***Thymbra capitata* EO (1% w/w)**	Room temperature (20-25 ºC)	-16,83%
D.O ***Thymbra capitata* EO (1% w/w)**	40 °C	-56,26%

3.3.10. Cellular Toxicity

Due to its most promising characteristics, vaginal sheet D was selected for toxicity testing. Before performing cytotoxicity tests, we confirmed that there were no interferences due to the conversion of MTT by *Thymbra capitata* essential at tested concentrations. The absorbance determined in assays using killed cells was close to zero, indicating there were no conversion of MTT due to non-cellular mechanisms. Furthermore, these pre-tests highlighted the importance of the washing-step with PBS to totally remove the test samples before adding MMT solution. It is vital to confirm by microscopy monitoring that cell detachment after washing steps did not occur. Positive control (SDS 2%) presented viability lower than 5%. Results for viability of solvent controls indicate that solvents used on the sample's extraction and to prevent phase separation do not cause a significant reduction of cell viability. We tested

the cytotoxicity of the active substance (*Thymbra capitata* EO – figure 15), the matrix of excipients (base formulation D) and the vaginal sheet with essential oil D.O (figure 17).

In all samples, we verified a decrease of cellular viability with increasing concentration of products, as expected (figure 15, figure 17).

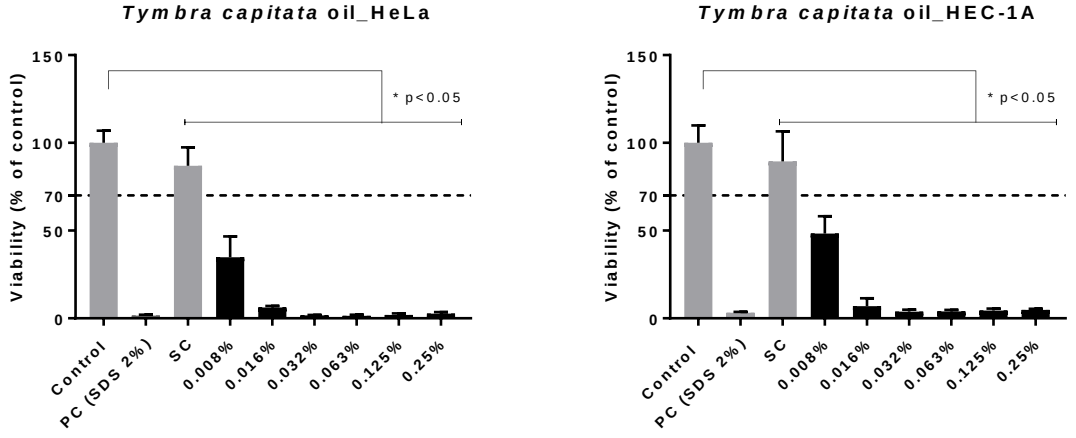

Figure 15. Cellular viability profile (MTT assay) *of Thymbra capitata* essential oil (0.25% to 0.008% v/v, corresponding to 2.5 µL/mL to 0.08 µL/mL). Cell viability is represented as percentage of the control treated only with culture media. Results are presented as the mean and standard deviations from 3 independent experiments. Horizontal bars represent statistical difference from negative control ($p<0.05$).

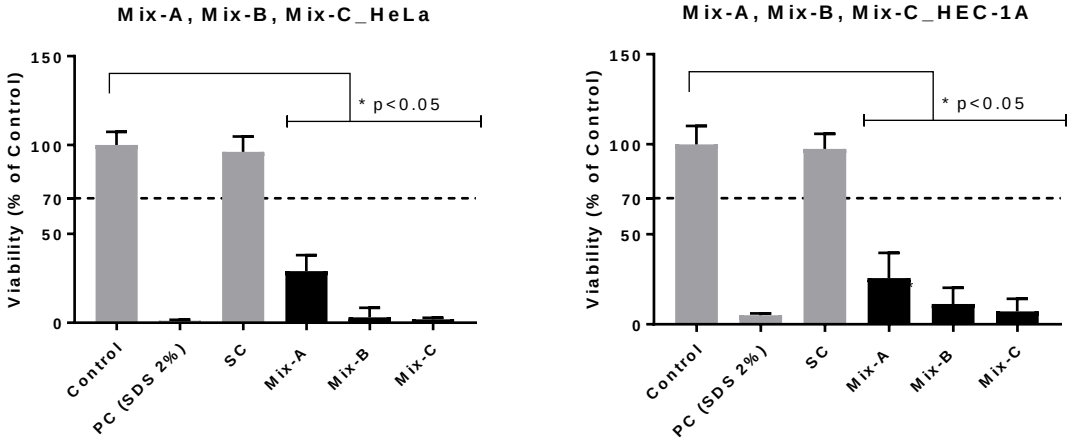

Figure 16. Cellular viability profile (MTT assay) of Mixture A (0.08 µL/mL Carvacrol + 0.32 µL/mL Linalool), Mixture B (0.02 µL/mL Carvacrol + 1.25µL/mL Linalool), Mixture-C (0.04 µL/mL Carvacrol + 2.5 µL/mLp-cymeno + 1.25 µL/mL Linalool) and solvent control (SC). Cell viability is represented as percentage of the control treated only with culture media. Results are presented as the mean and standard deviations from 3 independent experiments. Horizontal bars represent statistical difference from negative control ($p<0.05$).

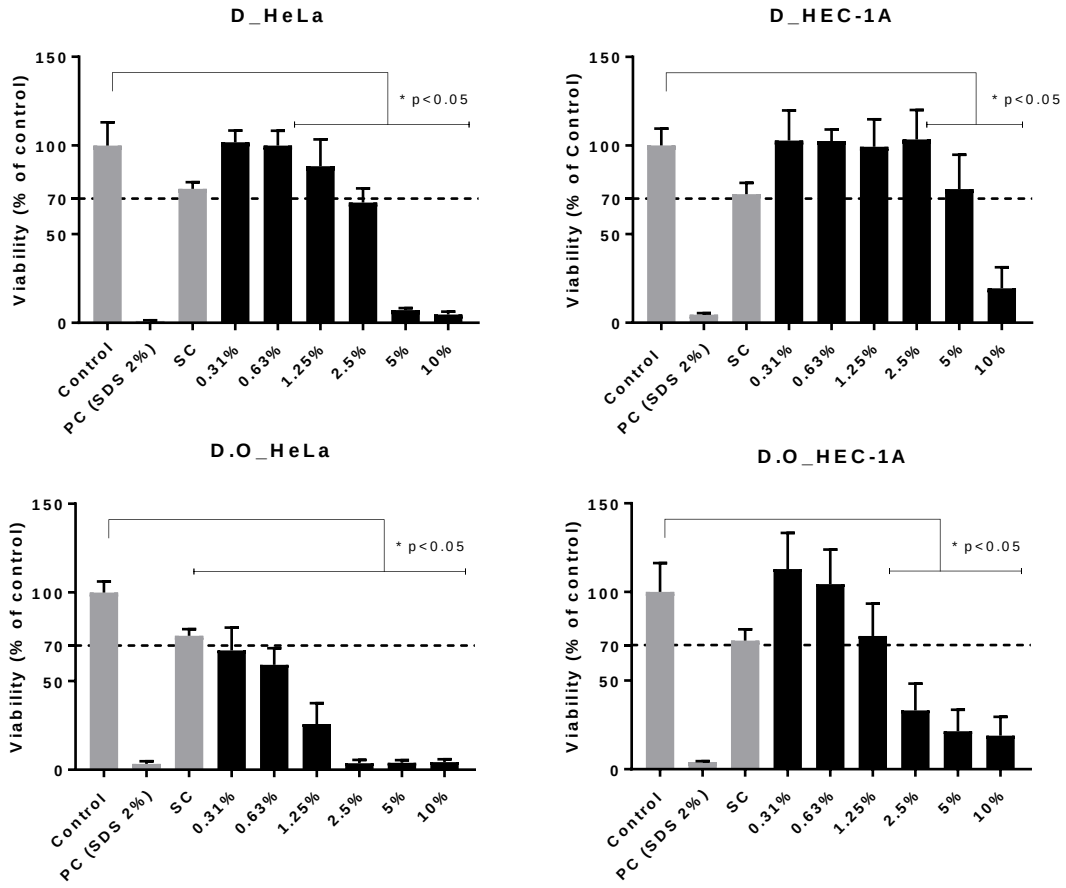

Figure 17. Cellular viability profile (MTT assay) of base formulation D tested in HeLa cell line and HEC-1A cell line and formulation D with oil tested in HeLa cell line and HEC-1A cell line. Cell viability is represented as percentage of the control treated only with culture media. Results are presented as the mean and standard deviations from 3 independent experiments. Horizontal bars represent statistical difference from negative control ($p<0.05$).

At all tested concentrations, *T. capitata* EO caused significant decrease on cellular viability (figure 15), possibly due to its potent activity and high hydrophobicity which can disintegrate the cellular membrane. Previous studies report that *T. capitata* EO exhibited a potent antibacterial activity against *Gardnerella vaginalis* planktonic cells isolated from women diagnosed with BV (MIC 0.16 µL/mL, corresponding to 0.016% v/v) [147]. MIC values for *Gardnerella* sp. UM241 biofilms of *Thymbra capitata* EO have been reported in the range 0.04 to 0.08 µL/mL and MLC values in the range 0.08 to 0.16 µL/mL [324]. At the concentration 0.32 µL/mL, *T. capitata* EO was cytotoxic for both cell lines.

We also evaluated the cytotoxicity of mixtures of components of *Thymbra capitata* EO which presented potent antimicrobial effect on *G. vaginalis* biofilms [324]. All mixtures caused a decrease of cellular viability and mixture A (0.08 µL/mL Carvacrol + 0.32 µL/mL Linalool) was the less toxic (figure 16).

The base formulation D was non-toxic for HeLa cell line at concentrations 0.31%, 0.63% and 1.25% w/v (corresponding to 0.031%, 0.0063 and 0.0125% of *Thymbra capitata* EO) and also non-toxic at 0.31% to 5% for HEC-1A cell line but revealed significant toxicity at higher concentrations for both cell lines. Considering the vaginal sheet with *T. capitata* EO (1% w/w), it

was non-toxic for HEC-1A at concentrations 0.31%, 0.63% and 1.25% (corresponding to 0.031%, 0.0063 and 0.0125% of *Thymbra capitata* EO).

The cytotoxicity of vaginal sheet D.O can be compared to the cytotoxicity using the same cell lines of Dalacin V® (a commercial semi-solid product also used for the treatment of BV) reported by Machado et al [253]. Dalacin V® was toxic, presenting lower viability than vaginal sheet D.O in both cell lines, including at lower concentrations of formulation. Moreover, Dalacin V® was also toxic when tested in *ex-vivo* model, exhibiting viabilities about 20% at product concentrations 5% and 20% w/v [253]. Thus, although this product was not biocompatible at some concentrations considering these models, it is available for the treatment of BV, since the benefits of its use for patients outweigh the possible risks. This relationship must be carefully evaluated also for the prototype here presented.

The cell lines included in this cytotoxicity study represent two different epithelia of the female genital reproductive tract. Comparing cell lines, we can observe that the assays performed upon HEC-1A cell lines conducted to higher viabilities than those performed on HeLa cells. HeLa cells were shown to be more sensitive to both the formulation and the EO contact, comparing to HEC-1A. Similar results were already reported in previous studies [253]. Gali et al studied the toxicity of some excipients and active pharmaceutical excipients using various cell lines and only slight differences in sensitivity among cell lines were observed. They reported that HEC-1A formed a stratified epithelia with a thickness of 4 to 5 cell layers (representative of intermediate between the endo- and ectocervical epithelia) and it could lead to a lower toxicity comparing to monolayer epithelia [322]. Therefore, it is vital to include different cell lines or models in the toxicity study of pharmaceutical products to achieve a more complete safety study.

3.3.11. Vaginal irritation – SkinEthic™ *Reconstructed Human Vaginal Epithelium* model

Due to the higher sensitivity of the cellular model, further cytotoxicity testing was performed in a three-dimensional model with histological resemblance to the vaginal epithelium (figure 18). In fact, tissue model is more robust and complete, although more expensive so less used in academic research.

The solvent used to dilute *T. capitata* EO and the mixture, sesame oil, do not cause a significative reduction of cellular viability, as expected.

Thymbra capitata essential oil at 0.32 μL/mL diluted in sesame oil was non-toxic when tested in tissue model, although it provided viabilities less than 10% when tested in monolayer cellular models, in both cell lines.

Moreover, mix-A was biocompatible in tissue model.

In both models (cellular or tissue), D (10% w/v) presented higher viability than D.O. (10% w/v) indicating that the *Thymbra capitata* EO contributes to the overall toxicity of the product at this concentration.

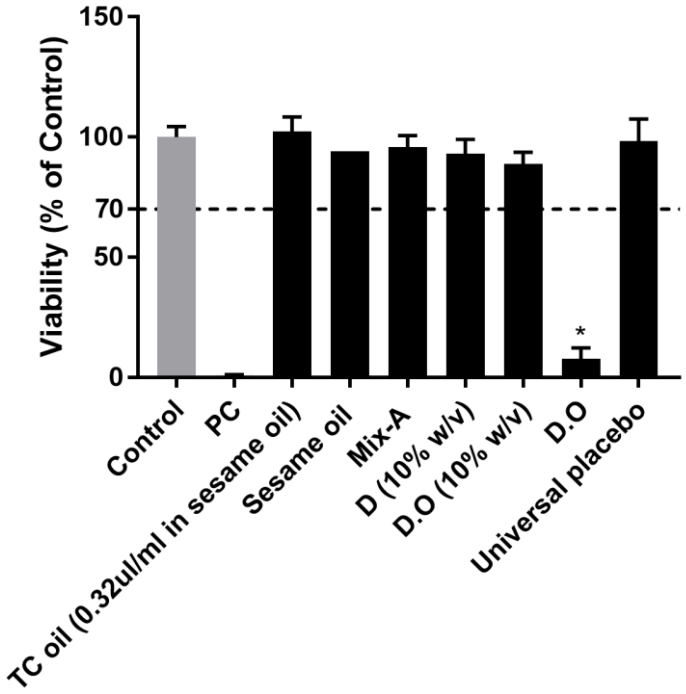

Figure 18. Cellular viability evaluated on *Reconstructed Human Vaginal Epithelium* model (MTT assay) of *Thymbra capitata* essential oil 0.32 µL/mL (diluted in sesame oil), sesame oil (solvent control), Mixture A (0.08 µL/mL Carvacrol + 0.32 µL/mL Linalool diluted in sesame oil), vaginal sheet D (10% w/v), vaginal sheet D with *Thymbra capitata* essential oil (10% w/v), vaginal sheet D with *Thymbra capitata* essential oil directly tested and universal placebo. Results are presented as the mean and bars represent standard deviations from 3 independent experiments. * represent statistical difference from negative control ($p<0.05$).

Vaginal sheet D (10% w/v) and D.O (10% w/v, corresponding to 0.1% w/v of *Thymbra capitata* EO) were biocompatible according to this model. Comparing to the universal placebo, vaginal sheet D.O was significantly more toxic. This direct application over the tissue corresponds to an extreme testing procedure assumed as a worst scenario. However, *in vivo*, vaginal sheet will not be in contact with vaginal epithelium during 24 h, since it gradually absorbs the vaginal simulant (that was not applied proportionally in this test), becoming diluted on it. So, *in vivo*, this toxicity may not occur since vaginal sheet will not be in contact with epithelium undiluted over this extended period. Intermediate toxicity may be expected when considering that only part of the formulation may swell through this contact. It should be considered that this formulation is not designed for chronic use and that the overall environment of the vaginal cavity is altered in BV.

3.3.12. Vaginal irritation - Hen's Egg Test-Chorioallantoic Membrane Assay (HET-CAM)

Vaginal sheets D and D.O were also tested in fine particles and diluted in saline solution (NaCl 0.9%). Vaginal sheets D and D.O tested in fine particles were defined as severe irritants. The hard nature and irregular and pointed shape of the particles may have influenced these results. Thus, the irritation potential obtained may result not only from the irritation caused by

the constituents of the formulation (such as the hygroscopic nature of glycerin and gelatin), but also from a mechanical effect of damage to the vessels when the sample is removed for viewing the endpoints, since there was adherence of the fine particles to the CAM [268]. Moreover, when vaginal sheets were divided into fine particles, the superficial area was obviously significantly higher than the entire sheet and then the hygroscopic characteristics of formulations (conferred mainly by gelatin and glycerine) became more evident, and it can lead to higher irritant potential. To confirm this influence on the results, the sheets D and D.O were diluted in saline solution, which is a negative control considered to be a non-severe irritant. Therefore, the irritant potential obtained in these samples may result only from the effect of the sheets' components at the tested final concentration. Vaginal sheets D and D.O diluted on saline solution were considered as non-severe irritants (IS 5 ± 0 for D and 8 ± 0 for D.O) (table 19). Concordant results were found to vaginal sheet D and D.O (10% w/v) using the tissue model.

Table 19. Irritation Score and classification of samples. Results are presented as mean values ± standard deviation (SD), n = 3

		IS (mean ± standard deviation)	**Classification**
Controls	NaCl 0.9% (w/v)	0 ± 0	Non-severe irritant
	NaOH (0.1N)	20 ± 1	Severe irritant
	SDS 1% (w/v)	10 ± 0	Severe irritant
Tested product	D (fine particles)	11 ± 1	Severe irritant
	D.O (fine particles)	11 ± 1	Severe irritant
	D 10% w/v in saline solution	5 ± 0	Non-severe irritant
	D.O 10% w/v in saline solution	8 ± 0	Non-severe irritant

For vaginal sheet D diluted on saline solution, it was observed lysis (slight and limited) at timepoint 0.5 min. For vaginal sheet D with oil it was observed lysis at timepoint 0.5 min and haemorrhage at timepoint 5 min (figure 19). The addition of *Thymbra capitata* oil to the base formulation increased the irritant potential.

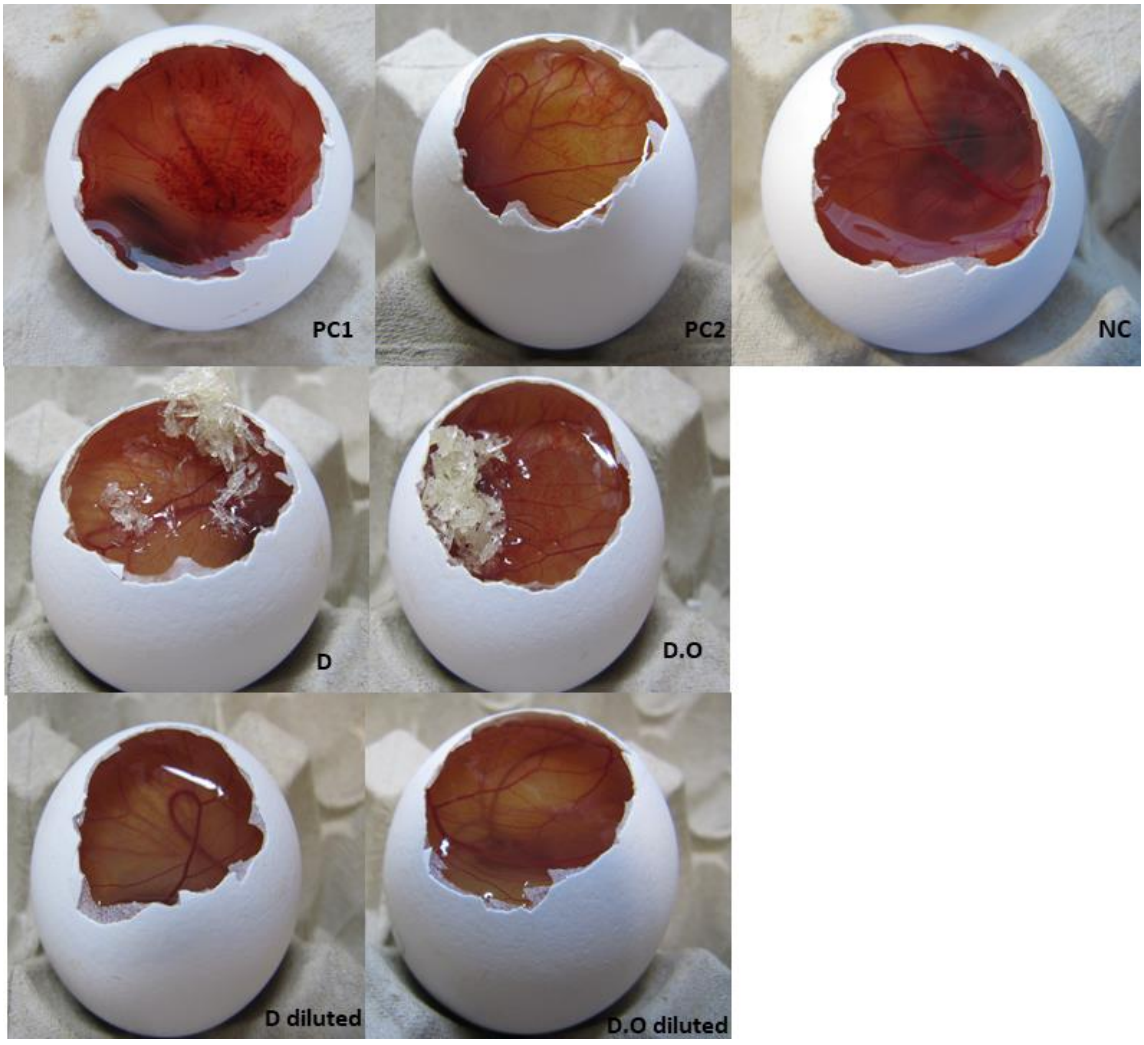

Figure 19. Irritation potential. Representative photographs (from n=3) for observation of the different endpoints at 5 min. PC1: Positive control (NaOH 0.1N) - observation of lysis, haemorrhage and coagulation (egg 2). PC2: Positive control (SDS 1%) - observation of lysis and haemorrhage (egg 1). NC: Negative control (NaCl 0,9%) - none of the endpoints being observed (egg 3). D: base formulation D finely divided – observation of lysis and haemorrhage (egg 3). D diluted: base formulation D diluted on mVFS (10% w/v) – observation of lysis (egg 2). D.O: formulation D with oil finely divided – observation of lysis and haemorrhage (egg 3). D.O. diluted: formulation D with oil diluted on mVFS (10% w/v) – observation of lysis and haemorrhage (egg 2).

3.3.13. Evaluation of the vaginal sheet D.O efficacy against *Gardnerella* species biofilms

Machado et al reported the potent bactericidal effect of *Thymbra capitata* EO against several clinical isolates of *Gardnerella spp*. planktonic cells (MIC and MLC values 0.16 µL/mL). The ability of *Thymbra capitata* EO to disrupt biofilms of several *Gardnerella spp*. strains was also reported, being effective in the biofilm biomass reduction at the range 0.16 to 0.64 µL/mL. [147]. Although recent advances in the genetic classification of *Gardnerella* spp. brought changes to the classification of bacteria formerly known as *Gardnerella vaginalis,* these results support the overall interest of this essential oil for bacteria involved in BV in concentrations up to 0.64 µL/mL [302].

Furthermore, recent results from our research group showed the potent antimicrobial activity of two EO from *Thymbra capitata* against a clinical isolate of *Gardnerella* sp. recovered

from cases of BV (MIC value of 0.04 and 0.08 µL/mL and MLC 0.08 and 0.16 µL/mL). Both *Thymbra capitata* EO, at 0.16 µL/mL caused a biofilm mass reduction of 20-30% on a 48 h biofilm of *Gardnerella* sp. UM241. *Thymbra capitata* EO (batch collected in Carvoeiro, Portugal) at 0.16 µL/mL exhibited a biomass reduction of 20-30% in *G. vaginalis* UM137, *G. piotii* UM035 and *G. swisinskii* GS 9838-1, being less effective against *G. leopoldii* UGent 09.48 (40% biomass reduction) [324].

We tested the efficacy of vaginal sheet D.O against *Gardnerella* spp. biofilms, to simulate a closer to *in vivo* scenario, since the polymicrobial dense and structured biofilms associated with BV are known to protect bacterial cells from the activity of antimicrobials, contributing to therapeutic failure. BV polymicrobial biofilms are primarily constituted by *Gardnerella vaginalis* clusters that initially adhere to vaginal epithelium and then facilitate the association and growth of other bacteria including *Atopobium vaginae*, *Mobiluncus* spp. and *Prevotella* spp. with a synergic effect in the reestablishment of infection [3,104,106,107,147,295,296]. Since, to date, the role of each species of *Gardnerella* in the reestablishment of infection is not totally clarified, (mostly due to the recent classification of species previously referred to as *Gardnerella vaginalis*) we included 4 species of *Gardnerella* for a more complete study considering several possible preponderant BV caused bacteria [302]. So, we evaluated the efficacy of the vaginal sheet containing *Thymbra capitata* EO upon *Gardnerella* spp. cells organized in biofilms. The tested concentrations considering the EO component were 0.32 µL/mL and 0.08 µL/mL, selected considering previous studies and the cytotoxicity results upon 3D models obtained in this study.

Our results showed that vaginal sheet D.O at 0.32 µL/mL of *T. capitata* EO was able to significantly reduce bacterial load of all tested *Gardnerella* species, being less efficient in *G. piotti* (figure 20). For all strains except *G. piotti* vaginal sheet D.O at 0.32 µL/mL of *T. capitata* EO was able to reduce the number of CFU below the limit of detection/quantification of the method. Our results from the vaginal irritation study using SkinEthic™ Reconstructed Human Vaginal Epithelium model indicated that this concentration (0.32 µL/mL of *T. capitata* EO) was biocompatible. For *G. leopoldi* 0.08 µL/mL were still able to reduce bacterial load. The goal of this study was not specifically to define the MLC of the formulation but to assess its ability to reduce the viability of the cells below the limit of quantification with the defined dose of oil that was not toxic to the 3D model. We can conclude that the formulation did not impair the effect of *Thymbra capitata* EO against cells of *Gardnerella spp. vaginalis* organized in biofilms. For most of the tested strains a dose-response curve is clearly observed. The *Gardnerella* species tested showed different sensibilities for vaginal sheet D.O. This fact should be considered in the rational design of new products being particularly relevant in the context of BV etiology, since the BV-related biofilms are polymicrobial. Thus, the concentration of *Thymbra capitata* EO to be incorporated in the drug delivery system should be effective against several BV related bacteria, to guarantee the success of the treatment.

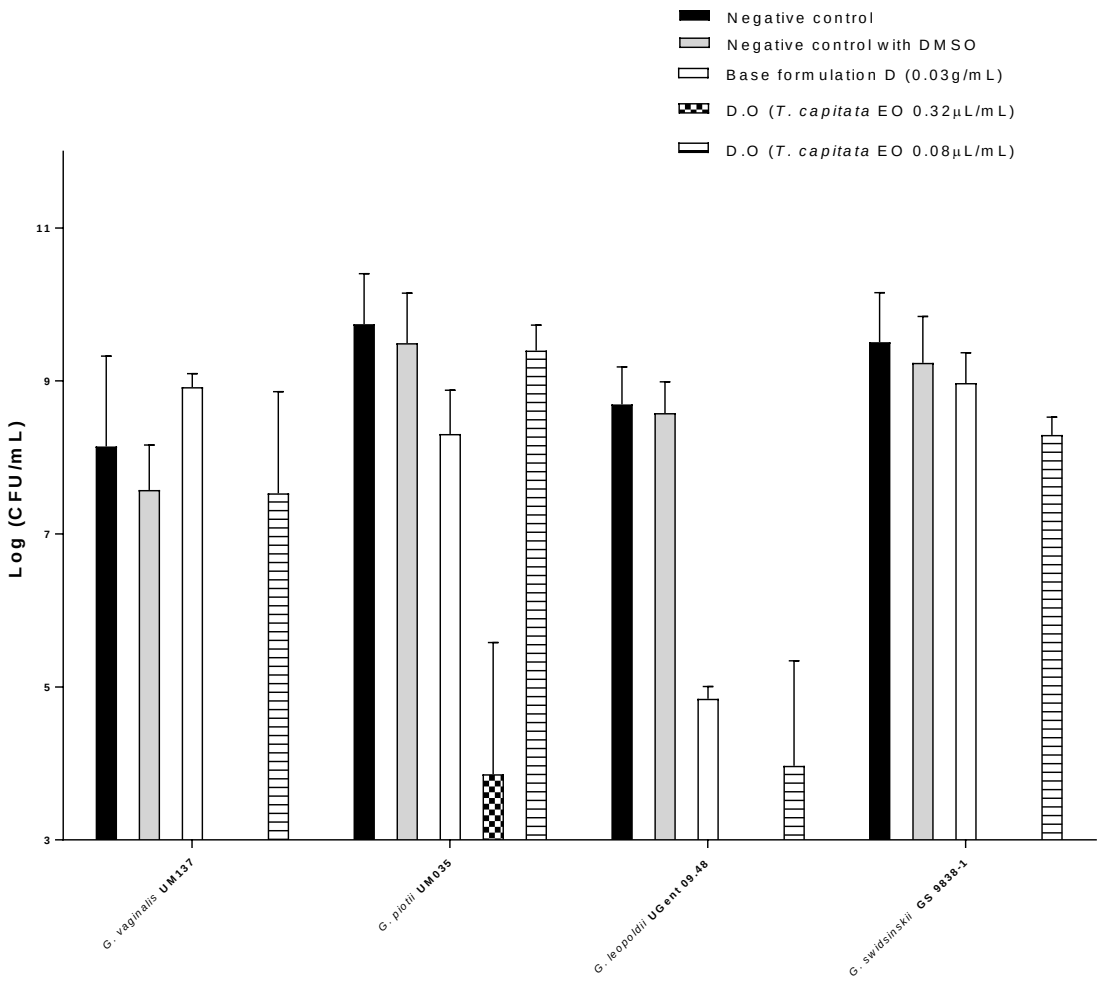

Figure 20. Activity of vaginal sheet D.O with *T. capitata* EO on 48h biofilm of four different species of Gardnerella at 0.32 µL/mLand 0.08 µL/mL, assessed as biofilm cells culturability. Limit of detection of the method corresponds to 3 Log (CFU/mL). Results from 3 independent assays (n=2 technical replicates)

In the absence of EO (base formulation tested at the same concentration that corresponds to the dilution of DO needed to achieve 0.32 µL/mL of EO) there was no direct inhibition on *G. vaginalis*, *G. piotti* or *G. swidsinskii* cells culturability from biofilms. However, for *G. leopoldi*, the base formulation D was able to reduce around 4 Logs CFUs detected. These results show that the strategies of including chitosan and the buffer solution in the formulation did not improve the efficacy profile of the overall formulation, for this specific outcome, upon most of the tested species but, indeed, some species may be sensitive to these effects. This is especially important since the polymicrobial biofilm of BV is envisaged. Also, it is important to highlight that the composition defined for the base intends to target the disease and not only the pathogen. For example, decreasing the pH of the vagina may be important to promote the reestablishment of the vaginal flora through beneficial growth of the protective microbiota. Also absorption of fluids may enhance the effect of the EO delivered through the formulation. The concentration of formulation that corresponds to 0.32 µL/mL of *T. capitata* EO is 0.03 g/mL. This is much lower than the concentration assessed for compatibility with the 3D model (0.1

g/mL of base formulation corresponding to 1.07 µL/mL). Furthermore, the dose showed as biocompatible corresponds to a 10% dispersion of the final product. These results may indicate that the concentration of *T. capitata* EO in the vaginal sheet is too high. Therefore, it would be interesting to optimize the proportion of excipients: *T. capitata* EO, decreasing the concentration of *T. capitata* EO in vaginal sheets, allowing to maintain the effect of active substance and simultaneously promoting the synergic effect of excipients. This may be achieved by diluting the EO in sesame oil before coating of vaginal sheets.

3.4. Conclusion

The rational design of vaginal sheets with *Thymbra capitata* EO involved strategies in the field of pharmaceutical technology to potentiate the treatment of BV through complementary and synergic action of therapeutic agent and excipients. The inclusion of LA buffer in formulations represents an additional mechanism to re-establish the healthy saprophytic flora, contributing for treatment efficacy and preventing recurrence. Besides, the inclusion of bioadhesive excipients will allow an intimate and prolonged contact of formulations with epithelium, promoting the efficacy of treatment. Furthermore, the excipient chitosan was included due to its bioadhesive properties and also due to its antimicrobial effect against agents that cause vaginal infection, so it can improve therapeutic success, although it can be species dependent.

All vaginal sheets developed in this study exhibited sensorial characteristics that anticipate a simple and comfortable application for women, allowing them to be easily wrapped around a finger and inserted, with limited expected leakage after application, promoting the user's compliance.

Vaginal sheet D, composed by water, lactic acid, sodium lactate, gelatin, glycerin, chitosan coated with *Thymbra capitata* EO presented promising technological characteristics and the best predictable *in vivo* performance. D.O presented the higher buffer capacity and ability to absorb mVFS among all vaginal sheets with EO and one of the bests bioadhesive profiles. Moreover, vaginal sheet D.O exhibited flexibility that allow it to be easily rolled and handled without breaking and structure (hardness and thickness) that allows the insertion.

This formulation was also able to hinder biofilm cells culturability of BV pathogens. These results show that this product acts simultaneously and immediately in relieving the preponderant symptom (abundant vaginal discharge with unpleasant odor) and in inhibiting the pathogen. Effective concentrations were much lower than the concentration tested as biocompatible in the toxicity studies (0.03g/mL for efficacy and 0.1 g/mL tested as biocompatible). Dose dependent toxicity was observed for this vaginal sheet that indicate that the concentration of EO may be reduced. Nevertheless, this product was not developed for a chronic use, but for a short time period of treatment, so controlled risk of minor irritation may be accepted if it is limited and completely reversed at the end of the short treatment.

4. Concluding remarks and future perspectives

The aims proposed in the beginning of this thesis were achieved. New therapeutic strategies to treat topically the two commonest vaginal infections were explored and scientific knowledge which allows to better manage these two clinical conditions with high prevalence and impact was generated. Therefore, this work further opened pathways of research that can continue to be studied in the academia to be applied in the pharmaceutical industry to improve the performance of the developed formulations. Through this pathway new effective and safe therapeutic options for the treatment of vaginal infections and for preventing recurrence may reach the market substantially improving women's health.

Although these formulations presented promising results in assays performed which simulate "close-to-use" *in vivo* conditions, in pre-clinical phase more complete and exhaustive characterization shall be conducted in the future.

The prototype gel C (5% sodium bicarbonate, 1% carbomer and 94% water) was shown effective in inhibiting *C. albicans'* growth and it was also effective against other species of *Candida*. The low viscosity and low firmness, predicts good spreading and coating of the epithelium and, in the other hand, the mucoadhesive characteristics and the reduced volume of administration may promote leakage and prolong the residence time. Despite presenting high pH and being hyperosmolar, the *in vivo* dilution in vaginal fluid can correct these parameters to values closer to physiologic ranges, and these characteristics seem not to affect the safety of the product. Furthermore, it shall be considered that the product was designed for treatment purposes and not for chronic use.

The prototype vaginal sheet D with *T. capitata* EO presented sensorial characteristics that allow to fold it around one finger and predict comfortable insertion. Importantly, D.O exhibited an excellent capacity to absorb vaginal fluid simulant, providing an effect that may immediate relief excessive vaginal discharge with unpleasant odor, which will give comfort and promote the compliance of treatment. Besides, it brings together additional mechanisms that may potentiate the treatment, such as the buffer capacity, the antimicrobial activity of chitosan and the bioadhesive profile.

Vaginal sheet D.O is now being characterized for antimicrobial activity against BV-related bacteria planktonic cells. As future perspective, it will be interesting to evaluate the efficacy and safety of vaginal sheet D.O containing lower concentrations of *T. capitata* EO (such as 0.50 and 0.25% w/w) to decrease the proportion EO:Base formulation for a better understanding of the formulation's contribution to antimicrobial activity.

Moreover, the stability of vaginal sheet D.O shall continue to be monitored over time considering the quantification of *T. capitata* EO using gas chromatography with flame ionization detection (GC/FID) to assure that the product might be labelled with an expiration period of at least 3 years (important for commercial purposes).

Soon, a multilayer vaginal sheet strategy with a sequential dispersion profile will be explored, in which one of the layers will be the prototype presented here and the other layer will contain inorganic clays, which are excipients with a high capacity to absorb odor. So, besides treating the infection with *T. capitata* essential oil the multilayer sheet will immediately provide comfort to users by effectively eliminate the rotten fish odor of the fluid. This multilayer vaginal sheet will combine the function of treatment and simultaneously alleviate symptoms.

References

[1] J. Ravel, P. Gajer, Z. Abdo, G.M. Schneider, S.S.K. Koenig, S.L. McCulle, S. Karlebach, R. Gorle, J. Russell, C.O. Tacket, R.M. Brotman, C.C. Davis, K. Ault, L. Peralta, L.J. Forney, Vaginal microbiome of reproductive-age women, Proc. Natl. Acad. Sci. 108 (2011) 4680–4687. https://doi.org/10.1073/pnas.1002611107.

[2] M.N. Anahtar, D.B. Gootenberg, C.M. Mitchell, D.S. Kwon, Cervicovaginal Microbiota and Reproductive Health : The Virtue of Simplicity, Cell Host Microbe. 23 (2018) 159–168. https://doi.org/10.1016/j.chom.2018.01.013.

[3] S.J. Kroon, J. Ravel, W. Huston, Cervicovaginal microbiota, women's health, and reproductive outcomes, Fertil. Steril. 110 (2018) 327–336. https://doi.org/10.1016/j.fertnstert.2018.06.036.

[4] S. Borges, J. Silva, P. Teixeira, The role of lactobacilli and probiotics in maintaining vaginal health, Arch. Gynecol. Obstet. 289 (2013) 479–489. https://doi.org/10.1007/s00404-013-3064-9.

[5] A. Javed, F. Parvaiz, S. Manzoor, Bacterial vaginosis: An insight into the prevalence, alternative regimen treatments and it's associated resistance patterns, Microb. Pathog. 127 (2018) 21–30. https://doi.org/10.1016/j.micpath.2018.11.046.

[6] S. Kovachev, Defence factors of vaginal lactobacilli, Crit. Rev. Microbiol. 44 (2018) 31–39. https://doi.org/10.1080/1040841X.2017.1306688.

[7] A. V. Sgibnev, E.A. Kremleva, Vaginal protection by H2O2-producing lactobacilli, Jundishapur J. Microbiol. 8 (2015) 4–8. https://doi.org/10.5812/jjm.22913.

[8] G. Tachedjian, D.E. O'Hanlon, J. Ravel, The implausible "in vivo" role of hydrogen peroxide as an antimicrobial factor produced by vaginal microbiota, Microbiome. 6 (2018) 3–7. https://doi.org/10.1186/s40168-018-0418-3.

[9] N. Recine, E. Palma, L. Domenici, M. Giorgini, L. Imperiale, C. Sassu, A. Musella, C. Marchetti, L. Muzii, P.B. Panici, Restoring vaginal microbiota: biological control of bacterial vaginosis. A prospective case – control study using Lactobacillus rhamnosus BMX 54 as adjuvant treatment against bacterial vaginosis, Arch. Gynecol. Obstet. 293 (2016) 101–107. https://doi.org/10.1007/s00404-015-3810-2.

[10] H.M.E. Willems, S.S. Ahmed, J. Liu, Z. Xu, B.M. Peters, Vulvovaginal candidiasis: A current understanding and burning questions, J. Fungi. 6 (2020) 1–20. https://doi.org/10.3390/jof6010027.

[11] F. Blostein, E. Levin-Sparenberg, J. Wagner, B. Foxman, Recurrent vulvovaginal candidiasis, Ann. Epidemiol. 27 (2017) 575-582.e3. https://doi.org/10.1016/j.annepidem.2017.08.010.

[12] S. Soni, P. Horner, M. Rayment, N. Pinto-Sander, N. Naous, A. Parkhouse, D. Bancroft, C. Patterson, H. Fifer, British Association for Sexual Health and HIV national guideline

for the management of vulvovaginal candidiasis, Int. J. STD AIDS. 30 (2019) 938–950. https://doi.org/10.1177/0956462419825948.

[13] Centers for Disease Control and Prevention, Sexually Transmitted Diseases Treatment Guidelines: Vulvovaginal Candidiasis, (2015). https://www.cdc.gov/std/tg2015/candidiasis.htm (accessed January 16, 2020).

[14] A. Matheson, D. Mazza, Recurrent vulvovaginal candidiasis: A review of guideline recommendations, Aust. New Zeal. J. Obstet. Gynaecol. 57 (2017) 139–145. https://doi.org/10.1111/ajo.12592.

[15] J. Sherrard, J. Wilson, G. Donders, W. Mendling, J.S. Jensen, 2018 European (IUSTI/WHO) International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge, Int. J. STD AIDS. 29 (2018) 1258–1272. https://doi.org/10.1177/0956462418785451.

[16] P. Faria-Gonçalves, J. Rolo, C. Gaspar, R. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, A. Palmeira-de-Oliveira, Virulence Factors as Promoters of Chronic Vulvovaginal Candidosis: A Review, Mycopathologia. 186 (2021) 755–773. https://doi.org/10.1007/s11046-021-00592-8.

[17] R. Palmeira-de-Oliveira, A. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, New strategies for local treatment of vaginal infections, Adv. Drug Deliv. Rev. 92 (2015) 105–122. https://doi.org/10.1016/j.addr.2015.06.008.

[18] C. Rodríguez-Cerdeira, M.C. Gregorio, A. Molares-Vila, A. López-Barcenas, G. Fabbrocini, B. Bardhi, A. Sinani, E. Sánchez-Blanco, R. Arenas-Guzmán, R. Hernandez-Castro, Biofilms and vulvovaginal candidiasis, Colloids Surfaces B Biointerfaces. 174 (2019) 110–125. https://doi.org/10.1016/j.colsurfb.2018.11.011.

[19] B. Gonçalves, C. Ferreira, C.T. Alves, M. Henriques, J. Azeredo, S. Silva, Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors, Crit. Rev. Microbiol. 42 (2016) 905–927. https://doi.org/10.3109/1040841X.2015.1091805.

[20] J.D. Sobel, Recurrent vulvovaginal candidiasis, Am. J. Obstet. Gynecol. 214 (2016) 15–21. https://doi.org/10.1016/j.ajog.2015.06.067.

[21] O. Makanjuola, F. Bongomin, S.A. Fayemiwo, An update on the roles of non-albicans candida species in vulvovaginitis, J. Fungi. 4 (2018). https://doi.org/10.3390/jof4040121.

[22] T.C. Felix, D.V.D. de Brito Röder, R. dos Santos Pedroso, Alternative and complementary therapies for vulvovaginal candidiasis, Folia Microbiol. (Praha). 64 (2019) 133–141. https://doi.org/10.1007/s12223-018-0652-x.

[23] J.D. Sobel, R. Sobel, Current treatment options for vulvovaginal candidiasis caused by azole-resistant Candida species, Expert Opin. Pharmacother. 19 (2018) 971–977. https://doi.org/10.1080/14656566.2018.1476490.

[24] S.Y. Chew, L.T.L. Than, Vulvovaginal candidosis: Contemporary challenges and the future of prophylactic and therapeutic approaches, Mycoses. 59 (2016) 262–273. https://doi.org/10.1111/myc.12455.

[25] E. Sitterle, A.T. Coste, T. Obadia, C. Maufrais, M. Chauvel, N. Sertour, D. Sanglard, A. Puel, C.D. Enfert, M. Bougnoux, Large-scale genome mining allows identification of neutral polymorphisms and novel resistance mutations in genes involved in Candida albicans resistance to azoles and echinocandins, 11 (2020) 1–14. https://doi.org/10.1093/jac/dkz537.

[26] J.A. Hendrickson, C. Hu, S.L. Aitken, N. Beyda, Antifungal Resistance: a Concerning Trend for the Present and Future, Curr. Infect. Dis. Rep. 21 (2019). https://doi.org/10.1007/s11908-019-0702-9.

[27] A.T. Nishimoto, C. Sharma, P.D. Rogers, Molecular and genetic basis of azole antifungal resistance in the opportunistic pathogenic fungus Candida albicans, J. Antimicrob. Chemother. (2019) 1–14. https://doi.org/10.1093/jac/dkz400.

[28] R. Prasad, R. Nair, A. Banerjee, Multidrug transporters of Candida species in clinical azole resistance, Fungal Genet. Biol. 132 (2019) 103252. https://doi.org/10.1016/j.fgb.2019.103252.

[29] K.E. Pristov, M.A. Ghannoum, Resistance of Candida to azoles and echinocandins worldwide, Clin. Microbiol. Infect. 25 (2019) 792–798. https://doi.org/10.1016/j.cmi.2019.03.028.

[30] C.A. Muzny, J.R. Schwebke, Biofilms: An Underappreciated Mechanism of Treatment Failure and Recurrence in Vaginal Infections, Clin. Infect. Dis. 61 (2015) 601–606. https://doi.org/10.1093/cid/civ353.

[31] C. Iavazzo, I.D. Gkegkes, I.M. Zarkada, M.E. Falagas, Boric acid for recurrent vulvovaginal candidiasis: The clinical evidence, J. Women's Heal. 20 (2011) 1245–1255. https://doi.org/10.1089/jwh.2010.2708.

[32] F. De Seta, M. Schmidt, B. Vu, M. Essmann, B. Larsen, Antifungal mechanisms supporting boric acid therapy of Candida vaginitis, J. Antimicrob. Chemother. 63 (2009) 325–336. https://doi.org/10.1093/jac/dkn486.

[33] M. Pointer, Benjamin R; Boyer, Michel; Schmidt, Boric acid destabilizes the hyphal cytoskeleton and inhibits invasive growth of Candida albicans, Yeast. (2008) 191–198. https://doi.org/10.1002/yea.

[34] A. Powell, K.G. Ghanem, L. Rogers, A. Zinalabedini, R.M. Brotman, J. Zenilman, S. Tuddenham, Clinicians' Use of Intravaginal Boric Acid Maintenance Therapy for Recurrent Vulvovaginal Candidiasis and Bacterial Vaginosis, Sex. Transm. Dis. 46 (2019) 810–812. https://doi.org/10.1097/olq.0000000000001063.

[35] S. Guaschino, F. De Seta, A. Sartore, G. Ricci, D. De Santo, M. Piccoli, S. Alberico, Efficacy of maintenance therapy with topical boric acid in comparison with oral

itraconazole in the treatment of recurrent vulvovaginal candidiasis, Am. J. Obstet. Gynecol. 184 (2001) 598–602. https://doi.org/10.1067/mob.2001.111938.

[36] J.D. Sobel, W. Chaim, V. Nagappan, D. Leaman, Treatment of vaginitis caused by Candida glabrata: Use of topical boric acid and flucytosine, Am. J. Obstet. Gynecol. 189 (2003) 1297–1300. https://doi.org/10.1067/S0002-9378(03)00726-9.

[37] D. Ray, R. Goswami, U. Banerjee, V. Dadhwal, D. Goswami, P. Mandal, V. Sreenivas, N. Kochupillai, Prevalence of Candida glabrata and its response to boric acid vaginal suppositories in comparison with oral fluconazole in patients with diabetes and vulvovaginal candidiasis, Diabetes Care. 30 (2007) 312–317. https://doi.org/10.2337/dc06-1469.

[38] S.R. Fan, X.P. Liu, In vitro fluconazole and nystatin susceptibility and clinical outcome in complicated vulvovaginal candidosis, Mycoses. 54 (2011) 501–505. https://doi.org/10.1111/j.1439-0507.2010.01887.x.

[39] T.M. Brandolt, G.B. Klafke, C.V. Gonçalves, L.R. Bitencourt, A.M.B. de Martinez, J.F. Mendes, M.C.A. Meireles, M.O. Xavier, Prevalence of Candida spp. in cervical-vaginal samples and the in vitro susceptibility of isolates, Brazilian J. Microbiol. 48 (2017) 145–150. https://doi.org/10.1016/j.bjm.2016.09.006.

[40] F. Choukri, M. Benderdouche, P. Sednaoui, In vitro susceptibility profile of 200 recent clinical isolates of Candida spp. to topical antifungal treatments of vulvovaginal candidiasis, the imidazoles and nystatin agents, J. Mycol. Med. 24 (2014) 303–307. https://doi.org/10.1016/j.mycmed.2014.05.001.

[41] S. Fan, X. Liu, C. Wu, L. Xu, J. Li, Vaginal Nystatin Versus Oral Fluconazole for the Treatment for Recurrent Vulvovaginal Candidiasis, Mycopathologia. 179 (2015) 95–101. https://doi.org/10.1007/s11046-014-9827-4.

[42] X. Zhang, T. Li, X. Chen, S. Wang, Z. Liu, Nystatin enhances the immune response against Candida albicans and protects the ultrastructure of the vaginal epithelium in a rat model of vulvovaginal candidiasis, BMC Microbiol. 18 (2018) 1–11. https://doi.org/10.1186/s12866-018-1316-3.

[43] N. Majdabadi, M. Falahati, F. Heidarie-Kohan, S. Farahyar, P. Rahimi-Moghaddam, M. Ashrafi-Khozani, T. Razavi, S. Mohammadnejad, Effect of 2-Phenylethanol as Antifungal Agent and Common Antifungals (Amphotericin B, Fluconazole, and Itraconazole) on Candida Species Isolated from Chronic and Recurrent Cases of Candidal Vulvovaginitis, Assay Drug Dev. Technol. 16 (2018) 141–149. https://doi.org/10.1089/adt.2017.837.

[44] M. Prażyńska, E. Gospodarek, In Vitro Effect of Amphotericin B on Candida albicans, Candida glabrata and Candida parapsilosis Biofilm Formation, Mycopathologia. 177 (2014) 19–27. https://doi.org/10.1007/s11046-014-9727-7.

[45] M. El-Azizi, N. Farag, N. Khardori, Antifungal activity of amphotericin B and voriconazole against the biofilms and biofilm-dispersed cells of Candida albicans

employing a newly developed in vitro pharmacokinetic model, Ann. Clin. Microbiol. Antimicrob. 14 (2015) 1–9. https://doi.org/10.1186/s12941-015-0083-3.

[46] C.F. Rodrigues, S. Silva, J. Azeredo, M. Henriques, Candida glabrata's recurrent infections: biofilm formation during Amphotericin B treatment, Lett. Appl. Microbiol. 63 (2016) 77–81. https://doi.org/10.1111/lam.12600.

[47] E.M. Maiolo, U.F. Tafin, O. Borens, A. Trampuz, Activities of fluconazole, caspofungin, anidulafungin, and amphotericin b on planktonic and biofilm candida species determined by microcalorimetry, Antimicrob. Agents Chemother. 58 (2014) 2709–2717. https://doi.org/10.1128/AAC.00057-14.

[48] L. Hanson, L. Vandevusse, M. Jermé, C.L. Abad, N. Safdar, Probiotics for Treatment and Prevention of Urogenital Infections in Women: A Systematic Review, J. Midwifery Women's Heal. 61 (2016) 339–355. https://doi.org/10.1111/jmwh.12472.

[49] A. López-Moreno, M. Aguilera, Vaginal Probiotics for Reproductive Health and Related Dysbiosis: Systematic Review and Meta-Analysis, J. Clin. Med. 10 (2021) 1461. https://doi.org/10.3390/jcm10071461.

[50] A. Shenoy, A. Gottlieb, Probiotics for oral and vulvovaginal candidiasis: A review, Dermatol. Ther. 32 (2019). https://doi.org/10.1111/dth.12970.

[51] J.H.H.M. van de Wijgert, M.C. Verwijs, Lactobacilli-containing vaginal probiotics to cure or prevent bacterial or fungal vaginal dysbiosis: a systematic review and recommendations for future trial designs, BJOG An Int. J. Obstet. Gynaecol. 127 (2020) 287–299. https://doi.org/10.1111/1471-0528.15870.

[52] H. Jeng, T. Yan, J. Chen, Treating vaginitis with probiotics in non-pregnant females: A systematic review and meta-analysis, Exp. Ther. Med. (2020) 3749–3765. https://doi.org/10.3892/etm.2020.9090.

[53] W.J.Y. Chee, S.Y. Chew, L.T.L. Than, Vaginal microbiota and the potential of Lactobacillus derivatives in maintaining vaginal health, Microb. Cell Fact. 19 (2020) 1–24. https://doi.org/10.1186/s12934-020-01464-4.

[54] E.F.M. Oerlemans, G. Bellen, I. Claes, T. Henkens, C.N. Allonsius, S. Wittouck, M.F.L. van den Broek, S. Wuyts, F. Kiekens, G.G.G. Donders, S. Lebeer, Impact of a lactobacilli-containing gel on vulvovaginal candidosis and the vaginal microbiome, Sci. Rep. 10 (2020) 1–10. https://doi.org/10.1038/s41598-020-64705-x.

[55] R.D. Wagner, S.J. Johnson, Probiotic lactobacillus and estrogen effects on vaginal epithelial gene expression responses to Candida albicans, J. Biomed. Sci. 19 (2012) 1–8. https://doi.org/10.1186/1423-0127-19-58.

[56] T. Andersen, E. Mishchenko, G.E. Flaten, J.U.E. Sollid, S. Mattsson, I. Tho, N. Škalko-basnet, Chitosan-Based Nanomedicine to Fight Genital Candida Infections : Chitosomes, Mar. Drugs. 15 (2017) 1–12. https://doi.org/10.3390/md15030064.

[57] N.L. Calvo, S. Sreekumar, L.A. Svetaz, M.C. Lamas, B.M. Moerschbacher, D. Leonardi, Design and characterization of chitosan nanoformulations for the delivery of antifungal agents, Int. J. Mol. Sci. 20 (2019). https://doi.org/10.3390/ijms20153686.

[58] A. Palmeira-De-Oliveira, M.P. Ribeiro, R. Palmeira-De-Oliveira, C. Gaspar, S. Costa-De-Oliveira, I.J. Correia, C. Pina Vaz, J. Martinez-De-Oliveira, J.A. Queiroz, A.G. Rodrigues, Anti-Candida activity of a chitosan hydrogel: Mechanism of action and cytotoxicity profile, Gynecol. Obstet. Invest. 70 (2010) 322–327. https://doi.org/10.1159/000314023.

[59] A. Silva-Dias, A. Palmeira-De-Oliveira, I.M. Miranda, J. Branco, L. Cobrado, M. Monteiro-Soares, J.A. Queiroz, C. Pina-Vaz, A.G. Rodrigues, Anti-biofilm activity of low-molecular weight chitosan hydrogel against Candida species, Med. Microbiol. Immunol. 203 (2014) 25–33. https://doi.org/10.1007/s00430-013-0311-4.

[60] F. Strains, W. Lo, F. Deng, C. Chang, C. Lin, Synergistic Antifungal Activity of Chitosan with Fluconazole against Candida albicans, Candida tropicalis, and Fluconazole-Resistant Strains, (2020) 22–24.

[61] V.H.S. Araujo, M.P.C. de Souza, G.C. Carvalho, J.L. Duarte, M. Chorilli, Chitosan-based systems aimed at local application for vaginal infections, Carbohydr. Polym. 261 (2021). https://doi.org/10.1016/j.carbpol.2021.117919.

[62] A. Palmeira-de-Oliveira, L.A. Passarinha, C. Gaspar, R. Palmeira-de-Oliveira, B. Sarmento, J. Martinez-de-Oliveira, C. Pina-Vaz, A.G. Rodrigues, J.A. Queiroz, The relationship between Candida species charge density and chitosan activity evaluated by ion-exchange chromatography, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (2011) 3749–3751. https://doi.org/10.1016/j.jchromb.2011.07.034.

[63] R.A. Costa Silva, C.R. da Silva, J.B. de Andrade Neto, A.R. da Silva, R.S. Campos, L.S. Sampaio, F.B.S.A. do Nascimento, B. da Silva Gaspar, S.G. da Cruz Fonseca, M.A.A. Josino, T.B. Grangeiro, D.M. Gaspar, D.F. de Lucena, M.O. de Moraes, B.C. Cavalcanti, H.V. Nobre Júnior, In vitro anti-Candida activity of selective serotonin reuptake inhibitors against fluconazole-resistant strains and their activity against biofilm-forming isolates, Microb. Pathog. 107 (2017) 341–348. https://doi.org/10.1016/j.micpath.2017.04.008.

[64] W. Gu, D. Guo, L. Zhang, D. Xu, S. Sun, The synergistic effect of azoles and fluoxetine against resistant Candida albicans strains is attributed to attenuating fungal virulence, 2016. https://doi.org/10.1128/AAC.03046-15.

[65] A.S. Oliveira, J. Martinez-de-Oliveira, G.G.G. Donders, R. Palmeira-de-Oliveira, A. Palmeira-de-Oliveira, Anti-Candida activity of antidepressants sertraline and fluoxetine: effect upon pre-formed biofilms, Med. Microbiol. Immunol. 207 (2018) 195–200. https://doi.org/10.1007/s00430-018-0539-0.

[66] A.S. Oliveira, C.A. Gaspar, R. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, A. Palmeira-

de-Oliveira, Anti-Candida activity of fluoxetine alone and combined with fluconazole: A synergistic action against fluconazole-resistant strains, Antimicrob. Agents Chemother. 58 (2014) 4224–4226. https://doi.org/10.1128/AAC.02623-13.

[67] A. Palmeira-De-Oliveira, A.R. Ramos, C. Gaspar, R. Palmeira-De-Oliveira, P. Gouveia, J. Martinez-De-Oliveira, In vitro anti-Candida activity of lidocaine and nitroglycerin: Alone and combined, Infect. Dis. Obstet. Gynecol. 2012 (2012). https://doi.org/10.1155/2012/727248.

[68] E. González-Burgos, M.P. Gómez-Serranillos, Natural Products for Vulvovaginal Candidiasis Treatment: Evidence from Clinical Trials, Curr. Top. Med. Chem. 18 (2018) 1324–1332. https://doi.org/10.2174/1568026618666181002111341.

[69] A. Palmeira-de-Oliveira, L. Salgueiro, R. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, C. Pina-Vaz, J.A. Queiroz, A.G. Rodrigues, Anti- Candida Activity of Essential Oils, Mini-Reviews Med. Chesmistry. 9 (2009) 1292–1305. https://doi.org/10.2174/138955709789878150.

[70] E. Bona, S. Cantamessa, M. Pavan, G. Novello, N. Massa, A. Rocchetti, G. Berta, E. Gamalero, Sensitivity of Candida albicans to essential oils: are they an alternative to antifungal agents?, J. Appl. Microbiol. 121 (2016) 1530–1545. https://doi.org/10.1111/jam.13282.

[71] N.H. Abd Ellah, A.S. Shaltout, S.M.M. Abd El Aziz, A.M. Abbas, H.G. Abd El Moneem, E.M. Youness, A.F. Arief, M.F. Ali, B.N. Abd El-hamid, Vaginal suppositories of cumin seeds essential oil for treatment of vaginal candidiasis: Formulation, in vitro, in vivo, and clinical evaluation, Eur. J. Pharm. Sci. 157 (2021) 105602. https://doi.org/10.1016/j.ejps.2020.105602.

[72] R.S. Francisconi, P.M.M. Huacho, C.C. Tonon, E.A.F. Bordini, M.F. Correia, J. de C.O. Sardi, D.M.P. Spolidorio, Antibiofilm efficacy of tea tree oil and of its main component terpinen-4-ol against Candida albicans, Braz. Oral Res. 34 (2020) 1–9. https://doi.org/10.1590/1807-3107BOR-2020.VOL34.0050.

[73] M. Di Vito, P. Mattarelli, M. Modesto, A. Girolamo, M. Ballardini, A. Tamburro, M. Meledandri, F. Mondello, In Vitro Activity of Tea Tree Oil Vaginal Suppositories against Candida spp. and Probiotic Vaginal Microbiota, Phyther. Res. 29 (2015) 1628–1633. https://doi.org/10.1002/ptr.5422.

[74] A. Mertas, A. Garbusińska, E. Szliszka, A. Jureczko, M. Kowalska, W. Król, The influence of tea tree oil (Melaleuca alternifolia) on fluconazole activity against fluconazole-resistant candida albicans strains, Biomed Res. Int. 2015 (2015). https://doi.org/10.1155/2015/590470.

[75] A. Rosato, C. Vitali, D. Gallo, L. Balenzano, R. Mallamaci, The inhibition of Candida species by selected essential oils and their synergism with amphotericin B, Phytomedicine. 15 (2008) 635–638. https://doi.org/10.1016/j.phymed.2008.05.001.

[76] S. Zakeri, S. Esmaeilzadeh, N. Gorji, Z. Memariani, R. Moeini, A. Bijani, The effect of Achillea Millefolium L. on vulvovaginal candidiasis compared with clotrimazole: A randomized controlled trial, Complement. Ther. Med. 52 (2020) 102483. https://doi.org/10.1016/j.ctim.2020.102483.

[77] N. Saghafi, M. Karjalian, M. Ghazanfarpour, I. Khorsand, H. Rakhshandeh, M. Mirteimouri, M. Babakhanian, T. Khadivzadeh, M.J. Najafzadeh, A. Ghorbani, L. Pourali, S. Bahman, The effect of a vaginal suppository formulation of dill (Anethum graveolens) in comparison to clotrimazole vaginal tablet on the treatment of vulvovaginal candidiasis, J. Obstet. Gynaecol. (Lahore). 38 (2018) 985–988. https://doi.org/10.1080/01443615.2018.1432578.

[78] O. Romero-Cerecero, A.L. Islas-Garduño, A. Zamilpa, J. Tortoriello, Effectiveness of Ageratina pichinchensis Extract in Patients with Vulvovaginal Candidiasis. A Randomized, Double-Blind, and Controlled Pilot Study, Phyther. Res. 31 (2017) 885–890. https://doi.org/10.1002/ptr.5802.

[79] I.R.G. Capoci, P.D.S. Bonfim-Mendonça, G.S. Arita, R.R.D.A. Pereira, M.E.L. Consolaro, M.L. Bruschi, M. Negri, T.I.E. Svidzinski, Propolis is an efficient fungicide and inhibitor of biofilm production by vaginal Candida albicans, Evidence-Based Complement. Altern. Med. 2015 (2015). https://doi.org/10.1155/2015/287693.

[80] S.J.J.M. van Riel, C.M.J.G. Lardenoije, G.J. Oudhuis, N.A.J. Cremers, Treating (Recurrent) vulvovaginal candidiasis with medical-grade honey—concepts and practical considerations, J. Fungi. 7 (2021). https://doi.org/10.3390/JOF7080664.

[81] F. Ahangari, A. Farshbaf-Khalili, Y. Javadzadeh, M. Adibpour, B. Sadeghzadeh Oskouei, Comparing the effectiveness of Salvia officinalis, clotrimazole and their combination on vulvovaginal candidiasis: A randomized, controlled clinical trial, J. Obstet. Gynaecol. Res. 45 (2019) 897–907. https://doi.org/10.1111/jog.13918.

[82] J.T. Andrade, G. Fantini de Figueiredo, L.F. Cruz, S. Eliza de Morais, C.D.F. Souza, F.C.H. Pinto, J.M.S. Ferreira, M.G. de F. Araújo, Efficacy of curcumin in the treatment of experimental vulvovaginal candidiasis, Rev. Iberoam. Micol. 36 (2019) 192–199. https://doi.org/10.1016/j.riam.2019.01.003.

[83] N. Abouali, E. Moghimipour, A. Mahmoudabadi, F. Namjouyan, Z. Abbaspoor, The effect of curcumin-based and clotrimazole vaginal cream in the treatment of vulvovaginal candidiasis, J. Fam. Med. Prim. Care. 8 (2019) 3920–3924. https://doi.org/10.4103/jfmpc.jfmpc.

[84] J. Shao, L. Xiong, M.X. Zhang, T.M. Wang, C.Z. Wang, Report : Synergism of sodium bicarbonate and baicalin against clinical Candida albicans isolates via broth microdilution method and checkerboard, Pak. J. Pharm. Sci. 32 (2019).

[85] F.A.C.G. de Sousa, T.C. Paradella, C.Y. Koga-Ito, A.O.C. Jorge, Effect of sodium bicarbonate on Candida albicans adherence to thermally activated acrylic resin, Braz.

Oral Res. 23 (2009) 381–385. https://doi.org/10.1590/s1806-83242009000400006.

[86] V. Letscher-Bru, C.M. Obszynski, M. Samsoen, M. Sabou, J. Waller, E. Candolfi, Antifungal Activity of Sodium Bicarbonate Against Fungal Agents Causing Superficial Infections, Mycopathologia. 175 (2013) 153–158. https://doi.org/10.1007/s11046-012-9583-2.

[87] H.S. Johal, T. Garg, G. Rath, A.K. Goyal, Advanced topical drug delivery system for the management of vaginal candidiasis, Drug Deliv. 23 (2016) 550–563. https://doi.org/10.3109/10717544.2014.928760.

[88] G. Chindamo, S. Sapino, E. Peira, D. Chirio, M. Gallarate, Recent advances in nanosystems and strategies for vaginal delivery of antimicrobials, Nanomaterials. 11 (2021) 1–29. https://doi.org/10.3390/nano11020311.

[89] P. Bassi, G. Kaur, Bioadhesive vaginal drug delivery of nystatin using a derivatized polymer: Development and characterization, Eur. J. Pharm. Biopharm. 96 (2015) 173–184. https://doi.org/10.1016/j.ejpb.2015.07.018.

[90] N.V. Gupta, S. Natasha, A. Getyala, R.S. Bhat, Bioadhesive vaginal tablets containing spray dried microspheres loaded with clotrimazole for treatment of vaginal Candidiasis, Acta Pharm. 63 (2013) 359–372. https://doi.org/10.2478/acph-2013-0027.

[91] B. Albertini, N. Passerini, M. Di Sabatino, B. Vitali, P. Brigidi, L. Rodriguez, Polymer-lipid based mucoadhesive microspheres prepared by spray-congealing for the vaginal delivery of econazole nitrate, Eur. J. Pharm. Sci. 36 (2009) 591–601. https://doi.org/10.1016/j.ejps.2008.12.009.

[92] N. Carbone, A. Fuochi, V; Zienska, E. Musumeci, A. Bonaccorso, C. Puglia, G. Petronio, P. Furneri, Dual-drugs delivery in Solid Lipid Nanoparticles for the treatment of Candida albicans mycosis, Colloids Surfaces B Biointerfaces. (2019). https://doi.org/https://doi.org/10.1016/j.colsurfb.2019.110705.

[93] P.A. Lucena, T.L. Nascimento, M.P.N. Gaeti, R.I. De Ávila, L.P. Mendes, M.S. Vieira, D. Fabrini, A.C. Amaral, E.M. Lima, In vivo vaginal fungal load reduction after treatment with itraconazole-loaded polycaprolactone-nanoparticles, J. Biomed. Nanotechnol. 14 (2018) 1347–1358. https://doi.org/10.1166/jbn.2018.2574.

[94] J.L. Soriano-Ruiz, A.C. Calpena-Capmany, C. Cañadas-Enrich, N.B. de Febrer, J. Suñer-Carbó, E.B. Souto, B. Clares-Naveros, Biopharmaceutical profile of a clotrimazole nanoemulsion: Evaluation on skin and mucosae as anticandidal agent, Int. J. Pharm. 554 (2019) 105–115. https://doi.org/10.1016/j.ijpharm.2018.11.002.

[95] R. Song, F. Yan, M. Cheng, F. Dong, Y. Lin, Y. Wang, B. Song, Ultrasound-assisted preparation of exopolysaccharide/nystatin nanoemulsion for treatment of vulvovaginal candidiasis, Int. J. Nanomedicine. 15 (2020) 2027–2044. https://doi.org/10.2147/IJN.S241134.

[96] D.S. Shaker, S. Ismail, S. Hamed, E.M. El-Shishtawy, Butoconazole nitrate vaginal

sponge: Drug release and antifungal efficacy, J. Drug Deliv. Sci. Technol. 48 (2018) 274–287. https://doi.org/10.1016/j.jddst.2018.09.011.

[97] N.H. Abd Ellah, J.A. Abdel-Aleem, M.N. Abdo, O.F. Abou-Ghadir, K.M. Zahran, H.F. Hetta, Efficacy of ketoconazole gel-flakes in treatment of vaginal candidiasis: Formulation, in vitro and clinical evaluation, Int. J. Pharm. 567 (2019). https://doi.org/10.1016/j.ijpharm.2019.118472.

[98] N. Nematpour, P. Moradipour, M.M. Zangeneh, E. Arkan, M. Abdoli, L. Behbood, The application of nanomaterial science in the formulation a novel antibiotic: Assessment of the antifungal properties of mucoadhesive clotrimazole loaded nanofiber versus vaginal films, Mater. Sci. Eng. C. 110 (2020). https://doi.org/10.1016/j.msec.2020.110635.

[99] T. Ci, L. Yuan, X. Bao, Y. Hou, H. Wu, H. Sun, D. Cao, X. Ke, Development and anti-Candida evaluation of the vaginal delivery system of amphotericin B nanosuspension-loaded thermogel, J. Drug Target. 26 (2018) 829–839. https://doi.org/10.1080/1061186X.2018.1434660.

[100] S.S. Deshkar, V.K. Palve, Formulation and development of thermosensitive cyclodextrin-based in situ gel of voriconazole for vaginal delivery, J. Drug Deliv. Sci. Technol. (2018) 277–285. https://doi.org/10.1016/j.jddst.2018.11.023.

[101] A. Falconi-McCahill, Bacterial Vaginosis: A Clinical Update with a Focus on Complementary and Alternative Therapies, J. Midwifery Womens. Health. (2019) 1–14. https://doi.org/10.1111/jmwh.13013.

[102] D. Machado, J. Castro, A. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, N. Cerca, Bacterial Vaginosis Biofilms : Challenges to Current Therapies and Emerging Solutions, Front. Microbiol. 6 (2016) 1–13. https://doi.org/10.3389/fmicb.2015.01528.

[103] R. Palmeira-De-Oliveira, P. Duarte, A. Palmeira-De-Oliveira, J. Das Neves, M.H. Amaral, L. Breitenfeld, J. Martinez-De-Oliveira, Women's experiences, preferences and perceptions regarding vaginal products: Results from a cross-sectional web-based survey in Portugal, Eur. J. Contracept. Reprod. Heal. Care. 20 (2015) 259–271. https://doi.org/10.3109/13625187.2014.980501.

[104] G. Leyva-Gómez, M.L. Del Prado-Audelo, S. Ortega-Peña, N. Mendoza-Muñoz, Z. Urbán-Morlán, M. González-Torres, M.G. Del Carmen, G. Figueroa-González, O.D. Reyes-Hernández, H. Cortés, Modifications in vaginal microbiota and their influence on drug release: Challenges and opportunities, Pharmaceutics. 11 (2019) 1–22. https://doi.org/10.3390/pharmaceutics11050217.

[105] C.S. Bradshaw, J.D. Sobel, Current Treatment of Bacterial Vaginosis — Limitations and Need for Innovation, J. Infect. Dis. 214 (2016). https://doi.org/10.1093/infdis/jiw159.

[106] C.S. Bradshaw, R.M. Brotman, Making inroads into improving treatment of bacterial vaginosis – striving for long-term cure, BMC Infect. Dis. 15 (2015) 1–12. https://doi.org/10.1186/s12879-015-1027-4.

[107] N.M. Gilbert, W.G. Lewis, G. Li, D.K. Sojka, J.B. Lubin, A.L. Lewis, Gardnerella vaginalis and Prevotella bivia Trigger Distinct and Overlapping Phenotypes in a Mouse Model of Bacterial Vaginosis, J. Infect. Dis. (2019) 1–10. https://doi.org/10.1093/infdis/jiy704.

[108] H. Verstraelen, A. Swidsinski, The biofilm in bacterial vaginosis: implications for epidemiology , diagnosis and treatment: 2018 update, Curr. Opin. Infect. Dis. 32 (2019) 38–42. https://doi.org/10.1097/QCO.0000000000000516.

[109] S. Reiter, S.K. Spadt, Bacterial vaginosis: a primer for clinicians, Postgrad. Med. 131 (2019) 8–18. https://doi.org/10.1080/00325481.2019.1546534.

[110] R. Amsel, P. Totten, C. Spiegel, K. Chen, D. Eschenbach, K. Holmes, Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic., Am. J. Med. 74 (1983) 14–22. https://doi.org/10.1016/0002-9343(83)91112-9.

[111] R.P. Nugent, M. Krohn, S. Hillier, Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation, J. Clin. Microbiol. 29 (1991) 297–301.

[112] S.S. Olmsted, L.A. Meyn, L.C. Rohan, S.L. Hillier, Glycosidase and Proteinase Activity of Anaerobic Gram-Negative Bacteria Isolated From Women With Bacterial Vaginosis, Sex. Transm. Dis. 30 (2003) 257–261. https://doi.org/10.1097/00007435-200303000-00016.

[113] A. Machado, N. Cerca, The influence of biofilm formation by Gardnerella vaginalis and other anaerobes on bacterial vaginosis, J. Infect. Dis. (2015) 1–17. https://doi.org/10.1093/infdis/jiv338.

[114] World Health Organization, Guidelines for the Management of Sexually Transmitted Infections, (2004). http://apps.who.int/medicinedocs/en/d/Jh2942e/2.4.html (accessed January 13, 2020).

[115] Clinical Effectiveness Group British Association for Sexual Health and HIV, UK National Guideline for the management of Bacterial Vaginosis 2012, (2012). https://www.bashh.org/documents/4413.pdf (accessed January 13, 2020).

[116] Centers for Disease Control and Prevention, Sexually Transmitted Diseases Treatment Guidelines, (2015). https://www.cdc.gov/std/tg2015/bv.htm (accessed January 13, 2020).

[117] Australian STI Management guidelines for use in primary care, (2018). http://www.sti.guidelines.org.au/sexually-transmissible-infections/infections-associated-with-sex/bacterial-vaginosis#management (accessed January 13, 2020).

[118] M.A.B. Petrina, L.A. Cosentino, L.K. Rabe, S.L. Hillier, Susceptibility of bacterial vaginosis (BV)-associated bacteria to secnidazole compared to metronidazole , tinidazole and clindamycin, Anaerobe. 47 (2017) 115–119. https://doi.org/10.1016/j.anaerobe.2017.05.005.

[119] O.O. Oduyebo, R.I. Anorlu, F.T. Ogunsola, The effects of antimicrobial therapy on bacterial vaginosis in non-pregnant women, Cochrane Database Syst. Rev. (2009). https://doi.org/10.1002/14651858.CD006055.pub2.

[120] C.S. Bradshaw, A.N. Morton, J. Hocking, S.M. Garland, M.B. Morris, L.M. Moss, L.B. Horvath, I. Kuzevska, C.K. Fairley, High Recurrence Rates of Bacterial Vaginosis over the Course of 12 Months after Oral Metronidazole Therapy and Factors Associated with Recurrence, J. Infect. Dis. 193 (2006) 1478–1486. https://doi.org/10.1086/503780.

[121] C. Gottschick, S.P. Szafranski, B. Kunze, H. Sztajer, Screening of Compounds against Gardnerella vaginalis Biofilms, PLoS One. 11 (2016). https://doi.org/10.1371/journal.pone.0154086.

[122] G.G.G. Donders, S. Guaschino, K. Peters, R. Tacchi, V. Lauro, V. Study, A multicenter , double-blind , randomized, placebo-controlled study of rifaximin for the treatment of bacterial vaginosis, Int. J. Gynecol. Obstet. 120 (2013) 131–136. https://doi.org/10.1016/j.ijgo.2012.08.022.

[123] F. Cruciani, E. Biagi, M. Severgnini, C. Consolandi, F. Calanni, G. Donders, P. Brigidi, B. Vitali, Development of a microarray-based tool to characterize vaginal bacterial fluctuations: application to a novel antibiotic treatment for bacterial vaginosis, Antimicrob. Agents Chemother. (2015) 1–30. https://doi.org/10.1128/AAC.00225-15.

[124] L. Laghi, G. Picone, F. Cruciani, F. Calanni, G. Donders, B. Vitali, L. Laghi, G. Picone, F. Cruciani, P. Brigidi, F. Calanni, G. Donders, F. Capozzi, Rifaximin Modulates the Vaginal Microbiome and Metabolome in Women Affected by Bacterial Vaginosis, Antimicrob. Agents Chemother. 58 (2014) 3411–3420. https://doi.org/10.1128/AAC.02469-14.

[125] P. Nyirjesy, J. Schwebke, Secnidazole: next-generation antimicrobial agent for bacterial vaginosis treatment, Future Microbiol. 13 (2018) 507–524. https://doi.org/10.2217/fmb-2017-0270.

[126] J.R. Schwebke, F.G. Morgan, J. Facog, W. Koltun, P. Nyirjesy, A phase-3, double-blind, placebo-controlled study of the effectiveness and safety of single oral doses of secnidazole 2 g for the treatment of women with bacterial vaginosis, Am. J. Obstet. Gynecol. 217 (2017). https://doi.org/10.1016/j.ajog.2017.08.017.

[127] S.L. Hillier, P. Nyirjesy, A.S. Waldbaum, J.R. Schwebke, F.G. Morgan, N.A. Adetoro, C.J. Braun, Secnidazole Treatment of Bacterial Vaginosis, Obs. Ginecol. 130 (2017) 379–386. https://doi.org/10.1097/AOG.0000000000002135.

[128] T.A. Hooven, T.M. Randis, S.R. Hymes, R. Rampersaud, A.J. Ratner, Retrocyclin inhibits Gardnerella vaginalis biofilm formation and toxin activity Thomas, J. Antimicrob. Chemother. 67 (2012) 2870–2872. https://doi.org/10.1093/jac/dks305.

[129] S.S. Rajan, V.L. Cavera, X. Zhang, Y. Singh, M.L. Chikindas, P.J. Sinko, Poly(ethylene glycol) (PEG)-based hydrogels for the controlled release of the antimicrobial subtilosin, for prophylaxis of bacterial vaginosis, Antimicrob. Agents Chemother. 58 (2014) 2747–

2753. https://doi.org/10.1128/AAC.02446-14.

[130] H. Verstraelen, R. Verhelst, K. Roelens, M. Temmerman, Antiseptics and disinfectants for the treatment of bacterial vaginosis: A systematic review, BMC Infect. Dis. 12 (2012). https://doi.org/10.1186/1471-2334-12-148.

[131] W. Mendling, E.R. Weissenbacher, S. Gerber, V. Prasauskas, P. Grob, Use of locally delivered dequalinium chloride in the treatment of vaginal infections: a review, Arch. Gynecol. Obstet. 293 (2016) 469–484. https://doi.org/10.1007/s00404-015-3914-8.

[132] E.R. Weissenbacher, G. Donders, V. Unzeitig, B.M. Tejada, S. Gerber, M. Halaska, J. Spacek, A Comparison of Dequalinium Chloride Vaginal Tablets (Fluomizin) and Clindamycin Vaginal Cream in the Treatment of Bacterial Vaginosis: A Single-Blind , Randomized Clinical Trial of, Gynecol. Obstet. Invest. 73 (2011) 8–15. https://doi.org/10.1159/000332398.

[133] B. Molteni, A. D'Antuono, P. Bandini, G. Sintini, E. Barcellona, A. Agnello, M. Milani, Efficacy and tolerability of a new chlorhexidine-based vaginal gel in vaginal infections, Curr. Med. Res. Opin. 20 (2015) 849–853. https://doi.org/10.1185/030079904125003692.

[134] N. Hübner, J. Siebert, A. Kramer, Octenidine Dihydrochloride , a Modern Antiseptic for Skin , Mucous Membranes, Skin Pharmacol. Physiol. (2010) 244–258. https://doi.org/10.1159/000314699.

[135] A. Swidsinski, V. Loening-Baucke, S. Swidsinski, H. Verstraelen, Polymicrobial Gardnerella biofilm resists repeated intravaginal antiseptic treatment in a subset of women with bacterial vaginosis : a preliminary report, Arch. Gynecol. Obstet. 291 (2015) 605–609. https://doi.org/10.1007/s00404-014-3484-1.

[136] C. Gottschick, Z.-L. Deng, M. Vital, C. Masur, C. Abels, D.H. Pieper, M. Rohde, W. Mendling, I. Wagner-Dobler, Treatment of biofilms in bacterial vaginosis by an amphoteric tenside pessary-clinical study and microbiota analysis, Microbiome. 119 (2017) 1–15. https://doi.org/10.1186/s40168-017-0326-y.

[137] A. Palmeira-de-Oliveira, B. Silva, R. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, L. Salgueiro, Are plant extracts a potential therapeutic approach for genital infections ?, Curr. Med. Chem. 20 (2013) 2914–2928. https://doi.org/10.2174/09298673113209990007.

[138] M. Azadpour, M. Dolatian, F. Mojab, M. Nasiri, B. Ezatpour, Y. Sahranavard, H. Shakiba, R. B, K. Ghanati, The Effect of Prangos Ferulacea Vaginal Cream on Accelerating the Recovery of Bacterial Vaginosis : A Randomized Controlled Clinical Trial ., Int. J. Community Based Nurs. Midwifery. 6 (2018) 100–110.

[139] M. Masoudi, S. Miraj, M. Rafieian-Kopaei, Comparison of the Effects of Myrtus Communis L , Berberis Vulgaris and Metronidazole Vaginal Gel alone for the Treatment of Bacterial Vaginosis, J. Clin. Diagnostic Res. 10 (2016) 4–7.

https://doi.org/10.7860/JCDR/2016/17211.7392.

[140] M. Masoudi, M.R. Kopaei, S. Miraj, Comparison between the efficacy of metronidazole vaginal gel and Berberis vulgaris (Berberis vulgaris) combined with metronidazole gel alone in the treatment of bacterial vaginosis, Eletronic Physician. (2016) 2818–2827. https://doi.org/10.19082/2818.

[141] M. Masoudi, S. Miraj, M. Rafieian-Kopaei, A comparison of the efficacy of metronidazole vaginal gel and Myrtus (Myrtus communis) extract combination and metronidazole vaginal gel alone in the treatment of recurrent bacterial vaginosis, Avicenna J. Phytomedicine. 7 (2017) 129–136.

[142] K. Abdali, L. Jahed, S. Amooee, M. Zarshenas, H. Tabatabaee, R. Bekhradi, Comparison of the Effect of Vaginal Zataria multiflora Cream and Oral Metronidazole Pill on Results of Treatments for Vaginal Infections including Trichomoniasis and Bacterial Vaginosis in Women of Reproductive Age, Biomed Res. Int. (2015) 1–7. https://doi.org/10.1155/2015/683640.

[143] F. Murina, F. Vicariotto, S. Di Francesco, Thymol , eugenol and lactobacilli in a medical device for the treatment of bacterial vaginosis and vulvovaginal, New Microbiol. 41 (2018) 220–224.

[144] Z. Pazhohideh, S. Mohammadi, N. Bahrami, F. Mojab, P. Abedi, E. Maraghi, The effect of Calendula officinalis versus metronidazole on bacterial vaginosis in women: A double - blind randomized controlled trial, J. Adv. Pharm. Technol. Res. 9 (2018) 15–19. https://doi.org/10.4103/japtr.JAPTR.

[145] F. Boselli, E. Petrella, A. Campedelli, M. Muzi, V. Rullo, L. Ascione, R. Papa, G. Saponati, Efficacy and Tolerability of Fitostimoline (Vaginal Cream , Ovules , and Vaginal Washing) and of Benzydamine Hydrochloride (Tantum Rosa Vaginal Cream and Vaginal Washing) in the Topical Treatment of Symptoms of Bacterial Vaginosis, ISRN Obstet. Gynecol. 2012 (2012). https://doi.org/10.5402/2012/183403.

[146] N. Baery, A.G. Nejad, M. Amin, S. Mahroozade, R. Mokaberinejad, S. Bioos, M. Anushiravani, J. Aliasl, M.K. Darmiyan, G. Amin, Effect of vaginal suppository on bacterial vaginitis based on Persian medicine (Iranian traditional medicine): a randomised double blind clinical study, J. Obstet. Gynaecol. (Lahore). (2018) 1–5. https://doi.org/10.1080/01443615.2018.1445706.

[147] D. Machado, C. Gaspar, A. Palmeira-de-Oliveira, C. Cavaleiro, L. Salgueiro, J. Martinez-de-Oliveira, N. Cerca, Thymbra capitata essential oil as potential therapeutic agent against Gardnerella vaginalis biofilm-related infections, Future Microbiol. 12 (2017) 407–416. https://doi.org/10.2217/fmb-2016-0184.

[148] H.-T. Trinh, I.-A. Lee, Y.-J. Hyun, D.-H. Kim, Artemisia princeps Pamp . Essential Oil and Its Constituents Eucalyptol and α -terpineol Ameliorate Bacterial Vaginosis and Vulvovaginal Candidiasis in Mice by Inhibiting Bacterial Growth and NF- κ B Activation,

Planta Med. 77 (2011) 1996–2002. https://doi.org/10.1055/s-0031-1280094.

[149] P.C. Braga, M.D. Sasso, M. Culici, A. Spallino, Inhibitory activity of thymol on native and mature Gardnerella vaginalis biofilms: in vitro study, Arzneimittelforschung. 60 (2010) 675–681. https://doi.org/10.1055/s-0031-1296346.

[150] J.A. Simoes, L.G. Bahamondes, R.P.S. Camargo, V.M.N. Alves, L.J.D. Zaneveld, D.P. Waller, J. Schwartz, M. Callahan, C.K. Mauck, A pilot clinical trial comparing an acid-buffering formulation (ACIDFORM gel) with metronidazole gel for the treatment of symptomatic bacterial vaginosis, Br. J. Clin. Pharmacol. 61 (2005) 211–217. https://doi.org/10.1111/j.1365-2125.2005.02550.x.

[151] M. Milani, E. Barcellona, A. Agnello, Efficacy of the combination of 2g oral tinidazole and acidic buffering vaginal gel in comparison with vaginal clindamycin alone in bacterial vaginosis : a randomized , investigator-blinded , controlled trial, Eur. J. Obstet. Gynecol. Reprod. Biol. 109 (2003) 67–71. https://doi.org/10.1016/S0301-2115(02)00478-5.

[152] J. Harwell, T. Moench, K. Mayer, S. Chapman, I. Rodriguez, S. Cu-uvin, A Pilot Study of Treatment of Bacterial Vaginosis with a Buffering Vaginal Microbicide, J. Women's Heal. 12 (2003) 255–259. https://doi.org/10.1089/154099903321667591.

[153] S.M. Prutting, J.D. Cerveny, Boric Acid Vaginal Suppositories: A Brief Review, Infect. Dis. Obstet. Gynecol. 6 (1998) 191–194. https://doi.org/10.1002/(SICI)1098-0997(1998)6:4<191::AID-IDOG10>3.0.CO;2-6.

[154] K. Van Slyke, V.P. Michel, M.F. Rein, The boric acid powder treatment of vulvovaginal candidiasis., J. Am. Coll. Heal. Assoc. ISSN. 30 (1981) 107–109. https://doi.org/10.1080/01644300.1981.10393050.

[155] J.M. Marrazzo, J.C. Dombrowski, M.R. Wierzbicki, C. Perlowski, A. Pontius, D. Dithmer, J. Schwebke, Safety and Efficacy of a Novel Vaginal Anti-infective , TOL-463 , in the Treatment of Bacterial Vaginosis and Vulvovaginal Candidiasis: A Randomized , Single-blind , Phase 2 , Controlled Trial, Clin. Infect. Dis. 68 (2018) 803–809. https://doi.org/10.1093/cid/ciy554.

[156] O. Reichman, R. Akins, J.D. Sobel, Boric Acid Addition to Suppressive Antimicrobial Therapy for Recurrent Bacterial Vaginosis, Sex. Transm. Dis. 36 (2009) 732–734. https://doi.org/10.1097/OLQ.0b013e3181b08456.

[157] A. Fiorilli, B. Molteni, M. Milani, Successful treatment of bacterial vaginosis with a policarbophil-carbopol acidic vaginal gel : results from a randomised double-blind , placebo-controlled trial, Eur. J. Obstet. Gynecol. Reprod. Biol. 120 (2005) 202–205. https://doi.org/10.1016/j.ejogrb.2004.10.011.

[158] V.N. Krasnopolsky, V.N. Prilepskaya, F. Polatti, N. V Zarochentseva, Efficacy of Vitamin C Vaginal Tablets as Prophylaxis for Recurrent Bacterial Vaginosis: A Randomised, Double-Blind, Placebo-Controlled Clinical Trial, J. Clin. Med. Res. 5 (2013) 309–315. https://doi.org/10.4021/jocmr1489w.

[159] M. Bahamondes, P. Portugal, E. Brolazo, J. Simões, L. Bahamondes, Use of a lactic acid plus lactoserum intimate liquid soap for external hygiene in the prevention of bacterial vaginosis recurrence after metronidazole oral treatment., Rev. Assoc. Med. Bras. 57 (2011) 415–420. https://doi.org/10.1590/s0104-42302011000400015.

[160] D.C. Decena, J.T. Co, R.M. Manalastas, E. Palaypayon, C.S. Padolina, J.M. Sison, L.A. Dancel, M.A. Lelis, Metronidazole with Lactacyd vaginal gel in bacterial vaginosis, J. Obstet. Gynaecol. Res. 32 (2006) 243–251. https://doi.org/10.1111/j.1447-0756.2006.00383.x.

[161] R.L. Holley, H.E. Richter, R.E. Varner, L. Pair, J.R. Schwebke, A randomized , double-blind clinical trial of vaginal acidification versus placebo for the treatment of symptomatic bacterial vaginosis ., Sex. Transm. Dis. 31 (2004) 236–238. https://doi.org/10.1097/01.OLQ.0000118423.20985.E7.

[162] A.J.P. Boeke, J.H. Dekker, J. Eijk, P.J. Kostense, P.D. Bezemer, Effect of lactic acid suppositories compared with oral metronidazole and placebo in bacterial vaginosis : a randomised clinical trial, Genitourinaly Med. 69 (1993) 388–393.

[163] J.M. Macklaim, J.C. Clemente, R. Knight, G.B. Gloor, G. Reid, Changes in vaginal microbiota following antimicrobial and probiotic therapy, Microb. Ecol. Health Dis. 26 (2015). https://doi.org/10.3402/mehd.v26.27799.

[164] J.-M. Kim, Y.J. Park, Probiotics in the Prevention and Treatment of Postmenopausal Vaginal Infections: Review Article, J. Menopausal Med. 23 (2017) 139–145. https://doi.org/10.6118/jmm.2017.23.3.139.

[165] P. Heczko, A. Tomusiak, P. Adamski, A.J. Jakimiuk, G. Stefanski, A. Mikolajczyk-Cichonka, M. Suda-Szczurek, M. Strus, Supplementation of standard antibiotic therapy with oral probiotics for bacterial vaginosis and aerobic vaginitis: a controlled trial, BMC Womens. Health. 15 (2015) 1–12. https://doi.org/10.1186/s12905-015-0246-6.

[166] R.C.R. Martinez, S.A. Franceschini, M.C. Patta, S.M. Quintana, B.C. Gomes, E.C.P. Martinis, G. Reid, Improved cure of bacterial vaginosis with single dose of tinidazole (2 g), Lactobacillus rhamnosus GR-1 , and Lactobacillus reuteri RC-14 : a randomized , double-blind , placebo-controlled trial, Can. J. Microbiol. 55 (2009) 133–138. https://doi.org/10.1139/W08-102.

[167] Anukam K, Osazuwa E, Ahonkahai I, Ngwu M, Osemene G, Bruce AW, Reid G, Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic Lactobacillus rhamnosus GR-1 and placebo controlled trial, Microbes Infect. 8 (2006) 1450–1454. https://doi.org/10.1016/j.micinf.2006.01.003.

[168] M.C. Terraf, L.M. Mendoza, M.S. Juárez Tomás, C. Silva, M.E.F. Nader-Macías, Phenotypic surface properties (aggregation, adhesion and biofilm formation) and presence of related genes in beneficial vaginal lactobacilli, J. Appl. Microbiol. 117 (2014) 1761–1772. https://doi.org/10.1111/jam.12642.

[169] P.R. Gregorio, M.S.J.T. Tomás, M.C.L. Terraf, E.F. Nader-Macías, In vitro and in vivo effects of beneficial vaginal lactobacilli on pathogens responsible for urogenital tract infections, J. Med. Microbiol. 63 (2014) 685–696. https://doi.org/10.1099/jmm.0.069401-0.

[170] P. Hutt, E. Lapp, J. Stsepetova, I. Smidt, H. Taelma, N. Borovkova, H. Oopkaup, A. Ahelik, T. Roop, D. Hoidmets, K. Samuel, A. Salumets, R. Mandar, Characterisation of probiotic properties in human vaginal lactobacilli strains, Microb. Ecol. Health Dis. 27 (2016). https://doi.org/10.3402/mehd.v27.30484.

[171] J. Castro, A.P. Martins, M.E. Rodrigues, N. Cerca, Lactobacillus crispatus represses vaginolysin expression by BV associated Gardnerella vaginalis and reduces cell cytotoxicity, Anaerobe. 50 (2018) 60–63. https://doi.org/10.1016/j.anaerobe.2018.01.014.

[172] C.M.A. Santos, M.C. V Pires, T.L. Le, M.L. Rodriguez, A.K.S. Martins, Z.P. Hern, L.S. Miranda, F.S. Martins, J.R. Nicoli, J.R. Nicoli, Selection of Lactobacillus strains as potential probiotics for vaginitis treatment, Microbiology. 162 (2016) 1195–1207. https://doi.org/10.1099/mic.0.000302.

[173] M. Gunawardana, M. Mullen, J. Yoo, P. Webster, J.A. Moss, M.M. Baum, Sustained Delivery of Commensal Bacteria from Pod- intravaginal Rings Running title : Delivery of Probiotic Bacteria from Intravaginal Rings, Antimicrob. Agents Chemother. (2014). https://doi.org/10.1128/AAC.02542-13.

[174] G. Zárate, M.E. Nader-Macias, Viability and biological properties of probiotic vaginal lactobacilli after lyophilization and refrigerated storage into gelatin capsules, Process Biochem. 41 (2006) 1779–1785. https://doi.org/10.1016/j.procbio.2006.03.024.

[175] M.S. Juárez Tomás, P.R. De Gregorio, M.C. Leccese Terraf, M.E.F. Nader-Macías, Encapsulation and subsequent freeze-drying of Lactobacillus reuteri CRL 1324 for its potential inclusion in vaginal probiotic formulations, Eur. J. Pharm. Sci. 79 (2015) 87–95. https://doi.org/10.1016/j.ejps.2015.08.010.

[176] H. Tan, Y. Fu, C. Yang, J. Ma, Effects of metronidazole combined probiotics over metronidazole alone for the treatment of bacterial vaginosis: a meta-analysis of randomized clinical trials, Arch. Gynecol. Obstet. 295 (2017) 1331–1339. https://doi.org/10.1007/s00404-017-4366-0.

[177] H. Huang, L. Song, W. Zhao, Effects of probiotics for the treatment of bacterial vaginosis in adult women : a meta-analysis of randomized clinical trials, Arch. Gynecol. Obstet. 289 (2014) 1225–1234. https://doi.org/10.1007/s00404-013-3117-0.

[178] B.M. Ngugi, A. Hemmerling, E. Bukusi, G. Kikuvi, J. Gikunju, S. Shiboski, D. Fredricks, C.R. Cohen, Effects of BV-Associated Bacteria and Sexual Intercourse on Vaginal Colonization with the Probiotic Lactobacillus crispatus CTV-05, Sex. Transm. Dis. 38 (2012) 1020–1027. https://doi.org/10.1097/OLQ.0b013e3182267ac.

[179] P. Mastromarino, S. Macchia, L. Meggiorini, V. Trinchieri, L. Mosca, M. Perluigi, C. Midulla, Effectiveness of Lactobacillus -containing vaginal tablets in the treatment of symptomatic bacterial vaginosis, Clin. Microbiol. Infect. 15 (2008). https://doi.org/10.1111/j.1469-0691.2008.02112.x.

[180] F. Vicariotto, L. Mogna, M. Del Piano, Effectiveness of the Two Microorganisms Lactobacillus Formulated in Slow-release Vaginal Tablets , in Women Affected by Bacterial Vaginosis A Pilot Study, J. Clin. Gastroenterol. 48 (2014) 106–112. https://doi.org/10.1097/MCG.0000000000000226.

[181] A. Cianci, E. Cicinelli, V. De Leo, F. Fruzzetti, G. Massaro, A. Bulfoni, F. Parazzini, A. Perino, A. Cianci, E. Cicinelli, V. De Leo, F. Fruzzetti, G. Massaro, A. Bulfoni, F. Parazzini, A.P. Observational, Observational prospective study on Lactobacillus plantarum P 17630 in the prevention of vaginal infections , during and after systemic antibiotic therapy or in women with recurrent vaginal or genitourinary infections, J. Obstet. Gynaecol. (Lahore). 0 (2018) 1–4. https://doi.org/10.1080/01443615.2017.1399992.

[182] J.M. Bohbot, E. Darai, F. Bretelle, G. Brami, C. Daniel, J.M. Cardot, Efficacy and safety of vaginally administered lyophilized Lactobacillus crispatus IP 174178 in the prevention of bacterial vaginosis recurrence, J. Gynecol. Obstet. Hum. Reprod. 47 (2017) 81–86. https://doi.org/10.1016/j.jogoh.2017.11.005.

[183] S. Pendharkar, E. Brandsborg, L. Hammarström, H. Marcotte, P. Larsson, Vaginal colonisation by probiotic lactobacilli and clinical outcome in women conventionally treated for bacterial vaginosis and yeast infection, BMC Infect. Dis. (2015) 1–12. https://doi.org/10.1186/s12879-015-0971-3.

[184] V. Rousseau, J.P. Lepargneur, C. Roques, M. Remaud-simeon, F. Paul, Prebiotic effects of oligosaccharides on selected vaginal lactobacilli and pathogenic microorganisms, Anaerobe. 11 (2005) 145–153. https://doi.org/10.1016/j.anaerobe.2004.12.002.

[185] I. Coste, P. Judlin, J. Lepargneur, S. Bou-antoun, Safety and Efficacy of an Intravaginal Prebiotic Gel in the Prevention of Recurrent Bacterial Vaginosis : A Randomized Double-Blind Study, Obstet. Gynecol. Int. (2012). https://doi.org/10.1155/2012/147867.

[186] S.L. Collins, A. McMillan, S. Seney, C. van der Veer, R. Kort, M.W. Sumarah, G. Reid, Evaluation of lactitol, lactulose, raffinose, and oligofructose for maintenance of a Lactobacillus-dominated vaginal microbiota establishes a promising prebiotic candidate, Apllied Environ. Microbiol. 84 (2017). https://doi.org/10.1128/AEM.02200-17.

[187] I. Coste, P. Judlin, J. Lepargneur, S. Bou-antoun, Safety and Efficacy of an Intravaginal Prebiotic Gel in the Prevention of Recurrent Bacterial Vaginosis : A Randomized Double-Blind Study, Obstet. Gynecol. Int. 2012 (2012). https://doi.org/10.1155/2012/147867.

[188] M. Dirkx, M.P. Boyer, P. Pradhan, A. Brittingham, W.A. Wilson, Expression and characterization of a β -fructofuranosidase from the parasitic protist Trichomonas

vaginalis, BMC Biochem. 15 (2014) 1–8. https://doi.org/10.1186/1471-2091-15-12.

[189] A. Pino, G. Giunta, C.L. Randazzo, S. Caruso, C. Caggia, A. Cianci, Bacterial biota of women with bacterial vaginosis treated with lactoferrin : an open prospective randomized trial, Microb. Ecol. Health Dis. 28 (2017). https://doi.org/10.1080/16512235.2017.1357417.

[190] R. Russo, E. Karadja, F. De Seta, Evidence-based mixture containing Lactobacillus strains and lactoferrin to prevent recurrent bacterial vaginosis: a double blind , placebo controlled , randomised clinical trial ., Benef. Microbes. 10 (2019) 19–26. https://doi.org/10.3920/BM2018.0075.

[191] K. Otsuki, N. Imai, Effects of lactoferrin in six patients with refractory bacterial vaginosis, Biochem. Cell Biol. 23 (2016). https://doi.org/10.1139/bcb-2016-0051.

[192] S. Hakimi, F. Farhan, A. Farshbaf, K. Parvin, The effect of prebiotic vaginal gel with adjuvant oral metronidazole tablets on treatment and recurrence of bacterial vaginosis : a triple - blind randomized controlled study, Arch. Gynecol. Obstet. (2017). https://doi.org/10.1007/s00404-017-4555-x.

[193] F. Polatii, Bacterial Vaginosis , Atopobium vaginae and Nifuratel, Curr. Clin. Pharmacol. 7 (2012) 36–40. https://doi.org/10.2174/157488412799218824.

[194] G. Togni, V. Battini, A. Bulgheroni, F. Mailland, M. Caserini, W. Mendling, In Vitro Activity of Nifuratel on Vaginal Bacteria : Could It Be a Good Candidate for the Treatment of Bacterial Vaginosis?, Antimicrob. Agents Chemother. 55 (2011) 2490–2492. https://doi.org/10.1128/AAC.01623-10.

[195] S. Xu, V.L. Cavera, M.A. Rogers, Q. Huang, K. Zubovskiy, M.L. Chikindas, Benzoyl Peroxide Formulated Polycarbophil/Carbopol 934P Hydrogel with Selective Antimicrobial Activity , Potentially Beneficial for Treatment and Prevention of Bacterial Vaginosis, Infect. Dis. Obstet. Gynecol. 2013 (2013). https://doi.org/10.1155/2013/909354.

[196] A. Algburi, S. Zehm, V. Netrebov, R. Weeks, K. Zubovskiy, M.L. Chikindas, Benzoyl Peroxide Inhibits Quorum Sensing and Biofilm Formation by Gardnerella vaginalis 14018, Infect. Dis. Obstet. Gynecol. (2018) 1–9. https://doi.org/10.1155/2018/1426109.

[197] S.R. Hymes, T.M. Randis, T.Y. Sun, A.J. Ratner, DNase Inhibits Gardnerella vaginalis Bio fi lms In Vitro and In Vivo, J. Infect. Dis. Infect. 207 (2013) 1491–1497. https://doi.org/10.1093/infdis/jit047.

[198] R. Palmeira-de-Oliveira, P. Duarte, A. Palmeira-de-Oliveira, J. Neves, M. Amaral, L. Breitenfeld, J. Martinez-de-Oliveira, What do Portuguese Women Prefer Regarding Vaginal Products? Results from a Cross-Sectional Web-Based Survey, Pharmaceutics. 6 (2014) 543–556. https://doi.org/10.3390/pharmaceutics6040543.

[199] V. Chervinets, I. Chervinets, V. Bondarenko, I. Stol'nikova, A. Samoukina, E. Mikhailova, A. Albulov, [Clinical effect of chitosan in bacterial vaginosis therapy], Zh Mikrobiol

Epidemiol Immunobiol. 5 (2011) 76–79.

[200] K.K. Kandimalla, E. Borden, R.S. Omtri, S.P. Boyapati, M. Smith, Ability of Chitosan Gels to Disrupt Bacterial Biofilms and Their Applications in the Treatment of Bacterial Vaginosis, J. Pharm. Sci. (2013) 2–7. https://doi.org/10.1002/jps.

[201] M.W. Jøraholmen, N. Škalko-basnet, G. Acharya, P. Basnet, Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections, PHASCI. (2015). https://doi.org/10.1016/j.ejps.2015.09.007.

[202] L. Perioli, V. Ambrogi, L. Venezia, C. Pagano, M. Ricci, C. Rossi, Chitosan and a modified chitosan as agents to improve performances of mucoadhesive vaginal gels, Colloids Surfaces B Biointerfaces. 66 (2008) 141–145. https://doi.org/10.1016/j.colsurfb.2008.06.005.

[203] E.-S. Ibrahim, S. Ismail, G. Fetih, O. Shaaban, K. Hassanein, N.H. Abdellah, Development and characterization of thermosensitive pluronic-based metronidazole in situ gelling formulations for vaginal application, Acta Pharm. 62 (2012) 59–70. https://doi.org/10.2478/v10007-012-0009-y.

[204] O.M. Shaaban, G.N. Fetih, N.H. Abdellah, S. Ismail, M.A. Ibrahim, E.A. Ibrahim, Pilot randomized trial for treatment of bacterial vaginosis using in situ forming metronidazole vaginal gel, J. Obstet. Gynaecol. Res. 37 (2011) 874–881. https://doi.org/10.1111/j.1447-0756.2010.01457.x.

[205] V.K. Singh, A. Anis, I. Banerjee, K. Pramanik, M.K. Bhattacharya, K. Pal, Preparation and characterization of novel carbopol based bigels for topical delivery of metronidazole for the treatment of bacterial vaginosis, Mater. Sci. Eng. C. 44 (2014) 151–158. https://doi.org/10.1016/j.msec.2014.08.026.

[206] Z. Vanic, O. Planinsek, N. Skalko-Basnet, I. Tho, Tablets of pre-liposomes govern in situ formation of liposomes: Concept and, Eur. J. Pharm. Biopharm. 88 (2014) 443–454. https://doi.org/10.1016/j.ejpb.2014.06.003.

[207] Z. Vanic, J. Hurler, K. Ferderber, P.G. Gasparovic, N. Skalko-Basnet, J. Filipovic-Grcic, Novel vaginal drug delivery system: deformable propylene glycol liposomes-in-hydrogel, J. Lipossome Res. 24 (2014) 27–36. https://doi.org/10.3109/08982104.2013.826242.

[208] Z. Vanic, A. Hafner, M. Bego, N. Skalko-Basnet, Characterization of various deformable liposomes with metronidazole, Drug Dev. Ind. Pharm. 39 (2013) 481–488. https://doi.org/10.3109/03639045.2012.670247.

[209] D.B. Patel, J.K. Patel, Liposomal Drug Delivery of Metronidazole for the Local Treatment of Vaginitis, Int. J. Od Pharm. Sci. Nanotechnol. 2 (2009) 421–427.

[210] Z. Pavelic, N. Skalko-Basnet, I. Jalsenjak, Characterisation and in vitro evaluation of bioadhesive liposome gels for local therapy of vaginitis, Int. J. Pharm. 301 (2005) 140–148. https://doi.org/10.1016/j.ijpharm.2005.05.022.

[211] T. Andersen, Z. Vanic, G.E. Flaten, S. Mattson, I. Tho, N. Skalko-Basnet, Pectosomes and Chitosomes as Delivery Systems for Metronidazole: The One-Pot Preparation Method, Pharmaceutics. 5 (2013) 445–456. https://doi.org/10.3390/pharmaceutics5030445.

[212] R.M. Machado, A. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, R. Palmeira-de-Oliveira, Vaginal Films for Drug Delivery, J. Pharm. Sci. 102 (2013) 2069–2081. https://doi.org/10.1002/jps.

[213] N. Dobaria, R. Mashru, Design and in vitro evaluation of a novel bioadhesive vaginal drug delivery system for clindamycin phosphate, Pharm. Dev. Technol. 15 (2010) 405–414. https://doi.org/10.3109/10837450903262058.

[214] U. Hani, S. Bhat, M. Younus Pasha, Formulation Design and Evaluation of Bioadhesive Vaginal Films of Metronidazole for Vaginal Candidiasis, Lat. Am. J. Pharm. 31 (2012) 84–90. https://doi.org/10.1371/journal.pone.0154086.

[215] G. Verstraete, L. Vandenbussche, S. Kasmi, L. Nuhn, D. Brouckaert, J. Van Renterghem, W. Grymonpré, V. Vanhoorne, T. Coenye, B.G. De Geest, T. De Beer, J.P. Remon, C. Vervaet, Thermoplastic polyurethane-based intravaginal rings for prophylaxis and treatment of (recurrent) bacterial vaginosis, Int. J. Pharm. 529 (2017) 218–226. https://doi.org/10.1016/j.ijpharm.2017.06.076.

[216] M. Pathak, M. Turner, C. Palmer, A.G.A. Coombes, Evaluation of polycaprolactone matrices for the intravaginal delivery of metronidazole in the treatment of bacterial vaginosis, J. Biomater. Appl. 29 (2014) 354–363. https://doi.org/10.1177/0885328214528256.

[217] H. Verstraelen, C. Vervaet, J. Remon, Rationale and Safety Assessment of a Novel Intravaginal Drug-Delivery System with Sustained DL-Lactic Acid Release , Intended for Long-Term Protection of the Vaginal Microbiome, PLoS One. 11 (2016) 1–15. https://doi.org/10.1371/journal.pone.0153441.

[218] E. Lastauskiene, A. Zinkevičiene, I. Girkontaite, A. Kaunietis, V. Kvedariene, Formic acid and acetic acid induce a programmed cell death in pathogenic Candida species, Curr. Microbiol. 69 (2014) 303–310. https://doi.org/10.1007/s00284-014-0585-9.

[219] T. Matare, P. Nziramasanga, L. Gwanzura, V. Robertson, Experimental germ tube induction in candida albicans: An evaluation of the effect of sodium bicarbonate on morphogenesis and comparison with pooled human serum, Biomed Res. Int. 2017 (2017) 8–13. https://doi.org/10.1155/2017/1976273.

[220] P.C.G. Faria-Gonçalves, Molecular epidemiology and evaluation of Candida spp . persistence in the vaginal mucosa , in chronic cases of vulvovaginal candidosis, University of Beira Interior, 2022.

[221] F. Valentini-Mioso, T.T. Maske, M.S. Cenci, N. Boscato, T. Pereira-Cenci, Chemical hygiene protocols for complete dentures: A crossover randomized clinical trial, J. Prosthet. Dent. 121 (2019) 83–89. https://doi.org/10.1016/j.prosdent.2017.12.022.

[222] L.J. Cibley, L.J. Cibley, Cytolytic vaginosis, Am. J. Obstet. Gynecol. 165 (1991) 1245–1249. https://doi.org/10.1016/S0002-9378(12)90736-X.

[223] M.H. Hutti, C. Hoffman, Cytolytic vaginosis: an overlooked cause of cyclic vaginal itching and burning., J. Am. Acad. Nurse Pract. 12 (2000) 55–57. https://doi.org/10.1111/j.1745-7599.2000.tb00284.x.

[224] R. Soares, P. Vieira-Baptista, S. Tavares, Cytolytic vaginosis: an underdiagnosed pathology that mimics vulvovaginal candidiasis, Acta Obs. Ginecol Port. 11 (2017) 106–112.

[225] Z. Hu, W. Zhou, L. Mu, L. Kuang, M. Su, Y. Jiang, Identification of cytolytic vaginosis versus vulvovaginal candidiasis, J. Low. Genit. Tract Dis. 19 (2015) 152–155. https://doi.org/10.1097/LGT.0000000000000076.

[226] T. Fujii, A. Udy, E. Licari, L. Romero, R. Bellomo, Sodium bicarbonate therapy for critically ill patients with metabolic acidosis: A scoping and a systematic review, J. Crit. Care. 51 (2019) 184–191. https://doi.org/10.1016/j.jcrc.2019.02.027.

[227] M.M. Adeva-Andany, C. Fernández-Fernández, D. Mouriño-Bayolo, E. Castro-Quintela, A. Domínguez-Montero, Sodium bicarbonate therapy in patients with metabolic acidosis, Sci. World J. 2014 (2014). https://doi.org/10.1155/2014/627673.

[228] K. Hopper, Is Bicarbonate Therapy Useful?, Vet. Clin. North Am. - Small Anim. Pract. 47 (2017) 343–349. https://doi.org/10.1016/j.cvsm.2016.09.005.

[229] M.A. Beloso C, Souto J, Fábregat M, Romanelli G, Javiel G, Use of sodium bicarbonate and blood gas monitoring in diabetic ketoacidosis: a review, World J. Diabetes. 9 (2018) 157–164. https://doi.org/10.5321/wjs.v2.i3.40.

[230] R.W. Haines, C.J. Kirwan, J.R. Prowle, Managing Chloride and Bicarbonate in the Prevention and Treatment of Acute Kidney Injury, Semin. Nephrol. 39 (2019) 473–483. https://doi.org/10.1016/j.semnephrol.2019.06.007.

[231] L. Vanessa Maria Fagundes, J.B. Pinheiro, M.X. Pisani, E. Watanabe, R. Freitas de Souza, H. de Freitas Oliveira Paranhos, C.H. Lovato-Silva, In vitro antimicrobial activity of an experimental dentifrice based on Ricinus Communis, Braz. Dent. J. 25 (2014) 191–196. https://doi.org/10.1590/0103-6440201302382.

[232] M.M. Salles, M.M. Badaró, C.N.F. de Arruda, V.M.F. Leite, C.H.L. da Silva, E. Watanabe, V. de C. Oliveira, H. de F.O. Paranhos, Antimicrobial activity of complete denture cleanser solutions based on sodium hypochlorite and Ricinus communis – A randomized clinical study, J. Appl. Oral Sci. 23 (2015) 637–6342. https://doi.org/10.1590/1678-775720150204.

[233] C.N.F. de Arruda, M.M. Salles, M.M. Badaró, V. de Cássia Oliveira, A.P. Macedo, C.H. Silva-Lovato, H. de Freitas Oliveira Paranhos, Effect of sodium hypochlorite and Ricinus communis solutions on control of denture biofilm: A randomized crossover clinical trial, J. Prosthet. Dent. 117 (2017) 729–734. https://doi.org/10.1016/j.prosdent.2016.08.035.

[234] M.M. Badaró, V.M.F. Leite-Fernandes, L.T. Martin, V. de C. Oliveira, E. Watanabe, H. de F. de O. Paranhos, C.H. Silva-Lovato, Antibiofilm activity of an experimental ricinus communis dentifrice on soft denture liners, Braz. Dent. J. 30 (2019) 252–258. https://doi.org/10.1590/0103-6440201902326.

[235] A. Zida, S. Bamba, A. Yacouba, R. Ouedraogo-Traore, R.T. Guiguemdé, Anti- Candida albicans natural products, sources of new antifungal drugs: A review, J. Mycol. Med. 27 (2017) 1–19. https://doi.org/10.1016/j.mycmed.2016.10.002.

[236] J. Suurbaar, R. Mosobil, A.M. Donkor, Antibacterial and antifungal activities and phytochemical profile of leaf extract from different extractants of Ricinus communis against selected pathogens, BMC Res. Notes. 10 (2017) 1–6. https://doi.org/10.1186/s13104-017-3001-2.

[237] M.M. Salles, V. de C. Oliveira, R.F. Souza, C.H.L. Silva, H. de F.O. Paranhos, Antimicrobial action of sodium hypochlorite and castor oil solutions for denture cleaning - in vitro evaluation, Braz. Oral Res. 29 (2015) 1–6. https://doi.org/10.1590/1807-3107BOR-2015.vol29.0104.

[238] M.C. Valera, L.E. Maekawa, L.D. de Oliveira, A.O.C. Jorge, É. Shygei, C.A.T. Carvalho, In vitro antimicrobial activity of auxiliary chemical substances and natural extracts on Candida albicans and Enterococcus faecalis in root canals, J. Appl. Oral Sci. 21 (2013) 118–123. https://doi.org/10.1590/1678-7757201302135.

[239] D.H. Owen, D.F. Katz, A vaginal fluid simulant, Contraception. 59 (1999) 91–95.

[240] R.M. Machado, A. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, R. Palmeira-de-Oliveira, Vaginal semisolid products: Technological performance considering physiologic parameters, Eur. J. Pharm. Sci. 109 (2017) 556–568. https://doi.org/10.1016/j.ejps.2017.09.009.

[241] J. das Neves, M.H. Amaral, M.F. Bahia, Performance of an in vitro mucoadhesion testing method for vaginal semisolids: Influence of different testing conditions and instrumental parameters, Eur. J. Pharm. Biopharm. 69 (2008) 622–632. https://doi.org/10.1016/j.ejpb.2007.12.007.

[242] J. das Neves, R. Palmeira-de-Oliveira, A. Palmeira-de-Oliveira, F. Rodrigues, B. Sarmento, Vaginal Mucosa and Drug Delivery, Mucoadhesive Mater. Drug Deliv. Syst. (2014) 99–132. https://doi.org/10.1002/9781118794203.ch05.

[243] A. Aka-Any-Grah, K. Bouchemal, A. Koffi, F. Agnely, M. Zhang, M. Djabourov, G. Ponchel, Formulation of mucoadhesive vaginal hydrogels insensitive to dilution with vaginal fluids, Eur. J. Pharm. Biopharm. 76 (2010) 296–303. https://doi.org/10.1016/j.ejpb.2010.07.004.

[244] E. Baloglu, Z.A. Senyigit, S.Y. Karavana, A. Bernkop-Schnürch, Strategies to prolong the intravaginal residence time of drug delivery systems, J. Pharm. Pharm. Sci. 12 (2009) 312–336. https://doi.org/10.18433/j3hp41.

[245] B.E. Lai, Y.Q. Xie, M.L. Lavine, A.J. Szeri, D.H. Owen, D.F. Katz, Dilution of microbiocide gels with vaginal fluid and semen simulants: effect on rheological properties and coating flow, Wiley Intersci. 95 (2006) 798–809. https://doi.org/10.1002/jps.

[246] E. Adriaens, J.P. Remon, Mucosal irritation potential of personal lubricants relates to product osmolality as detected by the slug mucosal irritation assay, Sex. Transm. Dis. 35 (2008) 512–516. https://doi.org/10.1097/OLQ.0b013e3181644669.

[247] A.R. Cunha, R.M. Machado, A. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, J. Das Neves, R. Palmeira-de-Oliveira, Characterization of commercially available vaginal lubricants: A safety perspective, Pharmaceutics. 6 (2014) 530–542. https://doi.org/10.3390/pharmaceutics6030530.

[248] Prista, L.N., Alves, A.C., Morgado, R., Tecnologia Farmacêutica, 4th, II vo ed., Fundação Calouste Gulbenkian, Lisboa, 1996.

[249] C.M. Caramella, S. Rossi, F. Ferrari, M.C. Bonferoni, G. Sandri, Mucoadhesive and thermogelling systems for vaginal drug delivery, Adv. Drug Deliv. Rev. 92 (2015) 39–52. https://doi.org/10.1016/j.addr.2015.02.001.

[250] N.B. Dobaria, A.C. Badhan, R.C. Mashru, A novel itraconazole bioadhesive film for vaginal delivery: Design, optimization, and physicodynamic characterization, AAPS PharmSciTech. 10 (2009) 951–959. https://doi.org/10.1208/s12249-009-9288-0.

[251] M.C. Bonferoni, P. Giunchedi, S. Scalia, S. Rossi, G. Sandri, C. Caramella, Chitosan Gels for the Vaginal Delivery of Lactic Acid : Relevance of Formulation Parameters to Mucoadhesion and Release Mechanisms, Am. Assoc. Pharm. Sci. 7 (2006) 3–10.

[252] NCCLS, Método de Referência para Testes de Diluição em Caldo para Determinação da Sensibilidade de Leveduras à Terapia Antifúngica : Norma Aprovada – Segunda Edição, 2012.

[253] R.M. Machado, A. Palmeira-de-Oliveira, L. Breitenfeld, J. Martinez-de-Oliveira, R. Palmeira-de-Oliveira, Optimization and Application of In Vitro and Ex Vivo Models for Vaginal Semisolids Safety Evaluation, J. Pharm. Sci. 108 (2019) 3289–3301. https://doi.org/10.1016/j.xphs.2019.05.026.

[254] F. Denizot, R. Lang, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, J. Immunol. Methods. 89 (1986) 271–277. https://doi.org/10.1016/0022-1759(86)90368-6.

[255] M. V. Berridge, P.M. Herst, A.S. Tan, Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction, Biotechnol. Annu. Rev. 11 (2005) 127–152. https://doi.org/10.1016/S1387-2656(05)11004-7.

[256] International Standard Biological evaluation of medical devices - Part 5: Tests for in vitro Cytotoxicity, in: ISO 10993-5, 3rd ed., Geneva, Switzerland, 2009. https://doi.org/10.1021/es0620181.

[257] R. Palmeira-de-Oliveira, R.M. Machado, J. Martinez-de-Oliveira, A. Palmeira-de-Oliveira, Testing vaginal irritation with the hen's egg test-chorioallantoic membrane assay, ALTEX. 35 (2018) 495–503. https://doi.org/10.14573/altex.1710091.

[258] Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), ICCVAM-Recommended Test Method Protocol: Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) Test Method, 2010. https://doi.org/10.3390/ijms13089959.

[259] ANVISA, Guia de estabilidade de produtos cosméticos, Editora AN, Brasília, 2004.

[260] I.F. Almeida, M.F. Bahia, Evaluation of the physical stability of two oleogels, Int. J. Pharm. 327 (2006) 73–77. https://doi.org/10.1016/j.ijpharm.2006.07.036.

[261] IFSCC, The fundamentals of stability testing, Weymouth, Dorset, England, 1992.

[262] European Medicines Agency, ICH Topic Q1E: Evaluation of Stability Data, 2003. https://doi.org/10.32388/yokp53.

[263] T. Zaveri, K.A. Powell, K.M. Guthrie, A.J. Bakke, G.R. Ziegler, J.E. Hayes, Qualitative exploration of intrinsic and extrinsic factors that influence acceptability of semisoft vaginal suppositories, BMC Womens. Health. 18 (2018) 1–17. https://doi.org/10.1186/s12905-018-0657-2.

[264] K.M. Morrow, K. Underhill, J.J. van den Berg, S. Vargas, R.K. Rosen, D.F. Katz, User-identified gel characteristics: a qualitative exploration of perceived product efficacy of topical vaginal microbicides., Arch. Sex. Behav. 43 (2014) 1459–1467. https://doi.org/10.1007/s10508-013-0235-5.

[265] F. Acarturk, Mucoadhesive Vaginal Drug Delivery Systems, Recent Pat. Drug Deliv. Formul. 3 (2009) 193–205. https://doi.org/10.2174/187221109789105658.

[266] D.F. Katz, A. Yuan, Y. Gao, Vaginal drug distribution modeling, Adv. Drug Deliv. Rev. 92 (2015) 2–13. https://doi.org/10.1016/j.addr.2015.04.017.

[267] M.M. Adeva-Andany, N. Carneiro-Freire, C. Donapetry-García, E. Rañal-Muíño, Y. López-Pereiro, The importance of the ionic product for water to understand the physiology of the acid-base balance in humans, Biomed Res. Int. 2014 (2014). https://doi.org/10.1155/2014/695281.

[268] R.C. Rowe, P.J. Sheskey, M.E. Quinn, Handbook of pharmaceutical Excipients, sixth edit, Pharmaceutical Press, USA, 2009.

[269] S. Salah, G.E.A. Awad, A.I.A. Makhlouf, Improved vaginal retention and enhanced antifungal activity of miconazole microsponges gel: Formulation development and in vivo therapeutic efficacy in rats, Eur. J. Pharm. Sci. 114 (2018) 255–266. https://doi.org/10.1016/j.ejps.2017.12.023.

[270] P.R. Varges, C.M. Costa, B.S. Fonseca, M.F. Naccache, P.R. De Souza Mendes, Rheological characterization of carbopol ® dispersions in water and in water/glycerol

solutions, Fluids. 4 (2019). https://doi.org/10.3390/fluids4010003.

[271] E. Amabebe, D.O.C. Anumba, The vaginal microenvironment: The physiologic role of Lactobacilli, Front. Med. 5 (2018) 1–11. https://doi.org/10.3389/fmed.2018.00181.

[272] D. Singh Malik, N. Mital, G. Kaur, Topical drug delivery systems: A patent review, Expert Opin. Ther. Pat. 26 (2016) 213–228. https://doi.org/10.1517/13543776.2016.1131267.

[273] U. Molander, I. Milsom, P. Ekelund, D. Mellström, O. Eriksson, Effect of oral oestriol on vaginal flora and cytology and urogenital symptoms in the post-menopause, Maturitas. 12 (1990) 113–120. https://doi.org/10.1016/0378-5122(90)90089-O.

[274] B. Xiao, X. Niu, N. Han, B. Wang, P. Du, R. Na, C. Chen, Predictive value of the composition of the vaginal microbiota in bacterial vaginosis , a dynamic study to identify recurrence-related flora, Sci. Rep. (2016). https://doi.org/10.1038/srep26674.

[275] J.L. Martino, S.H. Vermund, Vaginal douching: Evidence for risks or benefits to women's health, Epidemiol. Rev. 24 (2002) 109–124. https://doi.org/10.1093/epirev/mxf004.

[276] A. Suresh, A. Rajesh, R.M. Bhat, Y. Rai, R. Bhat, Cytolytic vaginosis: A review, Indian J Sex Transm Dis. 30 (2009) 48–50. https://doi.org/10.4103/0253-7184.55490.

[277] Y. Gao, A. Yuan, O. Chuchuen, A. Ham, K.H. Yang, D.F. Katz, Vaginal deployment and tenofovir delivery by microbicide gels, Drug Deliv. Transl. Res. 5 (2015) 279–294. https://doi.org/10.1007/s13346-015-0227-1.

[278] WHO/UNFPA/FHI360 Advisory note: Use and procurement of additional lubricants for male and female condoms, 2012.

[279] S. Ayehunie, Y.Y. Wang, T. Landry, S. Bogojevic, R.A. Cone, Hyperosmolal vaginal lubricants markedly reduce epithelial barrier properties in a three-dimensional vaginal epithelium model, Toxicol. Reports. 5 (2018) 134–140. https://doi.org/10.1016/j.toxrep.2017.12.011.

[280] E.M. Wilkinson, P. Łaniewski, M.M. Herbst-Kralovetz, R.M. Brotman, Personal and Clinical Vaginal Lubricants: Impact on Local Vaginal Microenvironment and Implications for Epithelial Cell Host Response and Barrier Function, J. Infect. Dis. 220 (2019) 2009–2018. https://doi.org/10.1093/infdis/jiz412.

[281] C.J. Lacey, S. Woodhall, Z. Qi, S. Sawant, M. Cowen, S. McCormack, S. Jiang, Unacceptable side-effects associated with a hyperosmolar vaginal microbicide in a phase 1 trial, Int. J. STD AIDS. 21 (2010) 714–717. https://doi.org/10.1258/ijsa.2010.010215.

[282] T.K. Drake, T. Shah, J.J. Peters, A. Wax, D.F. Katz, Measuring dilution of microbicide gels with optical imaging, PLoS One. 8 (2013) 8–14. https://doi.org/10.1371/journal.pone.0082213.

[283] G.P. Andrews, L. Donnelly, D.S. Jones, R.M. Curran, R.J. Morrow, A.D. Woolfson, R.K. Malcolm, Characterization of the rheological, mucoadhesive, and drug release properties of highly structured gel platforms for intravaginal drug delivery, Biomacromolecules. 10

(2009) 2427–2435. https://doi.org/10.1021/bm9003332.

[284] M.H. Henderson, G.M. Couchman, D.K. Walmer, J.J. Peters, D.H. Owen, M.A. Brown, M.L. Lavine, D.F. Katz, Optical imaging and analysis of human vaginal coating by drug delivery gels, Contraception. 75 (2007) 142–151. https://doi.org/10.1016/j.contraception.2006.08.014.

[285] J.B. da Silva, S.B. de S. Ferreira, A.V. Reis, M.T. Cook, M.L. Bruschi, Assessing mucoadhesion in polymer gels: The effect of method type and instrument variables, Polymers (Basel). 10 (2018) 1–19. https://doi.org/10.3390/polym10030254.

[286] O. Chuchuen, J.R. Maher, M.H. Henderson, M. Desoto, L.C. Rohan, A. Wax, D.F. Katz, Label-free analysis of tenofovir delivery to vaginal tissue using co-registered confocal Raman spectroscopy and optical coherence tomography, PLoS One. 12 (2017) 1–15. https://doi.org/10.1371/journal.pone.0185633.

[287] M. Rojewska, M. Olejniczak-Rabinek, A. Bartkowiak, A. Snela, K. Prochaska, J. Lulek, The wettability and swelling of selected mucoadhesive polymers in simulated saliva and vaginal fluids, Colloids Surfaces B Biointerfaces. 156 (2017) 366–374. https://doi.org/10.1016/j.colsurfb.2017.05.042.

[288] M.T. Cook, M.B. Brown, Polymeric gels for intravaginal drug delivery, J. Control. Release. 270 (2018) 145–157. https://doi.org/10.1016/j.jconrel.2017.12.004.

[289] K. Kumar, N. Dhawan, H. Sharma, S. Vaidya, B. Vaidya, Bioadhesive polymers: Novel tool for drug delivery, Artif. Cells, Nanomedicine Biotechnol. 42 (2014) 274–283. https://doi.org/10.3109/21691401.2013.815194.

[290] S. Garg, K. Tambwekar, K. Vermani, A. Garg, C. Kaul, L. Zaneveld, Compendium of pharmaceutical excipientes for vaginal formulations, Pharm Techn Drug Deliv. 25 (2001).

[291] M.T. Sánchez, M.A. Ruiz, H. Castán, M.E. Morales, A novel double-layer mucoadhesive tablet containing probiotic strain for vaginal administration: Design, development and technological evaluation, Eur. J. Pharm. Sci. 112 (2018) 63–70. https://doi.org/10.1016/j.ejps.2017.11.006.

[292] S. Rençber, S.Y. Karavana, Z.A. Şenyiğit, B. Eraç, M.H. Limoncu, E. Baloğlu, Mucoadhesive in situ gel formulation for vaginal delivery of clotrimazole: formulation, preparation, and in vitro/in vivo evaluation, Pharm. Dev. Technol. 22 (2017) 551–561. https://doi.org/10.3109/10837450.2016.1163385.

[293] 5.1.3. Efficacy of antimicrobial preservation, in: Eur. Pharmacopoeia 10.0, n.d.

[294] M. Tomás, A. Palmeira-de-Oliveira, S. Simões, J. Martinez-de-Oliveira, R. Palmeira-de-Oliveira, Bacterial vaginosis: Standard treatments and alternative strategies, Int. J. Pharm. 587 (2020) 119659. https://doi.org/10.1016/j.ijpharm.2020.119659.

[295] N. Machado, D; Castro, J; Palmeira-de-Oliveira, Ana; Martinez-de-Oliveira, José; Cerca,

Bacterial Vaginosis Biofilms : Challenges to Current Therapies and Emerging Solutions, Fontiers Microbiol. 6 (2016). https://doi.org/10.3389/fmicb.2015.01528.

[296] J. Castro, A.S. Rosca, P. Cools, M. Vaneechoutte, N. Cerca, Gardnerella vaginalis enhances Atopobium vaginae viability in an in vitro model, Front. Cell. Infect. Microbiol. 10 (2020) 83. https://doi.org/10.3389/fcimb.2020.00083.

[297] J. Castro, A.S. Rosca, C.A. Muzny, N. Cerca, Atopobium vaginae and prevotella bivia are able to incorporate and influence gene expression in a pre-formed gardnerella vaginalis biofilm, Pathogens. 10 (2021) 1–16. https://doi.org/10.3390/pathogens10020247.

[298] C.A. Muzny, C.M. Taylor, W.E. Swords, A. Tamhane, D. Chattopadhyay, N. Cerca, J.R. Schwebke, An Updated Conceptual Model on the Pathogenesis of Bacterial Vaginosis, J. Infect. Dis. 220 (2019) 1399–1405. https://doi.org/10.1093/infdis/jiz342.

[299] A.S. Rosca, J. Castro, Â. França, M. Vaneechoutte, N. Cerca, Gardnerella Vaginalis Dominates Multi-Species Biofilms in both Pre-Conditioned and Competitive In Vitro Biofilm Formation Models, Microb. Ecol. (2021). https://doi.org/10.1007/S00248-021-01917-2.

[300] J. Castro, D. Machado, N. Cerca, Unveiling the role of Gardnerella vaginalis in polymicrobial Bacterial Vaginosis bio films : the impact of other vaginal pathogens living as neighbors, ISME J. (2019) 24–26. https://doi.org/10.1038/s41396-018-0337-0.

[301] A.S. Rosa, J. Castro, L.G.V. Sousa, N. Cerca, Gardnerella and vaginal health: the truth is out there, FEMS Microbiol. Rev. 14 (2020). https://doi.org/10.1093/femsre/fuz027.

[302] M. Vaneechoutte, A. Guschin, L. Van Simaey, Y. Gansemans, F. Van Nieuwerburgh, P. Cools, Emended description of Gardnerella vaginalis and description of gardnerella leopoldii sp. Nov., gardnerella piotii sp. nov. and Gardnerella swidsinskii sp. nov., with delineation of 13 genomic species within the genus Gardnerella, Int. J. Syst. Evol. Microbiol. 69 (2019) 679–687. https://doi.org/10.1099/ijsem.0.003200.

[303] Council of Europe, European Pharmacopoeia, 7th ed., European Directorate for the Quality of Medicines and Healthcare of the Council of Europe, Strasbourg, France, 2010.

[304] R.M. Machado, M. Tomás, A. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, R. Palmeira-de-Oliveira, The vaginal sheet: an innovative form of vaginal film for the treatment of vaginal infections, Drug Dev. Ind. Pharm. 46 (2020) 135–145. https://doi.org/10.1080/03639045.2019.1711386.

[305] G.A. Martau, M. Mihai, D.C. Vodnar, The use of chitosan, alginate, and pectin in the biomedical and food sector-biocompatibility, bioadhesiveness, and biodegradability, Polymers (Basel). 11 (2019). https://doi.org/10.3390/polym11111837.

[306] M.W. Jøraholmen, A. Bhargava, K. Julin, M. Johannessen, N. Škalko-Basnet, The antimicrobial properties of chitosan can be tailored by formulation, Mar. Drugs. 18 (2020) 1–15. https://doi.org/10.3390/md18020096.

[307] N. Lupo, B. Fodor, I. Muhammad, M. Yaqoob, B. Matuszczak, A. Bernkop-Schnürch, Entirely S-protected chitosan: A promising mucoadhesive excipient for metronidazole vaginal tablets, Acta Biomater. 64 (2017) 106–115. https://doi.org/10.1016/j.actbio.2017.10.014.

[308] J.A. Robinson, M.A. Marzinke, R.P. Bakshi, E.J. Fuchs, C.L. Radebaugh, W. Aung, H.M.L. Spiegel, J.S. Coleman, L.C. Rohan, C.W. Hendrix, Comparison of Dapivirine Vaginal Gel and Film Formulation Pharmacokinetics and Pharmacodynamics (FAME 02B), AIDS Res. Hum. Retroviruses. 33 (2017) 339–346. https://doi.org/10.1089/AID.2016.0040.

[309] G. Regev, S.K. Patel, B.J. Moncla, J. Twist, B. Devlin, L.C. Rohan, Novel Application of Hot Melt Extrusion for the Manufacturing of Vaginal Films Containing Microbicide Candidate Dapivirine, AAPS PharmSciTech. 20 (2019) 1–11. https://doi.org/10.1208/s12249-019-1442-8.

[310] A. Akil, H. Agashe, C.S. Dezzutti, B.J. Moncla, S.L. Hillier, B. Devlin, Y. Shi, K. Uranker, L.C. Rohan, Formulation and characterization of polymeric films containing combinations of antiretrovirals (ARVs) for HIV prevention, Pharm. Res. 32 (2015) 458–468. https://doi.org/10.1007/s11095-014-1474-4.

[311] T. Gong, W. Zhang, M.A. Parniak, P.W. Graebing, B. Moncla, P. Gupta, K.M. Empey, L.C. Rohan, Preformulation and Vaginal Film Formulation Development of Microbicide Drug Candidate CSIC for HIV Prevention, J. Pharm. Innov. 12 (2017) 142–154. https://doi.org/10.1007/s12247-017-9274-0.

[312] Y.L. Traore, M. Fumakia, J. Gu, E.A. Ho, Dynamic mechanical behaviour of nanoparticle loaded biodegradable PVA films for vaginal drug delivery, J. Biomater. Appl. 32 (2018) 1119–1126. https://doi.org/10.1177/0885328217739451.

[313] F. Notario-Pérez, R. Cazorla-Luna, A. Martín-Illana, J. Galante, R. Ruiz-Caro, J. das Neves, M.D. Veiga, Design, fabrication and characterisation of drug-loaded vaginal films: State-of-the-art, J. Control. Release. 327 (2020) 477–499. https://doi.org/10.1016/j.jconrel.2020.08.032.

[314] W. Zhang, M.A. Parniak, S.G. Sarafianos, M.R. Cost, L.C. Rohan, Development of a vaginal delivery film containing EFdA, a novel anti-HIV nucleoside reverse transcriptase inhibitor, Int. J. Pharm. 461 (2014) 203–213. https://doi.org/10.1016/j.ijpharm.2013.11.056.

[315] F. Notario-Pérez, A. Martín-Illana, R. Cazorla-Luna, R. Ruiz-Caro, L.M. Bedoya, J. Peña, M.D. Veiga, Development of mucoadhesive vaginal films based on HPMC and zein as novel formulations to prevent sexual transmission of HIV, Int. J. Pharm. 570 (2019) 118643. https://doi.org/10.1016/j.ijpharm.2019.118643.

[316] N.L. Calvo, L.A. Svetaz, V.A. Alvarez, A.D. Quiroga, M.C. Lamas, D. Leonardi, Chitosan-hydroxypropyl methylcellulose tioconazole films: A promising alternative dosage form

for the treatment of vaginal candidiasis, Int. J. Pharm. 556 (2019) 181–191. https://doi.org/10.1016/j.ijpharm.2018.12.011.

[317] K. Ghosal, A. Ranjan, B.B. Bhowmik, A novel vaginal drug delivery system: Anti-HIV bioadhesive film containing abacavir, J. Mater. Sci. Mater. Med. 25 (2014) 1679–1689. https://doi.org/10.1007/s10856-014-5204-6.

[318] C. Grammen, G. Van Den Mooter, B. Appeltans, J. Michiels, T. Crucitti, K.K. Ariën, K. Augustyns, P. Augustijns, J. Brouwers, Development and characterization of a solid dispersion film for the vaginal application of the anti-HIV microbicide UAMC01398, Int. J. Pharm. 475 (2014) 238–244. https://doi.org/10.1016/j.ijpharm.2014.08.054.

[319] S. Ayehunie, Y.Y. Wang, T. Landry, S. Bogojevic, R.A. Cone, Hyperosmolal vaginal lubricants markedly reduce epithelial barrier properties in a three-dimensional vaginal epithelium model, Toxicol. Reports. 5 (2018) 134–140. https://doi.org/10.1016/j.toxrep.2017.12.011.

[320] M. Hu, T. Zhou, C.S. Dezzutti, L.C. Rohan, The Effect of Commonly Used Excipients on the Epithelial Integrity of Human Cervicovaginal Tissue, AIDS Res. Hum. Retroviruses. 32 (2016) 992–1004. https://doi.org/10.1089/aid.2016.0014.

[321] World Health, Examination and processing of human semen, World Health. Edition, V (2010) 286. https://doi.org/10.1038/aja.2008.57.

[322] Y. Gali, O. Delezay, J. Brouwers, N. Addad, P. Augustijns, T. Bourlet, H. Hamzeh-Cognasse, K.K. Ari??n, B. Pozzetto, G. Vanham, In vitro evaluation of viability, integrity, and inflammation in genital epithelia upon exposure to pharmaceutical excipients and candidate microbicides, Antimicrob. Agents Chemother. 54 (2010) 5105–5114. https://doi.org/10.1128/AAC.00456-10.

[323] P. Bassi, G. Kaur, Polymeric films as a promising carrier for bioadhesive drug delivery: Development, characterization and optimization, Saudi Pharm. J. 25 (2017) 32–43. https://doi.org/10.1016/j.jsps.2015.06.003.

[324] L. Sousa, J. Castro, C. Cavaleiro, L. Salgueiro, M. Tomás, R. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, N. Cerca, Thymbra capitata antimicrobial activity and synergistic effects of Carvacrol, α - Terpinene,γ - Terpinene, ρ -Cymene and Linalool against several species of the genus Gardnerella, Sci. Rep. (2021) 2021.

[325] Test No. 439: In Vitro Skin Irritation - Reconstructed Human Epidermis Test Method, n.d. https://doi.org/10.1787/9789264203884-en.

[326] D. Karakaş, F. Ari, E. Ulukaya, The MTT viability assay yields strikingly false-positive viabilities although the cells are killed by some plant extracts, Turkish J. Biol. 41 (2017) 919–925. https://doi.org/10.3906/biy-1703-104.

[327] R. Bruggisser, K. Von Daeniken, G. Jundt, W. Schaffner, H. Tullberg-Reinert, Interference of plant extracts, phytoestrogens and antioxidants with the MTT tetrazolium assay, Planta Med. 68 (2002) 445–448. https://doi.org/10.1055/s-2002-

32073.

[328] D. Tien, R.L. Schnaare, F. Kang, G. Cohl, T.J. McCormick, T.R. Moench, G. Doncel, K. Watson, R.W. Buckheit, M.G. Lewis, J. Schwartz, K. Douville, J.W. Romano, In vitro and in vivo characterization of a potential universal placebo designed for use in vaginal microbicide clinical trials, AIDS Res. Hum. Retroviruses. 21 (2005) 845–853. https://doi.org/10.1089/aid.2005.21.845.

[329] M.D. Fan, L.F. Kramzer, S.L. Hillier, J.C. Chang, L.A. Meyn, L.C. Rohan, Preferred Physical Characteristics of Vaginal Film Microbicides for HIV Prevention in Pittsburgh Women, Arch. Sex. Behav. 46 (2017) 1111–1119. https://doi.org/10.1007/s10508-016-0816-1.

[330] K.M. Guthrie, L. Rohan, R.K. Rosen, S.E. Vargas, J.G. Shaw, D. Katz, E.M. Kojic, A.S. Ham, D. Friend, K.W. Buckheit, R.W. Buckheit, Vaginal film for prevention of HIV: using visual and tactile evaluations among potential users to inform product design, Pharm. Dev. Technol. 23 (2018) 311–314. https://doi.org/10.1080/10837450.2017.1339085.

[331] T. Zaveri, K.A. Powell, K.M. Guthrie, A.J. Bakke, G.R. Ziegler, J.E. Hayes, Qualitative exploration of intrinsic and extrinsic factors that influence acceptability of semisoft vaginal suppositories, BMC Womens. Health. 18 (2018) 1–17. https://doi.org/10.1186/s12905-018-0657-2.

[332] R. Palmeira-De-Oliveira, P. Duarte, A. Palmeira-De-Oliveira, J. Das Neves, M.H. Amaral, L. Breitenfeld, J. Martinez-De-Oliveira, Women's experiences, preferences and perceptions regarding vaginal products: Results from a cross-sectional web-based survey in Portugal, Eur. J. Contracept. Reprod. Heal. Care. 20 (2015) 259–271. https://doi.org/10.3109/13625187.2014.980501.

[333] C. Turek, F.C. Stintzing, Stability of essential oils: A review, Compr. Rev. Food Sci. Food Saf. 12 (2013) 40–53. https://doi.org/10.1111/1541-4337.12006.

[334] A. Figueiredo, J. Barroso, L. Pedro, L. Salgueiro, M. Miguel, M. Faleiro, Portuguese Thymbra and Thymus Species Volatiles: Chemical Composition and Biological Activities, Curr. Pharm. Des. 14 (2008) 3120–3140. https://doi.org/10.2174/138161208786404218.