Beneficial Microbes, 2023; 14(2): 143-152

Impact of *Lactobacillus crispatus*-containing oral and vaginal probiotics on vaginal health: a randomised double-blind placebo controlled clinical trial

R. Mändar[1,2*], G. Sõerunurk[1], J. Štšepetova[1], I. Smidt[1,2], T. Rööp[1], S. Kõljalg[1], M. Saare[3], K. Ausmees[3], D.D. Le[4], M. Jaagura[5], S. Piiskop[6], H. Tamm[2] and A. Salumets[2,7,8]

[1]Department of Microbiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Ravila 19, 50411 Tartu, Estonia; [2]Competence Center on Health Technologies, Teaduspargi tn 13, 50411 Tartu, Estonia; [3]MediTA Clinic, Teguri 37b, 51013 Tartu, Estonia; [4]Faculty of Public Health, University of Medicine and Pharmacy, Hue University, 06 Ngo Quyen, Hue 53000, Vietnam; [5]Institute of Genomics, University of Tartu, Riia 23b, 51010 Tartu, Estonia; [6]Chemi-Pharm AS, Tänassilma tee 11, Tänassilma, 76406 Harju maakond, Estonia; [7]Division of Obstetrics and Gynecology, Department of Clinical Science, Intervention and Technology, Karolinska Institutet and Karolinska University Hospital, 171 77 Stockholm, Sweden; [8]Department of Obstetrics and Gynaecology, Institute of Clinical Medicine, University of Tartu, L. Puusepa 8, 50406 Tartu, Estonia; reet.mandar@ut.ee

Received: 26 July 2022 / Accepted: 20 December 2022

RESEARCH ARTICLE

Abstract

Health of reproductive tract is tightly associated with balance of microbial communities in this area. Bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) represent common disturbances of vaginal communities. Vaginal discharge due to BV or VVC is a very frequent reason for visiting gynaecologist. We aimed to evaluate the impact of the novel evidence-based probiotics on BV and VVC patients. The study group included 89 BV and 93 VVC patients (aged 18-50 years) who were recruited into randomised double-blind placebo-controlled two-arm parallel trial. The patients of each diagnosis group received oral or vaginal probiotic capsules, or placebo capsules during 3 months. A probiotic capsule contained two (DSM32717 and DSM32720, in case of BV) or three (DSM32720, DSM32718 and DSM32716, in case of VVC) *Lactobacillus crispatus* strains. Vaginal, intestinal and general health was monitored weekly by questionnaire. Blood analyses were done in the beginning and at the end of trial. Vaginal samples were collected monthly, microscopic and molecular analyses were performed. The study revealed that both oral and vaginal capsules reduced the signs and symptoms in BV patients. Remarkable improvement was noted in Nugent score, amount and smell of discharge, but also in itching/irritation. Consumption of vaginal probiotics significantly increased the lactobacilli counts in their vagina while mean proportion of some BV-related bacteria decreased. In VVC patients, both oral and vaginal capsules lowered the combined score of two most important symptoms, amount of discharge and itching/irritation. In conclusion, the novel formulations of evidence-based well-focused probiotic *L. crispatus* strains are effective against BV and VVC being suitable for both vaginal and oral administration.

Clinical trial registration: ISRCTN34840624, BioMed Central

Keywords: vaginal health, probiotic, *Lactobacillus crispatus*, bacterial vaginosis, vulvovaginal candidiasis

1. Introduction

Health of reproductive tract is tightly associated with balance of microbial communities in this area. Microorganisms living in the genital tract of healthy women form a complex and dynamic ecosystem where, on one hand, different bacterial species coexist, interact and compete for space and nutrients with many other microorganisms, and on the other hand, these microorganisms interact with host and environmental factors. The healthy female microbial communities are dominated by lactobacilli that play an important role in maintaining an optimal pH level and

preventing urogenital diseases but also ensuring successful reproduction (Chen *et al.*, 2021; Cribby *et al.*, 2008; Hickey *et al.*, 2012). The most prevalent vaginal lactobacilli include homofermentative species *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus gasseri* and *Lactobacillus jensenii* (Drell *et al.*, 2013; Ravel *et al.,* 2011).

A frequent disturbance of vaginal microbiome, bacterial vaginosis (BV) is characterised by disappearance of protective lactobacilli and appearance of polymicrobial community, dominated by *Gardnerella vaginalis, Mobiluncus mulieris*, *Atopobium vaginae,* mycoplasmas and other bacteria. BV can be associated with unpleasant symptoms like foul-smelling discharge but it may also contribute to reproductive failure via several mechanisms – BV-associated bacteria may ascend and cause pelvic inflammatory disease-related closure of fallopian tubes, cytokine imbalance may disturb implantation and increase miscarriage rate (Onderdonk *et al.*, 2016; Ravel *et al.*, 2021; Van Oostrum *et al.*, 2013). Vulvovaginal candidiasis (VVC) is another frequent disorder of vaginal microbial communities characterised by increase of *Candida* spp. counts in vaginal community as well as the hyphal transformation of the yeast that results in milky or curdy discharge and vulvovaginal irritation. The disease has been associated with changes in environmental and host immune status but also shifts in protective function of lactobacilli (Gonçalves *et al.*, 2015; Pytka *et al.*, 2019; Sobel, 2016). Vaginal discharge due to BV or VVC is a very frequent reason for visiting gynaecologist.

Both conditions tend to recur in many women while frequent use of antimicrobials (metronidazole or clindamycin for BV, antifungals for candidiasis) may be associated with side effects, drug resistance and negative effect on microbiome (Van de Wijgert *et al.*, 2020). Therefore, alternative and more safe treatment options are needed, one of the options being probiotic use. Probiotics are beneficial microorganisms that provide health benefits when consumed in suitable amounts (Hill *et al.*, 2014). Competence Centre on Health Technologies (CCHT, Tervisetehnoloogiate Arenduskeskus AS) has been working on development of novel lactobacillar probiotics for balancing vaginal microbiome. The strains were selected from a collection of vaginal lactobacilli (www.eemb.ut.ee/eng/crep_english_introduction_list.php) that was established during the previous clinical studies conducted by CCHT. The strains were derived from vaginal microbiota of Estonian women. The strains were allocated to thorough *in vitro* testing for several functional properties (lactic acid and hydrogen peroxide production, antagonistic activity against urinary tract and genital tract pathogens, autoaggregative and adhesive ability, proteome pattern), technological properties (lyophilisation, shelf-life, growth curves) and safety (absence of haemolytic activity and transferrable antibiotic resistance). As the result, a set of most promising vaginal *L. crispatus* strains was selected for further clinical trials (Hütt *et al.*, 2016; Mändar *et al.*, 2018; Palmiste, 2017; Smidt *et al.*, 2015; Štšepetova *et al.*, 2017). Main results of the *in vitro* experiments of the finally selected strains are given in Supplementary Table S1. During the first clinical trial, forty healthy volunteer women were recruited to evaluate safety, tolerability and preliminary efficacy of the selected strains (Rostok *et al.*, 2019). In this randomised double-blind placebo-controlled trial, the probiotic oral capsules were well-tolerated. Most participants reported no changes in vaginal discharge, while in three subjects, the discharge had changed from abnormal to normal. In addition, the intake of capsules resulted in decrease of Nugent score and reduction of *G. vaginalis* counts.

The aim of the current study was to evaluate the activity of the above-described evidence-based probiotics (*L. crispatus* strains DSM32717, DSM32720, DSM32718 and DSM32716) on BV and VVC patients in the clinical trial in terms of both clinical and microbiological improvement.

2. Material and methods

Study subjects

The clinical trial was carried out in collaboration with CCHT (Tartu, Estonia) and MediTA Clinic (Tartu, Estonia) in 2016-2019. The study group included BV and VVC patients who were recruited during their regular appointments due to vaginitis (Figure 1).

Inclusion criteria for all patients were as follows: a desire to participate and age 18-50 years. Exclusion criteria for all patients were as follows: pregnancy, breastfeeding, sexually transmitted diseases, chronic diseases (cardiovascular diseases, diabetes), acute or chronic infectious diseases, food allergies, antibiotic or anti-inflammatory drug use during last month.

Specific inclusion criteria for BV patients included current episode of BV according to complaints and Amsel criteria (Amsel *et al.*, 1983); specific exclusion criteria for BV patients included current episode of VVC. Specific inclusion criteria for VVC patients included current episode of VVC according to complaints, clinical picture and yeast culture; specific exclusion criteria for VVC patients included current episode of BV. The subjects were told to refrain from using probiotic dairy products (yoghurts, kefirs, etc.) and probiotic capsules during the trial period. Probiotic-free dairy products were allowed.

Ethical considerations

Participation in the study was voluntary. Written informed consent was obtained from all subjects and all were informed that they could withdraw from study at any

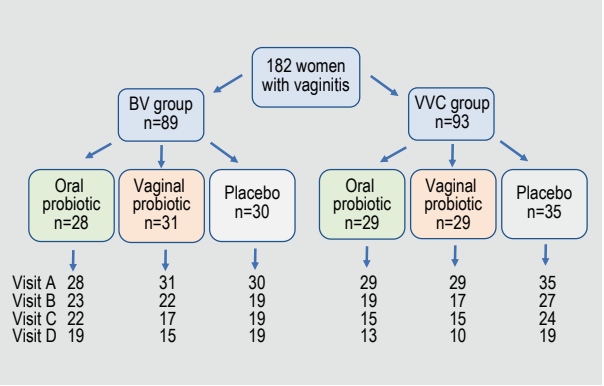

Figure 1. The participants' flow chart. In case of bacterial vaginosis (BV) patients, we removed from further analysis 18 women who dropped out before the second visit due to antibiotic treatment, pregnancy, positive sexually transmitted infections test and unwillingness to continue the trial. In addition, seven women were removed due to low Nugent score at first visit although BV had been diagnosed according to Amsel criteria. In case of vulvovaginal candidiasis (VVC) patients, we removed from further analysis 28 women who dropped out before the second visit due to antibiotic treatment, pregnancy, positive STI test and unwillingness to continue the trial. In addition, two women were removed due to missing samples. Unwillingness to continue the trial was mostly associated with the following reasons: too big oral capsules were difficult to swallow, vaginal administration was not liked, poor effect (was mostly associated with placebo capsules).

time. The study protocol was approved by the Research Ethics Committee of the University of Tartu (Permission No 256/T-13, 15.02.2016).

Study material and protocol

The eligible participants, 182 women were recruited into randomised double-blind placebo-controlled two-arm parallel trial (ISRCTN34840624, BioMed Central). The patients of each diagnosis group were randomly allocated to one of three subgroups who received oral or vaginal probiotic capsules, or placebo capsules (Figure 1). The oral capsules were enteric-coated capsules while the vaginal capsules were gelatin capsules. The oral placebo capsules contained maltodextrin that is commonly used in probiotic industry.

A probiotic capsule contained two (in case of BV) or three (in case of VVC) different *Lactobacillus crispatus* strains (in total 3×10^{10} cfu per capsule). The BV mixture contained the strains DSM32717 and DSM32720, and the VVC mixture contained the strains DSM32720, DSM32718 and DSM32716. The strains had passed careful *in vitro* testing (Hütt *et al.*, 2016; Smidt *et al.*, 2015; Štšepetova *et al.*, 2017) and the best strains were selected for clinical trials. In the first clinical trial, their safety and tolerability were evaluated on healthy volunteers (Rostok *et al.*, 2019). In addition, the shelf-life of the freeze-dried bacterial powders of the selected strains was tested. Viability of the strains remained stable (within one logarithm) within one year at -20 °C and -80 °C, six months at +4 °C and one month at room temperature. Hence, these capsulated probiotics can be stored in a regular refrigerator for up to 6 months. Selection and properties of the strains are described in a patent application (Mändar *et al.*, 2018).

All participants consumed one type of capsule during three months, 20 days per month (intermenstrual period), one capsule per day. The participants had four trial visits, of them first and last at the gynaecologist while 2nd and 3rd at study coordinator. During the first and last visit, vaginal and blood samples were collected at gynaecologist's appointment while during 2nd and 3rd visit the participants had to bring the self-collected vaginal samples. In addition, during the first visit the study questionnaire was filled that contained questions about lifestyle, reproductive and general health. During the trial period, the participants had to fill weekly a short questionnaire about their vaginal, intestinal and general health (Figure 2).

Clinical and basic laboratory investigations

The study subjects were clinically investigated during the first and last visit by one gynaecologist (M.S.) who assessed their reproductive and general health. In addition, the blood samples were collected at these visits. The blood tests included haemogram, high-sensitive C-reactive-protein (hs-CRP), markers of liver and kidney (ASAT, ALAT, eGFR, creatinine) and hormones (oestradiol, progesterone). In addition, common sexually transmitted infections (STI) were tested at the first visit (*Neisseria*

Month	Week	Visit	Samples and tests
		A	Vaginal and blood samples, study questionnaire
1	0		Weekly questionnaire
	1		Weekly questionnaire
	2		Weekly questionnaire
	3		Weekly questionnaire
		B	Vaginal samples
2	0		Weekly questionnaire
	1		Weekly questionnaire
	2		Weekly questionnaire
	3		Weekly questionnaire
		C	Vaginal samples
3	0		Weekly questionnaire
	1		Weekly questionnaire
	2		Weekly questionnaire
	3		Weekly questionnaire
		D	Vaginal and blood samples

Figure 2. The trial scheme.

gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium, Trichomonas vaginalis). Blood indices and STIs were determined by standard laboratory methods using certified assays in Synlab Eesti OÜ (Tallinn, Estonia).

Vaginal, gastrointestinal and general health monitoring

Assessment of vaginal, gastrointestinal and general health using self-reported questionnaires was performed throughout the trial. Vaginal discharge questions included amount, consistency, colour and odour of discharge, and itchiness. Gastrointestinal questions including the presence and severity of abdominal pain, bloating, gastric reflux, nausea, vomiting, flatulence and stool consistency was rated using scale from no complaints to severe symptoms. General health was assessed according to general feeling score and questions about viral infections and allergy.

Collection of vaginal samples

During the first and last visit, the vaginal samples were collected by gynaecologist. During the 1st and 2nd visits, the participants were given oral and written instructions by the gynaecologist or trial coordinator for collecting the vaginal samples in the morning before the next scheduled visit. The participants were instructed to insert two swabs with the tip up into the vagina 5-7 cm, where participants gently rotated the swabs and placed one swab back into transport tub and the second swab was used to make a smear onto microscope slide. During the visit, the samples were delivered to the laboratory immediately, where they were subsequently frozen at -80 °C.

Microscopic evaluation of the slides

The microscope slides were alcohol-fixed and Gram stained. The Gram-stained slides were examined using oil immersion at 1,000× magnification. Nugent score was detected as described earlier (Nugent *et al.*, 1991). Briefly, the quantity of three bacterial morphotypes (*Lactobacilli, Gardnerella/Bacteroides* and *Mobiluncus*), were recorded and translated into a rank score. Overgrowth of *Gardnerella*/*Bacteroides* and *Mobiluncus* is associated with bacterial vaginosis and so higher counts of these bacteria give higher rank scores. Conversely, higher counts of lactobacilli give lower rank scores. The rank scores from each type of bacteria were added together to determine an overall Nugent score, a standard measure of vaginal microbial communities. Clue cells, yeasts and white blood cells were recorded as well.

Molecular methods

Bacterial and fungal DNA from vaginal samples was extracted using a PureLink™ Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, US) according to manufacturer's instructions. qPCR method was used to quantify the key microorganisms *Lactobacillus* spp., *Candida* spp. and *Gardnerella vaginalis* in vaginal secretion. Next-generation sequencing was applied to evaluate vaginal microbiome composition in BV patients. Details of molecular methods are given in Supplementary Table S2.

Statistical analysis

Statistical analysis was performed using Past 4.03 (www.uio.no). All measurements of clinical data and scores were given as means and standard deviation. A statistical evaluation of the significance of the differences in vaginal score values at different sampling times was performed by using the Kruskal-Wallis test, followed by Dunn's method. Wilcoxon paired signed-rank test and Chi square test were applied to reveal dynamics in clinical markers and vaginal microorganisms. Mann-Kendall trend test was used to reveal time trends. Differences were considered statistically significant if the P-value was <0.05.

3. Results

Population data

Altogether 182 women were initially screened and enrolled into study, of them 89 with BV and 93 with VVC. The subjects of both diagnosis groups were randomised into 3 subgroups receiving oral or vaginal probiotic mixture, or placebo (Figure 1). Reasons for drop-out included antibiotic treatment, pregnancy, positive STI test or unwillingness to continue the trial. In total, 53 women of BV group and 42 women of VVC group completed the full cycle of the study while 127 women completed at least four weeks and two visits (of them 64 BV and 63 VVC patients). These 127 women were included into further analysis. Baseline characteristics of the study subjects at recruitment are presented in Table 1, no significant differences between the groups were found. In addition, the consumption of probiotic or placebo capsules did not have significant impact on general feeling and intestinal health, neither on blood markers (Supplementary Table S3).

Impact of probiotics on vaginal health in bacterial vaginosis patients

Both oral and vaginal capsules reduced the signs and symptoms in BV patients. Table 2 indicates decrease of Nugent score in both probiotic groups while no statistically significant change was seen in placebo group. The most remarkable shift was observed during first 4 weeks of the trial where proportion of women with score ≥7 was reduced to 26% (in oral capsule group) and 18% (in vaginal capsule group). This was not associated with the consumption of antibacterial soap (used by 18% of women).

Table 1. Baseline characteristics of study subjects.

	Patients with bacterial vaginosis				Patients with vulvovaginal candidiasis			
Characteristics	**Oral capsules**	**Vaginal capsules**	**Placebo capsules**	***P*-value**	**Oral capsules**	**Vaginal capsules**	**Placebo capsules**	***P*-value**
Age (years)	39.09±21.04	39.23±9.04	40.37±13.33	0.959	33.38±9.93	35.79±11.13	39.15±10.37	0.182
BMI[1] (kg/m^2)	25.29±3.92	26.89±4.77	26.30±5.19	0.516	24.47±4.83	23.40±3.58	25.51±5.55	0.377
Education								
Gymnasium or university	12 (54.6%)	11 (50.0%)	8 (42.1%)	0.726	8 (42.1%)	8 (47.1%)	14 (51.9%)	0.807
Vocational or basic	10 (45.4%)	11 (50.0%)	11 (57.9%)		11 (57.9%)	9 (52.9%)	13 (48.1%)	
Menarche								
<13 years	8 (36.4%)	10 (45.4%)	7 (36.8%)	0.790	6 (31.6%)	4 (23.5%)	8 (29.6%)	0.856
≥13 years	14 (63.6%)	12 (54.6%)	12 (63.2%)		13 (68.4%)	13(76.5%)	19 (70.4%)	
Regular menstruation								
No	11 (50.0%)	10 (47.6%)	9 (50.0%)	0.985	7 (43.8%)	6 (42.9%)	6 (25.0%)	0.374
Yes	11 (50.0%)	11 (52.4%)	9 (50.0%)		9 (56.2%)	8 (57.1%)	18 (75.0%)	
Intimate hygiene devices used[2]								
No	8 (36.4%)	13 (59.1%)	10 (52.6%)	0.301	9 (47.4%)	8 (47.1%)	14 (51.9%)	0.936
Yes	14 (63.6%)	9 (40.9%)	9 (47.4%)		10 (52.6%)	9 (52.9%)	13 (48.1%)	
Pregnancy in anamnesis								
No	4 (18.2%)	4 (18.2%)	4 (21.1%)	0.965	6 (31.6%)	3 (17.6%)	5 (20.0)	0.551
Yes	18 (81.8%)	18 (81.8%)	15 (78.9%)		13 (68.4%)	14 (82.4%)	20 (80.0%)	
Married or in relation								
No	5 (22.7%)	3 (14.3%)	3 (15.8%)	0.742	7 (36.8%)	2 (25.0%)	5 (18.5%)	0.375
Yes	17 (77.3%)	18 (85.7%)	16 (84.2%)		12 (63.2%)	12 (75.0%)	22 (81.5%)	
Contraception in last 2 months								
No	0	0	0	0.643	1 (5.3)	3 (17.6)	2 (7.4)	0.479
Intrauterine device	6 (30.0)	9 (42.9)	8 (42.1)		5 (26.3)	7 (41.2)	10 (37.0)	
Others	14 (70.0)	12 (57.1)	11 (57.9)		13 (68.4)	7 (41.2)	15 (55.6)	

[1] BMI = body mass index.
[2] Vaginal rinses, vaginal devices from pharmacy, antibacterial soap, tampons, others.

Table 2. Dynamics of Nugent score values (mean ± SD) in bacterial vaginosis patients during the trial.[1]

Trial visits	Oral capsules	Vaginal capsules	Placebo capsules
Visit A	7.6±1.8^{d,e}	7.6±1.4^{f,g}	7.1±2.0
Visit B	4.4±3.0^{a,d}	4.4±2.5^{b,f}	7.0±1.8^{a,b}
Visit C	4.7±2.7^{c,e}	5.7±2.3	6.9±2.9^{c}
Visit D	5.3±3.6	5.3±2.7 ^{g}	6.0±3.9
P-value[2]	<0.001	<0.001	NS

[1] Dunn's method ($P<0.05$; two similar letters indicate the pair having difference). SD = standard deviation.
[2] Kruskal-Wallis test; NS = not significant.

Dynamics in self-assessed symptoms are shown in Supplementary Figure S1. Remarkable improvement was noted in amount and smell of discharge, but also in itching/irritation that is not typical sign for BV. Change of all these parameters was statistically significant in case of vaginal probiotics while in case oral probiotics, the two first parameters were just on significance level.

Changes in the PCR-detected key microorganisms are shown in Table 3. Consumption of vaginal probiotics significantly increased the lactobacilli counts in vagina. Decrease of *G. vaginalis* counts was more pronounced in case of oral probiotics, however, it remained statistically insignificant.

Changes in the NGS-detected vaginal microbial communities are shown on Figure 3 and Supplementary Figure S2 and S3. No significant changes in alpha diversity indices were observed. Though above significance level,

Table 3. Quantification of *Lactobacillus* sp., *Gardnerella vaginalis* and *Candida* sp. in the vaginal samples.

Treatment	Counts of key microorganisms					
	Bacterial vaginosis patients					
	***Lactobacillus* sp.**			***Gardnerella vaginalis***		
	In recruitment	After 4 weeks	*P*-value	In recruitment	After 4 weeks	*P*-value
Oral probiotic	4.76 (2.62-5.28)	3.36 (2.71-5.71)	0.301	4.18 (2.92-4.66)	3.58 (2.88-4.38)	0.171
Vaginal probiotic	3.93 (2.97-4.49)	4.82 (3.72-5.40)	0.028	3.94 (3.31-4.34)	3.94 (2.34-4.46)	0.614
Placebo	6.15 (4.76-6.43)	5.68 (5.25-6.34)	0.935	3.30 (1.66-4.85)	3.99 (3.06-4.57)	0.903
	Vulvovaginal candidiasis patients					
	***Lactobacillus* sp.**			***Candida* sp.**		
	In recruitment	After 4 weeks	*P*-value	In recruitment	After 4 weeks	*P*-value
Oral probiotic	5.61 (4.80-5.95)	5.63 (4.95-5.98)	0.809	<1 (0-1.4)	0 (0-0.6)	0.178
Vaginal probiotic	5.23 (5.00-5.51)	5.66 (4.95-5.83)	0.135	<1 (0-1.1)	<1 (0-1.4)	0.570
Placebo	5.58 (4.76-5.76)	5.36 (4.85-5.82)	0.648	<1 (0-0.1)	0 (0-0.4)	0.158

[1] The data are presented as $\log_{10}$ (cfu/g) median and the interquartile range (IQR). Wilcoxon paired signed-rank test was used. Bold values indicate significance.

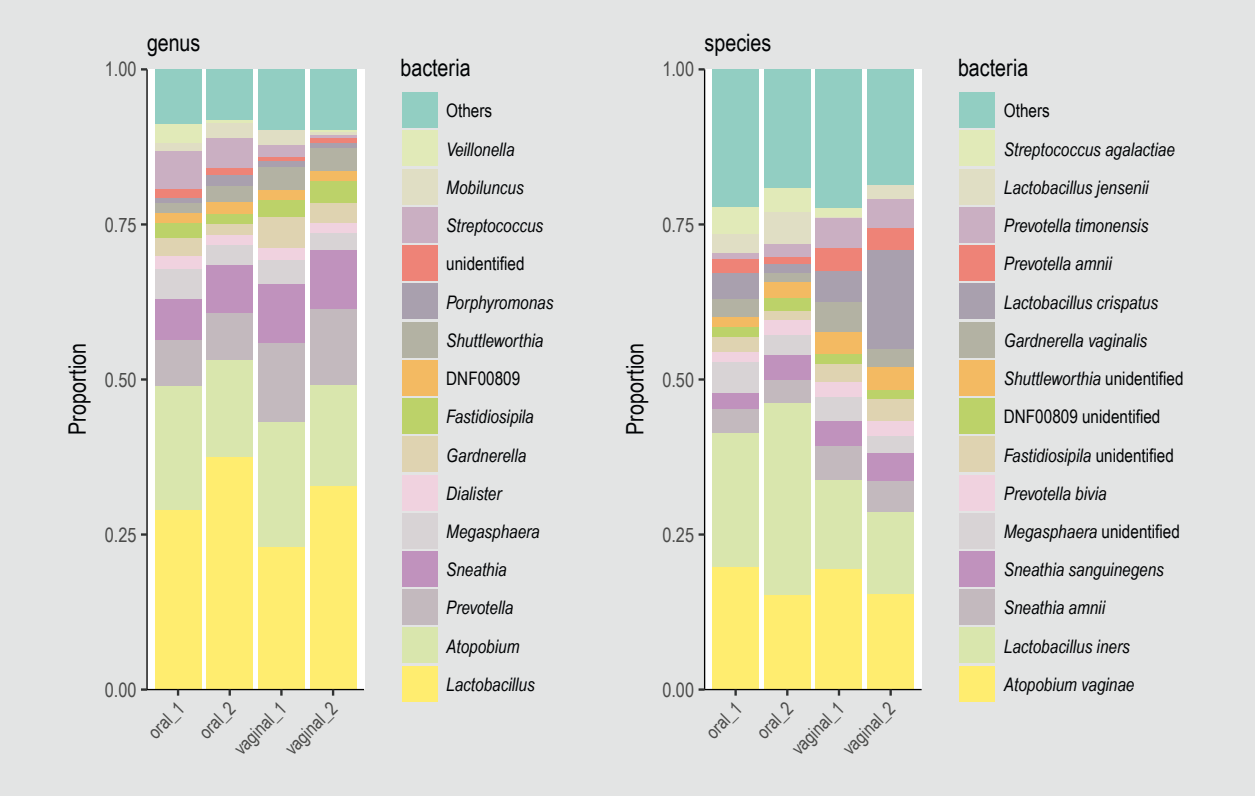

Figure 3. Dynamics of vaginal microbial communities in BV-patients on genus (A) and species level (B). oral_1 and oral_2 indicate patients consuming oral capsules, vaginal_1 and vaginal_2 indicate patients consuming vaginal capsules, 1 – beginning of trial, 2 – after four weeks. The order of the bacterial species in the legend is the same as in the bars.

mean proportion of lactobacilli increased in case of both consumption modes. It was interesting to note that the increase occurred at the expense of *L. crispatus* in the vaginal capsule group while at the expense of *L. iners* in the oral capsule group. Mean proportion of some BV-related bacteria decreased, being significant in case of *Mobiluncus* (P=0.008 vaginal capsules) and *Megasphaera* (P=0.011 oral, P=0.056 vaginal capsules). In addition, the mean proportion of *Aerococcus* (P=0.044 oral capsules) and *Ureaplasma* (P=0.045 oral capsules) decreased while that of *Corynebacterium* increased (P=0.005 oral, P=0.059 vaginal capsules).

Impact of probiotics on vaginal health in vulvovaginal candidiasis patients

Dynamics of two most important symptoms, amount of discharge and itching/irritation was assessed. The combined score of these two symptoms is presented in Table 4 showing that both oral and vaginal capsules relieved the symptoms while no statistically significant change of combined score was seen in placebo group. Decrease of amount of discharge was more expressed in case of oral capsules while that of itching/irritation in case of vaginal capsules, both being statistically significant (Supplementary Figure S1).

4. Discussion

Our randomised double-blind placebo controlled clinical trial revealed that novel probiotic *L. crispatus* strains were effective in relieving the signs and symptoms of BV and VVC while consumed orally or vaginally.

Lactobacilli comprise the highest proportion of microorganisms in healthy vagina (Ravel *et al.*, 2011). They possess several beneficial properties that help to ensure vaginal health (production of lactic acid, hydrogen peroxide and several bacteriocins, antagonistic activity against opportunistic and pathogenic microorganisms, stimulation of local immunity and disruption of biofilms) and therefore they have been generally considered the gatekeepers of the vaginal ecosystem. Their disappearance or substantial decline leads to diseases like BV or VVC. This is the reason why lactobacilli are used to develop probiotics in order to combat these microecological disorders. No adverse effects have been reported in clinical trials on vaginitis patients that makes this option even more promising (Homayouni *et al.*, 2014).

Table 4. Dynamics of summary score (amount of discharge + itching/irritation) in vulvovaginal candidiasis patients during the trial.[1,2]

Week	Oral capsules	Vaginal capsules	Placebo capsules
1	4.3±2.2	3.1±1.8	3.9±2.1
2	3.9±1.9	4.1±2.0[a]	3.4±2.1
3	2.9±1.9	2.2±1.6	3.0±2.2
4	2.4±1.9	2.1±1.9[a]	2.9±2.2
P-value[3]	0.045	0.006	NS

[1] Amount of discharge was scored from 0 to 4: 0 – missing or abnormally dry, 1 – minor (normal), 2 – medium, 3 – high, 4 – very high. Itching/irritation was assessed 0 (no) or 3 (yes). The two scores were summarised.
[2] Values are mean ± standard deviation. Dunn's method (P<0.05; two similar letters indicate the pair having difference).
[3] Kruskal-Wallis test. NS = not significant.

Development of novel targeted and effective probiotics needs large strain collection and comprehensive *in vitro* screening of their phenotypical properties. There exists significant strain level specificity for certain antimicrobial properties among cervicovaginal lactobacilli, indicating that the presence of a particular species in the vaginal microbiota is not sufficient to determine its benefit to the host. A full repertory of antimicrobial properties should be evaluated in choosing vaginal microbiota-associated *Lactobacillus* isolates for the development of live biotherapeutic strategies (Atassi *et al.*, 2019). Our strains have passed careful *in vitro* testing (Hütt *et al.*, 2016; Smidt *et al.*, 2015; Štšepetova *et al.*, 2017) as well as the clinical trial where their safety and tolerability were evaluated (Rostok *et al.*, 2019). In addition, we have tested shelf-life of the freeze-dried powders of the selected strains at four temperatures revealing that the capsulated probiotics can be stored at refrigerator up to 6 months.

To date, clinical testing of novel vaginal probiotics is not well standardised, and there exist studies describing clinical trials without some essential data like acronyms of specific probiotic strains, doses, impact on the microbiome, well defined diagnoses etc. According to a recent review, the doses ranged from $\geq 10^7$ cfu/day to 2.5×10^{10} cfu/day, but were highly variable regarding the treatment duration timing (Lopéz-Moreno and Aguilera, 2021). Substantial heterogeneity in products, trial methodologies and outcome measures do not provide sufficient evidence for or against recommending probiotics for the treatment of vaginitis. Therefore, well-designed randomised controlled trials with standardised methodologies and larger patient size have been suggested (Senok *et al.*, 2009). The majority of clinical trials yielding positive results have been performed with high doses of lactobacilli suggesting that, beside strain characteristics, the amount of exogenously applied lactobacilli could have a role in the effectiveness of the product (Mastromarino *et al.*, 2013).

Another questionable topic related to vaginal probiotics includes species composition and origin of lactobacilli. A lot of studies have used the lactobacilli that are not adapted to live in vaginal communities, like non-vaginal lactobacilli species (that are mostly characteristic of gut) and/or strains isolated from intestinal tract (Husain *et al.*, 2020; Koirala *et al.*, 2020; Oerlemans *et al.*, 2020; Pino *et al.*, 2021;

Vitali *et al.*, 2012; Yang *et al.*, 2020). In low number of cases vaginal species (*L. crispatus, L. jensenii, L. gasseri*) have been suggested for vaginal health (Laue *et al.*, 2018; Molin *et al.*, 2004; Nivoliez, 2012). At the same time, compared with other species of *Lactobacilli,* strains of *L. crispatus* provide the best balance of beneficial species-specific properties to sustain vaginal eubiosis with respect to the production of H_2O_2, bacteriocins and lactic acid, including the most beneficial ratio of the l- and d-isomers, and the protonated form of lactic acid. Therefore, the future vaginal probiotic research should be centred around *L. crispatus* (Lamont *et al.*, 2020).

We performed a clinical trial on vaginitis patients diagnosed with BV or VVC. Two different mixtures of vaginal *L. crispatus* strains derived from vaginal microbiota were selected for these patients according to the previous laboratory testing. The patients of each diagnosis group received either oral or vaginal capsules containing the specific mixture (3×10^{10} cfu per capsule), or placebo. Safety and effectivity of the daily doses 10^9-10^{11} cfu have been previously suggested (Hütt *et al.,* 2011; Mombelli and Gismondo, 2000). The oral capsules were enteric-coated, to ensure the viability of lactobacilli until distal part of intestinal tract. The vaginal capsules were gelatin-coated, to ensure their quick solubility in vaginal environment. These lactobacilli strains had formerly passed a clinical trial on healthy volunteer women where their safety and tolerability were evaluated (Rostok *et al.*, 2019). While administering these novel probiotics to the vaginitis patients, we observed the improvement in both objective and subjective health criteria.

In case of BV patients, we evaluated the dynamics of Nugent score that decreased in both oral and vaginal consumption mode. No statistically significant change of score was seen in placebo group. Interestingly, the most remarkable decrease in both probiotic groups was noted during the first month of the trial. The trial seemed too long for some participants and the capsules were not used as regularly during the following months. Significant improvement was noted also in the volume and odour of the discharge as well as irritation. Again, some 'trend towards fatigue' was noted in these parameters during the second and third months. Therefore, we chose to show the immediate (4-week) effect in some tables and figures. In addition, the time point B included the highest number of patients, in comparison with C and D where low number of participants may increase the randomness of the results. Positive effect of novel probiotics on vaginal microbial communities was confirmed by microbiological analyses, both PCR- and NGS-based.

Most of probiotics for women's health have been developed and tested in BV patients while much less in VVC patients (Oerlemans *et al.*, 2020). VVC has complicated pathogenesis involving host immune defects, interrupted vaginal epithelium and microbiological dysbiosis. Significant increase in the number of yeasts and their transformation to hyphae are observed that provoke inflammatory reaction. In addition, women with VVC have a less abundant *Lactobacillus* population, especially as concerns hydrogen peroxide secreting strains. Inhibitory effect of lactobacilli on the formation of mycelium and yeast adhesion has been revealed (Gonçalves *et al.*, 2016; Pytka *et al.*, 2019). We selected a special set of strains that possessed antagonistic activity (during *in vitro* testing) against *Candida albicans* that is the most common causative agent of VVC, and against *Candida glabrata* that is less common but more drug resistant species. In the current trial, we evaluated the dynamics of two most important complaints, amount of discharge and itching/irritation. Both parameters showed decreasing tendency. Decrease of discharge was more pronounced in case of oral capsules while itching/irritation in vaginal capsules. If we summarised the scores of these two parameters, we could see statistically significant decrease of complaints in both administration mode groups. Hence, the numerous women with recurrent VVC may benefit from this formulation.

Mild improvement of signs and symptoms was noted also in placebo group. Since both BV and VVC are the diseases of ecological nature that are significantly related to microbiome balance and the factors influencing this balance, then some improvement over time is a natural phenomenon. The probiotics can speed up this process and alleviate the complaints sooner. They could have a preventive effect too, that needs to be confirmed in carefully designed trials.

Administration mode can vary in the probiotics intended to improve vaginal health. Both vaginal and oral modes have been used (De Vrese *et al.*, 2019). Vaginally administered probiotics reach to the spot of action without the delay, therefore more pronounced benefits can be expected. At the same time, consumption of oral products is more convenient and numerous studies have shown its positive effects. For example, oral administration has resulted in reduced relative abundance of *G. vaginalis* counts (Koirala *et al.*, 2020), reduced Nugent score (Yang *et al.*, 2020), modulation of the vaginal microbiota and cytokine secretion (Vitali *et al.*, 2012), normalisation of vaginal parameters (Laue *et al.*, 2018; Strus *et al.*, 2012), reduced vaginal group B streptococci colonisation and improvement of pregnancy outcome (Liu *et al.*, 2020). We used both administration modes in parallel in separate study groups and found oral administration promising in case of both diagnoses. This is an important result indicating the perspective where each patient can select the most suitable and convenient pharmaceutical form. A recent review also indicated that pre- and probiotic beneficial effects can be delivered topically or systemically (Al-Ghazzewi and Tester, 2016).

In conclusion, the novel formulations of evidence-based well-focused probiotic *L. crispatus* strains are effective

against BV and VVC being suitable for both vaginal and oral administration.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2022.0091.

Figure S1. Dynamics of self-assessed symptoms in BV and VVC patients during the trial.

Figure S2. Dynamics of alpha diversity of vaginal microbiota in BV-group on phylum, genus and species levels.

Figure S3. Dynamics of vaginal microbial communities in BV-patients on genus and species level.

Table S1. Main functional parameters of the *Lactobacillus crispatus* strains.

Table S2. Details of molecular methods.

Table S3. Effect of three-month consumption of probiotic *Lactobacillus crispatus* or placebo on blood markers in vaginitis patients.

Acknowledgements

This study was supported by Enterprise Estonia (grants No. EU48695); Estonian Research Council (grant No. IUT34-19 and PRG1076), Horizon 2020 innovation grant (ERIN, grant no. EU952516) and Estonian Ministry of Education and Research (grant No. KOGU-HUMB). The design, conduct, and analysis of the study were completed independently by the investigators. The authors are grateful to Dr Tanel Kaart for a fruitful discussion.

Conflict of interest

R. Mändar, I. Smidt, H, Tamm and A. Salumets work part-time at CCHT that is the patent holder. No conflict was declared by other authors.

References

Al-Ghazzewi, F.H. and Tester, R.F., 2016. Biotherapeutic agents and vaginal health. Journal of Applied Microbiology 121: 18-27.

Amsel, R., Totten, P.A., Spiegel, C.A., Chen, K.C., Eschenbach, D. and Holmes, K.K., 1983. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. American Journal of Medicine 74: 14-22.

Atassi, F., Pho Viet Ahn, D.L. and Lievin-Le Moal, V., 2019. Diverse expression of antimicrobial activities against bacterial vaginosis and urinary tract infection pathogens by cervicovaginal microbiota strains of *Lactobacillus gasseri* and *Lactobacillus crispatus*. Frontiers in Microbiology 10: 2900.

Chen, X., Lu, Y., Chen, T. and Li, R., 2021. The female vaginal microbiome in health and bacterial vaginosis. Frontiers in Cellular and Infection Microbiology 11: 631972.

Cribby, S., Taylor, M. and Reid, G., 2008. Vaginal microbiota and the use of probiotics. Interdisciplinary Perspectives on Infectious Diseases 2008: 256490.

De Vrese, M., Laue, C., Papazova, E., Petricevic, L. and Schrezenmeir, J., 2019. Impact of oral administration of four *Lactobacillus* strains on Nugent score – systematic review and meta-analysis. Beneficial Microbes 10: 483-496.

Drell, T., Lillsaar, T., Tummeleht, L., Simm, J., Aaspõllu, A., Väin, E., Saarma, I., Salumets, A., Donders, G.G. and Metsis, M., 2013. Characterization of the vaginal micro- and mycobiome in asymptomatic reproductive-age Estonian women. PLoS ONE 8: e54379.

Gonçalves, B., Ferreira, C., Alves, C.T., Henriques, M., Azeredo, J. and Silva, S., 2016. Vulvovaginal candidiasis: epidemiology, microbiology and risk factors. Critical Reviews in Microbiology 42: 905-27.

Hickey, R.J., Zhou, X., Pierson, J.D., Ravel, J. and Forney, L.J., 2012. Understanding vaginal microbiome complexity from an ecological perspective. Translational Research 160: 267-82.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C. and Sanders, M.E., 2014. Expert consensus document. The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology 11: 506-14.

Homayouni, A., Bastani, P., Ziyadi, S., Mohammad-Alizadeh-Charandabi, S., Ghalibaf, M., Mortazavian, A.M. and Mehrabany, E.V., 2014. Effects of probiotics on the recurrence of bacterial vaginosis: a review. Journal of Lower Genital Tract Diseases 18: 79-86.

Husain, S., Allotey, J., Drymoussi, Z., Wilks, M., Fernandez-Felix, B.M., Whiley, A., Dodds, J., Thangaratinam, S., McCourt, C., Prosdocimi, E.M., Wade, W.G., de Tejada, B.M., Zamora, J., Khan, K. and Millar, M., 2020. Effects of oral probiotic supplements on vaginal microbiota during pregnancy: a randomised, double-blind, placebo-controlled trial with microbiome analysis. BJOG 127: 275-284.

Hütt, P., Lapp, E., Štšepetova, J., Smidt, I., Taelma, H., Borovkova, N., Oopkaup, H., Ahelik, A., Rööp, T., Hoidmets, D., Samuel, K., Salumets, A. and Mändar, R., 2016. Characterisation of probiotic properties in human vaginal lactobacilli strains. Microbial Ecology in Health and Disease 27: 30484.

Hütt, P., Kõll, P., Stsepetova, J., Alvarez, B., Mändar, R., Krogh-Andersen, K., Marcotte, H., Hammarström, L. and Mikelsaar, M., 2011. Safety and persistence of orally administered human *Lactobacillus* sp. strains in healthy adults. Beneficial Microbes 2: 79-90.

Koirala, R., Gargari, G., Arioli, S., Taverniti, V., Fiore, W., Grossi, E., Anelli, G.M., Cetin, I. and Guglielmetti, S., 2020. Effect of oral consumption of capsules *containing Lactobacillus paracasei* LPC-S01 on the vaginal microbiota of healthy adult women: a randomized, placebo-controlled, double-blind crossover study. FEMS Microbiology Ecology 96: fiaa084.

Lamont, R.F., Bagge, J.R., Vinter, C.A. and Jørgensen, J.S., 2020. Re: Effects of oral probiotic supplements on vaginal microbiota during pregnancy: a randomised, double-blind, placebo-controlled trial with microbiome analysis. BJOG 127: 303.

Laue, C., Papazova, E., Liesegang, A., Pannenbeckers, A., Arendarski, P., Linnerth, B., Domig, K.J., Kneifel, W., Petricevic, L. and Schrezenmeir, J., 2018. Effect of a yoghurt drink containing *Lactobacillus* strains on bacterial vaginosis in women – a double-blind, randomised, controlled clinical pilot trial. Beneficial Microbes 9: 35-50.

Liu, Y., Huang, Y., Cai, W., Li, D., Zheng, W., Xiao, Y., Liu, Y., Zhao, H. and Pan, S., 2020. [Effect of oral *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 on vaginal Group B *Streptococcus* colonization and vaginal microbiome in late pregnancy]. Nan Fang Yi Ke Da Xue Xue Bao 40: 1753-1759.

López-Moreno, A. and Aguilera, M., 2021. Vaginal probiotics for reproductive health and related dysbiosis: systematic review and meta-analysis. Journal of Clinical Medicine 10: 1461.

Mändar, R., Hütt, P., Smidt, I., Štšepetova, J., Lapp, E., Rostok, M., Sõerunurk, G., Rööp, T., Hoidmets, D., Tamm, H., Kolk, M., Piiskop, S., Palmiste, T., Koort, K. and Salumets, A., 2008. *Lactobacillus crispatus* probiotics. Patent application. Competence Centre on Health Technologies; Priority number: 1816425.1. Priority date: 9.10.2018. Other numbers: EP19202134.3A, WO2020074547A8, JP2022512692A.

Mastromarino, P., Vitali, B. and Mosca, L., 2013. Bacterial vaginosis: a review on clinical trials with probiotics. New Microbiologica 36: 229-38.

Molin, G., Ahrne, S., Jeppsson, B., Vasquez, A. and Berbbren, A., 2004. Probiotic *Lactobacillus* strains for improved vaginal health. Pat. US20080268006A1.

Mombelli, B. and Gismondo, M.R., 2000. The use of probiotics in medical practice. International Journal of Antimicrobial Agents 16: 531-6.

Nivoliez, A., 2012. Novel strain of *Lactobacillus crispatus*. Pat. US20150190438A1.

Nugent, R.P., Krohn, M.A. and Hillier, S.L., 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. Journal of Clinical Microbiology 29: 297-301.

Oerlemans, E.F.M., Bellen, G., Claes, I., Henkens, T., Allonsius, C.N., Wittouck, S., van den Broek, M.F.L., Wuyts, S., Kiekens, F., Donders, G.G.G. and Lebeer, S., 2020. Impact of a lactobacilli-containing gel on vulvovaginal candidosis and the vaginal microbiome. Scientific Reports 10: 7976.

Onderdonk, A.B., Delaney, M.L. and Fichorova, R.N., 2016. The human microbiome during bacterial vaginosis. Clinical Microbiology Reviews 29: 223-38.

Palmiste, T., 2017. Proteome analysis of *Lactobacillus crispatus* strains with probiotic potential. MSc thesis.

Pino, A., Rapisarda, A.M.C., Vitale, S.G., Cianci, S., Caggia, C., Randazzo, C.L. and Cianci, A., 2021. A clinical pilot study on the effect of the probiotic *Lacticaseibacillus rhamnosus* TOM 22.8 strain in women with vaginal dysbiosis. Scientific Reports 11: 2592.

Pytka, M., Kordowska-Wiater, M. and Jarocki, P., 2019. Microbiome of the women's genital system. Advancements of Microbiology 58: 227-236.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., McCulle, S.L., Karlebach, S., Gorle, R., Russell, J., Tacket, C.O., Brotman, R.M., Davis, C.C., Ault, K., Peralta and L., Forney, L.J., 2011. Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences of the USA 108 Suppl. 1: 4680-7.

Ravel, J., Moreno, I. and Simón, C., 2021. Bacterial vaginosis and its association with infertility, endometritis, and pelvic inflammatory disease. American Journal of Obstetrics and Gynecology 224: 251-257.

Rostok, M., Hütt, P., Rööp, T., Smidt, I., Štšepetova, J., Salumets, A. and Mändar, R., 2019. Potential vaginal probiotics: safety, tolerability and preliminary effectiveness. Beneficial Microbes 10: 385-393.

Senok, A.C., Verstraelen, H., Temmerman, M. and Botta, G.A., 2009. Probiotics for the treatment of bacterial vaginosis. Cochrane Database of Systematic Reviews 4: CD006289.

Smidt, I., Kiiker, R., Oopkaup, H., Lapp, E., Rööp, T., Truusalu, K., Štšepetova, J., Truu, J. and Mändar, R., 2015. Comparison of detection methods for vaginal lactobacilli. Beneficial Microbes 6: 747-51.

Sobel, J.D., 2016. Recurrent vulvovaginal candidiasis. American Journal of Obstetrics and Gynecology 214: 15-21.

Strus, M., Chmielarczyk, A., Kochan, P., Adamski, P., Chełmicki, Z., Chełmicki, A., Pałucha, A. and Heczko, P.B., 2012. Studies on the effects of probiotic Lactobacillus mixture given orally on vaginal and rectal colonization and on parameters of vaginal health in women with intermediate vaginal flora. European Journal of Obstetrics, Gynecology, and Reproductive Biology 163: 210-5.

Štšepetova, J., Taelma, H., Smidt, I., Hütt, P., Lapp, E., Aotäht, E. and Mändar, R., 2017. Assessment of phenotypic and genotypic antibiotic susceptibility of vaginal *Lactobacillus* sp. Journal of Applied Microbiology 123: 524-534.

Van de Wijgert, J.H.H.M., Verwijs, M.C., Agaba, S.K., Bronowski, C., Mwambarangwe, L., Uwineza, M., Lievens, E., Nivoliez, A., Ravel, J. and Darby, A.C., 2020. Intermittent lactobacilli-containing vaginal probiotic or metronidazole use to prevent bacterial vaginosis recurrence: a pilot study incorporating microscopy and sequencing. Scientific Reports 10: 3884.

Van Oostrum, N., De Sutter, P., Meys, J. and Verstraelen, H., 2013. Risks associated with bacterial vaginosis in infertility patients: a systematic review and meta-analysis. Human Reproduction 28: 1809-15.

Vitali, B., Cruciani, F., Baldassarre, M.E., Capursi, T., Spisni, E., Valerii, M.C., Candela, M., Turroni, S. and Brigidi, P., 2012. Dietary supplementation with probiotics during late pregnancy: outcome on vaginal microbiota and cytokine secretion. BMC Microbiology 12: 236.

Yang, S., Reid, G., Challis, J.R.G., Gloor, G.B., Asztalos, E., Money, D., Seney, S. and Bocking, A.D., 2020. Effect of oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 on the vaginal microbiota, cytokines and chemokines in pregnant women. Nutrients 12: 368.