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# Evidence-based Mixture containing Lactobacillus Strains and Lactoferrin to Prevent Recurrent Bacterial Vaginosis: A Double Blind, Placebo Controlled, Randomised Clinical Trial - Russo et al. 2019

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# Evidence-based mixture containing *Lactobacillus* strains and lactoferrin to prevent recurrent bacterial vaginosis: a double blind, placebo controlled, randomised clinical trial

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**RESEARCH ARTICLE**

## Abstract

Bacterial vaginosis (BV) is the most common cause of vaginal discomfort in women. It is characterised by abnormal vaginal microbiota with a depletion of lactobacilli and predominance of anaerobic microorganisms, mainly *Gardnerella vaginalis* and *Atopobium vaginae*. Although antibiotics represent an effective therapeutic option in the short-term, recurrent infections still remain a serious problem. Nowadays, evidence exists about the efficacy of probiotics for the management of BV. The aim of the current double blind, randomised clinical trial was to assess the efficacy of a probiotic mixture, including *Lactobacillus acidophilus* GLA-14 and *Lactobacillus rhamnosus* HN001, in combination with bovine lactoferrin, as adjuvant therapy to metronidazole in women with recurrent BV. In particular, normalisation of Nugent score, remission of symptoms and recurrences during a six-months follow-up were assessed. 48 adult women received metronidazole (500 mg twice daily) for 7 days and randomly assigned to take simultaneously either probiotics plus lactoferrin or placebo (2 capsules/day for 5 days followed by 1 capsule/day for 10 consecutive days; induction phase). The verum or placebo administration (1 capsule/day for 10 consecutive days) was repeated each month (maintenance phase) during the six months of follow-up starting the first day of menstrual cycle since the menstrual blood increases the vaginal pH and contributes to increase the risk of recurrences. The results showed that symptoms (vaginal discharge and itching), Nugent score and recurrence rate were significantly improved by probiotics mixture in association with lactoferrin. This alternative approach may represent a safe and effective remedy for the restoration of healthy vaginal microbiota in preventing recurrent BV.

**Keywords:** *Lactobacillus acidophilus*, *Lactobacillus rhamnosus,* bovine lactoferrin, bacterial vaginosis

## 1. Introduction

Bacterial vaginosis (BV) is one of the great conundrums in sexual and reproductive health. BV is considered to be the most common cause of vaginal infection among both pregnant and non-pregnant women and prevalence between 4.9 and 36.0% have been reported from European and American studies (Cristiano *et al.*, 1996; Kasaro *et al.*, 2017; Unemo *et al.,* 2017). When present, symptoms typically include abnormal vaginal discharge and unpleasant fishy odour. Microbiologically, BV is characterised by a dramatic shift of vaginal microflora which comprises the loss of beneficial bacteria (lactobacilli) and a simultaneous proliferation of anaerobic bacteria including *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus* spp., *Bacteroides* spp., and *Prevotella* spp. (Verstraelen *et al.*, 2004).

Despite the most recent discoveries related to the aetiology of BV, the current treatment is still directed toward alleviation of symptoms through reduction of BV-associated bacteria overgrowth and restoration of normal vaginal flora (Pirotta *et al.*, 2009). Conventionally, BV is treated with antibiotics, such as metronidazole, clindamycin or tinidazole. Recurrent bacterial vaginosis (RBV) is defined as three or more documented episodes in 12 months, and women with RBV should be offered long-term maintenance

therapy as typically done for recurrent candidiasis (Sobel, 2006). Despite the high cure rates achieved in some studies, very high BV recurrence rates and relevant treatment side effects have been reported. The low efficacy of antibiotics in preventing recurrences is thought might be related to their inability to fully eradicate BV vaginal biofilms-associated bacteria (Machado *et al.,* 2016). An alternative approach to treat BV is by modulating the vaginal microbiota, for example, by using probiotics. In the human vagina, certain *Lactobacillus* strains can act as probiotics, preventing the growth of BV-associated bacteria through two main mechanisms: the inhibition of pathogens adhesion to vaginal epithelium and the production of antimicrobial compounds like hydrogen peroxide ($H_2O_2$), lactic acid and bacteriocins (Bertuccini *et al.*, 2017). Many pharmaceutical formulations containing either oral or vaginal probiotic lactobacilli strains were well tolerated and able to reduce BV symptoms, improve the vaginal microflora profile, and reduce recurrence rate (Cianci *et al.,* 2008; Larsonn *et al.,* 2008; Mastromarino *et al.,* 2009). In contrast, other clinical trials have not detected any significant improvement in BV management (Falagas *et al.*, 2007). Alternatively, probiotics have been proposed as adjuvants to antibiotic therapy. Several combinations of metronidazole, clindamycin or tinidazole with probiotic preparations have shown promising results in the treatment of BV as they have been associated with high cure rates, low recurrence or quick re-establishment of a healthy vaginal microflora.

Lactoferrin is a glycoprotein naturally present in milk and other biological fluids of mammals including vaginal secretions and cervix mucus. It is mainly involved in the host defense against pathogen microorganisms. Recent findings showed that both oral and vaginal administration of bovine lactoferrin improved the vaginal microbiota composition in women with BV by increasing the lactobacilli and decreasing the pathogen bacteria associated with BV including *G. vaginalis* (Otsuki and Imai, 2017; Otsuki *et al.*, 2014; Pino *et al.*, 2017).

The aim of our study is to evaluate the efficacy of a probiotic *Lactobacillus* mixture in combination with bovine lactoferrin (Respecta® complex) given orally as adjuvant to drug therapy with oral metronidazole in adult women with RBV. In particular, microbiological cure rate (Nugent score), clinical cure rate, and recurrence rate in a six-month follow-up were assessed.

## 2. Materials and methods

### Study design

The study was performed in accordance with Good Clinical Practices in conducting human clinical trials and the World Medical Association (WMA) Declaration of Helsinki regarding the Ethical Principles for Medical Research Involving Human Subjects adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964 and its following amendments. The study protocol was approved by the National Commission on Bioethics of Drugs and Medical Devices (Bucharest, approval number 2DM/26.01.2016, 14 March 2016).

A double-blinded, prospective, randomised pilot clinical trial was performed from May 2016 to January 2017 in four outpatient gynaecological clinics in Romania. 48 adult women (age 18-50 years) with symptomatic BV, Nugent score >7 and documented anamnestic history of RBV were enrolled in the study. Other inclusion criteria were willingness to use condom as contraceptive method, take the investigational products and collaborate in the completing the study procedures. Women were excluded from the study if they were participating to other clinical trials, suffered from vaginal infections different from BV or other pathologies (i.e. immunodeficiency, diabetes, oestrogen-dependent tumours, etc.), consuming probiotics or foods containing probiotics. Moreover, pregnant or breastfeeding women, under drug therapy with both systemic or local drugs different from those prescribed by the protocol, with hypersensitivity or allergy to antibiotics or any ingredient of investigational products, using vaginal douching were not considered for being enrolled in the current trial.

Each participating woman was informed about the study protocol, procedures and investigational product as well as on potential risks and benefits deriving from the treatment. All recruited women signed an informed consent before participating in the study. Women were randomly divided into 2 groups (24 subjects per group) according to the supplementary treatment (either verum or placebo) to the antibiotic, based on a computer randomised 1:1 scheme.

All participating women attended 6 visits (from T0 to T5) at the gynaecologic centres at recruitment (T0), 1 week (T1), 2 weeks (T2), 1 month (T3), 4 months (T4) and 6 months (T5) after initial treatment or induction phase. In each clinical examination, 2 vaginal swabs (for vaginal culture and Nugent score) were collected. During the first visit (baseline), patient medical history was recorded, informed consent was signed and women were allocated into the two study arms. Visits from T1 to T5 were devoted to check the efficacy and safety of the investigational products. The symptoms evaluated (vaginal discharge, itching) were self-reported by the patients as present or absent during gynaecologic visits.

The evaluation of efficacy was based on clinical cure rate of BV symptoms (defined as absence of vaginal discharge or itching), microbiological cure rate (defined as remission and vaginal microbiota improvement by restoration of healthy Nugent score with range 0-3), and overall cure rate (absence of symptoms and Nugent score <7). In addition, recurrence

rate was calculated for both groups as percentage of women presenting with symptoms and Nugent score more than 3 during the 6 months follow-up period. Safety was assessed by recording all side effects (i.e. adverse events, serious adverse events, and suspected unexpected serious adverse reactions). An adverse event was considered severe if it was fatal, life threatening, required hospitalisation, or led to permanent injury.

### Treatment

Induction phase: all enrolled women were treated with standard antibiotic therapy (metronidazole 500 mg twice daily for 7 days) and randomly assigned to one of two study arms receiving either verum (Respecta) or placebo (2 capsules/day for 5 days followed by further 10 consecutive days at the dosage of 1 capsule/day) by oral intake (acute treatment). Respecta and placebo administration started simultaneously to the metronidazole therapy (on day 1).

Maintenance phase: during follow-up period (6 months), all women ingested 1 capsule of Respecta or placebo per day, for 10 consecutive days per month starting on the first day of menstrual cycle (prophylactic treatment). In fact, the menstrual blood increases the vaginal pH and contributes to increase the risk of RBV. Moreover, starting the prophylactic cycles at the first day of the menstrual phase facilitated the protocol scheme and the compliance of patients.

Respecta is registered and manufactured as a medical device class IIa by Giellepi S.p.A. Health Science (Lissone, Italy) and contains a proprietary lactobacilli mixture ($5\times10^9$ cfu per capsule) including *Lactobacillus acidophilus* GLA-14 (BCCM/LMG Bacteria Collection, LMG S-29159) and *Lactobacillus rhamnosus* HN001 (American Tissue Culture Collection, ATCC Number: SD5675) in combination with bovine lactoferrin RCX™ (50 mg).

Placebo was an identical capsule containing the inactive ingredient maltodextrin (100 mg); both products were produced with the same excipients. Compliance was determined by assessing returned packages.

### Samples collection and laboratory procedures

During each visit, two vaginal swabs were collected from all women by careful rotation of sterile cotton swab on the vaginal sidewall. One swab was immediately inserted into transport media and transferred to the laboratory for the microbiological analysis (culture for excluding yeasts, bacteria or trichomonas) and the second one was used to perform the fresh smear on a glass slide analysed by optical microscopy after fixation and Gram staining and Nugent score was calculated according to method described by Nugent *et al.* (1991).

### Statistical analysis

Descriptive statistics was used for the demographics and clinical evaluations. Inferential statistics was used to compare the statistical differences between study arms (verum and placebo) for the following variables: vaginal discharge, itching and Nugent score. Student's *t*-test and Chi square test were used to compare the statistical differences. The statistically significance was set at $P<0.05$.

## 3. Results

### Demographic characteristics

After signing the inform consent, each woman was evaluated for eligibility criteria. Medical history and demographic data were collected. Mean age was 35.4±9.2 and 36.7±7.7 years; weight: 61.0±8.4 and 63.4±5.6 kg; height: 165.0±5.2 and 168.0±7.4 cm; body mass index: 22.5 and 22.5 kg/m$^2$ for Respecta and placebo respectively. No significant differences were observed between the 2 study arms. Compliance was 100% for all participants.

### Safety evaluation

No woman experienced adverse events during the study period.

### Clinical cure rate

#### *Vaginal discharge*

After one week of treatment (T1), a significant improvement of vaginal discharge was shown in patients in both arms. Such an effect is not surprising because it is likely due to the antibiotic efficacy (metronidazole 500 mg twice daily for one week), simultaneously to either Respecta or placebo. However, only in the Respecta arm, vaginal discharge improved significantly during the follow-up period at T4 and T5. In particular, women without vaginal discharge after Respecta treatment in comparison to placebo were 87.5 vs 37.5% at T4 and 70.8 vs 33.3% at T5 ($P<0.05$ Respecta vs placebo). Study results are shown in Figure 1.

#### *Itching*

After the metronidazole treatment (T1), a significant improvement of itching was shown in either Respecta or placebo arms. However, only Respecta usage improved itching significantly during the follow-up period at T4 and T5. In particular, women without itching after Respecta treatment in comparison to placebo were 87.5 vs 20.8% at T4 and 70.5 vs 20.8% at T5, respectively ($P<0.05$ Respecta vs placebo). Study results are shown in Figure 2.

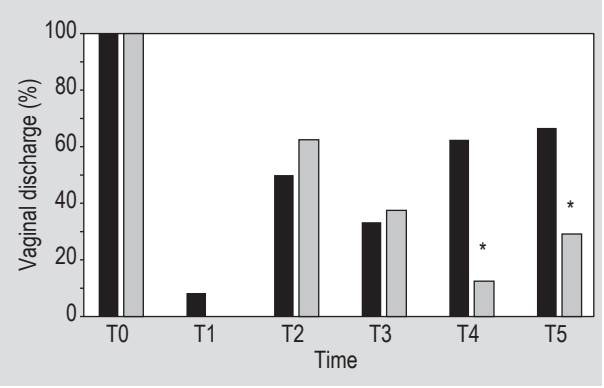


**Figure 1. Resolution of vaginal discharge in women treated with Respecta® (grey) or placebo (black). *$P$<0.05 Respecta vs placebo. T0 = baseline; T1 = 1 week; T2 = 2 weeks; T3 = 1 month; T4 = 4 months; T5 = 6 months. T2 corresponds to the end of induction phase (2 weeks of treatment); T5 corresponds to the end of follow-up.**

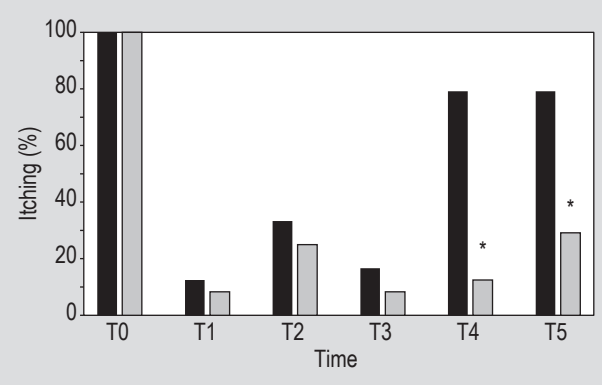


**Figure 2. Resolution of vaginal itching in women treated with Respecta® (grey) or placebo (black). *$P$<0.05 Respecta vs placebo. T0 = baseline; T1 = 1 week; T2 = 2 weeks; T3 = 1 month; T4 = 4 months; T5 = 6 months. T2 corresponds to the end of induction phase; T5 corresponds to the end of follow-up.**

### Microbiological cure rate (Nugent score)

The Nugent score was improved in the Respecta group compared to the placebo group with statistically significant difference between the two study arms during the entire study period ($P$<0.05, $P$<0.01 and $P$<0.001), except at T1 and T4. Results are shown in Table 1.

### Overall cure rate

Respecta intake during maintenance phase improved BV symptoms with reduction of Nugent score as shown by the overall cure rate (no symptoms and Nugent score <7) and a significant difference between the two study arms was present at each visit until the end of the follow-up. In particular, the overall cure rate of Respecta vs placebo was 83.33 vs 70.83% at T1, 66.67 vs 45.83% at T2, 79.17 vs 58.33% at T3, 75.00 vs 33.33% at T4 and, finally, 83.33 vs 37.50% at T5 (end of follow-up). Results are shown in Table 2.

### Recurrence rate

When we considered recurrent episodes of BV as presence of symptoms and Nugent score more than 3 during 6 months of follow-up, at the end of the study (T5), women who took Respecta experienced fewer recurrences (29.17%) than women treated with placebo (58.33%); such reduction was statistically significant ($P$<0.05). The interim analysis of recurrence rate at 1 month (25.00 vs 45.83%) and at 4 months (33.33 vs 87.50%) of follow-up showed that new episodes of BV were significantly reduced in the Respecta arm only. Results are shown in Table 3. The rate of women free of recurrence and treated with Respecta is different from the overall cure rate because we considered recurrent patient also a woman without symptoms but with intermediate Nugent score (dysbiosis).

## 4. Discussion

Qualitative studies showed that BV is associated with a substantial negative impact on self-esteem, sexual relationships, and quality of life. *G. vaginalis* is virtually always present at high concentrations in women who have BV. However, it is also detected frequently in healthy women and in some cases, the number of *G. vaginalis* can even reach $10^7$-$10^8$ cfu/ml also in absence of BV, so the most important maker of BV to look at is the ratio of logarithm concentration *Lactobacillus* spp. and *G. vaginalis*.

Nowadays, the 'gold standard' for BV diagnosis is considered the Nugent's scoring system, grading vaginal flora as normal with Nugent score (NS)=0-3, intermediate vaginal flora with NS=4-6 or BV with NS=7-10. Presently, metronidazole is considered to be the drug of choice for BV treatment (Centers for Disease Control and Prevention, 2015). Recommended first line treatment for BV includes also topical clindamycin cream. Despite the fact that high cure rates were achieved during some clinical investigations, very high BV recurrence rates and relevant treatment side effects have been also reported (Abdali *et al.*, 2015; Chavoustie *et al.*, 2015; Schwebke and Desmond, 2011). Antibiotic treatment (clindamycin or metronidazole) success rates of about 50% have been described for BV and recurrence rates can exceed 50% in the following 6-12 months after cessation of treatment. From these data, it remains uncertain whether recurrence is due to reinfection or relapse. Current treatment guidelines do not define BV as a sexually transmitted infection or recommend partner treatment. In fact, also with the treatment of sexual partner the recurrence rates remain stable as shown by a recent Cochrane systematic review (Amaya-Guio *et al.*, 2016).

**Table 1. Microbiological cure rate as Nugent scores in women treated with Respecta® or placebo.[1]**

| Time[2] | Respecta | | | | | | Placebo | | | | | | Respecta vs placebo |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ≤3 | | 4-6 | | ≥7 | | ≤3 | | 4-6 | | ≥7 | | *P*-value |
| | n | % | n | % | n | % | n | % | n | % | n | % | |
| T0 | 0/24 | 0 | 0/24 | 0 | 24/24 | 100 | 0/24 | 0 | 0/24 | 0 | 24/24 | 100 | – |
| T1 | 15/24 | 62.5 | 9/24 | 37.5 | 0/24 | 0 | 9/24 | 37.5 | 13/24 | 54.2 | 2/24 | 8.3 | *P*>0.05 |
| T2 | 13/24 | 54.2 | 11/24 | 45.8 | 0/24 | 0 | 5/24 | 20.8 | 18/24 | 75.0 | 1/24 | 4.2 | *P*<0.05 |
| T3 | 15/24 | 62.5 | 9/24 | 37.5 | 0/24 | 0 | 5/24 | 20.8 | 18/24 | 75.0 | 1/24 | 4.2 | *P*<0.01 |
| T4 | 9/24 | 37.5 | 15/24 | 62.5 | 0/24 | 0 | 6/24 | 25.0 | 18/24 | 75.0 | 0/24 | 0 | *P*>0.05 |
| T5 | 20/24 | 83.3 | 4/24 | 16.7 | 0/24 | 0 | 5/24 | 20.8 | 19/24 | 79.2 | 0/24 | 0 | *P*<0.001 |

[1] Results are shown as ratio between the number of women with Nugent score and total women. Results were evaluated by Chi-square test; the significance was set for *P*<0.05 (Respecta vs placebo).
[2] T0 = baseline; T1 = 1 week; T2 = 2 weeks; T3 = 1 month; T4 = 4 months; T5 = 6 months. T2 corresponds to the end of induction phase; T5 corresponds to the end of follow-up.

**Table 2. Overall cure rate showing the number of women without any symptoms of bacterial vaginosis (BV).**

| Time[1] | Respecta® | | Placebo | | Respecta vs placebo |
|---|---|---|---|---|---|
| | Women (n) | Rate (%) | Women (n) | Rate (%) | *P*-value |
| T0 | 0/24 | 0 | 0/24 | 0 | – |
| T1 | 20/24 | 83.33 | 17/24 | 70.83 | *P*<0.01 |
| T2 | 16/24 | 66.67 | 11/24 | 45.83 | *P*<0.01 |
| T3 | 19/24 | 79.17 | 14/24 | 58.33 | *P*<0.01 |
| T4 | 18/24 | 75.00 | 8/24 | 33.33 | *P*<0.01 |
| T5 | 20/24 | 83.33 | 9/24 | 37.50 | *P*<0.01 |

[1] T0 = baseline; T1 = 1 week; T2 = 2 weeks; T3 = 1 month; T4 = 4 months; T5 = 6 months. T2 corresponds to the end of induction phase; T5 corresponds to the end of follow-up.

**Table 3. Recurrence rate shown as the number of women who experienced at least one recurrence of bacterial vaginosis (BV) during the follow-up (6 months).**

| Time[1] | Respecta® | | Placebo | | Respecta vs placebo |
|---|---|---|---|---|---|
| | Women (n) | Rate (%) | Women (n) | Rate (%) | *P*-value |
| T3 | 6/24 | 25.00 | 11/24 | 45.83 | *P*<0.05 |
| T4 | 8/24 | 33.33 | 21/24 | 87.50 | *P*<0.05 |
| T5 | 7/24 | 29.17 | 14/24 | 58.33 | *P*<0.05 |

[1] T3 = 1 month; T4 = 4 months; T5 = 6 months.

Most proposed non-antibiotic therapies for recurrent BV are vaginal or oral probiotics, which aim to restore the normal vaginal microbiota. Several trials evaluated the use of combined metronidazole and/or clindamycin therapy and vaginal probiotics to prevent BV recurrence. In fact, the poor efficacy of antibiotics to eradicate BV biofilms and restore a healthy lactobacilli population, had led to an increased interest in alternative and complementary approaches (Bohbot *al.*, 2018; Laue *et al.*, 2018).

Probiotics and freshly isolated lactobacilli both induced disruptive changes to the *Gardnerella* biofilms, raising the possibility that indigenous lactobacilli may perform this restorative function in women who maintain a normal vaginal microbiota over the duration of their pregnancy, or menstrual cycle. To date, meta-analyses on probiotics to treat and/or prevent BV show promising results, but more clinical studies are still necessary (Grin *et al.*, 2013; Senok *et al.*, 2009).

Lactoferrin might represent an alternative and effective remedy for preventing the BV since it is able to restore healthy vaginal microbiota mainly dominated by lactobacilli. In addition, Bertuccini *et al.* (2018) showed that bovine lactoferrin added to *L. acidophilus* GLA-14 and *L. rhamnosus* HN001, alone and in combination, was able to enhance the biofilm formed by those strains on both abiotic surface and HeLa cell monolayer. This is a very promising result providing the rationale for the lactoferrin and lactobacilli symbiotic mixture for vaginal health.

Jang *et al.* (2017) found that orally administered *L. acidophilus* GLA-14 and *L. rhamnosus* HN001 in association with bovine lactoferrin (Respecta complex), were able to reach and colonise the vagina in mice and attenuate BV experimentally induced in treated animals. In particular, it has been shown that this treatment was more effective against *G. vaginalis*-induced BV than intravaginal administration in mice. A previous clinical study showed that oral administration of Respecta led to a significant increase in vaginal colonisation by *L. acidophilus* GLA-14 and *L. rhamnsosus* HN001 in healthy women after two weeks of treatment; on the other hand, the placebo did not induce any shift in lactobacilli in the vagina of treated women. Interestingly, the levels of both strains increased one week after the end of oral mixture intake, suggesting a prolonged persistence of lactobacilli in the vagina (De Alberti *et al.*, 2015).

Our recent data (Russo *et al.*, 2018) showed that Respecta use for 15 consecutive days lead to significant vaginal colonisation by *L. acidophilus* GLA-14 and *L. rhamnosus* HN001 with the restoration of normal Nugent score (values 0-3) in women with dysbiosis or intermediate Nugent score (values 4-6).

Significant body of data supports the pathogenicity of *G. vaginalis* and the hypothesis that vaginal *Gardnerella* spp. biofilm could determine the failure of conventional antibiotic treatments and the high recurrence rate, strongly suggesting that other bacteria in the biofilm are opportunistic secondary invaders. Last but not least, bacteria in biofilms respond differently to antibiotic treatment when compared with their planktonic counterparts, and antibiotic resistance is postulated as one of the reasons for persistent and recurrent BV. Saunders *et al.* (2007) showed that in situations where BV biofilms occur, strains of *Lactobacillus* could have the ability to disrupt and dislodge these biofilms, and potentially reduce the need for antibiotics.

Vaginal delivery of the probiotic strains via oral route seems to be well documented in the light of the papers currently published in the literature (Cianci *et al.,* 2008; De Alberti *et al.,* 2015; Reid *et al.,* 2003; Russo *et al.,* 2018). The results from the current study highlighted the usefulness of lactobacilli (*L. acidophilus* GLA-14 and *L. rhamnosus* HN001) in association with bovine lactoferrin to reduce the risk of recurrences in women with recurrent BV. To this end, each month women took one capsule per day for 10 consecutive days for 6 months (follow-up). The decision to start prophylactic cycles at the first day of the menstrual phase was made not only to facilitate the protocol scheme and the compliance of patients but also after considering that the menstrual blood and the increasing pH in the early days of period constitute a sort of 'red flag' for patients at high risk of RBV. Several studies have explored the supplementation of different strains and/or combinations of lactobacilli (such as *Lactobacillus gasseri, L. rhamnosus, L. acidophilus*) in the prevention of recurrent BV. While Vujic *et al.* (2013) found that 6 weeks of probiotic treatment versus placebo may aid in restoration of normal vaginal flora; other studies support an adjuvant role for probiotics without replacement of the standard metronidazole therapy (Larsson *et al.*, 2008; Martinez *et al.*, 2009). A recent study showed that 17 women who consumed yogurt containing several lactobacilli strains (i.e. *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus crispatus* LbV 88, *L. gasseri* LbV 150N, *Lactobacillus jensenii* LbV 116 and *L. rhamnosus* LbV96) for 4 weeks after the treatment with metronidazole (500 mg twice daily for 7 days) were all BV-free based on Amsel criteria whereas only 11 out of 17 were BV-free in the placebo group. In addition, lactobacilli supplementation led to a significant improvement of both symptoms (i.e. discharge and odour) and Nugent score during the intervention period (Laue *et al.,* 2018). Anukam *et al.* (2006) after a 7-day treatment course with metronidazole administered a probiotic oral formulation containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 versus placebo for 30 days, and found an initial cure rate of 88 vs 40% ($P<0.001$) with a significant recovery of lactobacilli in vaginal swabs from the study group post treatment. Nevertheless, data about probiotics are so sparse as to prevent recommending them in women with RBV.

Our results are comparable with those obtained with prolonged courses of antibiotics but with much fewer environmental risks or the risk of developing resistance. The improvements obtained by using probiotics, must be considered alongside the known red flags or risk factors for BV such as diet, douching, type of contraception, menstrual cycle, etc. Furthermore, in order to sustain and maximise long-term benefits, RBV should be treated as a chronic condition requiring both acute and long-term treatments. The uncertain aetiology of BV, high rates of recurrence, sub-optimal treatment options, often insensitive and inconsistent clinical advice and distressing symptoms remain a major challenge for modern obstetrics and gynaecology. For all these reasons, it is not surprising that women are desperately trying all manner of self-help remedies in an attempt to prevent further BV recurrences, and doctors desperately looking for new therapeutic strategies to address it. Although many providers may think of vulvovaginal infections as fairly straightforward and easy to treat, treatment failures and recurrent infections occur commonly. Patients facing this type of infections are frequently offered repeated courses of the same ineffective regimens. However, for most women with chronic or recurrent vaginal infections, there are approaches to evaluation and treatment, which can yield more satisfactory outcomes. In this light, the use of probiotics may represent a valid therapeutic strategy.

## 5. Conclusions

The results of the current study support that repeated courses of orally administered probiotics in combination with lactoferrin (Respecta) are effective in the prevention of BV recurrences as showed by significant higher clinical and microbiological cure rates in the investigational group and a good safety profile as well. Moreover, a significant regression of Nugent score and resolution of symptoms such as vaginal discharge and itching were seen both during the induction phase (oral metronidazole for 7 days plus Respecta for 15 days) and the maintenance phase (10 consecutive days starting at first day of cycle for 6 months) in women with RBV after oral intake of selected probiotics and lactoferrin. Despite some limitations of our study, the investigated lactobacilli mixture in combination with bovine lactoferrin is a promising option as coadjuvant to antibiotics for the treatment of BV and maintenance cycles in women with RBV. Anyway, additional high-quality, larger RCT trials are necessary to reinforce the current available evidence.

## Acknowledgements

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## Conflict of interest

Rosario Russo is employed by Giellepi; he had no influence on the interpretation of results. Giellepi is the manufacturer of Respecta. The other Authors have no conflict of interest. Giellepi funded the clinical trial.

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# Study on the Effects of an Oral Lactobacilli and Lactoferrin Complex in Women with Intermediate Vaginal Microbiota - Russo et al. 2017

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GENERAL GYNECOLOGY

# Study on the effects of an oral lactobacilli and lactoferrin complex in women with intermediate vaginal microbiota

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## Abstract

**Purpose** In the vagina of healthy reproductive-aged women, several microbial species maintain a finely tuned mutualistic relationship with the host providing the first-line of defense against the colonization by opportunistic pathogens, which are the leading cause of dysbiosis or vaginal infections (bacterial vaginosis, vulvovaginal candidiasis, and aerobic vaginitis). The use of probiotic lactobacilli to prevent vaginal infections has a good rationale, and an excellent safety record, but so far only a few strains have been clinically proven to be effective, particularly to prevent BV. The aim of the clinical trial was to evaluate the changes in Nugent score in women with intermediate vaginal microbiota treated with oral *Lactobacillus acidophilus* GLA-14 and *Lactobacillus rhamnosus* HN001 mixture, in combination with bovine lactoferrin RCX™ (Respecta®) or placebo, for 15 days.

**Methods** Vaginal swabs were collected from each woman at baseline and at the end of probiotic treatment and analyzed by RT-PCR. Both symptoms of abnormal vaginal micorbiota and adverse effects were assessed throughout the study.

**Results** The results showed that oral intake of lactobacilli/lactoferrin mixture led to significant vaginal colonization by *L. acidophilus* GLA-14 and *L. rhamnosus* HN001 showing that both strains can colonize vagina following oral ingestion. The effect of such colonization is correlated with the restoration of normal Nugent score (values 0–3) and an improvement of symptoms of abnormal vaginal micorbiota including itching and discharge.

**Conclusions** Oral consumption of lactobacilli/lactoferrin complex corroborates the effectiveness of using lactobacilli for supporting vaginal health and provides a rational basis for future studies on vaginal infections.

**Keywords** *Lactobacillus acidophilus* · *Lactobacillus rhamnosus* · Lactoferrin · Respecta® · Nugent score

## Introduction

Vaginal genital tract is populated by several microbial species, mainly lactobacilli spp., living in equilibrium as a balanced microbiota responsible for the healthy status of vagina and the maintenance of acidic pH (pH < 4.5) [1, 2]. Due to their capability to produce lactic acid and other soluble molecules such as $H_2O_2$ and bacteriocins, lactobacilli provide also a defense against opportunistic and pathogenic microorganisms, such as *Gardnerella vaginalis* and *Atopobium vaginae*. When number of vaginal lactobacilli is reduced (usually below $10^6$ CFU/ml), the risk of vaginal infections increases significantly. Bacterial vaginosis (BV) is the most common cause of vaginal infection among childbearing women and its prevalence ranges between 4.9 and 36.0% according to European and American studies [3, 4]. BV is caused by bacteria (mainly anaerobic ones) able to adhere to vaginal epithelium, forming biofilm and producing enzymes such as cytolisin (vaginolisin) and sialidase [5]. *G. vaginalis* is virtually always present at high concentrations in women who have BV but it is also detected frequently in healthy women, so the ratio of logarithm concentration between *Lactobacillus* spp. and *G. vaginalis* should be considered as a reliable marker [6]. The most reliable methods to diagnose BV are Amsel criteria and Nugent score [7, 8]. In particular, Nugent score is a scoring system assessing the Gram stained preparations from vaginal smear by wet mount

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microscopy; it ranges from 0 to 10. Vaginal micorbiota is considered as normal if Nugent score (NS) is between 0 and 3; intermediate vaginal micorbiotia (IF) with NS between 4 and 6 and, finally, BV if NS is between 7 and 10. It is important to note that abnormal vaginal micorbiota or dysbiosis (Nugent score 4–6) shows almost the same characteristics of BV [9] in term of association with upper genital tract infections, sexually transmitted infections (STI) and unfavorable pregnancy outcomes [10, 11]. This condition is found in approximately 20% of clinically healthy women and could be considered a transitional step between normal micorbiota and bacterial vaginosis, which is related to several gynecologic or obstetric outcomes. Therefore, the early detection of vaginal disturbance and the proper management of the intermediate microbiota represents a pivotal matter especially for pregnant women [12].

The current study was performed to assess the degree and persistence of the vaginal colonization by probiotic strains from an orally administered mixture of lactobacilli containing *Lactobacillus acidophilus* GLA-14 and *Lactobacillus rhamnosus* HN001, in combination with bovine lactoferrin RCX™ (Respecta®). Its effect on parameters of vaginal health (e.g. total lactobacilli counts, vaginal pH and Nugent score) and subjective symptoms such as itching and vaginal discharge were also monitored.

## Patients and methods

The study was performed from October 2016 to January 2017 as a double blind, randomized, placebo controlled clinical trial with two parallel groups receiving either verum or placebo (Fig. 1).

Study product (verum) was a lactobacilli mixture (Respecta® class IIa medical device in Europe, studied and developed by Giellepi S.p.A. Health Science, Lissone, MB, Italy), formulated in capsules including $5 \times 10^9$ CFU

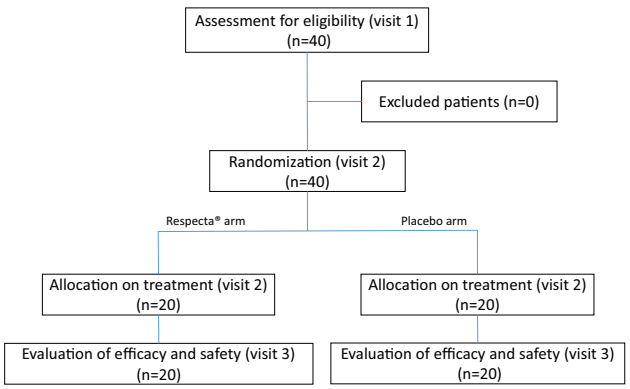


**Fig. 1** CONSORT flow diagram of the patients participating in the study

probiotic blend (*Lactobacillus acidophilus* GLA-14, LMG S-29159 and *Lactobacillus rhamnosus* HN001, AGAL NM07/09514), in combination with bovine lactoferrin RCX™ (50 mg). Placebo consisted of an identical capsule containing maltodextrin (100 mg); the excipients were the same in both verum and placebo.

The study was evaluated and approved by the National Committee of Bioethics for Medicines and Medical Devices, Bucharest (no. 3DM/16.02.2016). Clinical procedures have been completed in accordance with Good Clinical Practices and the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments regarding the ethical principles for medical research involving human subjects adopted during the 64th WMA General Assembly at Fortaleza in Brazil on October 2013.

Forty (40) women of reproductive age (18–50 years old) were enrolled with signs or symptoms of vaginitis/vaginosis. The symptoms (itching; vaginal discharge; burning or dryness; fishy odor) were reported from the patients during gynecologic visits using self-completion questionnaire for comprehensive assessment of the severity according to the following four level scale: absent; mild; moderate and severe.

Written informed consent was obtained from all individual participants included in the study. Patients were included if they: (1) need supplementation and/or restoration of the vaginal microbiota (Nugent score 4–6); (2) want to cooperate with investigators during the study and comply with the study procedures; and (3) abstain from sexual relations and vaginal douche during the study. The exclusion criteria were: (1) age < 18 and > 50 years old; (2) Nugent score different from 4 to 6 or other vaginal infections as candidiasis, BV or trichomoniasis; (3) hypersensitivity to any ingredient in the study product; (4) pregnancy, breastfeeding or menopause state; (5) the use of intrauterine device; (6) participation to other clinical trial which could influence urogenital tract micorbiota; (7) the use of any other oral or vaginal probiotics; (8) antibiotic therapy; (9) hormonal therapy.

The full study cycle consisted of three visits at gynecology centers. The first visit was devoted to initial screening to ascertain the inclusion/exclusion criteria; vaginal swabs were collected to define Nugent score and vaginal culture was included to exclude yeast, bacterial or protozoal vaginitis. After informed consent during second visit, all women with intermediate Nugent score were randomized into two groups (20 women per group) according to treatments (verum or placebo). For treatment allocation, a random number table list was created using SPSS program (version 20). The randomization codes were generated as a serial number of four digits indicating the treatment patient should receive (A or B). The randomization scheme ratio was of 1:1; each time a patient met the eligibility criteria, a randomization code was requested from the headquarter by the investigator

based on randomization codes distributed at that site. The randomization list was kept in a secure place with limited access and unknown to the clinical investigators and laboratory researchers. The randomization list was opened at the end of the study for study results presentation.

During the third visit (end of the treatment), data regarding efficacy and safety of the intervention were collected. Nugent score at the end of treatment in comparison to baseline values was evaluated and Real-Time PCR (RT-PCR) was carried out for detecting *L. acidophilus* and *L. rhamnosus* from vaginal swabs to assess whether the strains transferred from the gut to the vaginal site. The microbiologists able to read Gram staining were blinded.

All enrolled women ingested one capsule of verum/placebo per day for 15 days. Patients were not allowed to take any medicine, intravaginal products (e.g. spermicides, lubricants, etc.), or food that could interact with the study treatments during the entire study period. Participants utilizing/consuming the above-mentioned products were excluded from the evaluation and statistical analysis; women were only allowed to use a neutral detergent for intimate hygiene.

Nugent score is a Gram stain scoring system useful as screening method to differentiate normal vaginal micorobiota from bacterial vaginosis. Vaginal specimens were collected according to Good Clinical Practice from each woman by careful rotation of sterile cotton swab against sidewalls in the upper third area of the vagina. Each vaginal swab was used for doing a smear on a microscope slide and stained for Nugent score estimation. The Nugent score was calculated from the microscopic evaluation according to Nugent et al. [8]. Regarding the criteria for interpreting the results, Nugent score ranging from 0 to 3 indicates normal vaginal micorbiota; Nugent score from 4 to 6 indicates intermediate vaginal microbiota or dysbiosis; Nugent score from 7 to 10 indicates bacterial vaginosis. Collectively, Nugent score greater than three have been referred to as "abnormal" vaginal micorbiota.

The pH of secretions collected from the lateral vaginal wall was measured using paper strips, whose color indicator ranged from 3.5 to 5.2.

A second swab was collected for assessing lactobacilli colonization by RT-PCR analysis. Total DNA was extracted from vaginal swabs using the genomic DNA extraction PureLink Mini Kit (Thermo Fisher Scientific) and DNA Cleanup Micro kit according to the manufacturer protocols. Extracts purity was determined using a biophotometer (Eppendorf). In the following stage, the concentrations of total DNA were calculated in all samples and a quantitative PCR was performed using the highest concentration of DNA common to all samples (100 ng). In all PCR reactions, positive controls (DNA extracted from lactobacilli cultures) and negative controls were used for internal control amplification. RT-PCR results were reported as genome containing particles (GCP)/100 ng total DNA (number of copies of DNA lactobacilli in 100 ng of total DNA).

DNA amount of the two studied microorganisms was assessed by RT-PCR according to methods described by De Alberti et al. [13]. Two sets of primers were used; the first set of primers was LacidoF (50-TGCAAAGTGGTAGCG TAAGC-30) and LacidoR (50-CCTTTCCCTCACGGT ACTG-30), which was designed to amplify a 200 bp on the region between 16S rDNA and 32S rDNA of *L. acidophilus*. The second set of primers was LrhamF (50-TGCTTG CATCTTGATTTAATTTTG-30) and LrhamR (50-GGTTCT TGGATYTATGCGGTATTAG-30), which was designed to amplify a 120 bp on the region of the 16S rDNA of *L. rhamnosus*.

DNA amplification conditions were as follows: 3 min initial denaturation at 95 °C, 35 denaturation cycles at 95 °C for 30 s, 35 denaturation-annealing and annealing-elongation cycles at 60 °C for 30 s. A Quant Studio 3 Real Time-PCR System was used for RT-PCR analyses. RT-PCR was performed with TaqMan Multiplex Master Mix (Applied Biosystems, Thermo Fisher Scientific). After TaqMan amplification, a dissociation curve analysis was performed to confirm the absence of nonspecific amplification products.

Adverse events were classified as light, moderate or severe (an adverse event was considered severe if it was fatal, life threatening, requiring hospitalization, leading to permanent handicaps or congenital abnormalities).

Causality of any adverse events was considered as: correlated if the investigator had sufficient information and considered the symptom to be correlated to the study treatment. Uncorrelated if the investigator had sufficient information and considered the symptom not to be correlated to the study treatment. Unable to determine correlation if the investigator had insufficient information or was not able to determine the correlation or non-correlation of the symptom to the study treatment.

For normality testing, Shapiro–Wilk test was performed. Descriptive statistics was used in this study to present quantitative descriptions of different variables. Unpaired Student's *t* test was used for assessing differences between treatment groups. Chi-Square test was used for the analysis to tests the association between tested variables. The null hypothesis to be tested was no difference between the group treated with the lactobacilli/lactoferrin mixture compared to placebo group.

## Results

Demographic characteristics of the recruited women are presented in Table 1. No difference was observed between the two treated groups (Respecta® and placebo). Compliance was 100% for all participants in both study groups during

**Table 1** Demographic characteristics of participating women

| | Respecta® | Placebo |
|---|---|---|
| Sex (female, %) | 100 | 100 |
| Age (years ± SD) | 34.30 ± 8.58 | 34.75 ± 8.02 |
| Weight (kg ± SD) | 66.05 ± 9.04 | 64.70 ± 8.83 |
| Height (cm ± SD) | 170 ± 7.23 | 169 ± 8.53 |
| BMI (kg/m$^2$) | 22.7 ± 1.7 | 22.4 ± 1.2 |
| Method of contraception (%) | | |
| Oral contraception | 20 | 25 |
| Condom | 80 | 75 |

No difference was shown between the two treated groups (Respecta® and placebo)

**Table 2** Nugent score evolution from baseline to the end of treatment

| | Nugent score | | *P* value (Respecta® vs Placebo) |
|---|---|---|---|
| | Respecta® | Placebo | |
| Baseline | 5.09 ± 0.82 | 5.10 ± 0.72 | Not significant |
| End of treatment | 2.90 ± 0.34 | 5.14 ± 0.94 | 0.0110 |
| *P* value (baseline vs end of treatment) | 0.0004 | Not significant | |

Values are expressed as mean ± SD (standard deviation). The significance level was set at $P < 0.05$

the treatment. During clinical examinations, symptoms such as itching, vaginal discharge, fishy odor, burning and dryness were monitored in both groups. As expected, no woman showed burning sensation or dryness. Women taking lactobacilli/lactoferrin mixture experienced a general improvement of symptoms; in particular, itching and vaginal discharge decreased significantly at the end of treatment with Respecta® compared to baseline and placebo group ($P < 0.001$). Results are shown in Fig. 2.

Nugent score results are shown in Table 2. The intragroup analysis for the evaluation of Nugent score evolution from baseline to the end of the study showed that Nugent score was significantly improved (from intermediate to normal score) in the group treated with lactobacilli/lactoferrin mixture after 15 days treatment ($P = 0.0004$). On the other hand, the administration of placebo did not induce any modifications in Nugent score. Intergroup comparison at the end of treatment revealed that Nugent score was significantly improved after lactobacilli/lactoferrin mixture oral administration compared to placebo ($P = 0.0110$).

The mean vaginal pH varied between 4.42 and 4.09 in verum group and between 4.03 and 4.47 in the placebo group (Table 3). No statistical difference was observed neither between baseline and the end of the study in each arm, nor between the two arms.

RT-PCR was performed to assess vaginal colonization by both probiotic strains (*L. acidophilus* GLA-14 and *L. rhamnosus* HN001) after lactobacilli/lactoferrin mixture oral intake. The results are showed in Figs. 3 and 4 for *L. acidophilus* GLA-14 and *L. rhamnosus* HN001, respectively. Both strains increased significantly in the vagina of women in the active arm at the end of the study in comparison to baseline. On the contrary, vaginal colonization did not change significantly in the placebo group anytime. Intergroup comparison at the end of treatment revealed that lactobacilli vaginal colonization was significantly improved after lactobacilli/lactoferrin mixture oral administration compared to placebo ($P < 0.001$).

During the study period no adverse event occurred, neither correlated nor unrelated. Both products (verum and placebo) were well tolerated by all subjects participating in the study.

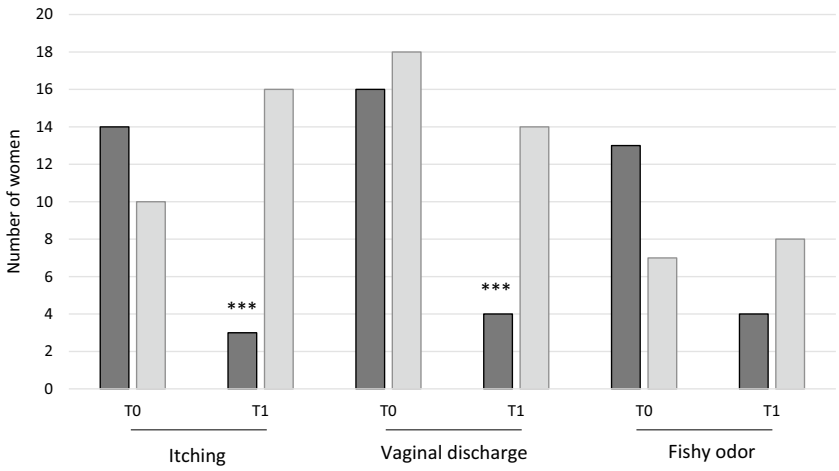


**Fig. 2** Vaginal symptoms (itching, vaginal discharge, fishy odor) evaluated at both baseline and the end of treatment in each group. In the graph, the number of women with at least one vaginal symptom is shown. ***$P < 0.001$ Respecta® vs placebo. Dark grey: Respecta®; Light grey: Placebo

**Table 3** Vaginal pH

| | pH | | *P* value (Respecta® vs Placebo) |
|---|---|---|---|
| | Respecta® | Placebo | |
| Baseline | 4.42 ± 0.70 | 4.03 ± 0.42 | Not significant |
| End of treatment | 4.09 ± 0.33 | 4.47 ± 1.03 | Not significant |
| *P* value (baseline vs end of treatment) | Not significant | Not significant | |

Values are expressed ad mean ± SD. The significance level was set at $P < 0.05$

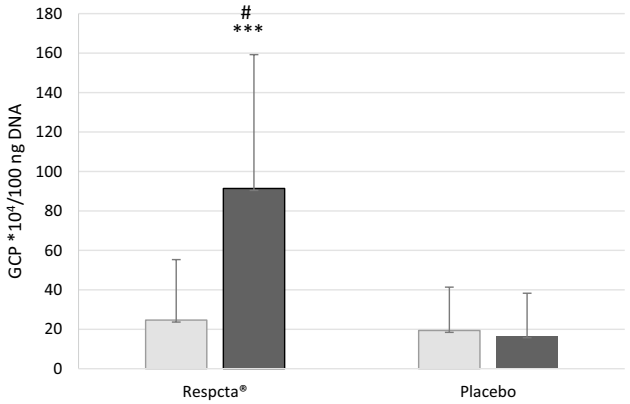


**Fig. 3** *Lactobacillus acidophilus* GLA-14 vaginal colonization. Values are expressed as mean ± SD. *GCP* genome containing particles. $^{\#}P < 0.01$ End of treatment vs baseline in the Respecta® group; $^{***}P < 0.001$ Respecta® vs placebo. Light grey: baseline; Dark grey: end of treatment

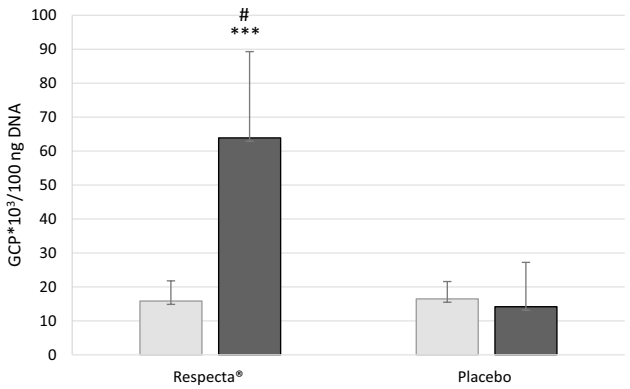


**Fig. 4** *Lactobacillus rhamnosus* HN001 vaginal colonization. Values are expressed as mean ± SD. *GCP* genome containing particles. $^{\#}P < 0.01$ End of treatment vs baseline in the Respecta® group; $^{***}P < 0.001$ Respecta® vs placebo. Light grey: baseline; Dark grey: end of treatment

## Discussion

The manipulation of vaginal microbiota by supplementation of probiotics represents a strong rationale for restoring the homeostasis of unbalanced vaginal micorbiota, which causes dysbiosis and vaginal infections [14, 15]. Nevertheless, not all probiotic strains are suitable for vaginal indications. To be effective, a strain must reach and colonize the human vagina. Substantial of experimental evidence exists about vaginal colonization by lactobacilli, also following oral intake [13, 16–18]. Unfortunately, also pathogenic bacteria (e.g. *Streptococcus agalactiae*, *Escherichia coli*, *Gardnerella vaginalis*) can move from the gastrointestinal tract to the lower uro-genital tract [19] thus causing diseases such as urinary tract infections (UTI), bacterial vaginosis (BV), aerobic vaginitis (AV) and vulvovaginal candidiasis (VVC).

Some studies have suggested that even a transient colonization through a short probiotic intervention may be important and sufficient to correct a non-balanced microbiota and this may be relevant in case of intermediate microbiota/dysbiosis [18] and BV during pregnancy [20].

Oral supplementation with probiotics, which may colonize the vagina, can be the first step in the prevention of further development of vaginal dysbiosis [21]. Jang et al. [22] found that orally administered *L. acidophilus* GLA-14 and *L. rhamnosus* HN001, in association with bovine lactoferrin, were able to reach and colonize the vagina in mice and attenuate bacterial vaginosis experimentally induced in treated animals. In particular, it has been shown that oral administration of the lactobacilli mixture was more effective against *G. vaginalis*-induced BV than intravaginal administration in mice. A previous clinical study showed that oral administration of lactobacilli mixture with bovine lactoferrin (Respecta®) led to a significant increase in vaginal colonization by *L. acidophilus* GLA-14 and *L. rhamnosus* HN001 in healthy women after 1 week of treatment; on the contrary, the placebo did not increased lactobacilli in the vagina of treated women. Interestingly, the levels of both strains increased 1 week after the end of oral probiotic intake, suggesting a prolonged persistence of lactobacilli in the vagina [13]. In addition, Strus and co-workers [21] showed also a significant vaginal colonization after oral administration of a lactobacilli mixture containing *L. fermentum* 57A, *L. plantarum* 57B, and *L. gasseri* 57C.

Following vaginal colonization, lactobacilli can counteract the proliferation of opportunistic or pathogenic microorganisms by different ways. Lactobacilli compete with other bacteria for both nutrients and adhesion sites onto the vaginal epithelium. In addition, they exert antimicrobial activities by producing lactic acid, which decreases

vaginal pH and produce soluble molecules including $H_2O_2$ and bacteriocins able to cause either microbicidal effects by disrupting bacteria membrane or inhibiting the activity of enzymes (i.e. sialidases and prolidases) needed for bacterial virulence [23, 24]. These characteristics must be considered as key parameters to select strains for probiotic vaginal therapy.

A recent in vitro study showed that *L. acidophilus* GLA-14 and *L. rhamnosus* HN001, exert antimicrobial effects against some pathogenic bacteria responsible for lower genital tract infections by inhibiting the proliferation of *Gardnerella vaginalis*, *Atopobium vaginae*, *Staphylococcus aureus* and *Escherichia coli* [25]. The hypothesized mechanism of action by which lactobacilli exert such an effect against pathogenic bacteria could be linked to the production of soluble antimicrobial compounds such as bacteriocins and lactic acid. Most likely, an additional mechanism by which lactobacilli reduce pathogen colonization is by competition and displacement on the vaginal tissue. Jang et al. [22] showed that *L. acidophilus* GLA-14 and *L. rhamnosus* HN001 significantly inhibited the adherence of *G. vaginalis* to HeLa cells (a human cervical cancer cell line) and pathogen growth in vitro. In addition, the authors demonstrated that either oral or intravaginal administration of a selected lactobacilli mixture, in combination with bovine lactoferrin, significantly inhibited *G. vaginalis*-induced epithelial cell disruption.

Abnormal vaginal microbiota (dysbiosis or intermediate Nugent score) appears less resilient to disturbance/symptom and more susceptible to disease. Ideally, 'intermediate microbiota' condition represents a turning point from a healthy status into BV, or conversely from BV to normal condition. Interestingly, women having a disturbed microbiota (intermediate Nugent score 4–6) are in most cases not treated with any antibiotic regimen because both the lack of symptoms and clear guideline in regards of the management of borderline situation. Considering that this category represents a 'pivot point' and can eventually lead to a serious range of complications including mid-trimester pregnancy loss, the 'classic' full-blown BV, a strategic probiotic supplementation may represent a safe method to re-establish a healthy vaginal environment.

The results from the current study indicate that the oral intake of selected probiotics for a short time (i.e. 15 days) increase significantly the vaginal levels of both lactobacilli species, *L. acidophilus* GLA-14 and *L. rhamnosus* HN001 and confirm the results from the study of De Alberti et al. [13]. This effect determines a significant regression of Nugent score from intermediate towards a normal value (0–3) and a resolution of symptoms such as itching and vaginal discharge reported in women with symptomatic vaginal dysbiosis.

No significant reduction on vaginal pH was observed after neither verum nor placebo treatment. Nevertheless, lactobacilli/lactoferrin mixture oral intake induced a positive trend towards lower pH values. This is not surprising considering the baseline value of pH were found < 4.5 in both groups (i.e. 4.42 and 4.03 in verum arm and placebo arm, respectively) and the short period of probiotic administration.

The results of this study suggest that oral supplementation with probiotics proven to colonize vagina can be the first step in the treatment of dysbiosis or intermediate Nugent score. The resolution of all these forms of abnormal microbiota will be a major challenge for future treatment studies. The new insights in the differential diagnosis of all types of abnormal vaginal microbiota have created renewed interest in screening and treating pregnant and non-pregnant women to prevent acquisition of STI or pregnancy complications as preterm labour [26].

Our results may suggest a potential risk reduction for women with elevated Nugent score. Furthermore, the lack of evidence-based clinical guidelines for the management of these subtypes of abnormal vaginal microbiota, such as intermediate Nugent score, needs to be addressed urgently.

The results found in this double-blind, placebo-controlled randomized study suggest that the tested lactobacilli mixture in combination with bovine lactoferrin may be a new candidate for vaginal dysbiosis and further studies are needed to analyze similar protocols in women with vaginal infections such as bacterial vaginosis or aerobic vaginitis.

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**Author contributions** RR: Project development; data analysis; manuscript writing. AE: Data management; clinical operations. FDS: Project development; data analysis; manuscript writing.

## Compliance with ethical standards

**Conflict of interest** RR is employed by Giellepi. He had no influence on the interpretation of results. AE and FDS have no conflict of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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# Lactobacilli Vaginal Colonisation after Oral Consumption of Respecta Complex: A Randomised Controlled Pilot Study - Alberti et al. 2015

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GENERAL GYNECOLOGY

# Lactobacilli vaginal colonisation after oral consumption of Respecta® complex: a randomised controlled pilot study

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**Abstract**
*Purpose* The aim of the current pilot study was to determine if oral consumption of a combination of two probiotics; *L. acidophilus* La-14, *L. rhamnosus* HN001, and bovine lactoferrin (Respecta® complex), would lead to the detection with molecular techniques of the consumed probiotic strains in the vagina.
*Methods* Healthy volunteers (40) consumed the study product twice daily for 2 weeks. Vaginal swabs were collected at 0, 1, 2 and 3 weeks and analysed for the consumed organisms by qPCR.
*Results* Vaginal *L. rhamnosus* and *L. acidophilus* levels were significantly increased on days 14 and 21. On days 14 and 21 a significant number of women had increased levels of vaginal *L. acidophilus* and on days 7 and 21 a significant number of women had increased levels of vaginal *L. rhamnosus*.
*Conclusions* Consumption of *L. acidophilus* La-14, *L. rhamnosus* HN001 in combination with bovine lactoferrin leads to vaginal detection; even 1 week after consumption was stopped. This provides a basis for future studies on urogenital tract health.

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**Keywords** Probiotics · Lactoferrin · Vaginal colonisation · Molecular techniques · *Lactobacillus*

## Introduction

The healthy human vagina is characterised by its colonisation with members of the genus *Lactobacillus*. The predominance of lactobacilli in the healthy vaginal microbiota forms also the basis of the so-called Nugent score [1]. The most commonly detected lactobacilli in the healthy vagina are *Lactobacillus crispatus*, *Lactobacillus jensenii* and *Lactobacillus gasseri*, and to a lesser extent *Lactobacillus inners*. As a reduced proportion of lactobacilli in the vaginal microbiota is associated with disease or increased disease risk; attempts have therefore been made to increase the levels of vaginal lactobacilli through the use of probiotics.

Probiotics have been tested for their effectiveness in preventing and treating bacterial vaginosis (BV). In particular *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* GR-1, and *Lactobacillus fermentum* RC-14 have been reported to be effective [2]; both orally and vaginally administered. For vaginal applications, probiotics are considered to provide antimicrobial activity, by reducing vaginal pH, producing $H_2O_2$ and potentially bacteriocins. Furthermore, vaginal probiotics are expected to adhere to vaginal epithelial cells and thereby exclude potential pathogens [3]. In order to do so, probiotics have to reach the vagina. Most studies documenting vaginal colonisation have used vaginal application of the probiotics [3] and have been documented to beneficially maintain the vaginal microbiota [4]. However, it is also considered possible for orally consumed probiotics to colonise the vagina from the rectum. This has, however, not been studied in any greater

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detail; to date only few studies have confirmed, after culturing and identification with molecular techniques, that oral consumption of lactobacilli leads to vaginal colonisation [5, 6]. Certainty on the ability of the consumed probiotics to reach the vagina can be expected to provide a basis for future clinical studies on e.g. risk reduction of BV with orally instead of vaginally administered probiotics [7] or as adjunct therapy in combination with antibiotics or antimycotics in the treatment of BV [8].

The aim of the current pilot study was therefore to determine if oral consumption of Respecta® complex, a combination of probiotics and lactoferrin, would lead to the detection with molecular techniques of the consumed probiotic strains in the vagina. To this end, Respecta® complex, which includes the combination of *L. acidophilus* La-14, *L. rhamnosus* HN001; and bovine lactoferrin RCX™, was consumed by healthy women and vaginal swabs taken at four time points.

## Volunteers and methods

The study was evaluated and approved by the Independent Ethics Committee for Non-Pharmacological Clinical Investigations (Genova, Approval 2012/03 dated June 28 2012) and performed according to the Declaration of Helsinki and its amendments (Ethical Principles for Medical Research Involving Human Subjects, adopted by the 18th WMA General Assembly Helsinki, Finland, June 1964 and amendments).

The primary outcome of the study was the detection of the consumed probiotics; *L. rhamnosus* HN001 and *L. acidophilus* La-14 from vaginal swabs. Secondary outcome was tolerance to the consumed product.

### Study design

The study took place at Farcoderm Poliambulatorio facilities in San Martino Siccomario (PV), Italy, from January to September 2013. The study was performed as a double blind, randomised, placebo controlled design with two parallel groups receiving either verum or placebo. The study was supervised by a certified gynaecologist and performed according to Good Clinical Practice. The volunteers consumed their assigned study product for 14 days. Vaginal swabs were taken at 0, 7, 14 and 21 days to be analysed for the consumed probiotics (see below).

### Volunteers

Forty volunteers were assessed for eligibility (Fig. 1); all assessed fulfilled inclusion and exclusion criteria (Table 1). Each participant was fully informed on study risks and benefits, aims, and procedures. An informed consent form was signed by each subject prior to participating in the study. Subjects were randomly assigned to receive either verum ($n = 20$) or placebo ($n = 20$).

### Study products

For allocation, a computer-generated, using PASS 11 statistical software (version 11.0.8 for Windows; PASS, LLC. Kaysville, UT, USA) restricted randomization list (biased coin using Efron's algorithm) was used. Volunteers, investigator and his collaborators were kept blind to products assignment. Sequentially numbered, opaque, and sealed envelopes, reporting the unblinded treatment allocation, where prepared for each subject and stored in a safe place by the in site Study Director.

Verum consisted of a capsule containing the proprietary Respecta® complex (Giellepi S.p.A. Health Science, Seregno, MB, Italy), which includes $5 \times 10^9$ CFU probiotics; *Lactobacillus acidophilus* La-14 (ATCC SD5212), and *Lactobacillus rhamnosus* HN001 (AGAL NM07/09514), and bovine lactoferrin RCX™. Placebo consisted of an identical capsule containing 100 mg maltodextrin; the excipient that was also used in the verum capsules. Volunteers were instructed to consume two capsules per day, after breakfast, for 14 days.

Compliance was determined by assessing returned packaging and by interviewing the volunteers.

### Vaginal swab analyses

Volunteers visited the clinic at the time of randomisation (baseline) and after 7 and 14 days of treatment. A further clinic visit by the subjects was 7 days after treatment stop (follow-up). During each clinic visit, the gynaecologist collected the medical history of the subjects, checked subjects eligibility to (continue to) participate in the study, measured vaginal pH and collected vaginal samples using a nylon flocked swab (eSwab liquid Amies, Copan). Subjects had to abstain from sexual intercourse 24 h prior to sample collection. Subjects were not allowed to use any intravaginal products (e.g. spermicides, lubricants, etc.) 24 h prior to sample collection, subjects were only allowed to use a neutral detergent, supplied by the sponsor, for intimate hygiene. Swabs were stored at −80 °C until analysis.

Vaginal pH was measured using chemical reagent strips (StrongStep® Bacterial vaginosis rapid test, Liming Bio-Products Co., Ltd).

DNA amount of the two study organisms was assessed by real-time PCR. Two sets of primers were used according to Zhang and co-workers [9]. The first set of primers was LacidoF (5′-TGCAAAGTGGTAGCGTAAGC-3′) and LacidoR (5′-CCTTTCCCTCACGGTACTG-3′), which was designed to

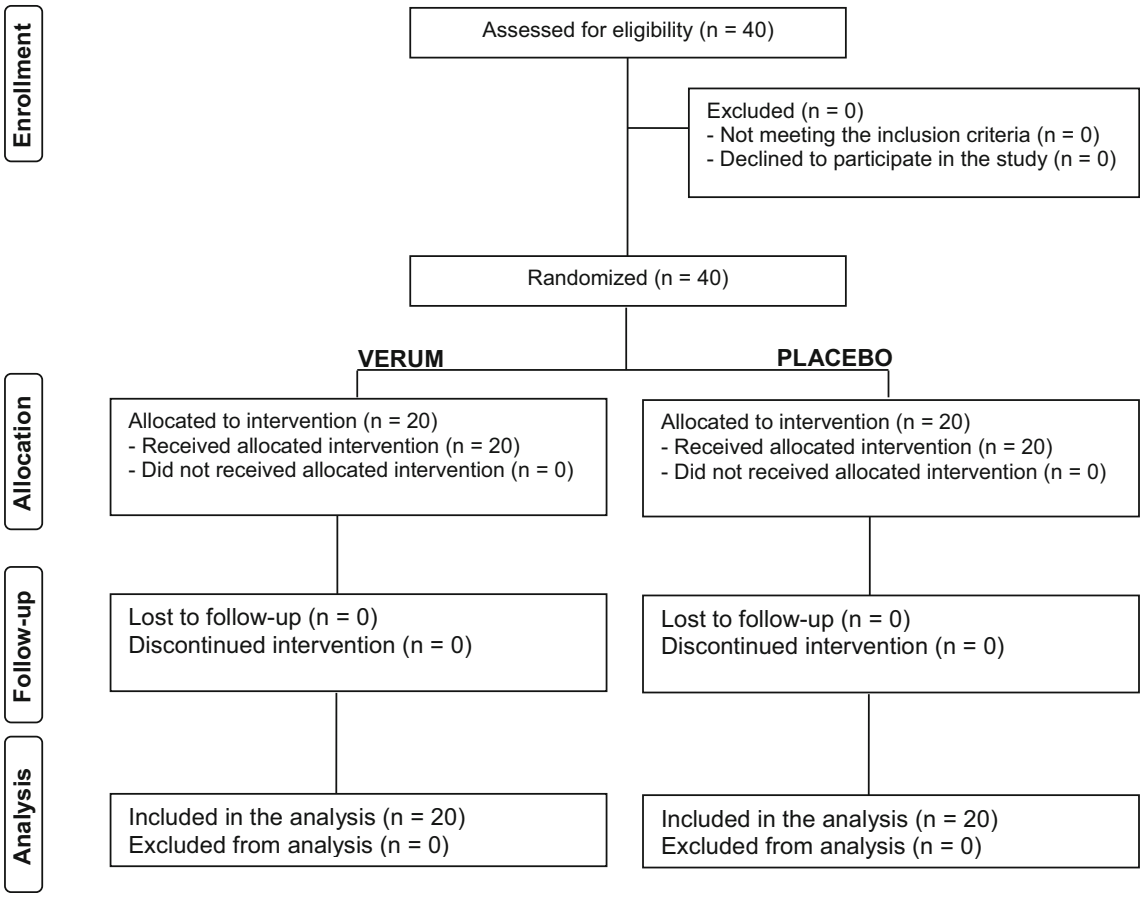


**Fig. 1** CONSORT flow diagram of the volunteers participating in the study

**Table 1** Inclusion and exclusion criteria for the volunteers participating in the study

| Inclusion criteria | Exclusion criteria |
|---|---|
| Female | Vaginal of urinary complaints |
| Age 18–50 years (fertile age) | Pregnant or planning pregnancy |
| Caucasian | Breast feeding |
| No vaginal infections within previous 12 month | History of vulvo-vaginal pathological conditions |
| Not recently participate in a similar study | Systemic or topical pharmacotherapy |
| No changes in daily routine | Use of vulvo-vaginal medication |
| Willingness to take dietary supplements | Acquired or congenital immune deficiency |
| Valid contraception for the duration of the study | Recent history of radiotherapy |
| Willingness to collaboration in completing the binding parts of the study protocol | Prolonged use of corticosteroids or other immune modulating medication |
| | Habitual use of probiotics |
| | Intolerance to any of the study products |
| | History of alcohol abuse |
| | History of drug abuse |
| | Unable to communicate with the investigator |

amplify a 200 bp on the region between 16S rDNA and 32S [23S] rDNA of *L. acidophilus*. The second set of primers was LrhamF (5′-TGCTTGCATCTTGATTTAATTTTG-3′) and LrhamR (5′-GGTTCTTGGATYTATGCGGTATTAG-3′), which was designed to amplify a 120 bp on the region of the 16S rDNA of *L. rhamnosus*. All DNA samples (125 µl of

liquid Amies swab preservation medium) were extracted using QIAamp DNA Kit (Qiagen, Milan, Italy), following the manufacturer's protocol for bacteria. Extracts purity was determined using a biophotometer (Eppendorf). DNA amplification conditions were as follows: 10 min denaturation at 95 °C, 40 denaturation-annealing cycles at 95 °C for 15 s, and annealing-elongation at 60 °C for 1 min. An ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) was used for real-time PCR analyses. Real-time PCR was performed with SYBR Green I (SsoFast Evagreen TM with low ROX, BIORAD), and each sample was analyzed in duplicate. After SYBR Green amplification, a dissociation curve analysis was performed to confirm the absence of non-specific amplification products.

Results are expressed as a change in copy number/swab [10] compared to base line. As a statistically significant change in recovered organisms may not always be a clinically relevant change, transient colonisation was arbitrarily defined as doubling the level of the probiotic species compared to base line.

### Adverse event analysis

Adverse events were classified as: light; moderate or severe.

Adverse events were considered severe when: fatal, life threatening, requiring hospitalisation, leading to permanent handicaps or congenital abnormalities.

Causality of any adverse events was considered as:

Correlated; the investigator has sufficient information and considers the symptom to be correlated to the study treatment. Uncorrelated; the investigator has sufficient information and considers the symptom not to be correlated to the study treatment. Unable to determine correlation; the investigator has insufficient information or is not able to determine the correlation or non-correlation of the symptom to the study treatment.

### Statistical analyses

Statistical analysis was carried out on the intention to treat population using NCSS 8 (version 8.0.4 for Windows; NCCS, LLC, Kaysville, UT, USA) running on Windows Server® 2008 R2 64 Edition. Data normality was checked using Skewness, Kurtosis, Omnibus, and Shapiro–Wilk W normality tests. Since data were not normally distributed: (a) Kruskal–Wallis one-way ANOVA was used to compare active and placebo data, (b) Fisher's exact test was used to determine transient colonization and (c) Mann–Whitney *U* test was used to compare follow up data. A power analysis was performed using software PASS 11—Professional (version 11.0.8). The analysis was performed using non-inferiority test set at $\alpha = 0.05$. Values are reported as mean ± standard error of mean (SEM).

## Results

### Volunteers

Baseline characteristics of the volunteers are presented in Table 2. No differences were observed between the two groups of volunteers. Compliance was 100 % for all volunteers.

### Vaginal recovery

Compared to baseline, vaginal *L. acidophilus* levels were significantly increased on days 14 and 21 in the probiotic group (Fig. 2; $p < 0.001$). Likewise, vaginal *L. rhamnosus* levels were increased on days 14 and 21 in the probiotic group (Fig. 2; $p < 0.001$).

In the placebo group, no significant change in vaginal *L. acidophilus* or *L. rhamnosus* levels was observed (Fig. 2).

Transient colonisation of *L. acidophilus* (i.e. at least double levels from base line) was observed on days 14 and 21 in 12 ($p = 0.0225$) and 16 ($p = 0.0001$) out of 20 women, respectively, in the probiotic group (Table 3). For

**Table 2** Baseline characteristics of the volunteers

| | Probiotic ($n = 20$) | Placebo ($n = 20$) |
|---|---|---|
| Sex (female, %) | 100 | 100 |
| Age (years ± SEM) | 33.2 ± 9.7 | 33.7 ± 7.4 |
| Weight (kg) | 60.7 ± 7.1 | 58.5 ± 8.6 |
| BMI (kg/m$^2$) | 22.9 ± 2.4 | 22.1 ± 2.6 |
| Method of contraception (%) | | |
| Oral contraception | 25 | 30 |
| Contraceptive patch | 10 | 5 |
| Intra-vaginal ring | 20 | 15 |
| Condom | 45 | 50 |
| Recent vaginal infections | 0 | 0 |
| Duration menstrual cycle (%) | | |
| 25–35 days | 90 | 80 |
| <25 days | 10 | 20 |
| >35 days | 0 | 0 |
| Duration of menstruation (%) | | |
| 3–7 days | 95 | 100 |
| <3 days | 0 | 0 |
| >7 days | 5 | 0 |
| Menstrual intensity (%) | | |
| 30–80 ml | 80 | 70 |
| <30 ml | 0 | 10 |
| >80 ml | 20 | 20 |

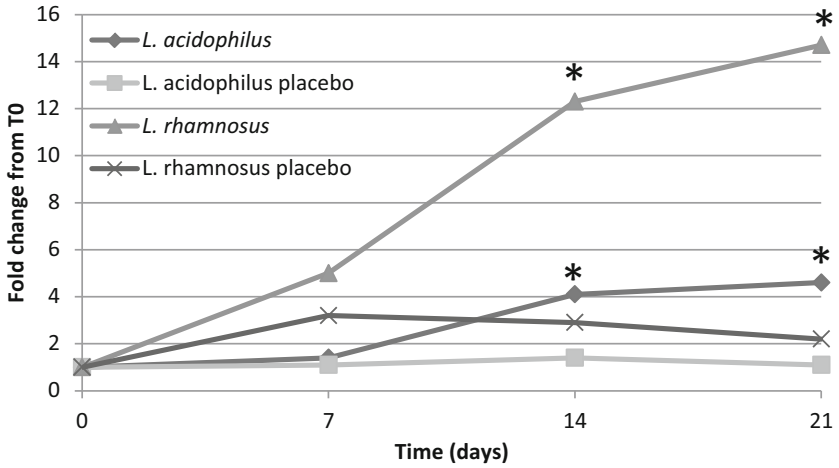


**Fig. 2** Vaginal recover of consumed *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* as determined by quantitative PCR. Results are expressed as the fold change from base line (i.e. 0 days = 1). *$p < 0.001$ compared to baseline

**Table 3** Women exhibiting transient colonisation of the species included in the study product; defined as at least a doubling in levels as compared to base line

| Treatment | Detected species | 7 days | 14 days | 21 days |
|---|---|---|---|---|
| Placebo ($n = 20$) | *L. acidophilus* | 0 | 4 | 0 |
| | *L. rhamnosus* | 9 | 9 | 9 |
| Verum ($n = 20$) | *L. acidophilus* | 4 | 12* | 16** |
| | *L. rhamnosus* | 16‡ | 15 | 17‡‡ |

Difference compared to placebo (Fisher's exact test): * $p = 0.0225$, ** $p = 0.0001$, ‡ $p = 0.0484$, ‡‡ $p = 0.0187$

*L. rhamnosus* transient colonisation was observed on days 7 and 21 in 15 ($p = 0.0484$) and 17 ($p = 0.0187$) out of 20 women, respectively, in the probiotic group (Table 3).

In the placebo group no significant number of women was observed to exhibit transient colonisation (Table 3).

### Vaginal pH

Over the course of the study, the mean vaginal pH varied between 4.06 and 4.24. No statistical difference was observed between the treatment groups or at any of the time points, Table 4.

### Post-study power calculation

The study was not sufficiently powered to detect a treatment effect at 7 days. However, at 14 and 21 days, the power was sufficient (requiring a minimum of 11 or 9 subjects per group for *L. acidophilus* La-14 and 9 and 7 subjects for *L. rhamnosus* HN001, respectively).

### Adverse events

During the study period no adverse event occurred; neither correlated nor unrelated. Both the active and the placebo products were well tolerated by all subjects participating in the study.

**Table 4** Vaginal pH

| Day | 0 | 7 | 14 | 21 |
|---|---|---|---|---|
| Verum | 4.16 ± 0.05 | 4.20 ± 0.05 | 4.24 ± 0.04 | 4.24 ± 0.04 |
| Placebo | 4.10 ± 0.04 | 4.06 ± 0.03 | 4.14 ± 0.04 | 4.10 ± 0.04 |

Data are mean ± SEM

## Discussion

The vaginal microbiota is of major importance to feminine health. Ravel and co-workers [11] observed five types of bacterial community clusters; women dominated by, *L. jensenii, L. crispatus, L. gasseri* or *Lactobacillus inners* and a fifth cluster that was not dominated by one species but exhibited a great diversity and organisms commonly associated with BV; *Gardnerella vaginalis* and *Prevotella* spp. This latter group also exhibited a higher Nugent score and higher pH. Hispanic and African American women are commonly exhibiting this latter microbiota type although they are asymptomatic for BV and levels of vaginal lactobacilli alone are not a sufficient indicator for vaginal health as ethnicity appears to play a role. In Caucasian and Asian women, however, a higher vaginal colonisation with lactobacilli does seem to correlate with health.

A way to increase the level of vaginal lactobacilli is through the use of probiotics; live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [12]. Several probiotics have been found to both increase the overall level of vaginal lactobacilli [3] and to aid in the treatment of bacterial vaginosis [13]. Two ways of administration are commonly applied; direct application through vagitories or indirect application through

oral consumption of probiotics. The latter option is preferred as it is more userfriendly, but has as disadvantage that it poses a hurdle to the probiotics as they have to survive gastrointestinal transit and subsequently colonise the vagina. While many probiotics have been documented to survive gastrointestinal transit, to date only one study has been conducted that investigated and documented that the consumed oral probiotics (*Lactobacillus fermentum* 57A, *Lactobacillus plantarum* 57B and *Lactobacillus gasseri* 57C) indeed subsequently transiently colonise the vagina [5].

The aim of the current pilot study was to investigate if indeed oral consumption of a probiotic and lactoferrin complex would lead to increased levels of the consumed species in the vagina. In the present study Caucasian women were recruited and administered with Respecta® complex (*L. acidophilus, L. rhamnosus* and lactoferrin RCX™). Lactoferrin RCX™ in the complex represents a component with prebiotic function able to promote the survival and proliferation of lactobacilli in the intestine [14].

The treatment increased levels of the species detected in the vagina of women consuming the strains in comparison to women in the placebo group. Interestingly, the levels were still increased after 21 days; i.e. 7 days after cessation of probiotic consumption. Furthermore, on days 7 and 14 significantly more women had increased levels of *L. rhamnosus* and *L. acidophilus*, respectively, as compared to the start of the intervention. On day 21 a significant number of women had increased levels of *L. rhamnosus* and *L. acidophilus*. The increased level and increased number of colonised women 7 days after cessation of probiotic consumption suggests a prolonged persistence of the strains. Also Strus and co-workers [5] noted a still increased number of women colonised by the consumed lactobacilli 10 days after consumption was seized; although this was significantly less than directly after the probiotic consumption. Bohbot and Cardot [6] also observed an increase in women colonised by the consumed species, but this did not reach statistical significance; most likely due to the small number of women in the study (two groups of 10). The current study is different from the previous two studies as it did not use any culturing step but solely used molecular techniques (qPCR), and thus is not at risk for bias, and was sufficiently powered.

The observed increase in the level of the probiotic species was not accompanied with a change in vaginal pH. This is, however, not surprising as the women were healthy and had a normal vaginal pH [15] at the start of the study.

In conclusion, consumption of *L. acidophilus* La-14 and *L. rhamnosus* HN001, leads to a significant increase in these species in comparison to base line and in comparison to women in the placebo group. Although it has been suggested that the intestine is a potential reservoir for both vaginal lactobacilli and pathogens [15], this is only the second time it is shown that consumption of probiotic lactobacilli leads to transient colonisation of the vagina; and the first time for the species tested; *L. acidophilus* and *L. rhamnosus*. It is especially important to note that the species remained increased even 1 week after consumption of the probiotics was stopped. The volunteers in the study were healthy; hence no conclusions can be drawn on the potential health benefits of the observed vaginal colonisation. However, the observations, together with the absence of adverse events, provide a strong basis for studying the health benefits of the strains; e.g. as adjunct therapy in treatment of BV or candidiasis, or in risk reduction for these conditions.

**Acknowledgments** We are grateful to the volunteers for their participation.

**Conflict of interest** The study was sponsored by Giellepi S.p.A.; Giellepi is the owner of the investigated product. RR and FT are employees of Giellepi. ACO is an employee of DuPont Nutrition & Health; DuPont manufactures and markets probiotics. The sponsor and the author had no influence on the interpretation of the results.

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# A Healthy Vaginal Microbiota Remains Stable during Oral Probiotic Supplementation: A Randomised Controlled Trial - Lyra et al. 2023

*microorganisms*

MDPI

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# A Healthy Vaginal Microbiota Remains Stable during Oral Probiotic Supplementation: A Randomised Controlled Trial

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**Abstract:** The primary objective of this randomised, placebo-controlled, triple-blind study was to assess whether orally consumed *Lactobacillus acidophilus* La-14 (La-14) and *Lacticaseibacillus rhamnosus* HN001 (HN001) colonise a healthy human vagina. Furthermore, potential effects on vaginal microbiota and immune markers were explored. Fifty women devoid of vaginal complaints (Nugent score 0–3 and vaginal pH $\leq$ 4.5) were randomised into a 2-week intervention with either La-14 and HN001 as the verum product or a comparable placebo. Vaginal swab samples were collected at baseline, after one and two weeks of intervention, and after a one-week follow-up, for assessing colonisation of the supplemented lactobacilli, vaginal microbiota, and six specific immune markers. Colonisation of *L. acidophilus* and *L. rhamnosus* was not observed above the assay detection limit (5.29 and 5.11 log 10 genomes/swab for *L. acidophilus* and *L. rhamnosus*, respectively). Vaginal microbiotas remained stable and predominated by lactobacilli throughout the intervention, and vaginal pH remained optimal (at least 90% of participants in both groups had pH 4.0 or 4.5 throughout the study). Immune markers elafin and human β-defensin 3 (HBD-3) were significantly decreased in the verum group ($p = 0.022$ and $p = 0.028$, respectively) but did not correlate with any microbiota changes. Adverse events raised no safety concerns, and no undesired changes in the vaginal microbiota or immune markers were detected.

**Keywords:** vaginal colonisation; microbiota; immune markers; lactobacilli; probiotics; *Lactobacillus acidophilus*; *Lacticaseibacillus rhamnosus*



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## 1. Introduction

The adult pre-menopausal vaginal microbiota is typically low in diversity and dominated by one or two lactobacilli species or by a mixture of non-lactobacilli genera that form distinct community state types (CSTs), which can be reliably assigned for sample sets originating from various populations [1–3]. The CSTs can be defined as CST I (*Lactobacillus crispatus* dominated), CST II (*Lactobacillus gasseri* dominated), CST III (*Lactobacillus iners* dominated), CST IV (non-lactobacilli dominated, such as *Gardnerella vaginalis* and *Atopobium vaginae*), and CST V (*Lactobacillus jensenii* dominated). In addition, hormonal changes during the female lifespan and the menstrual cycle, lifestyle, hygiene practices, and ethnicity can affect the composition and stability of the vaginal microbiota [4–6]. Lactobacilli including *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. iners* have been associated with a healthy vaginal microbiota [2,7], with *L. iners* also being linked to transitional and dysbiotic stages [7,8]. Non-lactobacilli genera/species, including *Prevotella* spp., *G. vaginalis*, *A. vaginae*, and *Mycoplasma hominis*, are found in a higher abundance when lactobacilli levels are

depleted among asymptomatic women and have been associated with bacterial vaginosis (BV) risk [7,9,10].

The vaginal immune system is part of the broader female reproductive tract mucosal immune system that is regulated differently during each phase of the menstrual cycle by the sex hormones [11]. Furthermore, different CSTs, disease states, and genetic variation influence the immunological responses in the vaginal mucosa–microbiota interface. For example, in a cohort of African women, more diverse microbial communities were correlated with higher inflammatory responses [12]. Imbalances of the vaginal microbiota are also characterised by elevated vaginal inflammatory responses. In response to microbiota and hormonal control, the vaginal mucosa secretes a variety of immunomodulatory molecules, including antimicrobial peptides (AMP), protease inhibitors, and immunoglobulins (Ig) [13,14]. One class of AMPs are human β-defensins (HBDs). HBD-1, HBD-2, and HBD-3 are cationic peptides that are active against common vaginal bacteria and pathogens [15]. Secretory leukocyte protease inhibitor (SLPI) and elafin are protease inhibitors which protect host tissue against overactive immune response and harbor antimicrobial and immunomodulatory activity [16]. In some studies, both SLPI and elafin have been reported to be decreased in BV [16,17]. IgA secreted by the cervix plays a key role in microbiota homeostasis on the vaginal mucosa by maintaining commensal bacteria and on the other hand targeting pathogens and preventing their access to vaginal mucosa [18,19].

Probiotic lactobacilli are an attractive and safe approach to support vaginal health as an adjunct or alternative to conventional antibiotic BV therapies [20,21]. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [22], with the definition permitting both oral and vaginal applications in relation to vaginal health. As a route, oral consumption can be considered more practical and appealing to the consumer, especially in a long-term prophylactic use. Orally consumed probiotics have shown beneficial effects in studies investigating BV (reduced Nugent score) and BV-associated symptoms in comparison to both placebo and conventional antibiotic treatment [23–25] and prolonged remission in recurrent BV as adjunct to conventional antibiotic therapy [26]. Vaginal colonisation by orally consumed probiotics has been reported both with strain-level detection [27–29] and as elevation of the supplemented species [23,30–32]. Additionally, on a community level, an increase in *Lactobacillus* spp. in a dysbiotic vaginal microbiota has been reported even without detectable colonisation in oral, intestinal, or vaginal microbiota samples [33].

In the vaginal tract, probiotics are considered to provide antimicrobial activity by reducing vaginal pH via lactic acid production, producing bacteriocins, and modulating local and systemic immune responses. Furthermore, vaginal probiotics are expected to adhere to vaginal epithelial cells and thereby exclude potential pathogens. However, information is lacking on the effects of probiotic strains on indigenous vaginal microbiota composition and potential immunomodulatory effects in a healthy vaginal tract. The probiotic strains selected for this investigation were *Lactobacillus acidophilus* La-14 (La-14) and *Lacticaseibacillus rhamnosus* HN001 (HN001). La-14 and HN001 included in the Respecta® complex (Giellepi S.p.A. Health Science, Seregno, MB, Italy, containing $5 \times 10^9$ colony forming units (CFU) of La-14 and HN001 and 50 mg of bovine lactoferrin per capsule with dosing varying from 1–2 daily capsules) have shown beneficial effects for vaginal health in randomised placebo-controlled clinical trials. These include elevating vaginal *L. acidophilus* and *L. rhamnosus* levels, reducing Nugent score, which reflects an increase in vaginal lactobacilli, and alleviating BV- and vulvovaginal candidiasis (VVC) associated symptoms [23,30,34]. The lactoferrin contained within the Respecta® complex is a glycoprotein able to both inhibit and enhance the growth of Lactobacillaceae and *Bifidobacterium* strains in vitro [35]. Vaginally applied lactoferrin (100 mg and 200 mg in vaginal pessaries for 10 days) has been reported to have a beneficial effect on the vaginal microbiota in a prospective randomised trial [36], and orally consumed lactoferrin (100 mg) may have potential for normalising the vaginal microbiota in comparison to ferrous sulphate as revealed in a pilot study on the use of lactoferrin for the prevention of preterm delivery [37]. However, the bacterial strain components of the Respecta® complex (La-14 and HN001) have

been reported to have an alike or enhanced efficacy compared to the combination of La-14, HN001, and lactoferrin in attenuating *G. vaginalis*-induced BV in mice, in inhibiting *G. vaginalis* growth, and in adherence to human HeLa cells in vitro [38], suggesting that lactoferrin is not essential for efficacy. In the mouse study, oral administration was more effective than vaginal application and both systemic and vaginal immune responses were activated [38]. La-14, HN001, and the combination of La-14 and HN001 showed adherence to cervical cells (HeLa cells) and were able to form biofilms in vitro [39]. Moreover, as a pre-requisite for vaginal colonisation after oral consumption, La-14 and HN001 have been reported to survive both through the human gastrointestinal tract [40,41] and the passage from the anus to the vaginal orifice [23,30]. Both *L. acidophilus* and *L. rhamnosus* have long been shown to be safe and suitable for human consumption and have been present in human food for decades [42,43].

The objectives of this clinical trial were to assess whether a two-week oral administration of La-14 and HN001 in a vegan capsule devoid of lactoferrin results in vaginal colonisation of the supplemented species and to characterise the potential effects on vaginal pH, microbiota composition, and immune markers. The target population included healthy premenopausal females devoid of vaginal symptoms, comparable to the study by De Alberti and colleagues [30], with additional Nugent testing at screening to confirm vaginal health with a non-subjective measure. Furthermore, the participants' menstrual cycle and ethnicity were considered in the study design to minimise confounding variation in the microbiota analyses.

## 2. Materials and Methods

### *2.1. Study Design*

This was a randomised, double-blind, placebo-controlled, parallel group, single center clinical trial performed at an independent clinical research site, CPS Research (Glasgow, UK), under the supervision of Gordon Crawford, MD, as the principal investigator. The trial included a screening phase, a two-week intervention, and a one-week follow-up with five visits: V1 (Day $-42$ to $-3$; screening), V2 (Day 0; baseline and randomisation), V3 (Day $7 \pm 1$; mid intervention), V4 (Day $14 \pm 1$; end of intervention), and V5 (Day $21 \pm 1$; after 1-week follow-up) (Figure 1). The trial was conducted in accordance with the World Medical Association (WMA) Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human use (ICH) harmonised guidelines for Good Clinical Practice (GCP) E6(R2), dated 9 November 2016, and with laws and regulations for clinical research in the United Kingdom. The trial was registered publicly at the ISRCTN registry prior to initiation (ISRCTN29375062).

After pre-screening, participants attended a screening visit (V1) 3 to 42 days prior to randomisation visit (V2) to allow for visits V2 to V5 to be held in between menses for participants with a natural menstrual cycle. During the screening visit, written informed consent was obtained from all participants before any study-specific procedures or assessments were performed. Screening procedures included assessment of eligibility per set criteria (Table 1), vital signs, a gynecological examination to exclude abnormalities or signs of infection, and collection of demographics (age, ethnicity, smoking, and alcohol consumption), relevant medical history (vaginal symptoms and health, menstrual cycle, pregnancies, relevant operations), height and weight, and conducts potentially affecting the vaginal microbiota (contraception method, sexual activity, multiple or single partners, practice of anal intercourse). To confirm eligibility, a urine pregnancy test, and vaginal swab tests for Nugent score, candidiasis, trichomoniasis, and vaginal pH were performed.

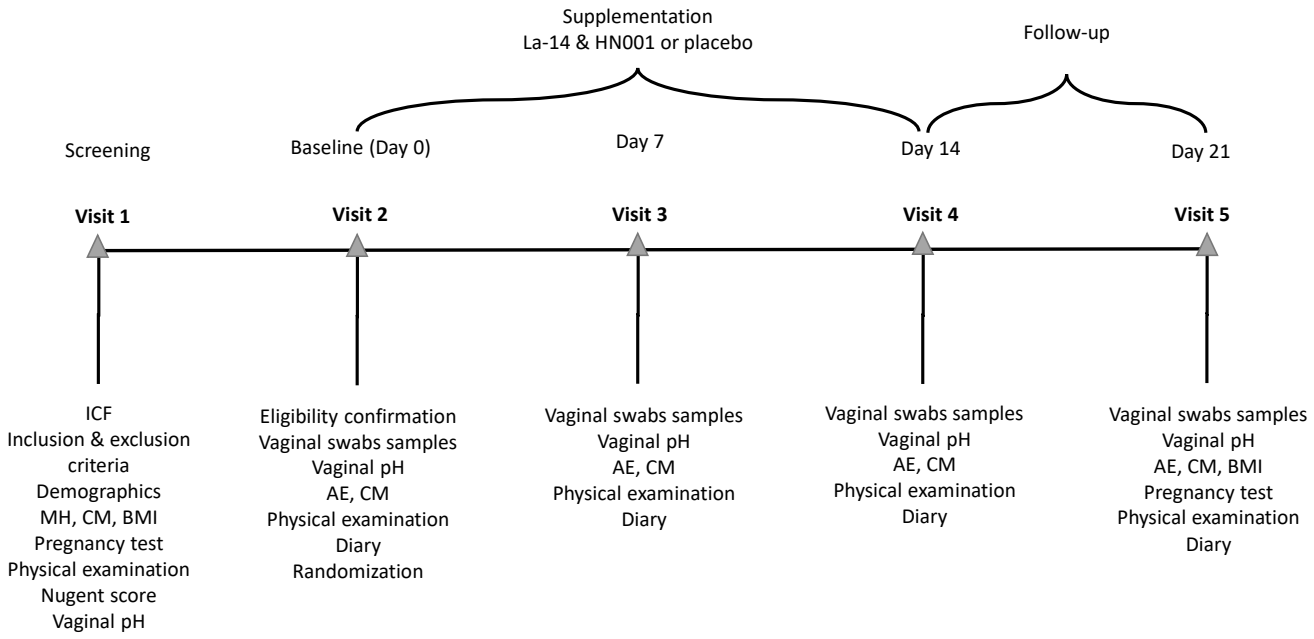


**Figure 1.** Schedule of events in the study. The screening visit (Visit 1) was held 3 to 42 days before randomisation (Visit 2) to allow for the study endpoints to be measured in between menses. Vaginal pH was measured, and vaginal swabs samples were collected for analysis of vaginal colonisation by the supplemented *Lactobacillus acidophilus* La-14 and *Lacticaseibacillus rhamnosus* HN001 and for assessment of the vaginal microbiota and immune markers at baseline (Visit 2) before investigational product (IP) consumption at weekly intervals during the intervention (Visit 3 and 4) and after a 1-week follow-up (Visit 5). Throughout the study, participants collected information on adverse events, concomitant medications, IP compliance, sexual behaviour, contraceptive method used, and menstrual bleeding days on a diary. A gynecological examination and vital signs' measurement were performed on each visit as the physical examination. AE, adverse event; BMI, body mass index; CM, concomitant medication; HN001, *Lacticaseibacillus rhamnsosus* HN001; ICF, informed consent form; La-14, *Lactobacillus acidophilus* La-14; MH, medical history.

During the study, participants were asked to continue their normal routines and lifestyle (including diet, exercise, choice of contraceptive method), to refrain from sexual intercourse, and use of intravaginal products (lubricants, spermicides) for 24 h preceding visits, and only to use a provided neutral detergent wash for intimate hygiene.

On V2, participants were randomly allocated to receive the investigational product (IP) and instructed to consume one capsule daily after breakfast from Day 1 to the day of V4 (a total of 13 to 15 days). IP compliance was set per protocol at $\geq$80% and assessed based on the number of returned capsules.

On visits V2 to V5 a gynecological examination was performed, and vital signs were measured. Adverse events (AEs) and updates of contraceptive method, sexual activity, and concomitant medications (CMs) were recorded. For outcome assessments, three vaginal swabs were collected for microbiota and immune marker analyses, and the vaginal pH (NutraBlast® Feminine pH Test Strips, NutraBlast, Pompano Beach, FL, USA) was measured. Between visits, participants collected information on AEs, CMs, IP compliance, sexual behaviour, contraceptive method used, and menstrual bleeding days on a paper diary. In case of menstrual bleeding on Day 20–22, the follow-up visit (V5) was postponed to immediately after the bleeding had ended. Pregnancy test was repeated on V5 as a safety precaution. All AEs were assessed for causality and severity by the investigator.

In case of COVID-19 related restriction to attend a visit on site, the study nurse or investigator contacted the participant, and a remote visit was held including collection of three vaginal self-swabs, AEs, CMs, and IP compliance.

**Table 1.** Inclusion and exclusion criteria.

| Inclusion Criteria | Exclusion Criteria |
| --- | --- |
| 1. Female<br>2. Age 18–50 years (fertile age)<br>3. Caucasian<br>4. No vaginal infections within previous 6 months<br>5. Has not participated in another investigational drug clinical trial within 1 month (30 days) or having received an investigational drug within the last month (30 days) before the start of screening<br>6. No significant changes in daily routines related to dietary/activity patterns<br>7. Willingness to take dietary supplements<br>8. Valid contraception for the duration of the study<br>9. Willingness to collaborate in completing the binding parts of the study protocol | 1. Vaginal or urinary complaints<br>2. Vaginal pH > 4.5<br>3. Pregnant or planning pregnancy<br>4. Breast feeding<br>5. History of vulvovaginal pathological conditions<br>6. Antibiotic usage during last 3 months<br>7. Oral corticosteroid usage during last 3 months<br>8. Use of vulvovaginal medication<br>9. Acquired or congenital immune deficiency<br>10. Recent history of radiotherapy<br>11. Prolonged use of corticosteroids or other immune modulating medication<br>12. Habitual use of probiotic supplementation<br>13. Menstrual irregularities including menopause<br>14. On-going diagnosed disease which, in the opinion of the investigator, makes the participant unfit for the study<br>15. Intolerance to any of the study products<br>16. History of alcohol abuse within 2 years<br>17. History of drug abuse within 2 years<br>18. Unable to communicate with the investigator<br>19. Nugent score > 3 (sampled during screening visit) |

### *2.2. Participants*

Premenopausal Caucasian women aged 18–50 years fulfilling eligibility criteria (Table 1) were recruited from the Glasgow (Scotland, UK) area between 10 November 2020 and 24 March 2021.

### *2.3. Intervention*

Participants were supplemented either with verum or placebo IP for two weeks. One verum capsule (V-Caps HMPC capsules, Lonza, Puebla, Mexico) contained $10^{10}$ CFU of La-14 (ATCC SD5212) and HN001 (ATCC SD5675) in a 4:1 ratio, potato maltodextrin as a carrier, and stearate and silicon dioxide as flow agents. The placebo capsules were identical in shape, texture, and taste to the verum capsules and contained potato maltodextrin adjusted to equal the weight of the verum capsule and the same amounts of stearate and silicon dioxide as added to the verum capsule. Both verum and placebo IP were manufactured and packaged using identical containers and labels by Danisco USA Inc. (Madison, WI, USA).

### *2.4. Outcomes*

Vaginal colonisation of *L. acidophilus* and/or *L. rhamnosus* at species level was assessed as the primary outcome and vaginal pH was followed as the secondary outcome. Exploratory outcomes included strain-specific vaginal colonisation (La-14 and HN001) and the assessment of vaginal microbiota composition and specific immune markers. AEs were collected as a safety outcome.

### *2.5. Sample Collection and Analysis*

Vaginal swabs were collected as dry swabs with the Copan FLOQswab Collection Kit (Copan diagnostics, Murietta, CA, USA) from the sidewalls in the upper third area of the vagina (approximately 5 cm past the introitus) by gently rotating the swab for 10 to 20 s. Before sampling, any excessive secretion or discharge was wiped out and skin contact was avoided. Samples were immediately frozen and stored at −80 °C and all shipments for analysis were conducted on dry ice (temperature range −90 to −20 °C).

#### 2.5.1. DNA Extraction

Vaginal swabs' flocked heads were clipped directly into a Lysis Matrix E bead beating tube (MP Biomedicals, Santa Ana, CA, USA). The tubes were beaten using a Precellys 24 Homogenizer (Bertin Instruments, Montigny-le-Brétonneux, France), twice for 60 s at 4500 RPM. The bead beating tubes were then spun at 14,000× g for 15 min, then 500 µL of the resulting lysate was carried forward with a FastDNA Spin Kit for Soil (MP Biomedicals) following the manufacturer's instructions. The purified DNA was quantified using Qubit HS dsDNA kit on Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

#### 2.5.2. Detection of Colonisation with Quantitative PCR

The vaginal swab DNA samples were analysed as a primary endpoint with absolute quantitative PCR (qPCR) assays for *L. acidophilus* [44] and *L. rhamnosus* [45], and as an exploratory endpoint for La-14 [46] and HN001. All qPCRs were run with a total reaction volume of 25 µL and 1 ng of sample DNA in triplicate using a QuantStudio 5 Real Time PCR System (Thermo Fisher Scientific), applying either 1 × SYBR FAST or 1 × Taq FAST Advanced master mix depending on the detection chemistry applied (Table 2). Standard curves ranging from 100 fg to 10 ng of La-14 or HN001 DNA were prepared as applicable, and a negative control selected based on optimisation runs (*Faecalibacterium prausnitzii* DSM 17677 for La-14 and *L. acidophilus* DGCC 8698 or *Lacticaseibacillus paracasei* DGCC 4981 for HN001) were included on each 96-well plate. Quantification of PCR amplification was performed using the QuantStudio Design and Analysis Software v1.5.1 (Thermo Fisher Scientific). The limit of detection (LOD) values were based on 1/2 the number of genomes calculated to be within the 100 fg standard applied. For the SYBR assays, the dissociation curves were analysed to control for non-specific amplification. to control for non-specific amplification.

**Table 2.** Quantitative PCR (qPCR) assays applied to assess colonisation of supplemented strains from vaginal swab samples. F, forward primer; R, reverse primer; P, probe.

| Primer/Probe (5′–3′) | Master Mix | Annealing Temperature |
|---|---|---|
| Primary outcome assays | | |
| *L. acidophilus* (species level) [44]<br>F: CCTTTCTAAGGAAGCGAAGGAT (400 nM)<br>R: ACGCTTGGTATTCCAAATCG (400 nM) | 1 × SYBR FAST | 60 °C |
| *L. rhamnosus* (species level) [45]<br>F: TGCTTGCATCTTGATTTAATTTTG (400 nM)<br>R: GGTTCTTGGATYTATGCGGTATTAG (400 nM) | 1 × SYBR FAST | 62 °C |
| Exploratory outcome assays | | |
| *L. acidophilus* La-14 (strain level) [46]<br>F: CCGGTTAATAAAATCTTTTCACCTTG (600 nM)<br>R: GCAGTTATTAATCGTGATTTGCATATAAATT (600 nM)<br>P: FAM-AGTTGATCAGTCAGCAAGTAGTGTTATGG-IowaBlack (300 nM) | 1 × Taq FAST Advanced | 60 °C |
| *L. rhamnosus* HN001 (strain level)<br>F: CTGGAGGAGATCACAACGACT (400 nM)<br>R: ATTGTCCCAACGCTGAATGC (400 nM)<br>P: FAM-TGAAGACAAGGTTGCGCCCTGTACACTGTTA-IowaBlack (200 nM) | 1 × Taq FAST Advanced | 60 °C |
| Post hoc analysis | | |
| *L. acidophilus* (species level) [47]<br>F: TGCAAAGTGGTAGCGTAAGC (400 nM)<br>R: CCTTTCCCTCACGGTACTG (400 nM) | 1 × SYBR FAST | 62 °C |

As a post hoc analysis, an alternative *L. acidophilus* assay previously successfully applied for detection of vaginal colonisation [23,30,47] was tested with a subset of the vaginal swab samples and DNA extracted from pure cultures of *L. crispatus* DSM 20584, *L. jensenii* DGCC 11796, *L. gasseri* ATCC 33323, and *L. acidophilus* strains La-14 ATCC SD5212, DGCC 8698, ATCC 4356, and DGCC 12900.

#### 2.5.3. Vaginal Microbiota Sequencing and Data Analysis

The microbiota populations from vaginal samples were analysed by MiSeq 16S amplicon sequencing of the V4 region (Azenta Genewiz, South Plainfield, NJ, USA) and QIIME2 (v. 2021.2) as previously described [48–50] and applied by Lehtoranta and colleagues [7]. Samples containing less than 9000 reads were removed from the study and reverse reads were trimmed at 180 bp and overlapping sequences were paired. Taxonomy was assigned to aligned amplicon sequence variants (ASVs) using 'q2-feature-classifier' (classify-sklearn) trained on the V4 region of sequences contained in the RDP Classifier (v. 2.13; training set No. 18 July 2020 release) [51]. Taxa compositions were reported as relative abundance (% of total sequences). Vaginal CSTs were assigned to the samples using the software package VALENCIA [3]. Alpha and beta diversities were analysed as described previously [7] applying Faith's Phylogenetic Diversity (PD) [52] and weighted UniFrac [53], respectively, and tested with Kruskal–Wallis and permutational multivariate ANOVA (PERMANOVA), respectively, with the latter visualised using principal coordinates analysis (PCoA) with the R (v. 3.4) 'ggplot2' package [54,55].

#### 2.5.4. Vaginal Immune Marker Analysis

Immune marker levels (HBD-1, HBD-2, HBD-3, SLPI, elafin and, IgA) were determined by ELISA from the eluants of the vaginal swabs collected from the participants at different time points using the Spectramax 250 ELISA analyzer (Molecular Devices, San Jose, CA, USA). Briefly, the vaginal swabs were reconstituted in ice-cold phosphate buffered saline (PBS) by vortexing and finally the eluant supernatants were aliquoted in Eppendorf tubes for subsequent ELISA analysis. HBD-1 and HBD-3 levels were measured using Human DEFB1 (Beta-defensin 1) and Human DEFB3 (Beta-defensin 3) ELISA Kits (MyBioSource, London, UK), respectively, and HBD-2 by Human DEFb2/DEFB2 ELISA kit (Elabscience). SLPI and elafin levels were analysed with the human ELISA kits (Hycult Biotech, Uden, The Netherlands). IgA was determined using Invitrogen Human IgA ELISA kit. All kits were used according to the manufacturer's instructions and analysed in duplicate on the Spectramax 250 ELISA analyzer using the software SOFTmax PRO v4.3.1 LS. The lower limit of quantification (LLOQ) was defined as the lowest point on the standard curve for each individual analyte.

### *2.6. Determination of Sample Size*

The sample size was calculated using nQuery software (version 8.5.2) with 90% power and a two-sample *t*-test assuming unequal variances and applying a two-sided significance level of 5%. For *L. acidophilus* the mean difference between the verum and placebo arms was assumed to be 60, with standard deviations of 30 (placebo) and 70 (verum) [23]. For *L. rhamnosus*, the mean difference between the verum and placebo arms was assumed to be 30, with standard deviations of 20 (placebo) and 33 (verum) using $10^4$ genome containing particles/100 ng DNA in qPCR results evaluation as the unit for both endpoints for the power calculation [23], although the unit in the analyses was calculated per swab [30] rather than per a mass unit [23] to account for the expected high variance in the amount of host DNA in the samples [56].

With the above assumptions, 40 evaluable randomised participants were needed regarding both primary endpoints. Assuming a 20% attrition rate, 50 participants (25/group) were to be randomised to the study.

### *2.7. Randomisation and Blinding*

Study participants were allocated to one of two intervention groups in equal proportions using randomly permuted blocks through a computer-generated process. All verum and placebo products were labelled with a randomisation number accordingly.

All volunteers, study site personnel, clinical CRO personnel, the monitor, and Sponsor laboratory personnel involved in the study conduct were kept blinded during the entire study period.

### *2.8. Statistical Analysis*

#### 2.8.1. Primary and Secondary Outcomes

For the primary endpoints, a repeated measures analysis of variance (RM-ANOVA)-model was to be used with fixed effects for supplementation group, repeating factor week (Week 1, Week 2) and the interaction between supplementation group and week. The participant was to be used as a random effect in the models. The contrasts between the verum group and the placebo group at the end of the 2-week intervention period was to be estimated from the model with 95% confidence intervals together with two-sided *p*-values for the null hypotheses. The secondary endpoint (change in vaginal pH) was analysed utilising a mixed effects cumulative logit model, including the same explanatory variables as the models for primary endpoint. A categorised change in vaginal pH (decrease/no changes/increase) was used as the response. The model was constructed to model the probability of lower ordered categories of vaginal pH.

In addition, sensitivity analyses with RM-ANCOVA model and responder analyses, as described in De Alberti et al., [30] were planned for the primary endpoints. No changes in the vaginal pH were expected to occur. No interim analyses of the study data were planned or performed. Intention-to-treat (ITT) population was a priori designated as the primary population of the study. The safety population consisted of all participants who were randomised and received at least one dose of the study product.

#### 2.8.2. Exploratory Outcomes

For the vaginal sequence data, differential taxa were tested for the main effect of group (including model adjustment for visit and random effect of participant or CST at baseline) using the R package 'ANCOM2' [57]. Taxa were tested at the family, genus, and ASV level in a participant-paired repeated measures model which included all visits. The analysis considered multiple sampling from each participant. The samples were also subset by visit and modelled separately for each time point. Additionally, differential taxa were evaluated for the main effect of CST at baseline (V2) with model adjustment for visit and random effect of subject. ANCOM2 uses raw ASV sequence counts that have been log-transformed after addition of a pseudocount as input. The log ratios of each taxon were compared to all the remaining taxa one at a time (i.e., ASV1/ASVXX). The test reports a W value analogous to effect size, which represents the number of pairwise tests (taxa ratios) where ASV1/ASVXX significantly differed. The ANCOM2 significance cut-off was set to $p < 0.05$ or $p < 0.1$ after false discovery rate (FDR) correction by the Benjamini–Hochberg method. Spearman correlation analyses were conducted using the R packages 'hmisc' and 'gplots' for individual visits. *P*-values were adjusted for multiple comparisons by Benjamini–Hochberg FDR as noted.

Differences in CST distribution between intervention groups at baseline and for change in CST by timepoint (V3, V3 and V4 together, V4 and V5) were checked with Fisher's exact test. CST distribution and ASV correlations were also assessed for demographic variables including Nugent score, age, body mass index (BMI), contraceptive method (hormonal/non-hormonal), and pregnancies (prior pregnancies/no prior pregnancies) with the Chi-Square test and Spearman correlation, respectively. In addition, the percentages of participants with over 1% increase or decrease from baseline in the prevalence of an ASV were tabulated and tested with Fisher's exact test as an effort to detect effects related to probiotic consumption.

For each immune marker, the differences between verum and placebo in change from baseline were analysed with a RM-ANCOVA-model. The models included fixed effects for supplementation group, repeating factor visit and the interaction between supplementation group and week. Baseline value was included in the model as a covariate. Participant was used as the random effect in the models. The contrasts between verum and placebo at different visits were estimated from the model including 95% confidence intervals (CIs) together with two-sided *p*s. Change from baseline used in the RM-ANCOVA model was calculated using logarithm transformed values. If model assumptions were not met, square root transformation was used when calculating the change from baseline. Model assumptions were confirmed testing residual normality using Shapiro–Wilk test. If normality assumptions were not met with either of the data transformation, nonparametric Wilcoxon rank sum test was applied by visit to compare group differences.

For statistical analysis of microbiota and immune markers, the groups were analysed together. The R package 'rmcorr' was used to correlate immune markers (all timepoints joined) and ASVs present in at least 40% of the participants using repeated measures and *P*s were adjusted for multiple comparisons by Benjamini–Hochberg FDR. Additionally, immune marker concentrations were compared with Wilcoxon test between participants grouped according to CST at baseline (regardless of intervention group).

## 3. Results

### *3.1. Participants*

A total of 50 participants were randomised and 47 participants completed the study. Of the participants, 1 was discontinued due to randomisation error and did not receive any IP, leaving 49 participants in the ITT and safety analyses (Figure 2). The two other discontinued participants were lost to follow-up after the 2-week visit and took at least one dose of study product. The groups were comparable according to demographics, characteristics of menstruation cycle, and obstetric history (Tables 3 and 4). There were no reports of pain in the lower abdomen, presence of vaginal inflammation, dryness, itchiness, stinging, sterility, or vulvovaginal infections reported in the history of the randomised participants and only one leucorrhea (placebo group) and one pelvic or abdominal pelvic pain (verum group).

**Table 3.** Demographic characteristics for the intention-to-treat population. BMI, body mass index.

| Item | Statistic | Placebo (n = 24) | Verum (n = 25) |
|---|---|---|---|
| Age (years) | Mean (SD) | 30.9 (6.89) | 32.4 (7.94) |
| | Median | 30.0 | 33.0 |
| | Min, Max | 21, 41 | 20,47 |
| BMI (kg/m$^2$) | Mean (SD) | 26.9 (6.94) | 26.5 (6.41) |
| | Median | 25.3 | 23.8 |
| | Min, Max | 16.8, 41.5 | 19.9, 43.6 |
| Smoking status | | | |
| Every day smoker | n (%) | 0 (0.0) | 2 (8.0) |
| Former smoker | n (%) | 7 (29.2) | 3 (12.0) |
| Never smoker | n (%) | 16 (66.7) | 16 (64.0) |
| Someday smoker | n (%) | 1 (4.2) | 4 (16.0) |
| Alcohol use | | | |
| No | n (%) | 4 (16.7) | 4 (16.0) |
| Yes | n (%) | 20 (83.3) | 21 (84.0) |
| Contraceptive method | | | |
| Hormonal | n (%) | 14 (58.3) | 9 (36.0) |
| Not hormonal | n (%) | 10 (41.7) | 16 (64.0) |
| Nugent score | | | |
| 0 | n (%) | 19 (79.2) | 16 (64.0) |
| 1 | n (%) | 3 (12.5) | 3 (12.0) |
| 2 | n (%) | 2 (8.3) | 2 (8.0) |
| 3 | n (%) | 0 (0.0) | 3 (12.0) |

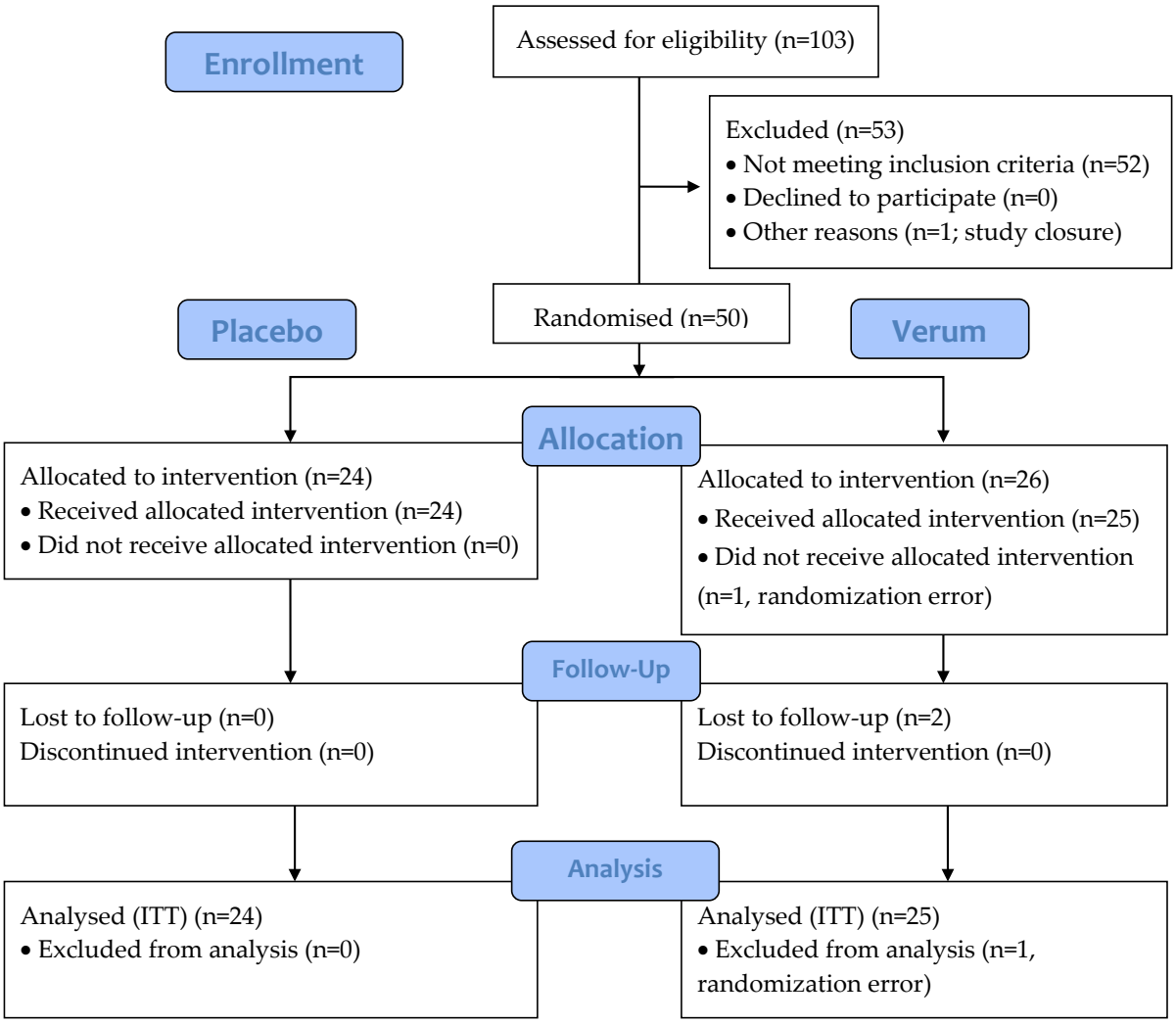


**Figure 2.** CONSORT flow diagram of the 21-day intervention study of triple-blind, randomised, and placebo-controlled design. Placebo: maltodextrin; Verum: $10^{10}$ CFU *Lactobacillus acidophilus* La-14 and *Lacticaseibacillus rhamnosus* HN001. CONSORT, consolidated standards of reporting trials; ITT, intention-to-treat.

### *3.2. Vaginal Colonisation and pH*

DNA extraction was successful from all samples, but the efficiency varied considerably, having a range of 206 to 32,400 ng/swab (median 5340 ng/swab, average 8405 ng/swab, SD 7863 ng/swab) for all 188 samples.

For the primary endpoint, there was no detection of *L. acidophilus* above the assay's LOD 5.29 Log10 genomes per swab in either group at any of the sampling timepoints (Table 5). Two participants in the placebo group showed clear amplification at the follow-up visitV5 for *L. rhamnosus*, although one of them was below LOD (Table 5). The strain-specific qPCR analyses conducted as exploratory outcomes did not detect any amplification above LODs 5.29 or 5.11 log10 genomes per swab for La-14 or HN001, respectively. Thus, no statistical analyses were performed for the primary or exploratory assays on colonisation. IP allocation was confirmed for all participants by culturing samples from the returned IP capsules after the study.

**Table 4.** Details on menstruation cycle and obstetric history for the intention-to-treat population.

| Item | Statistic | Placebo (n = 24) | Verum (n = 25) |
|---|---|---|---|
| Duration of menstruation (days) | n | 18 | 19 |
| | Mean (SD) | 4.83 (1.15) | 4.53 (1.07) |
| | Median | 5.00 | 5.00 |
| | Min, Max | 3.00, 7.00 | 2.00, 7.00 |
| Length of menstrual cycle (days) | n | 18 | 19 |
| | Mean (SD) | 27.61 | 28.47 |
| | Median | 28.00 | 28.00 |
| | Min, Max | 24.00, 29.00 | 25.00, 35.00 |
| Menarche age (years) | n | 24 | 25 |
| | Mean (SD) | 12.63 (1.53) | 13.04 (1.59) |
| | Median | 12.50 | 13.00 |
| | Min, Max | 10.00, 16.00 | 10.00, 16.00 |
| | | n (%) | n (%) |
| Intermenstrual bleeding | N/A | 5 (20.8) | 5 (20.0) |
| | No | 19 (79.2) | 20 (80.0) |
| Menstrual flow | Mild | 6 (25.0) | 5 (20.0) |
| | Moderate | 12 (50.0) | 14 (56.0) |
| Post-coital bleeding | No | 24 (100.0) | 25 (100.0) |
| Leucorrhea | No | 23 (95.8) | 25 (100.0) |
| | Yes | 1 (4.2) | 0 (0.0) |
| Number of pregnancies | 0 | 14 (58.3) | 12 (48.0) |
| | 1 | 4 (16.7) | 3 (12.0) |
| | 2 | 5 (20.8) | 7 (28.0) |
| | 3 | 1 (4.2) | 3 (12.0) |
| Operations | No | 22 (91.7) | 23 (92.0) |
| | Yes | 2 (8.3) | 2 (8.0) |
| Pain in the lower abdomen | No | 24 (100.0) | 25 (100.0) |
| Pelvic or abdominal pelvic pain | No | 24 (100.0) | 24 (96.0) |
| | Yes | 0 (0.0) | 1 (4.0) |
| Presence of vaginal inflammations, dryness, itchiness, stinging | No | 24 (100.0) | 25 (100.0) |
| Sterility | No | 24 (100.0) | 25 (100.0) |
| Vulvovaginal infections | No | 24 (100.0) | 25 (100.0) |

The *L. acidophilus* qPCR assay successfully applied earlier for detecting colonisation [23,30,38] amplified four *L. acidophilus* strains (La-14, DGCC 8698, ATCC 4356, and DGCC 12900) efficiently, but with two peaks repetitively present in the melt curve analysis (a main peak and a shoulder-like second peak). With *L. acidophilus* as standard, 82 °C (dissociation temperature of the main peak) was the dissociation temperature for standard-based quantification of samples in the qPCR analysis. On the other hand, vaginal swab samples (137/141 of the tested samples) and commensal vaginal species *L. crispatus*, *L. gasseri,* and *L. jensenii* all amplified with a uniform amplicon dissociating at 78 °C with a single peak, although PCR-efficiency was poorer. As the amplicon melting point for *L. acidophilus* was 4 °C higher than that of the samples, we were unable to quantify the samples with *L. acidophilus* as standard and had to reject the uniform amplification seen in the samples as background. With *L. crispatus* as standard, quantification of samples was possible and results could be calculated as the number of participants presenting an over 2-fold increase from baseline, as previously conducted by De Alberti and colleagues [30] (Table 6).

**Table 5.** Vaginal colonisation of *Lactobacillus acidophilus* and *Lacticaseibacillus rhamnosus* at species level shown as mean (SD) log 10 genomes/swab. The limit of detection (LOD) values were 5.29 log 10 genomes/swab and 5.11 log 10 genomes/swab for *L. acidophilus* and *L. rhamnosus*, respectively. V, Visit; V2, baseline visit; V3, after one week of intervention; V4, after two weeks of intervention; V5, after a one-week follow-up.

| Assay | Visit | Placebo (n = 20–23) | Verum (n = 23–25) |
|---|---|---|---|
| Genomes detected | | log 10 genomes/swab | log 10 genomes/swab |
| *L. acidophilus* | V2 | <5.29 (0.00) | <5.29 (0.00) |
| | V3 | <5.29 (0.00) | <5.29 (0.00) |
| | V4 | <5.29 (0.00) | <5.29 (0.00) |
| | V5 | <5.29 (0.00) | <5.29 (0.00) |
| *L. rhamnosus* | V2 | 5.12 (0.056) | <5.11 (0.000) |
| | V3 | <5.11 (0.017) | <5.11 (0.000) |
| | V4 | <5.11 (0.009) | <5.11 (0.000) |
| | V5 | 5.12 (0.048) | <5.11 (0.000) |

**Table 6.** Percentage of participants with an over 2-fold increase from baseline in lactobacilli copies per ng of DNA or swab. The analysis applied *Lactobacillus acidophilus* primers designed by Song et al., 2000, with *Lactobacillus crispatus* DSM 20,584 used as the standard. V, Visit; V3, after one week of intervention; V4, after two weeks of intervention.

| Group | % (*n*/*n*) of Participants with > 2-Fold Increase from Baseline in Genome Copies/ng | | % (*n*/*n*) of Participants with > 2-Fold Increase from Baseline in Genome Copies/swab | |
|---|---|---|---|---|
| | V3 | V4 | V3 | V4 |
| Verum | 36 (9/25) | 40 (10/25) | 40 (10/25) | 36 (9/25) |
| Placebo | 30 (7/23) | 15 (3/20) | 26 (6/23) | 30 (6/20) |

Vaginal pH remained stable over the intervention, being 4.0 or 4.5 for at least 95% of the participants during the intervention phase (Supplementary Table S1) and no statistically significant differences in category of pH change (decrease, no change, increase) were found between the intervention groups (Table 7).

**Table 7.** Prevalence of change in vaginal pH during the intervention and follow-up measured with NutraBlast® Feminine pH Test Strip (NutraBlast, Pompano Beach, FL, USA) on each visit. V, Visit; V3, after one week of intervention; V4, after two weeks of intervention; V5, after a one-week follow-up.

| Visit | Categorised Change in Vaginal pH from Baseline | Placebo (n = 22–24) | Verum (n = 23–25) |
|---|---|---|---|
| | | n (%) | n (%) |
| V3 | Decrease | 5 (20.8) | 5 (20.0) |
| | No change | 13 (54.2) | 16 (64.0) |
| | Increase | 6 (25.0) | 4 (16.0) |
| V4 | Decrease | 4 (16.7) | 8 (32.0) |
| | No change | 14 (58.3) | 14 (56.0) |
| | Increase | 4 (16.7) | 3 (12.0) |
| V5 | Decrease | 2 (8.3) | 10 (40.0) |
| | No change | 16 (66.7) | 8 (32.0) |
| | Increase | 5 (20.8) | 5 (20.0) |

### 3.3. Vaginal Microbiota

A total of 176 samples (90 verum and 86 placebo samples) had at least 1000 reads and were included in the analysis. For both groups and all timepoints, Lactobacillaceae was predominant, with *L. crispatus*/*acidophilus*, *L. iners*, and *L. jensenii* being the three most common ASVs within the sequence data (Figure 3; Supplementary Table S2). Community wise, CSTs I (*L. crispatus* dominated), II (*L. gasseri* dominated), and IV (*L. jensenii* dominated) were most prevalent (Table 8). There were no changes detected in the relative abundance of microbial families, genera, or ASVs between visits within the verum or placebo groups (ANCOM, $p > 0.1$), nor did any taxa differ when comparing between the groups at any visit including the baseline (ANCOM, $p > 0.1$). Similarly, the baseline CST distribution did not vary significantly between groups (Fisher's exact test $p$ 0.7547).

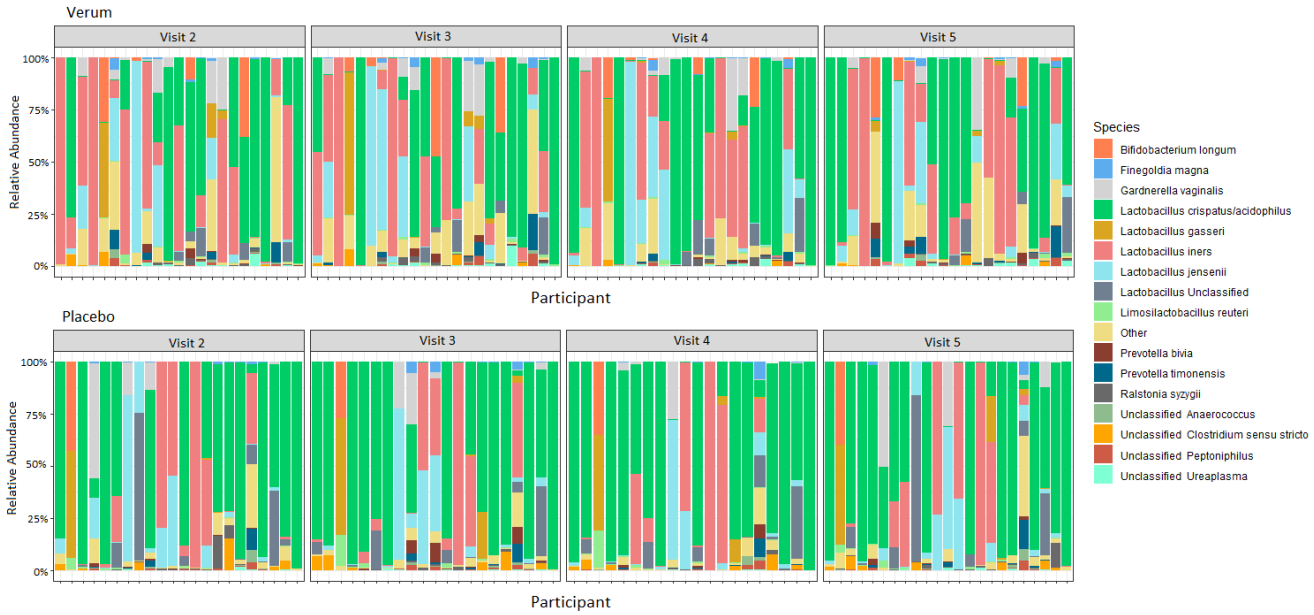


**Figure 3.** Relative abundance of species in the vaginal microbiota for verum and placebo participants consuming $10^{10}$ CFU *Lactobacillus acidophilus* La-14 and *Lactiplantibacillus rhamnosus* HN001, or maltodextrin daily for two weeks between Visits 2 and 4. Visit 2, baseline visit; Visit 3, after one week of intervention; Visit 4, after two weeks of intervention; Visit 5, after a one-week follow-up.

**Table 8.** Community state type (CST) distribution of sequenced samples per group and visit. V, visit; V2, baseline visit; V3, after one week of intervention; V4, after two weeks of intervention; V5, after a one-week follow-up.

| Group | Visit | Samples | CST I | CST II | CST III | CST IV-B | CST IV-A | CST V |
|---|---|---|---|---|---|---|---|---|
| | | n | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) |
| Verum | All visits | 90 | 38 (42.2) | 3 (3.3) | 26 (28.9) | 3 (3.3) | 5 (5.6) | 15 (16.7) |
| | V2 | 23 | 9 (39.1) | 1 (4.3) | 8 (34.8) | 1 (4.3) | 1 (4.3) | 3 (13.0) |
| | V3 | 23 | 8 (34.8) | 1 (4.3) | 5 (21.7) | 1 (4.3) | 3 (13.0) | 5 (21.7) |
| | V4 | 22 | 10 (45.5) | 1 (4.5) | 7 (31.8) | 0 (0.0) | 0 (0.0) | 4 (18.2) |
| | V5 | 22 | 11 (50.0) | 0 (0.0) | 6 (27.3) | 1 (4.5) | 1 (4.5) | 3 (13.6) |
| | Placebo | All visits | 86 | 56 (65.1) | 4 (4.7) | 14 (16.3) | 1 (1.2) | 2 (2.3) | 9 (10.5) |
| Placebo | All visits | 86 | 56 (65.1) | 4 (4.7) | 14 (16.3) | 1 (1.2) | 2 (2.3) | 9 (10.5) |
| | V2 | 22 | 13 (59.1) | 1 (4.5) | 4 (18.2) | 1 (4.5) | 2 (9.0) | 1 (4.5) |
| | V3 | 21 | 14 (66.7) | 1 (4.8) | 3 (14.3) | 0 (0.0) | 0 (0.0) | 3 (14.3) |
| | V4 | 20 | 14 (70.0) | 1 (5.0) | 3 (15.0) | 0 (0.0) | 0 (0.0) | 1 (5.0) |
| | V5 | 23 | 15 (65.2) | 1 (4.3) | 4 (17.4) | 0 (0.0) | 1 (4.3) | 2 (8.7) |

No difference was seen in within-sample species diversity (α-diversity) between groups or timepoints. For between-sample dissimilarity (β-diversity), the percent of variation that could be attributed to the study factors was mainly due to individual (77%) and, to a lesser extent, to CST grouping (10%), intervention (3%), BMI (3%), and Nugent score at screening (1.5%, and age (1.5%), whereas visit (time) did not have a significant influence on the observed diversity (Figure 4).

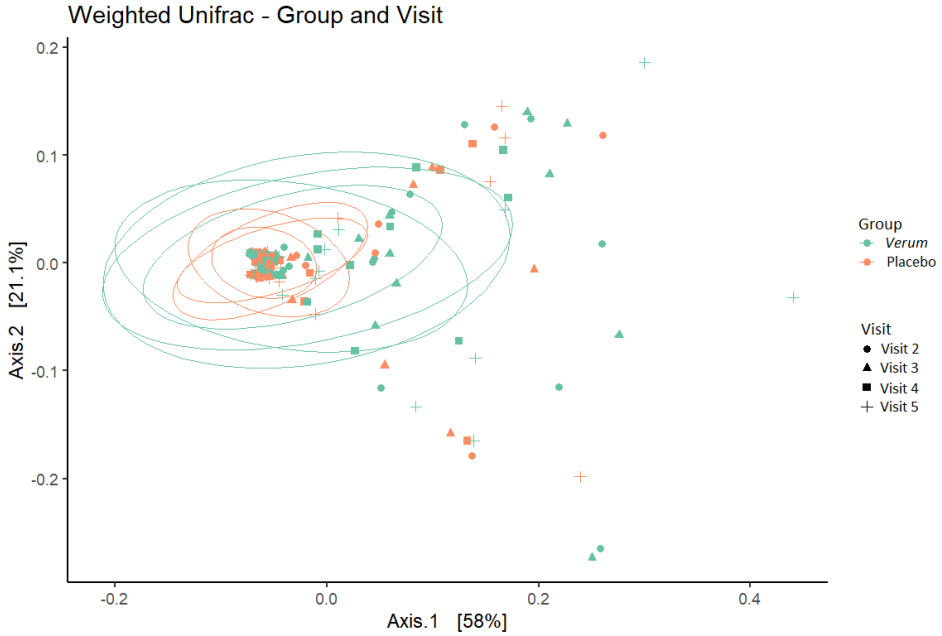


**Figure 4.** β-diversity (weighted UniFrac metric) depicting sample clustering of the vaginal microbiota samples from study participants by intervention group and visit in a principal coordinate analysis (PCoA) plot. Ellipses represent 95% confidence interval. Visit 2, baseline visit; Visit 3, after one week of intervention; Visit 4, after two weeks of intervention; Visit 5, after a one-week follow-up.

Abundances of families, genera, and ASVs were comparable between verum and placebo and remained stable over time. One participant from the placebo group, with *L. rhamnosus* also detected in qPCR, had ASVs assigned to *L. rhamnosus*. Table 9 shows the percentage of participants having an over 1% change in the relative abundance of ASVs assigned to *G. vaginalis* and predominant *Lactobacillus* during the intervention. The number of participants with over or under 1% change in ASV abundance did not differ significantly between groups or timepoints (Fisher's exact test).

With all samples combined (both groups and all visits) the relative abundance of ASVs assigned to *Prevotella, Finegoldia, Campylobacter ureolyticus,* and *Streptococcus anginosus* correlated positively, whereas ASVs within Lactobacillaceae did not show correlation. Of the demographic variables, BMI correlated positively with *Gardnerella vaginalis* and *Campylobacterium ureolyticus* ($p$ for Spearman correlation < 0.01 with analyses limited for ASVs present in at least 40% of participants).

### *3.4. Vaginal Immune Markers*

For the immune marker analysis (Table 10; Supplementary Figure S1), log-transformed data were used for SLPI, elafin, and IgA, whereas square root transformed data were used for HBD-1 and HBD-3 that did not fulfil the model assumptions for log transformation. HBD-2 had 17%, 39%, 34%, and 31% of the values below the LLOQ for timepoints V2, V3, V4, and V5, respectively, and was thus tested with non-parametric methods using 31.25 pg/mL (LLOQ divided by 2) imputed for the missing values before transformation. There were no significant differences regarding HBD-2 for any of the visits; hence, it is not included in the Table 10.

**Table 9.** Percentage of participants with over 1% change in relative abundance of amplicon sequence variants (ASVs) assigned to predominant *Lactobacillus* species or *Gardnerella vaginalis*. V, Visit; V3, after one week of intervention; V4, after two weeks of intervention; V5, after a one-week follow-up.

| Group | Visit | *N* | *Gardnerella vaginalis* | *Lactobacillus* spp. | *Lactobacillus crispatus* | *Lactobacillus jensenii* | *Lactobacillus gasseri* | *Lactobacillus iners* |
|---|---|---|---|---|---|---|---|---|
| Percentage of participants with >1% increase in the relative abundance of an ASV. n (%) | | | | | | | | |
| Verum | V3 | 23 | 4 (17) | 3 (13) | 5 (22) | 7 (30) | 3 (13) | 4 (17) |
| | V4 | 22 | 3 (14) | 4 (18) | 7 (32) | 7 (32) | 2 (9) | 6 (27) |
| | V5 | 22 | 4 (18) | 2 (9) | 8 (36) | 5 (23) | 0 (0) | 7 (32) |
| Placebo | V3 | 20 | 4 (20) | 3 (15) | 5 (25) | 4 (20) | 3 (15) | 3 (15) |
| | V4 | 19 | 2 (11) | 4 (21) | 8 (42) | 2 (11) | 2 (11) | 4 (21) |
| | V5 | 22 | 4 (18) | 3 (14) | 7 (32) | 5 (23) | 2 (9) | 2 (9) |
| Percentage of participants with >1% decrease in the relative abundance of an ASV. n (%) | | | | | | | | |
| Verum | V3 | 23 | 4 (17) | 2 (9) | 6 (26) | 1 (4) | 1 (4) | 9 (39) |
| | V4 | 22 | 5 (23) | 1 (5) | 5 (23) | 2 (9) | 0 (0) | 7 (32) |
| | V5 | 22 | 5 (23) | 1 (5) | 3 (14) | 4 (18) | 3 (14) | 7 (32) |
| Placebo | V3 | 20 | 0 (0) | 2 (10) | 6 (30) | 4 (20) | 0 (0) | 4 (20) |
| | V4 | 19 | 1 (5) | 1 (5) | 5 (26) | 5 (26) | 1 (5) | 3 (16) |
| | V5 | 22 | 2 (9) | 3 (14) | 7 (32) | 6 (27) | 1 (5) | 5 (23) |

**Table 10.** The effect of the supplementation on vaginal immune markers during the intervention (V3 and V4) and follow-up (V5). HBD, Human β-defensin; IgA, Immunoglobulin A; SLPI, Secretory leukocyte protease inhibitor; V, Visit; V3, after one week of intervention; V4, after two weeks of intervention; V5, after a one-week follow-up.

| Analysis Method | Parameter | Visit | Verum vs. Placebo | *p* |
|---|---|---|---|---|
| RM-ANCOVA (log-transformed) | SLPI ($p$ = 0.15) | V3 | −0.194 (−0.793, 0.406) | 0.52 |
| | | V4 | −0.148 (−0.657, 0.362) | 0.56 |
| | | V5 | −0.488 (−0.988, 0.013) | 0.056 |
| | Elafin ($p$ = 0.022) | V3 | −0.310 (−0.648, 0.028) | 0.071 |
| | | V4 | −0.305 (−0.626, 0.015) | 0.062 |
| | | V5 | −0.318 (−0.640, 0.004) | 0.051 |
| | IgA ($p$ = 0.82) | V3 | 0.038 (−0.401, 0.477) | 0.86 |
| | | V4 | −0.079 (−0.577, 0.420) | 0.75 |
| | | V5 | −0.083 (−0.625, 0.460) | 0.76 |
| RM-ANCOVA (square-root-transformed) | HBD-1 ($p$ = 0.97) | V3 | −1.48 (−11.78, 8.83) | 0.77 |
| | | V4 | 2.50 (−7.42, 12.43) | 0.61 |
| | | V5 | −1.51 (−9.40, 6.38) | 0.70 |
| | HBD-3 ($p$ = 0.028) | V3 | −6.34 (−24.72, 12.05) | 0.49 |
| | | V4 | −13.86 (−31.69, 3.97) | 0.12 |
| | | V5 | −22.70 (−39.03, −6.37) | 0.008 |

The RM-ANCOVA analysis showed statistically significant differences between the study groups for elafin ($p$ = 0.022) and HBD-3 ($p$ = 0.028) over all visits (Table 10). For elafin, the comparisons performed on single visits were not statistically significant ($p$-values ranging from 0.051 to 0.071; however, the $p$-values can be considered as a trend towards significance. The difference appeared to be consistent over all post-baseline visits, in an agreement with the

overall statistical difference. In addition, the results indicated a possible similar decreasing trend for another protease inhibitor SLPI ($p = 0.15$ overall and $p = 0.056$ at V5).

HBD-3 also showed an overall statistically significant decrease in verum compared to placebo with an increasing numerical trend in the difference between the groups adjusted for baseline. On the follow-up visit (V5), the difference between the groups was statistically significant ($p = 0.008$).

Correlations were assessed for immune markers and 16 ASVs that were present in at least 40% of the participants (i.e., *Lactobacillus* spp., *L. crispatus/acidophilus*, *L. jensenii*, *Limosilactobacillus reuteri*, *L. iners*, *Fenollaria massiliensis*, *Finegoldia magna*, *Streptococcus angiosus*, *Campylobacter ureolyticus*, *Prevotella bivia*, *Prevotella disiens*, *Prevotella timonensis*, *Peptoniphilus* spp., *Rastolnia syzgii*, and *G. vaginalis* presented by two separate ASVs). Statistically significant ($p < 0.05$) correlations were found for HBD-1, HBD-3, and IgA, but not for HBD-2, elafin, and SLPI, as detailed in Table 11; Supplementary Figure S2.

**Table 11.** Repeated measures correlations between normalised reads of 16 ASVs that were present in at least 40% of the participants and immune markers. The verum and placebo groups and all timepoints were joined for the analysis. Only significant results are shown. HBD, Human β-defensin; IgA, Immunoglobulin A; NS, not significant.

| | ***Finegoldia magna*** | ***Gardnerella vaginalis*** | ***Lactobacillus crispatus/acidophilus*** | ***Lactobacillus jensenii*** | ***Prevotella bivia*** |
|---|---|---|---|---|---|
| | | | **Average R-Value (*p*)** | | |
| HBD-1 | −0.173 (0.05) | NS | NS | NS | −0.231 (0.01) |
| HBD-3 | −0.2 (0.02) | −0.197 (0.03) | NS | −0.193 (0.03) | NS |
| IgA | NS | NS | −0.258 (0.004) | NS | NS |

When comparing levels of immune marker for each CST at baseline (verum and placebo grouped combined), a trend of higher SLPI and lower HBD-3 was seen in CST I compared to CST III (Wilcoxon test $p = 0.0918$ and $p = 0.1221$, respectively; Supplementary Figure S3). CST II and IV were not included in the analyses due to the prevalence of them at baseline being too low (Table 8).

### *3.5. Safety*

Only four participants had IP compliance < 80% (two in verum and two in placebo). IP stability was confirmed to be above target potency ($10^{10}$ CFU/capsule) before initiating the clinical phase and after the last participant had completed the study. There were no differences in vital signs, menstruation, or vaginal health between groups during the study. After randomisation, only one participant reported reddening of vulva and/or vagina (verum group). Otherwise, all the participants had normal smell of vaginal secretion, normal vaginal appearance, no menstrual bleeding, and no reddening of the vulva and/or vagina.

There were 34 AEs associated with the intervention recorded in 21 participants. These were balanced equally between the verum (17 events in 11 participants) and placebo (17 events in 10 participants) groups. A total of 11 AEs were classified as potentially related to the IP (for three participants in the placebo group and four participants in the verum group). The most common organ class affected by AEs was the gastrointestinal system (total 14 events). These were equally divided between verum (7) and placebo (7). There were no severe AEs or serious adverse events and no events requiring withdrawal from the study.

## 4. Discussion

A BV-associated microbiota resembles that of CST IV, with pronounced abundance of members from the genera *Gardnerella, Atopobium*, and *Prevotella*, whereas lactobacilli-predominance is associated with a healthy-like vaginal microbiota [7,9,10]. Both species richness and diversity elevate with BV and recurrent BV [7,10]. The Nugent score (0–10), which is used for BV diagnosis, correlates negatively with lactobacilli abundance and positively with microbial diversity [7]; when BV is treated with an antibiotic (5-day

metronidazole course), lactobacilli numbers start to elevate, especially *L. iners*, and the microbial diversity (α-diversity) is restored even within 8 days, with no significant differences between relative abundances of bacterial taxa remaining after 15 days.

These depleted vaginal lactobacilli levels, associated with BV and increased disease risk [58,59], can potentially be normalised with orally consumed probiotics [23–26], though further systematic research on the efficacy and safety of strain specific effects of probiotics is warranted [60]. For the probiotic strains applied for vaginal health, the ability to locally colonise the vagina has been considered a beneficial trait, although the colonisation may be transient and the mode of action in vivo remains obscured [20,21]. Thus, our objective in the present study was to assess the vaginal colonisation potential of two orally consumed probiotic strains in premenopausal women without vaginal complaints, and to evaluate whether there were any intervention-related alterations on a well-balanced commensal vaginal microbiota or immune marker profile.

Vaginal colonisation of La-14 or HN001 on species or strain level was not detected with the applied methods allowing detection of 5.29 and 5.11 Log10 genomes per swab, respectively (Table 5). Previously La-14 and HN001 have been shown to elevate vaginal *L. acidophilus* and *L. rhamnosus* levels in a comparable study setting with calculations based either on all DNA extracted from the vaginal swab (per swab) or on mass of DNA (per 100 ng of DNA) [23,30], neither allowing inference of the actual target DNA quantities per swab. In the study by Russo and colleagues [23], the baseline and end-of-intervention quantities can be estimated to have been approximately 2.2 and 2.8 Log10 genomes per ng of DNA for *L. rhamnosus* and 3.3 and 3.9 Log10 genomes per ng of DNA for *L. acidophilus*, respectively [23]. For orally supplemented *Lacticaseibacillus paracasei* LPC-S01, the vaginal colonisation on strain level was detected at approximately 4–6 logs below the level of total bacteria present in a vaginal sample, ranging up to approximately 5 Log10 cells/swab [27]. Moreover, Hertz and colleagues have published a non-controlled clinical study assessing the vaginal microbiota from 16 women with self-reported good health during oral probiotic consumption (*L. rhamnosus* PB01 and *L. gasseri* EB01) applying shotgun metagenomics [61]. From the intervention period samples, with discriminatory single nucleotide polymorphisms (DSNPs), supplemented strain associated reads were detected from 3/16 participants at or below 1.4% of the supplemented species-associated reads or 0.004% of the total microbiota, suggesting that if the strains were present, their abundance was very low. Unfortunately, the baseline samples were not assessed for DSNPs and there was no placebo control. However, comparison between units, studies, and even samples within a study is hindered by the high variance in DNA extraction efficiency from the vaginal swabs seen in our study (up to 150-fold difference) and in previous studies [56]. Regardless, we would also have expected to be able to detect potential vaginal colonisation with the assays we optimised and applied in our study, although at a low level.

For the *L. rhamnosus* assay we applied the same primers as were used in prior studies [23,30,38], but with a higher annealing temperature. For *L. acidophilus,* we applied a different assay [44] than the one used in prior studies [23,30,38] due to a persistent double-peak dissociation curve generated from a *L. acidophilus* standard (DGCC 8698 and La-14 both tested as the standard strain through extensive reaction condition optimisation steps) suggesting suboptimal performance of the earlier applied assay, i.e., that there were either two different amplicons being generated during the qPCR, or that the amplicon had a secondary DNA structure leading to a step-wise dissociation. The assay did, however, amplify DNA extracted from the clinical study vaginal swab samples and from pure cultures of *L. crispatus, L. gasseri,* and *L. jensenii,* resulting in a uniform single-peak dissociation curve. Due to a difference in the melting point temperatures of these two PCR products, quantification of the samples with *L. acidophilus* as the standard was not possible with SYBR chemistry. However, when we changed the standard to *L. crispatus* (likely *L. jensenii* or *L. gasseri* would have performed similarly as a standard), we were able to quantify the clinical samples (Table 6), Our results resemble those published by De Alberti and colleagues [30], albeit without showing a significant difference between groups, potentially due to the very

high vaginal lactobacilli levels confirmed already present at baseline in our study. Moreover, De Alberti and colleagues detected *L. rhamnosus* and *L. acidophilus* in both groups: placebo and verum [30]. The prior clinical studies detecting vaginal colonisation of *L. acidophilus* and *L. rhamnosus* after La-14 and HN001 consumption [23,30] do mention confirming the dissociation curve quality visually, but not comparing it with a standard. Thus, it remains unclear whether the detected elevation from baseline in the study by De Alberti et al. [30] and Russo et al. [23] could have reflected an increase in commensal *Lactobacillus* spp., including *L. crispatus*, *L. gasseri*, *L. jensenii*, and alike, rather than an increase solely of the supplemented species, *L. acidophilus*. The former would potentially have an even more significant health benefit, and, indeed, the product tested has been shown to reduce the Nugent score indicating an increase in commensal *Lactobacillus* spp. [23]. Moreover, the 50 mg of lactoferrin included in the Respecta® complex [23,30] may also contribute to the difference between groups in vaginal lactobacilli levels: in a 30-day pilot intervention study on pregnant women, a four times higher daily dose of orally supplemented lactoferrin without probiotics was shown to increase the prevalence of a normal vaginal microbiota, defined as the predominance of variable size lactobacilli in phase contrast microscopy of a vaginal swab [37].

We had recruited participants devoid of vaginal complaints (Nugent score 0–3 and vaginal pH $\leq$ 4.5) into an intervention with lactobacilli being orally supplemented as the test ingredient expecting the vaginal pH and microbiota to remain relatively stable. Both outcomes were followed to assess the safety of the verum IP and potential intervention-related fluctuations in a set-up not assessing treatment or prevention. The vaginal pH remained stable and at a healthy level throughout the study, being $\leq$ 4.5 for almost all participants in both groups at all study visits, although no statistically significant changes within a healthy pH range were detected (Supplementary Table S1; Table 7). The pH results corresponded with De Alberti et al. [30]. The vaginal microbiota was lactobacilli predominant in both groups, with no intervention related changes detected (Figure 3; Supplementary Table S2); this was also reflected by the high prevalence of lactobacilli dominated CSTs (I, II and V; Table 8). Numerically intriguing results were observed between groups for the percentage of participants with >1% reduction in *G. vaginalis* and *L. jensenii* ASVs for the benefit of the verum IP, but the difference was not statistically significant (Table 9). The positive correlation noted between non-lactobacilli species but not among different *Lactobacillus* spp. can be seen as characteristic of vaginal microbiota, which tend to be either a mixed type (CST IV) or predominated by one *Lactobacillus* species (CST types I, II, III, and IV) [1]. BMI, which had a wide range at baseline in the current study (Table 3), was found to correlate positively with *G. vaginalis* and *C. ureolyticus*, in alignment with earlier findings by Allen and colleagues [62]. Thus, restricting BMI range at recruitment or stratifying for BMI at randomisation could be advisable in future studies assessing vaginal microbiota.

The noted wide range in DNA extraction efficiency was likely due to varying amounts of host DNA carryover during DNA extraction and may have challenged detection of intervention-emergent changes among less abundant ASVs. DNA extracted from vaginal swab samples may contain over 95% of host DNA if host DNA is not removed during the extraction process [63]. As an example, 90% host DNA carryover already prevents detection of 16S rDNA reads that are present as 0.1–1% of total reads in a metagenomic sequence analysis [64]. Indeed, DNA extraction efficiency has been inversely linked with 16S rDNA sequencing-based microbial diversity in a study comparing different DNA extraction methods for vaginal swabs [65]. In our study, colonisation was not detected with the sequencing analysis either. For *L. acidophilus*, this would not have been possible as the 16S rDNA V4 region does not differentiate between *L. acidophilus* and *L. crispatus*, but since the V4 region distinguishes species relevant for vaginal microbial disturbances (e.g., *G. vaginalis*), it was selected.

We had aimed to repeat the results published by De Alberti and colleagues, reporting elevation of *L. acidophilus* and *L. rhamnosus* among women with no vaginal complaints dur-

ing a 2-week intervention with La-14 and HN001 [30]; thus, we also recruited participants devoid of vaginal symptoms. To add a non-subjective measure for vaginal health, we used Nugent score (0–3) as an inclusion criterion, although it had not been applied in the prior study. In our study, Nugent score was not measured over the course of the study, but both vaginal pH and the sequencing results indicate healthy vaginal microbiota throughout the intervention. A prior observational study assessing the vaginal microbiota before and after a 5-day metronidazole treatment for BV and that of healthy controls has shown a clear increase in the vaginal microbiota diversity already at Nugent 1–3 in comparison to Nugent 0 [7]. By chance, the screening visit Nugent scores of the eligible randomised participants were predominantly 0 (Nugent was 0 for 79.2% and 64.0% of verum and placebo participants, respectively), although Nugent 0–3 was permissible. Consequently, the study population in our study may have been especially challenging for detecting colonisation. Even when locally applied at a daily dose of $2 \times 10^6$ or $2 \times 10^8$ CFU for three days, *L. crispatus* CTV-05 colonisation has been shown to be significantly more likely during weekly follow-up visits among women initially devoid of commensal *L. crispatus* within their vaginal microbiota and not practicing unprotected vaginal intercourse [66]. Additionally, with vaginally applied probiotics, Marcotte and colleagues have reported colonisation to be more efficient among women with BV receiving antibiotic treatment than among women within the healthy control group devoid of BV and not subject to an antibiotic course [67], suggesting that perturbations in the vaginal microbiota enhance the colonisation efficiency of vaginally applied probiotics. Oral supplementation predisposes the vagina to much lower quantities of the supplemented strain(s) than local application; thus, comparable competitive and environmental factors may play an even more significant role. Although *L. acidophilus*, being a gut-oriented lactobacillus, adapts well to simulated vaginal fluid, it would unlikely be able to successfully compete with abundant endogenous vaginal lactobacilli such as *L. crispatus*, *L. gasseri*, and *L. jensenii* [68]. Aligned, Chen and colleagues found the effects of an oral probiotic to be more probable in the vaginal microbiota of participants with higher intraindividual variability within the vaginal microbiome already before the intervention commenced, whereas participants with a stable and lactobacilli-rich vaginal microbiota at baseline were more resilient to the effects of the probiotic [33]. The study followed 60 Chinese female participants for over a year with multiple baseline samples, and intervention and follow-up samples collected from the oral cavity, feces, and vagina. Colonisation was not shown; rather, a potential for the increase of commensal vaginal lactobacilli was indicated [33]. A further unplanned confounder was that our study took place between November 2020 and March 2021 in Glasgow, UK, while everyday life was greatly restricted due to the Covid-19 pandemic and participants were mostly home bound and obligated to social distancing. This almost certainly resulted in reducing intrusions to microbial balance, also in the vagina, due to less social and environmental contact, elevated hygiene practices, and potentially a less versatile diet [69], and may have further enhanced the resilience of participants to probiotic colonisation above detection limit.

The current study also provides insights on the effects of probiotics on the mucosal immune system modulation in a healthy vaginal tract. The participants in the verum group showed statistically significant decrease in elafin and HBD-3, with a similar trend in SLPI, when compared with the placebo (Table 10). Interestingly, reduction in vaginal SLPI has also been observed, albeit in vitro, with another strain: *L. rhamnosus* Lcr35 [70]. According to the literature, it is well established that the presence of *Lactobacillus* spp. in healthy vaginal microbiota has an important role in competitive exclusion of pathogenic bacteria, competition for nutrients, production of antimicrobial substances, and on the immune system [59,71]. In this context, Jiang et al. [72] showed a significant correlation between HBD-2 and HBD-3 and DNA levels of *L. jensenii*, as well as HBD-2 and DNA levels of *L. crispatus*, in cervicovaginal lavage samples from healthy women. In our study, we found significant correlation of HBD-3 with *L. jensenii* and of IgA with *L. crispatus* in 40% of participants irrespective of intervention/group (Table 11). In another study, Orfanelli et al. [73] investigated the association of SLPI concentrations with CST in healthy

women. They showed that median levels of SLPI was three times higher when the vaginal microbiota was dominant with *L. crispatus* than when it was dominant with *L. iners*. While analyzing the concentration of immune markers at baseline, combined for the verum and the placebo, we also observed a trend of higher SLPI and lower HBD-3 in CST I compared to CST III (Supplementary Figure S3). The other CSTs were not accounted for due to their low prevalence in the population. As mentioned above, commensal bacteria in the vagina represent one of the first lines of defense against vaginal infection, and some *Lactobacillus* species, in particular *L. crispatus*, play important roles in this defense, thus regulating the vaginal immune system [6]. Hence, the small decrease in immune marker levels of HBD-3 and SLPI in the verum group (Table 10) could hypothetically reflect a small change of *Lactobacillus* species composition (or metabolites produced) within the population that we could not detect with 16S sequencing (Figure 3). Indeed, in healthy vaginal mucosa, probiotics more likely promote immunological homeostasis rather than inflammation. Given the small sample size, large standard deviation, and stable vaginal health and microbiota, further exploration in a more plausible setting is needed to have a better understanding of the intervention effect on the immune markers.

Taken together, the high and constant lactobacilli predominance observed in the vaginal swab samples of the current study likely left less of a niche for non-commensal lactobacilli strains to colonise the vagina and increased the technical challenges in detecting any potential colonisation, if such existed. DNA extracted from vaginal swab samples are bound to have high but varying amounts of host DNA carryover and, if from healthy volunteers, commensal microbiota likely consisting predominantly of lactobacilli. This underlies the need for stringent optimisation procedures and interpretation of results when PCR-based analyses are applied to detect low levels of supplemented lactobacilli. The stability of the healthy-like vaginal microbiota and pH throughout the study highlights the safety of using La-14 and HN001 as over-the-counter supplements without the risk of jeopardising well-balanced vaginal microbiota. Vaginal immune markers, elafin, HBD-3, and SLPI, should be evaluated further in future probiotic intervention studies elucidating the effects of the supplementation on vaginal health. No safety concerns were raised regarding vital signs, menstruation, vaginal health, or AEs.

## 5. Conclusions

Colonisation of La-14 or HN001 was not observed above the detection limit 5.29 and 5.11 Log10 genomes per vaginal swab, respectively. Oral supplementation of La-14 and HN001 at $10^{10}$ CFU in a 4:1 ratio for two weeks had no aberrant effects on stable and healthy commensal vaginal microbiota and immunological homeostasis.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11020499/s1, Table S1: Prevalence of vaginal pH results measured with NutraBlast® Feminine pH Test Strip (NutraBlast, Pompano Beach, FL, USA ) on each visit; Table S2: Average, SD, minimum and maximum relative abundance values of amplicon sequence variants (ASVs) detected in at least 2 participants per group and visit in the 16S rDNA sequencing analysis; Figure S1: Immune marker box plots by group and visit; Figure S2: Significant correlations detected between immune markers and amplicon sequence variants (ASVs); Figure S3: Immune marker concentrations at baseline for community state types (CSTs).

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**Data Availability Statement:** Data generated during the study is not publicly available due to lack of consent from the study participants. The study is registered at https://www.isrctn.com/ (accessed on 14 February 2023) under identifier ISRCTN29375062.

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# Randomised Clinical Trial in Women with Recurrent Vulvovaginal Candidiasis: Efficacy of Probiotics and Lactoferrin as Maintenance Treatment - Russo et al. 2018

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# Randomised clinical trial in women with Recurrent Vulvovaginal Candidiasis: Efficacy of probiotics and lactoferrin as maintenance treatment

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## Summary

**Background**: Vulvovaginal candidiasis (VVC) is a recurrent vaginal condition in childbearing women.

**Objectives**: The aim of this study was to assess the efficacy of an oral formulation containing *Lactobacillus acidophilus* GLA-14, *Lactobacillus rhamnosus* HN001 and bovine lactoferrin on symptoms and recurrence of VVC as adjuvant therapy to topical clotrimazole.

**Patients/Methods**: Forty-eight women positive for *C. albicans*, symptoms of VVC and documented history of recurrences were randomised into 2 groups receiving verum or placebo (2 capsules/day for 5 days followed by 1 capsule/day for additional 10 days)a sa djuvant treatment to clotrimazole (induction phase) followed by a maintenance cycle of 6 months (1 capsule/day verum or placebo for 10 consecutive days eachm onth). Symptoms, overall cure rate and recurrence rate were assessed.

**Results**: After clotrimazole therapy, a significant improvement of symptoms was shown in both groups. However, only women treated with probiotics and lactoferrin showed a significant improvement of itching and discharge at 3 and 6 months. During the six-month follow-up, recurrences were significantly less in the intervention group vsp lacebo (33.3% vs 91.7% after 3 months and 29.2% vs 100% after 6 months).

**Conclusions**: The results show that the investigated lactobacilli mixture in combination with lactoferrin represents a safe and effective adjuvant approach for reducing symptomsa nd recurrences of RVVC.

KEYWORDS

Bovine lactoferrin, *Candida* spp., *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, Respecta®, vulvovaginal infections

## 1 | INTRODUCTION

Vaginitis is a common gynaecologic disorder that is responsible for 10 million office visits to physicians each year. In particular, fungal infection of the vulva and the vagina is estimated to be the second most common cause of inflammation after bacterial vaginosis.[1] About 75% of women during their reproductive age have at least one episode of vulvovaginal candidiasis (VVC)[2] and approximately half of them have two or more episodes. The most common pathogen is *Candida albicans*, which is isolated in 85% to 90% of all cases.[3] Asymptomatic colonisation with *Candida* spp. is also common. It can be found in about one-third of women without any symptoms and was identified in 70% of women during a 1-year observation period.[4] Recurrent vulvovaginal candidiasis (RVVC) is defined as the 4 or more episodes of symptomatic VVC within 1 year.[5] RVVC is less common than acute form, affecting up to 9% of women of reproductive age

but can greatly affect the quality of life of women, causing symptoms such as itching and soreness of the vulva, dyspareunia, dysuria and the classic "cottage cheese-like" discharge.[5] It is most commonly caused by *Candida albicans*, but other non-*albicans* species such as *C. glabrata*, *C. krusei*, *C. tropicals* and *C. parapsilosis*, although less frequent, are more commonly associated with recurrence.[6,7] An increased rate of vaginal colonisation represents a phase of susceptibility to RVVC and causes may be genetic, biologic, or behavioural: diabetes, atopy, antibiotic or corticosteroids use, vaginal douching, orogenital sex etc. Not only, but also some primary or idiopathic RVVC defines women in whom secondary precipitating events or triggering factors are not apparent and hence genetic factors are likely to play a dominant or exclusive mechanism.[8] It is proposed that both RVVC and VVC involve similar immunopathologies but that the triggers occur with greater sensitivity in individuals with RVVC. Women with RVVC (approximately 150 million worldwide) result in idiopathic chronic episodes of vaginal irritation that require antifungal maintenance therapy (eg, azoles) to partially control symptoms.[9] Although these treatments are typically effective at reducing organism burden and symptoms, the static function of azole activity and fungal recalcitrance to clearance are key factors resulting in recurrence. The most widely used form of treatment involves an intense induction therapy with antifungals, followed by an extended period of maintenance therapy for some months.[10-12] Despite this is being generally accepted as standard treatment, many variations exist including different therapeutic agents and durations.[13,14]

Current evidence suggests that in the case of induction and maintenance therapy with fluconazole, approximately 50% of patients experience relapse after 6 months.[15,16] Studies evaluating the effectiveness of probiotics in preventing RVVC have shown conflicting results. A report by De Seta et al[17] evaluated the effectiveness of local *Lactobacillus plantarum* P17630. One group of patients received the standard treatment with local clotrimazole for 3 days and the other group had additional probiotic capsules applied intravaginally for 6 days and then once weekly for 4 weeks. They concluded that local *Lactobacillus plantarum* P17630 offers potential benefit for resolution of vaginal discomfort but no data are showed for the recurrence rate. Results from a review study[18] support the effectiveness of both oral and local lactobacilli in preventing new episodes of vaginitis, particularly *Lactobacillus rhamnosus* GR-1, *Lactobacillus fermentum* RC- 14 and *Lactobacillus acidophilus*, whereas other studies did not prove the effectiveness of lactobacilli.

De Seta et al[19] published additional evidence supporting the use of specific lactobacilli able to reduced significantly the recurrence of *Candida* spp. infection. Until today, published studies show several limitations including the small sample size, absence of placebo group, high heterogeneity of probiotic strains, dose and treatment duration.[18] It should be emphasised that the various probiotic strains have different properties and different effects on *Candida* spp.; thus, results from studies testing one strain should not be extrapolated to other strains. Consequently, it is difficult to draw reliable conclusions from the existing studies. Prospective, randomised, double-blind, placebo-controlled trials able to assess vaginal colonisation and clinical outcomes are very few. A recent Cochrane review compared conventional antifungal drugs used as single treatment to probiotics as adjuvant therapy for enhancing short-term (5-10 days) clinical and mycological cure and relapse or recurrence episodes over time. Adjunctive treatment does not seem to influence the rate of long-term (within 1 to 3 months) clinical cure, long-term mycological cure, serious and non-serious side events. Up to the present, due to the low quality of data available, there is insufficient evidence for the use of probiotics either as adjuvants to conventional antifungal medicines or used alone for the treatment of VVC in non-pregnant women.[20]

In addition to lactobacilli, among a variety of natural substances, lactoferrin seems to be one of the most interesting compound for reducing the risk of vaginal candida infection. Lactoferrin is an iron-binding glycoprotein naturally present in mammals' secretions including milk and cervical mucus. It is stored in the secondary granules of neutrophils from where it is released in inflamed sites.[21,22] In vitro evidence showed that both human and bovine lactoferrin are able to inhibit the growth of *C. albicans* and *C. glabrata* as well as the production of inflammatory cytokines.[23,24] Recent findings showed that lactoferrin was able to improve the biofilm production of two lactobacilli strains cultured on HeLa cell monolayer thus enhancing their protective action against pathogen bacteria adhesion to genital cells.[25]

The aim of the current study was to evaluate clinical cure rate and recurrences of infection at 1, 3 and 6 months in women with RVVC treated with oral probiotics in combination with bovine lactoferrin as adjuvant therapy after an induction phase with clotrimazole.

## 2 | PATIENTS AND METHODS

### 2.1 | Study design and general procedures

The current double blinded, prospective, randomised, clinical trial was carried out in accordance with Good Clinical Practices and the World Medical Association (WMA) Declaration of Helsinki regarding the Ethical Principles for Medical Research Involving Human Subjects adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964 and its following amendments. The study protocol was approved by the National Commission on Bioethics of Drugs and Medical Devices (Bucharest, approval number 2DM/26.01.2016 14 March 2016).

Forty-eight women with positive cultures for *Candida* spp., symptomatic acute episode of VVC and with documented anamnestic history of recurrences confirmed by culture analysis were enrolled according to the inclusion and exclusion criteria (Table 1). Women were informed about the study protocol, procedures, investigational product and potential risks of treatment. After signing the informed consent, women were randomly divided into 2 groups (24 subjects per group) according to a computer randomised 1:1 scheme to receive either verum or placebo as supplementary treatment to antifungal drug with an induction scheme with topical clotrimazole. Figure 1 shows the study flow chart.

**TABLE 1** Inclusion and exclusion criteria

| Inclusion criteria | Exclusion criteria |
|---|---|
| Adult premenopausal women (age 18-50 years). | Vaginal infections different from VVC. |
| Symptomatic vulvovaginal candidiasis (VVC) and anamnestic documented history of recurrences. | Women treated with other probiotics or consuming food containing probiotics. |
| Sexually active women willing to use condom as contraceptive method. | Pregnant or breastfeeding women. |
| Signed informed consent. | Systemic or local drugs (not indicated in the study protocol). |
| Not participating in other clinical studies. | Other pathologies (immunodeficiency, diabetes, oestrogen-dependent tumours). |
| Willingness to take the investigational product or placebo. | Hypersensitivity or allergy to antibiotics or any ingredient of investigational product or placebo. |
| Willingness to collaborate in completing the study procedures. | Use of douching. |

During the screening visit before treatment allocation, the gynaecologist collected a vaginal swab from each woman by careful rotation of sterile cotton swab against sidewalls in the upper third area of the vagina for performing the culture analysis (culture for bacteria, yeast and trichomonas) for diagnostic confirmation of *Candida* spp. and gram stain (Nugent score) to exclude bacterial vaginosis. Six visits were scheduled during the study at participating gynaecological centres. During the first visit (T0) at baseline, patient medical history was recorded, informed consent was signed and women were allocated into the two study arms. Controls were performed 1 week (T1), 2 weeks (T2), 1 month (T3), 3 months (T4) and 6 months (T5) after initial antifungal treatment and were aimed to assess the efficacy and safety of the investigational products. The symptoms evaluated (itching and vaginal discharge) were self-reported by the patients as present or absent since the previous gynaecologic visit.

The evaluation of efficacy was based on clinical overall cure rate defined as the ratio between the number of women without vaginal discharge or itching and negative culture and all women present in the treatment group. In addition, recurrence rate was calculated for both groups as ratio between the number of women experiencing at least one symptom during the 6-month follow-up period, confirmed by culture, and all women present in the treatment group. Safety was assessed by recording all side effects (ie, adverse events, serious adverse events, and suspected unexpected serious adverse reactions). An adverse event was considered severe if it was fatal, life threatening, required hospitalisation, or led to permanent injury.

## 2.2 | Treatment

The investigational product, Respecta®, is registered and manufactured as a medical device class IIa by Giellepi S.p.A. Health Science (Lissone, MB, Italy). It contains a proprietary lactobacilli mixture ($5 \times 10^9$ CFU per capsule) including *Lactobacillus acidophilus* GLA-14 (BCCM/LMG Bacteria Collection, LMG S-29159) and *Lactobacillus rhamnosus* HN001

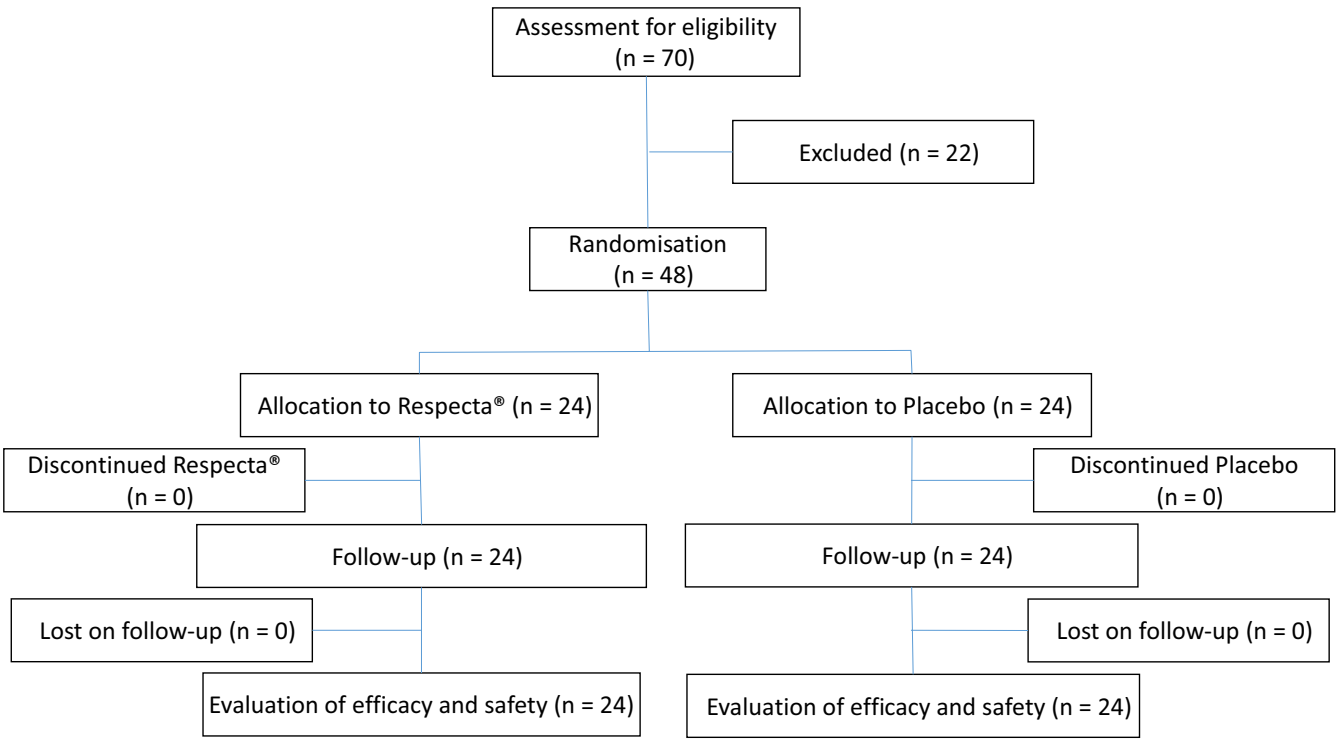


**FIGURE 1** Study flow chart

**TABLE 2** Demographic characteristics (data are expressed as mean ± standard deviation) of women participating in the clinical trial

| | Respecta® (n = 24) | Placebo (n = 24) |
|---|---|---|
| Age (year ± SD) | 36.5 ± 7.0 | 34.3 ± 7.8 |
| Weight (Kg ± SD) | 64.6 ± 7.9 | 62.4 ± 8.8 |
| Height (cm ± SD) | 166.0 ± 5.1 | 165.0 ± 5.5 |
| BMI (kg/m$^2$) | 23.3 | 22.7 |

BMI: body mass index.

(American Tissue Culture Collection, ATCC Number: SD5675) in combination with bovine lactoferrin RCX™ (50 mg). Placebo was an identical capsule containing the inactive ingredient maltodextrin (100 mg); both products were produced with the same excipients.

Treatment consisted in 2 different phases; during the induction phase, all enrolled women were treated with standard antifungal therapy (clotrimazole 100 mg daily for 7 days) and randomly assigned to one of two study arms receiving either verum (Respecta®) or placebo (2 capsules/day for 5 days followed by further 10 consecutive days at the dosage of 1 capsule/day) by oral intake (acute treatment). Respecta® or placebo was administered simultaneously to the antifungal drug. During the maintenance phase (6 months following the induction phase), all recruited women ingested 1 capsule of Respecta® or placebo per day, for 10 consecutive days per month in premenstrual phase (prophylactic treatment). This timeline was related to the consideration that the recurrence, typically, occurs in premenstrual or luteal phase. In fact, during premenstrual phase, for some hormonal and immunological reasons (high oestrogen, increasing progesterone, higher level of inflammatory cytokines), the vagina becomes more vulnerable to the pathogens. For these reasons, we decide to perform maintenance cycle with probiotics during a sort of "window period" at high risk of recurrences.[26,27]

Compliance was determined by assessing returned packages.

### 2.3 | Statistical analysis

Descriptive statistics was used for the demographics and clinical evaluations. Differences between the study arms were assessed by chi-square test. The statistical significance was set at $P < 0.05$.

## 3 | RESULTS

### 3.1 | Demographic characteristics

All recruited women completed the study and compliance was 100% for all participants. Table 2 shows demographic characteristics of women from both study arms. No significant differences were observed.

### 3.2 | Safety evaluation

No woman experienced adverse events during the study period.

### 3.3 | Clinical cure rate (itching and vaginal discharge)

#### 3.3.1 | Itching

After the clotrimazole treatment (T1), a significant improvement of itching was shown in both placebo and Respecta® arms. However, only this latter improved itching significantly during the follow-up period at T4 (3 months) and T5 (6 months). In particular, women without itching in the intervention group in comparison to placebo were 70.8% vs 8.3% at T4 and 83.3% vs 0% at T5, respectively ($P < 0.01$ Respecta® vs placebo). Study results are shown in Figure 2.

#### 3.3.2 | Vaginal discharge

After 1 week of treatment (T1), no women showed vaginal discharge in both arms of the study probably related to the antifungal therapy effects. Symptoms appeared in a few women from each of the groups within 1 week after the end of drug administration and no significant difference was recorded between verum or placebo at T2 and T3. On the contrary, it was only in the intervention group that vaginal discharge improved significantly during the follow-up period at T4 and T5. In particular, women treated with Respecta® remained vaginal discharge free in 66.7% of cases (vs 8.3% in placebo group) at T4 and in 70.8% of cases (vs 20.8% in placebo group) at T5 ($P < 0.01$ Respecta® vs placebo for both T4 and T5). Study results are shown in Figure 3.

### 3.4 | Overall cure rate

The investigational product during the maintenance phase improved the clinical overall cure rate showing the absence of any symptoms (neither itching nor vaginal discharge) and of yeasts indicated by culture from swab, with a significant difference between the two study arms at T4 and T5. In particular, the overall cure rate in the intervention group vs placebo was 66.7% vs 8.3% at T4 and 70.8% vs 0% at end of follow-up (T5; $^{**}P < 0.01$ Respecta® vs placebo). Results are shown in Figure 4.

### 3.5 | Recurrence rate

Significantly fewer recurrences were found in women treated with the investigational product during the 6-month follow-up in comparison to those who took the placebo. In particular, the recurrence rates, defined as presence of at least one VVC symptom confirmed by vaginal culture, were significantly lower in the intervention group than in placebo group during the maintenance phase, being 33.3% at T4 and 29.2% at T5 in the Respecta® group and 91.7% at T4 and 100% at T5 in the placebo arm ($^{**}P < 0.01$ Respecta® vs placebo). Results are shown in Figure 5.

## 4 | DISCUSSION

Cases of RVVC are hard to manage and require prolonged treatment.[11] International guidelines for the treatment of RVVC are

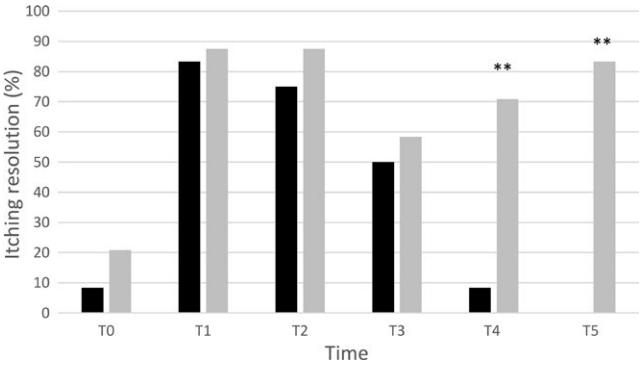


**FIGURE 2** Vaginal itching resolution. The graph shows the percentage of women without vaginal itching from baseline (T0) to the end of 6-month follow-up (T5). T1 = 1 week of therapy; T2 = 2 weeks of therapy; T3 = 1 month after the end of antifungal therapy; T4 = 3 months after the end of antifungal therapy. Black = placebo; Grey = Respecta®. ***P* < 0.01 Respecta® vs placebo

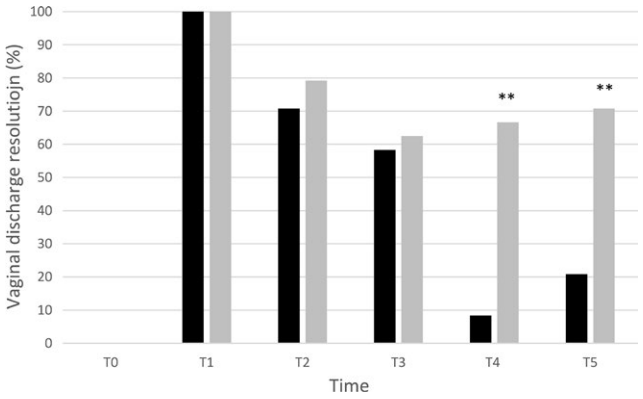


**FIGURE 3** Vaginal discharge resolution. The graph show the percentage of women without vaginal discharge from baseline (T0) to the end of 6-month follow-up (T5). T1 = 1 week of therapy; T2 = 2 weeks therapy; T3 = 1 month after the end of antifungal therapy; T4 = 3 months after the end of antifungal therapy. Black = placebo; Grey = Respecta®. ***P* < 0.01 Respecta® vs placebo

consistent. However, the suggested treatments are not particularly effective and a majority of women relapse following several types of treatment and the question of duration of therapy remains unclear. Despite most guidelines agreeing on six months of oral antifungal therapy as the appropriate treatment, the results from this regimen are disappointing. A longer course may be appropriate,[8,12] but this is not yet supported by large-scale studies. Therefore, further research is needed to address this issue. Generally, several factors have been shown to contribute to the acquisition and/or induction of a symptomatic recurrence or chronicity of vulvar candidiasis.[8] These include intestinal reservoir, persistent vaginal yeasts, sexual transmission,[28] reduced protective vaginal factors,[29] acquired acute hypersensitivity reaction[30] and host genetic susceptibility.[31,32] Most existing regimens in use for recurrent or chronic fungal vulvovaginal infections are empirical and not evidence based.[33] In 2004, data published

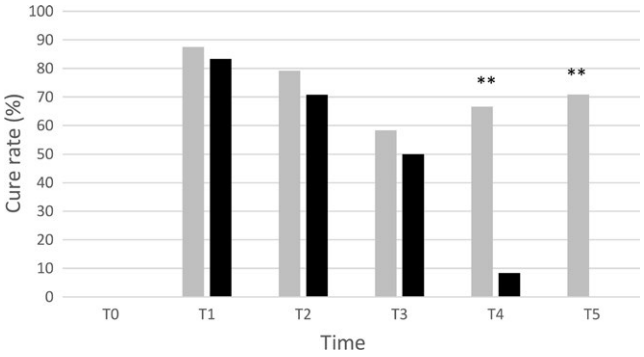


**FIGURE 4** Overall cure rate. The graph shows the rate (expressed as percentage) of women without any symptoms of VVC. Respecta® improved symptoms during the follow-up. T0 = baseline; T1 = 1 week of therapy; T2 = 2 weeks of therapy; T3 = 1 month after the end of antifungal therapy; T4 = 3 months after the end of antifungal therapy; T5 = 6 months after the end of antifungal therapy. Black = placebo; Grey = Respecta®. ***P* < 0.01 Respecta® vs placebo

from Sobel et al[15] concluded that long-term weekly treatment with fluconazole could reduce the rate of recurrence of symptomatic vulvovaginal candidiasis.

Principles of therapy include induction therapy, followed by a maintenance therapy for 6 months.[34] Therapy interruption may result in relapse in about 50% of patients. Alternative therapies should be considered for recalcitrant cases or with the aim to reduce azole use. In fact, a growing number of women are troubled by the high prevalence and recurrence rates of VVC and, although anti-candida agents are quite effective at providing clinical cure for VVC, resistance to the drugs is increasing. For these reasons, the ability of probiotics in maintaining and recovering the normal vaginal microbiota, and their potential ability to resist *Candida* spp. gives rise to the concept of using probiotics for the treatment of vaginal candidiasis.[35-37] Natural defense mechanisms against infections have been described in the vaginal milieu.[38] These include indigenous microbial flora such as lactobacilli, which are believed to interfere

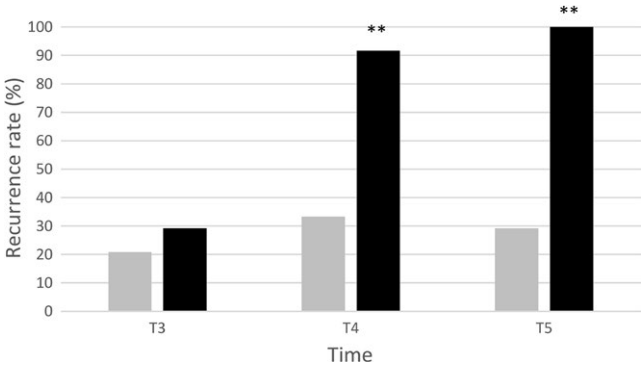


**FIGURE 5** Recurrence rate. The figure shows the rate (expressed as percentage) of women who experienced at least one recurrence of VVC during the follow-up (6 months). Respecta® reduced significantly the recurrences during 6 months. T3 = 1 month; T4 = 3 months; T5 = 6 months. Black = placebo; Grey = Respecta®. ***P* < 0.01 Respecta® vs placebo

with pathogens by various mechanisms. Extending the concept of lactobacilli as endogenous defense mechanisms, both oral and vaginal probiotics have been examined and have shown some efficacy in the context of urogenital health. The actual mechanism of action of lactobacilli in the vagina is probably multifactorial, including immunomodulation in the host, restoration of normal vaginal flora, and interference with pathogen colonisation by competing for both nutrients and adhesion sites on the host tissue.[39] Adherence on the vaginal epithelium is an important virulence factor of *C. albicans*. Co-aggregation of lactobacilli with *Candida* spp. may also be important for the prophylaxis against vaginal infections by preventing the binding to the receptors of the vaginal epithelium. Lactobacilli may block and prevent the *Candida* species' colonisation, adhesion, invasion and growth by producing antimicrobial substances like lactic acid, hydrogen peroxide and bacteriocin, which are toxic to *Candida* species.[40,41] De Alberti et al[42] showed that oral consumption of *L. acidophilus* GLA-14 and *L. rhamnosus* HN001 leads to a significant increase in vagina of these species in comparison to baseline and to women in the placebo group. Although it has been suggested that the intestine is a potential reservoir for both vaginal lactobacilli and pathogens (yeasts or bacteria), this study showed not only that consumption of probiotic lactobacilli leads to transient colonisation of the vagina but also that the species remained increased, in the vagina, even 1 week after consumption of the probiotics was stopped. These data have been recently confirmed in women with abnormal vaginal microbiota (Nugent score between 4 and 6).[43] The use of probiotics in augmenting normal bacterial populations is gradually achieving scientific acceptance. For a few years, probiotics have been used in clinical practice for the treatment of vulvovaginal inflammations and several evaluations have found that are effective against VVC with only few minor side effects,[44] while other studies demonstrated no efficacy in VVC.[15] Currently, there is no consensus on the use of probiotics for treating VVC. Therefore, it is necessary that rigorous randomised, placebo, controlled studies determine the effectiveness and safety of probiotic formulations for the treatment of VVC, and to identify strategic areas for future research.

Lactoferrin is a glycoprotein involved in the host protection against pathogen microorganisms, such as bacteria, viruses and yeasts, and its levels in vaginal secretions increase significantly during infections such as gonorrhoea, *Chlamydia*, cervicitis, bacterial vaginosis and trichomoniasis[45] thus highlighting its protective physiological role against pathogens and inflammation. Moreover, both the oral and vaginal administration of lactoferrin have been proven to improve the vaginal microbiota composition by increasing the number of lactobacilli, which contribute to hamper pathogen growth.[46,47] Evidence shows that it is able to reduce significantly the growth of *Candida albicans* and *C. glabrata*. Interestingly, lactoferrin exerts antibiofilm activity by interfering with *C. albicans* mainly at early stages.[48] Furthermore, Naidu et al[49] demonstrated that lactoferrin exerts both adhesion-blocking and detachment properties thus preventing the adhesion of both *C. albicans* and *C. glabrata* to vaginal epithelial cells collected from woman biopsies.

Our study examined the ability of an oral mixture of Lactobacilli with lactoferrin (Respecta®) to reduce the recurrence of VVC in a prospective, randomised, double blinded, clinical trial after a conventional therapy with clotrimazole in an induction scheme. Key findings indicated that although the cure rate in the short-term after conventional therapy with topical clotrimazole was comparable in the two groups, a maintenance treatment with probiotics and lactoferrin reduced recurrence rate of candidiasis significantly. In particular, our results showed a significant reduction of recurrence rate corresponding to 33.3% and 29.2% at T4 and T5, respectively, which is somewhat lower than other data reported in literature. The intention to treat women in the luteal phase could become an interesting proposal of maintenance scheme of treatment in patients with RVVC. Finally, the study indicated a modest, but discernable, late effect on vaginal symptoms that may be related to the potential anti-inflammatory and immunomodulatory effects of investigated lactobacilli and lactoferrin combination. Further studies with larger number of patients and related clinical and immunological outcomes will be mandatory to confirm this suggestive hypothesis.

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## CONFLICT OF INTEREST

Rosario Russo is employed by Giellepi; he had no influence on the interpretation of results. The other authors have no conflict of interest.

## ETHICAL APPROVAL

The clinical study was carried out in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study protocol was approved by the National Committee of Bioethics of Medicines and Medical Devices.

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***Lactoferrin 50mg***

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1 capsule OD for next 10 days

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1 capsule OD for first 10 days of menstrual cycle

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**Acid Resistant Hypromellose Capsule**
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