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ORIGINAL ARTICLE

Vaginal colonization of women after oral administration of *Lactobacillus crispatus* strain NTCVAG04 from the human microbiota

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ABSTRACT

BACKGROUND: The genomic approach has deeply changed the microbiology perspective, mainly concerning the microbioma identification. In this regard, some microbes colonize the healthy vagina. Vaginitis is a common gynecological ailment and includes bacterial vaginosis (BV), usually caused by local dysbiosis, such as a microbiota imbalance. Lactobacilli are the most prevalent bacteria colonizing the healthy vagina, so guaranteeing local eubiosis. In particular, vaginal colonization by *L. crispatus* is associated with low susceptibility to BV. Therefore, probiotics, such as life bacteria providing health advantages, are a current strategy in the prevention or treatment of vaginitis, including BV. However, there is a low level of evidence that probiotics after ingestion could really colonize the vagina. In particular, no study evidenced that *L. crispatus* after ingestion can colonize vagina. Therefore, the current study explored the capacity of Biovaginil® (NTC, Milan, Italy) dietary supplement containing Lactobacillus crispatus NTCVAG04 and vitamin A to colonize the gut and vagina in women with a history of vaginitis/vaginosis.

METHODS: Twenty fertile females (mean age 34.0 years) were enrolled in the study. Rectal and vaginal swabs were collected at baseline and after the first and second cycle of Biovaginil®. Each cycle lasted 14 days within two consecutive menstrual periods.

RESULTS: Seven women were excluded from the analysis because the samples were technically not evaluable. One woman dropped out because of mild adverse event. At the end of the study, nine women (75%) had positive rectal swab for *L. crispatus* NTCVAG04, and 8 of them also had positive vaginal swab.

CONCLUSIONS: The current study provided the first evidence that *L. crispatus* NTCVAG04, administered by two Biovaginil® courses, colonized both the gut and vagina. Moreover, the *L. crispatus* NTCVAG04 strain could be considered the archetype of a new class of oral probiotics that actively colonize the vagina, and that could be called "colpobiotics."

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KEY WORDS: Vaginitis; Bacterial vaginosis; Microbiota; Dysbiosis; *Lactobacillus crispatus*.

The genomic approach has deeply changed the microbiology perspective, mainly concerning the microbioma identification. In this regard, some microbes colonize the healthy vagina, realizing an environment unfavorable for pathogens. Unbalance composition of vaginal microbiota is a risk factor for vaginitis.[1] Vaginitis is an umbrella definition indicating several different types of vaginal inflammation, which commonly include: 1) bacterial vaginosis (BV), resulting from dysbiosis of the vaginal microbiota; 2) mycosis, usually caused by Candida albicans; 3) trichomoniasis, caused by a parasite usually transmitted by sexual intercourse; 4) vaginal atrophy, commonly occurring during menopause; 5) non-infectious vaginitis, depending on irritative factors.[2]

BV is a complicated and polymicrobial infection that mostly affects fertile, pregnant, and premenopausal women.[3] The epidemiologic prevalence ranges between 23-29%, with a relevant difference considering ethnicity, especially affecting sub-Saharan African and Hispanic women.[4] This epidemiologic inconsistency depends on different vaginal microbiota. Namely Lactobacillus spp. dominate (70-90%) the healthy vaginal microbiota and mount resistance to colonization by urogenital pathogens.[5] Many factors, including genetic, individual, and environmental aspects, modulate microbiota composition. Sexual habits, hormonal changes, menses, personal hygiene, diet, smoking, stress, infections, metabolic disorders (mainly diabetes mellitus), antibiotic therapy, and socio-economic conditions may significantly affect the vaginal eubiosis.[6] As a consequence of disrupted microbiota balance, BV results in bacterial overgrowth of pathogenic bacteria, mainly including Gardnerella vaginalis and *Peptostreptococcus anaerobius*.[5] In this regard, microbiological researches identified five community state types (CST) of the vaginal microbiota: CST I, dominated by *Lactobacillus crispatus*, CST II, dominated by *Lactobacillus gasseri*, CST III, characterized by *Lactobacillus iners* predominance, CST IV lacking lactobacilli and harboring anaerobic bacteria (*Gardnerella*, *Prevotella*, *Megasphera*, and *Sneathia*), and CST V dominated by *Lactobacillus jensenii*.[7] CST I characterizes the healthy vaginal microbiota; this fact underlines the relevance of *L. crispatus* in maintaining a protective milieu against pathogens.

From a clinical point of view, women suffering from BV usually complain of change in color, odor or amount of vaginal discharge, local itching and/or irritation, painful intercourse, dysuria, and light vaginal bleeding or spotting. These symptoms significantly affect the quality of life, relationship life, and the mood of women with BV; consequently, women with a frequently turn to gynecological consultation. Gynecologist diagnoses BV performing careful history, pelvic examination, lab testing, and vaginal pH assessment. The confirmed clinical diagnosis implies at least three positive Amsel criteria: pH >4.5, grey and homogeneous discharge, fishy smell of vaginal secretions, and presence of epithelial cells coated with bacterial at vaginal cytology.[8] The Nugent Scoring is the gold standard of laboratory diagnosis as it measures the microbiota composition of the vagina.[9] However, new molecular testing allows more precise identification of the bacterial composition of vaginal samples.[10]

From a pathophysiologic perspective, vaginal dysbiosis as well as being associated with BV and vulvovaginal candidiasis, is also a predisposing factor for sexually transmitted infections, pelvic inflammatory diseases, and during pregnancy causes neonatal morbidity, and mortality.[11] Therefore, BV requires prompt diagnosis and timely treatment.[12, 13] International guidelines recommend antibiotic therapy, namely metronidazole and clindamycin, per oral or vaginal route.[1, 14, 15] Antibiotics are effective as the cure rate ranges between 80-90%, but the BV recurrence is widespread, such as up to 70% after one year.[16] Moreover, antibiotics are associated with bacterial resistance and production/persistence of biofilm.[12] Therefore, an alternative strategy may favorably implement standard treatment. Based on the concept of dysbiosis's pathogenic role, the use of probiotics collected enthusiastic consents as could represent a physiologic way to contrast pathogens predominance avoiding antibiotic use/overuse.[17, 18] The FAO/WHO definition of a probiotic is "live microorganisms which when administered in adequate amounts confer a health benefit on the host" should be integrated with the concept of genus and strain specificity of each

probiotic.[19] In fact, the outcomes obtained by one probiotic cannot be generalized to all probiotics, but require the identification of the single species and strain used.

Lactobacilli provide numerous beneficial effects as they mainly produce lactic acid by fermentation of sugars, reducing the vaginal pH, and inhibiting the growth of many pathogenic microorganisms.[11, 20, 21] Moreover, lactobacilli produce hydrogen peroxide and bacteriocins to protect vaginal mucosa from pathogens, and stimulate the innate and adaptive immune response.[22] In this regard, *L. crispatus* abundance is strongly associated with a healthy vagina microbiota (CST I). Thus, it may be an ideal candidate to restore the unbalanced vaginal microbiota in women with BV.[17, 23]

Many systematic reviews and meta-analyses investigated the role of probiotics for BV.[24-26] Most of them concluded that probiotic supplementation might be effective for treating and preventing BV, but high-quality trials need to rigorously confirm the promising outcomes observed in clinical trials. In particular, there is modest evidence that probiotic administration results in vaginal colonization, and, as recently outlined, most studies did not distinguish endogenous lactobacilli from supplemented strains.[3] Moreover, probiotics were mostly administered intravaginally, but the European Community's recent regulatory provisions concerning medical devices do not allow the topical administration of live bacterial strains. Consequently, oral probiotics should be prescribed.

However, there is still no evidence that *L. crispatus*, orally administered, could really colonize vagina. Based on this background, we tested the hypothesis that oral *L. crispatus* could colonize the gut and consequently vagina, so providing the demonstration that a gut-vagina communication could occur. As proof of the concept, this study aimed at investigating the dual (vagina and rectum) colonization of oral *L. crispatus* NTCVAG04 in women with a history of vaginitis/vaginosis.

Materials and methods

The current trial was an open-label, non-randomized pilot study. The study included 20 women in reproductive age, with a regular menstrual cycle (28±4 days) and with a history of vaginitis/vaginosis. The inclusion criteria were: female gender, age >18 years, history of vaginitis/vaginosis, and informed consent. Exclusion criteria were: pregnancy, breastfeeding, and current antibiotic treatment for vaginitis/vaginosis.

The Ethic Committee of the Tuscany Region (CEAVSE) approved the study protocol (NTCVAG04_02-2018). Each subject signed an informed consent. The trial was registered in Siena University Hospital, internal code CE 14444_2019. The protocol was registered (first registration: 21/12/2020) and posted on ClinicalTrials.gov (Identifier: NCT04676503).

The scheduled visits included one at baseline, one at the end of the first treatment, and one at the end of the second treatment cycle. Treatment encompassed the oral ingestion of Biovaginil® (NTC, Milan, Italy), which is a notified food supplement formulated as 480 mg capsules. Each capsule contains *Lactobacillus crispatus* NTCVAG04 3 billion and vitamin A 120 µg.

The primary outcome was to identify the presence of *L. crispatus* NTCVAG04 in the rectum and vagina after oral administration by searching for specific DNA of the bacterial strain. Secondary outcomes were to evaluate the minimum duration of the oral administration necessary to determine the appearance of *L. crispatus* NTCVAG04 in the vagina and rectum, and to evaluate the tolerability of Biovaginil®.

The variables were: positivity for NTCVAG04 in rectal and vaginal swabs collected at the end of the first treatment cycle and positivity for NTCVAG04 in rectal and vaginal swabs collected at the end of the second treatment cycle.

The endpoints were: 1) number and percentage of positive rectal and vaginal swabs collected at the end of the first treatment cycle; 2) number and percentage of positive rectal and vaginal swabs collected at the end of the second treatment cycle; 3) number and percentage of positive rectal and vaginal swabs collected at the end of the second treatment cycle in comparison to results collected at the end of the first treatment cycle; 4) incidence, severity, and relationship to trial treatment of adverse events during the entire course of study.

All patients took Biovaginil® capsules. Two cycles of treatment were administered: the first

cycle was one capsule a day by mouth for 14 days, from day 7±2 to day 21±2 of the menstrual cycle in progress or following enrolment; the second cycle was one capsule a day by mouth for 14 days, from the first day to day 14±2 of the next menstrual cycle.

The administration schedule and follow-up duration were established on the basis of an empirical approach.

The first visit was performed on November 22nd, 2019 and the last visit at the end of May 2020.

Rectal and vaginal swabs were collected at each visit and further analyzed to identify *Lactobacillus crispatus* strain NTCVAG04.

A sterile dry rayon-tipped swab collected samples (*e.g.*, FL Medical, cat. no. 26061) and transferred them to the laboratory. Swabs were immediately processed in the laboratory.

Each dry swab was resuspended in 1 mL of saline and the suspension was centrifuged at 3500 rpm for 10 min. The presence of different bacterial species was assayed by plating 10 microliter of the suspension on the following selective solid media: blood columbia CNA mod (Biotec, Dueville, Vicenza, Italy), mannitol salt agar (Oxoid Ltd., Basingstoke, UK), MacConkey agar (Oxoid) and incubated for 24 hours at 37 °C in presence or absence of 5% CO_2. Samples were also plated on Schaedler and Rogosa agar (Oxoid) and incubated at 37 °C in an anaerobic chamber for 48 hours. Bacterial isolates were further identified by standard microbiological techniques.

Primers and probe used for the Taqman based Real Time PCR assay are: IF1141 (5'-TACTAAGAAGCATACTTAGAAAT-3'), IF1142 (5'-ACCTCACAAATTGTTACTTTAAT-3'), IF1145 (6-Fam-GTATCCTTAGAGCCACAAG-BHQ-1). Primers and probes were checked using BLAST to avoid unwanted cross-homology to non-targeted sequences of the NTCVAG04 genome.

PCR experiments were carried out essentially as already reported.[27]

The safety assessment consisted of evaluating all adverse events, such as any untoward medical occurrence associated with the use of an intervention in humans after providing written informed consent for participation in the study until the end of the study visit, whether considered intervention-related or not.

Statistical analysis

In consideration of the pilot study's characteristics, a specific sample size calculation has not been performed. No formal statistical hypothesis has been formulated. A descriptive statistical analysis of rectal and vaginal swabs for NTCVAG04 at the end of the first and second treatment cycles was performed.

Results

The current study explored the capacity of Biovaginil® dietary supplement containing *L. crispatus* NTCVAG04 and vitamin A) to colonize the gut and vagina in women with a history of vaginitis/vaginosis.

Detection and identification of NTCVAG04 and indigenous lactobacilli were performed by cultural and molecular assays. In order to set up a molecular assay based on a quantitative real-time PCR, the complete genome sequence of *L. crispatus* NTCVAG04 was determined (data not shown). Comparative genome analysis showed the presence of a 64-kb genetic element integrated in NTCVAG04 genome not present in the other sequenced *L. crispatus* strains. PCR primers and probe were designed to detect an unique and specific 212-bp DNA fragment located on the genetic element.

Twenty women (mean age 34.0 years, range 19-49) provided a valid written informed consent for participating in the study and were enrolled in the study. All were of Caucasian ethnicity. All of them had positive history for BV. The medical history reported that one subject had glaucoma, one polycystic ovary syndrome, two had previous laparoscopic surgery, and one essential hypertension.

The mean number of previous BV was 4.4, range 2-8). Twelve subjects used estroprogestinic combination.

All subjects completed the study, except one who discontinued the dietary supplement and the trial because she had an adverse event potentially related to the dietary supplement (Figure 1).

The evaluable population consisted of 13 wom-

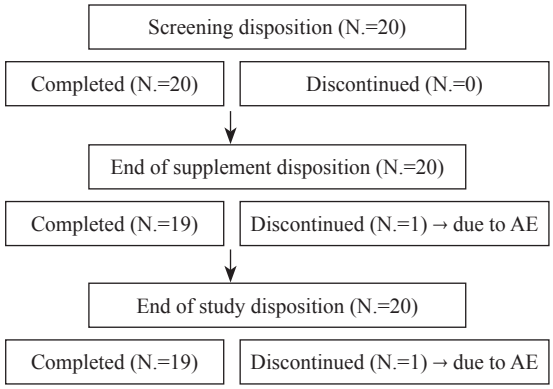

Figure 1.—Flow diagram of the study.

en because samples of seven subjects were not evaluable for technical reasons. As a result, the findings of 13 women were considered concerning the second visit. The findings of 12 subjects were considered concerning the third visit because one woman did not show up at the third visit.

Table I contains the findings of rectal swabs at the three visits. At the second visit, at the end of the first course, six subjects (46.15%) had positive rectal swabs for *L. crispatus* NTCVAG04. At the end of the trial, nine (75%) women had positive rectal swabs for *L. crispatus* NTCVAG04.

Table II provides the findings of vaginal swabs at the three visits. At the second visit, at the end of the first course, five subjects (41.6%) had positive vaginal swab for *L. crispatus* NTCVAG04 (one vaginal sample missed). At the end of the trial, eight (66.6%) women had positive vaginal swab for *L. crispatus* NTCVAG04.

All 20 patients had compliance higher than 80%. The mean was 98.0±2.84%, with a median of 100%, ranging from 91% to 100%.

None of the reported adverse events were severe, but one patient discontinued treatment for two suspected food supplement-related adverse events: swollen tongue and epiglottitis.

Discussion

The current study explored the possibility that oral delivered *Lactobacillus crispatus* could

TABLE I.—*Summary of rectal swabs results by visit: evaluable population.*

Parameter	Total (N.=13)
Lactobacillus crispatus NTCVAG04 in rectal swab at baseline	
Absent	13 (100%)
Lactobacillus crispatus NTCVAG04 in rectal swab at visit 2	
Absent	7 (53.85%)
Present	6 (46.15%)
Lactobacillus crispatus NTCVAG04 in rectal swab at visit 3	
Absent	3 (25.0%)
Present	9 (75.0%)

Percentages are computed on patients in the evaluable population. Only results of rectal swabs regarding the presence of *Lactobacillus crispatus* NTCVAG04 specific DNA was considered. Rectal swab results were considered as acceptable only if the "reason non-compliance-DNA" was empty. One patient did not perform visit 3.

TABLE II.—*Summary of vaginal swabs results by visit; evaluable population.*

Parameter	Total (N.=13)
Lactobacillus crispatus NTCVAG04 in vaginal swab at baseline	
Absent	13 (100.00%)
Lactobacillus crispatus NTCVAG04 in vaginal swab at visit 2	
Absent	7 (58.33%)
Present	5 (41.66%)
Missing	1
Lactobacillus crispatus NTCVAG04 in vaginal swab at visit 3	
Absent	4 (30.77%)
Present	8 (61.54%)

Percentages are computed on patients in the evaluable population. Only results of vaginal swabs regarding the presence of *Lactobacillus crispatus* NTCVAG04 specific DNA was considered. Vaginal swab results were considered as acceptable only if the "reason non-compliance-DNA" was empty. Missing category refers to not performed swabs or non-acceptable swab results in non-drop-out patients. One patient did not perform visit 3.

colonize both the digestive tract and vagina in women with a history of vaginitis/vaginosis. Two 14-day cycles of the dietary supplement Biovaginil® resulted in a 75% frequency of rectal colonization and 66.6% of vaginal colonization. As there was a progressive increase in vaginal colonization over time, it seems conceivable to speculate that the colonization rate could be even higher in case of a greater number of cycles.

This outcome was outstanding compared with the findings of a recent study, which reported a vaginal colonization rate of only 11% after a two-month administration of capsules containing *Lactobacillus rhamnosus* and *Lactobacillus reuteri* $5x10^9$ in pregnant women.[28] Notably, only *L. rhamnosus* colonized the vagina. This outcome confirmed that *Lactobacillus crispatus* commonly harbors the gut and vagina also in women with a history of vaginitis/vaginosis. This result was also consistent with a reported 27% rate of *L. crispatus* and *Lactobacillus jensenii* colonization after one-month treatment for BV with vaginal metronidazole gel.[29] Interestingly, the authors proposed that a low colonization rate could suggest the existence of a window of vulnerability during which there is an increased risk of reinfection recurrence.[29]

The high frequency of vaginal colonization, observed in the present study, could depend on the species of *Lactobacillus* genus; indeed, after a preliminary dose-finding study,[30] it was reported that *L. crispatus* $2x10^9$, topically administered by a vaginal applicator, colonized 61% of women after a course consisting of five initial consecutive days followed by a weekly application over two weeks.[31]

Therefore, restoring vaginal eubiosis, documented by lactobacilli abundance, should represent a crucial target in the management of BV. A recent meta-analysis confirmed that oral intake of a fixed mixture of four lactobacilli species, including *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. rhamnosus*, improved the microbial pattern in BV.[32] Furthermore, additional yogurt intake containing lactobacilli probiotic species (*L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. rhamnosus*) improved the recovery rate and symptoms of BV and tended to improve the vaginal microbial pattern evaluated by the Nugent Score.[33]

However, the choice of the probiotic should consider the specific species and strain and the route of administration. In this regard, *L. crispatus* claims the peculiar antimicrobial property, exhibiting killing, bacteriostatic, and antibiofilm activity against vaginal pathogens; moreover, it exerts competitive, excluding, and displacing activity against mucosal colonization by vaginal pathogens.[34] These microbiological characteristics reduced the recurrence rate and increase the time to recurrence, as recently documented in women with recurrent BV.[35]

On the other hand, it has to be noted that, to our knowledge, no study investigated the dual colonization, such as rectal and vaginal, of oral probiotics. Intestinal colonization has important relevance as the gut constitutes a reservoir for lactobacilli colonizing the vagina so ensuring the maintenance of vaginal eubiosis.[36]

Another interesting issue provided by the current study underlined the close bidirectional relationship between the gut and vagina. Namely, the documentation that oral Lactobacillus crispatus NTCVAG04 colonized the gut and vagina represented the proof of the concept that a gut-vagina communication exists. Namely, a bidirectional crosstalk has been previously prospected.[37] This concept is consistent with the well-known gut-lung axis and explains the pivotal role exerted by intestinal microbiota on the whole human body.

The gut-lung axis highlights that the gut microbiota intimately interacts with the immune system of both the intestinal and respiratory tracts.[38] There is evidence that oral probiotics may effectively improve and prevent respiratory infections and inflammatory diseases.[39] Oral probiotics arrive at the airways after the initial enteric absorption and immune processing by antigen-presenting cells.[40] Consequently, oral probiotics could directly colonize upper and lower airways passing through the bloodstream and lymphatics and indirectly stimulate innate and adaptive immunity by complex mechanisms, including mediators and cytokines release.[41]

Limitations of the study

However, the present study had some limitations, including the open design, the limited number of participants, the empiric choice of administration

schedule and follow-up duration, and the lack of clinical assessment. Further studies should address these unmet needs. On the contrary, the current study's strengths were the genomic approach to identify the specific strain *L. crispatus* NTCVAG04, and the documented proof of the simultaneous colonization of vagina and rectum.

Conclusions

The present study provided evidence that oral administration of *Lactobacillus crispatus* NTCVAG04 strain colonized the rectum and vagina in a large percentage of women with a history of vaginitis/vaginosis. This dual colonization confirmed the gut-vagina crosstalk, which deserves adequate studies, as recently outlined.[42] In particular, vaginal eubiosis is a fundamental requirement to maintain healthy microbiota and homeostasis. The current study suggests that a new probiotics class could be defined, such as the "colpobiotics" characterized by a peculiar tropism for the vaginal environment. In this context, *L. crispatus* NTCVAG04 strain could be envisaged as the archetype of colpobiotics. Indeed, *L. crispatus* is the leading component of the healthy vaginal microbiota, is associated with a low prevalence of BV, and its supplementation can restore vaginal eubiosis, preventing BV recurrence and relieving BV symptoms.

After all, it has been observed that the oral route is more acceptable for the patients than the vaginal route, increasing the compliance to the treatment. Therefore, oral *L. crispatus* NTCVAG04 treatment could represent a holistic approach to managing women with vaginitis/vaginosis.

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Conflicts of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Authors' contributions

Conceptualization: Francesco Iannelli, Gianni Pozzi, Vincenzo De Leo. Methodology: Elisa Lazzeri, Laura Governini. Investigation: Vincenzo De Leo, Laura Governini, Anna M. Cuppone, Lorenzo Colombini, Lucia Teodori. Writing – original draft preparation: Giorgio Ciprandi. Writing – review and editing: Vincenzo De Leo, Francesco Iannelli, Gianni Pozzi. All authors read and approved the final version of the manuscript.

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