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Assessment of Live Lactobacilli Recovery from Probiotic Products for Vaginal Application

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Abstract: The interest in the use of probiotics to treat and prevent vaginal infections is known. The new regulation of medical devices by the European Medical Agency (EMA) introduced big changes in Europe regarding probiotic products for vaginal application, as they are no longer considered as medical devices. As the future classification will be as drugs, it will stress the need to define robust and reliable pre-clinical in vitro testing in order to assess the quality, safety and efficacy of probiotics for human use. Before discussing the efficacy in human pathology, it is mandatory to evaluate the survival and multiplication potential of probiotic strains when brought into contact with vaginal fluid. In this work, our objective was to assess the recovery and stability profile of lactobacilli from six vaginal probiotic formulations brought in contact with specific culture media or vaginal fluid simulants (VFS). Overall, the recovery of viable lactobacilli cells from a modified vaginal fluid simulant (MVFS) solution was comparable to the recovery pattern obtained in standard culture medium. Therefore, we conclude that the MVFS seems to better simulate the conditions of the human vaginal fluid, in contrast with other simulants, and may be used to predict the viability of probiotics over time in the normal vaginal milieu. We discovered that each probiotic product has a unique profile that requires stand-alone studies in conditions that mimic the in vivo status in order to assess their preclinical effectiveness and promote their differential use by the medical community.

Keywords: lactobacilli; probiotic; vaginal fluid simulant; microbiome; recovery

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1. Introduction

The vaginal microbiome is constituted by a wide variety of microorganisms, comprising mostly bacteria of different species. Nonetheless, 70% of healthy women have *Lactobacillus*-dominated flora [1]. The four most frequent species that colonize the vagina of healthy women are *L. crispatus*, *L. jensenii*, *L. iners* and *L. gasseri* [2]. The acid pH of the healthy vaginal ecosystem, ranging between 4 and 4.5, determines the selection of microorganisms capable of colonization [3] and is crucial in inhibiting the proliferation of pathogens, like *Gardenerella* spp. or *Candida* spp. This characteristic results from the production of lactic acid by lactobacilli from the glycogen derived from vaginal epithelial cells [4]. Additionally, lactobacilli compete with pathogens for adhesion sites and nutrients and produce bacteriostatic and bactericidal products, which control the growth of pathogenic strains [4].

Due to these inherent biological characteristics, lactobacilli strains have been frequently used in probiotic formulations aiming to aid in the prevention and treatment of vaginal infections [4–7]. Currently, the focus on probiotic activity is related mostly to its activity in the presence of the multidrug resistance of many pathogens to antibiotics and to the in vitro and in vivo evidence of their safety and efficacy [8,9]. Moreover, the implementation of

a new regulation for medical devices in Europe introduced a challenge in the market of probiotics for vaginal application [10]. Indeed, while in the past these products were considered as medical devices, the European Pharmacopeia now considers the possibility that these products will be classified as drugs [11]. Therefore, in order to market a probiotic product for vaginal application in Europe, its efficacy and safety should be assessed in phase 1, 2 and 3 trials, like any other drug for vaginal application [12,13]. With these changes, since 2017, there are officially no longer any probiotic products for vaginal application available on the European market as, due to classification limitations, all the commercial trademarks had to be removed and have yet to be replaced. These changes stress the need to define robust and reliable testing methods to assess the safety and efficacy of probiotic products for vaginal application. Some of these methods are pre-clinical and quality control tests, performed in vitro, in conditions that could mimic their use in vivo, in order to anticipate their activity when used in clinical conditions.

The World Health Organization (WHO) defines probiotics as "living microorganisms which, when administered in adequate doses, confer benefit to the health of the host" [14]. Therefore, there is the premise of (1) the viability of microorganisms, (2) the appropriate dose and route of administration, (3) desired effect and (4) safety. With probiotics, the effective dose is directly related to and dependent on the number of living microorganisms that can be recovered from the formulation at the time of administration. The effectiveness of the product, besides its inherent antibacterial and epithelial cell adhesive qualities, relates to its ability to remain viable over time after local administration [7,8]. A 1990 study evaluating lactobacilli recovery from 16 marketed oral probiotic supplements showed that, in the majority of them, the living bacterial cell count did not correspond to the concentration claimed on the insert [15]. Additionally, these authors reported that 11 out of 16 products were identified as different species than described in the leaflet of the product [15]. Recent studies confirmed these results in up to 20% of dietary supplements marketed to restore the intestinal flora [16,17]. The recovery of live bacteria in vivo not only depends on the selection of the most appropriate strains but also the inclusion in the final product of compounds that stimulate proliferation and/or the effect of the probiotic microorganisms, the so-called prebiotics [8,9,18]. The use of prebiotics for vaginal application has been limited to carbohydrates [19], but interest has been rising and new products and ways to study these compounds in vitro are emerging [20]. Other probiotic-derived products of interest for vaginal application are postbiotics. Postbiotics are defined as "a preparation of inanimate microorganisms and/or its components that is beneficial to the health of the host" [21]; they have been recently applied with success in the treatment of patients with bacterial vaginosis [22]. Therefore, there is potential in the use of biotherapeutics (comprising pro-, pre- and postbiotics) in the treatment of vaginal infections and the improvement of vaginal health [19]. However, the changes in regulation emphasize the need to implement more rigorous quality control of biotherapeutics particularly for vaginal use, which, to our knowledge, has not yet been thoroughly performed. In this study, we aim to (i) evaluate the in vitro recovery of living lactobacilli from probiotic products designed for vaginal application; (ii) compare the experimental results with the claimed product information; (iii) assess the viability of living cells recovered over time (48 h); and (iv) compare the recovery of live lactobacilli on two types of simulated vaginal fluid solutions. We propose the introduction of quality control tests to assess products to be marketed for vaginal application as probiotic drugs.

2. Materials and Methods

2.1. Probiotic Formulations

In this study, only mono-species probiotic formulations intended for vaginal application were used. We acquired six formulations in Portuguese, Belgian, Swiss and Austrian pharmacies: Pregyn-S®, Isadin-α-barcillus®, Gynophilus®, Gynoflor®, Baciginal Is® and Baciginal Activ® (Supplementary Table S1). According to the regulation of pharmaceutical products and the manufacturers, one of them, Gynoflor®, is recognized by most European

authorities (e.g., www.infarmed.pt, accessed on 31 July 2023) as a drug, since it contains minimal amounts of estriol [23]. The remaining are classified as medical devices, as indicated on the package by the manufacturers [23]. The products were stored under the conditions described by the manufacturers. The excipients and species type present in each formulation are shown in Supplementary Table S1.

2.2. Assessment of Live Lactobacilli Cells from Probiotic Products

An in vitro method to assess the number of living lactobacilli cells recovered from vaginal probiotic products was adapted from classical microbiology methods. Briefly, one tablet/capsule of each probiotic product was dispersed in 6 mL of three different solutions: Man Rogosa Sharpe broth (MRS; Prolabo, Leuven, Belgium), which was used as a control, and two different solutions of vaginal fluid simulant previously reported: a classic preparation of vaginal fluid simulant (VFS-OK of Owen and Katz) [24] and a modified solution of vaginal fluid simulant (MVFS) [25]. The VFS-OK is composed of 3.51 g/L NaCl, 1.40 g/L KOH, 0.222 g/L $Ca(OH)_2$, 0.018 g/L bovine serum albumin, 2.00 g/L lactic acid, 1.00 g/L acetic acid, 0.16 g/L glycerol, 0.4 g/L urea and 5.0 g/L glucose; it has a pH of 4.2 and has been used in efficacy studies of vaginal drug delivery systems [25–27]. The enriched MVFS contains 3.50 g/L NaCl, 1.50 g/L KCl, 2 g/L bovine serum albumin, 2 g/L lactic acid, 1 g/L acetic acid, 0.5 g/L urea and 10 g/L glucose. Additionally, it contains 10 g/L glycogen, 0.25 g/L mucin, 1.064 g/L Tween-80 and 0.50 g/L-cysteine. The pH is adjusted at 4.25. It resembles the normal vaginal condition even better [25] and has mainly been used in studies with vaginal commensal microorganisms [28–30].

Suspensions of products fully dispersed in MRS, VFS-OK and MVFS were incubated at 37 °C in an atmosphere with 10% CO_2 and 21% O_2. Serial 1:10 dilutions of the suspensions were performed in sterile distilled water (10^{-1} to 10^{-6}) immediately after suspension (0 h) and after 6, 12, 24 and 48 h of incubation. A drop of 4 µL of each dilution was then inoculated in quadruplicate in MRS supplemented with agar (Pronadisa, Madrid, Spain), and the plates were incubated for 48 h under the same conditions of temperature and atmosphere as described above. After the incubation period, the colony-forming units (CFU) were counted. The procedure was repeated for each product in two independent experiments.

2.3. Analysis of the Results

Results are presented in logarithm, after calculating the average and standard deviation of CFU/unit at the different time points, in each of the VFS solutions compared to the control medium (MRS). The significance of the differences observed was evaluated by analyzing the results with the χ^2 test, with a significance level of 95%.

3. Results

The yield of all products in MRS was higher than what was claimed in the tablet by the pharmaceutical companies, except for one product (Table 1). From Pregyn-S®, 20 million fewer cells than the value claimed by the company were recovered (91% of the claimed value). For the remaining products, a surplus of 200 to 5000 million cells were recovered, compared to the numbers given on the inserts (Table 1). Also, after dispersion in VFS-OK, a higher recovery than that claimed by the companies was obtained for all products (Table 1). Regarding the dispersion at MVFS, at the initial time (t_0 h), the recovery was always higher than what was claimed by the companies, except for Pregyn-S ®, from which we obtained around 70% of the claimed value. Similarly, after 48 h of incubation in MRS, the recovery was superior to the label for all products except for Pregyn-S® (80% of the claimed value), while, after 48 h in VFS-OK, the recovery was lower than claimed in all but Baciginal IS ® and Isadin-α-barcilus®. Nonetheless, the recovery rate obtained was always superior to 70% of the claimed value (Table 1). After 48 h of incubation in MVFS, species were recovered in higher (Gynoflor® and Isadin-α-barcilus®), equal (Baciginal Activ® and Gynophilus®) or lower (Baciginal-IS® and Pregyn-S®) concentrations than the

value claimed by the companies (80% and 65% of the claimed value, respectively, for the latter two).

Table 1. Average recovery of probiotic cells (CFU/capsule, CFU/cp), in logarithm, obtained at t_0 h and t_{48} h. The claimed viability of the cells present in each product is also shown. Recovery values below the value claimed by the companies are shown in bold. MRS: MRS broth; VFS-OK: vaginal fluid simulant of Owen and Katz; MVFS: modified vaginal fluid simulant.

Product	Claimed Viable Cells per Unit (log)	MRS (t_0 h)	MRS (t_{48} h)	VFS-OK (t_0 h)	VFS-OK (t_{48} h)	MVFS (t_0 h)	MVFS (t_{48} h)	Species
Baciginal Activ®	9	9.80	9.35	9.89	8.78	9.22	8.73	*L. acidophilus*
Baciginal Is®	8	9.47	8.74	10.80	8.70	7.70	**6.33**	*L. acidophilus*
Gynoflor®	7	9.36	8.95	8.59	**5.59**	9.14	8.60	*L. acidophilus*
Gynophilus®	9	9.51	9.33	9.42	**8.42**	9.51	9.27	*L. rhamnosus*
Isadin α barcilus®	8	8.59	10.37	8.36	9.31	8.26	9.86	*L. plantarum*
Pregyn–S®	8.5	**7.7**	**6.80**	9.53	**6.21**	**6.00**	**5.48**	*L. acidophilus*

Overall, the recovery rate of cells differed in VFS-OK and MVFS, with higher recovery rates at t_0 h using VFS-OK (63–140% of the quantity obtained using MRS) when compared with MVFS (22.3–61.7% of the quantity obtained using MRS) (Table 2). The two exceptions were Gynoflor® and Gynophilus®, where a higher recovery was obtained from MVFS compared to MRS even after 48 h of incubation (Table 2). The recovery of lactobacilli from Baciginal Activ® and Baciginal IS ® in VFS-OK was also higher than from MRS (Table 2).

Table 2. Recovery of lactobacilli cells for each product in VFS and MVFS, expressed in comparison with the recovery of the cells obtained in MRS. Relevant differences are highlighted in bold. MRS: MRS broth; VFS-OK: vaginal fluid simulant of Owen and Katz; MVFS: modified vaginal fluid simulant.

Product	Recovery (% MRS) 0 h VFS-OK	Recovery (% MRS) 0 h MVFS	*p*-Value	Recovery (% MRS) 48 h VFS-OK	Recovery (% MRS) 48 h MVFS	*p*-Value
Baciginal Activ®	**63.3**	61.7	$p > 0.05$	**22.9**	14.3	$p > 0.05$
Baciginal Is®	**77**	31.95	***p* < 0.05**	**8.49**	3.93	$p > 0.05$
Gynoflor®	13.4	**76.15**	***p* < 0.05**	0.07	**28.5**	***p* < 0.05**
Gynophilus®	89.4	**92.3**	$p > 0.05$	11.6	**100**	***p* < 0.05**
Isadin α barcilus®	**120**	22.3	***p* < 0.05**	9	**29.4**	***p* < 0.05**
Pregyn–S®	**137.9**	53.8	***p* < 0.05**	0.97	**63.4**	***p* < 0.05**

Throughout the 48 h test, the recovery rate of viable cells from Baciginal Activ® and Baciginal Is® from VFS-OK and MVFS was gradually reduced (Figure 1A–G and Figure 1B–H, respectively). The recovery of Gynoflor® from MRS remained above the claimed value until t_6 h (Figure 1C), and, at t_{12} h, it decreased below the value claimed on the label; however, from MVFS, the recovery remained superior to the claimed value up to the end of the experiment at t_{48} h (Figure 1I). The recovery of lactobacilli from Gynophilus® from VFS-OK and MVFS remained superior during the first 24 h of the experiment but declined below the claimed value after 48 h in VFS-OK (Figure 1D,J), while the recovery of lactobacilli from Isadin α barcilus® showed an increased number of cells at t_{12} h and onwards (Figure 1E,K). The recovery of Pregyn-S® microorganisms from VFS-OK decreased after the first 6 h, becoming lower than the value described in the package of the product. When using MVFS to recover these microorganisms, this result was also observed from the first moment until the end of the experiment (Figure 1L).

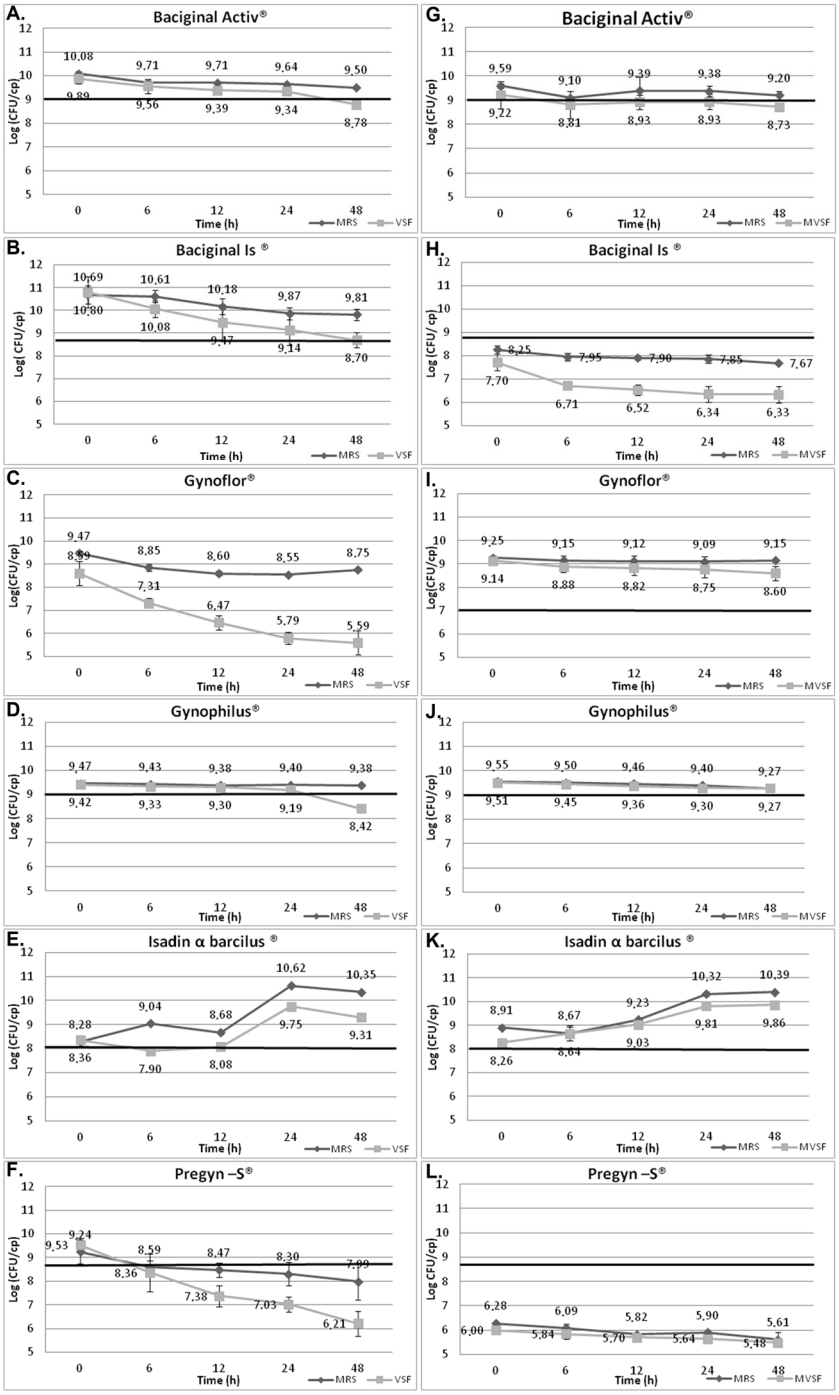

Figure 1. Recovery of lactobacilli from each product expressed in CFU/capsule (CFU/cp) in logarithm, in MRS/VFS-OK (**A**–**F**) and MRS/MVFS (**G**–**L**). CFU/capsule (CFU/cp) in logarithm alleged in the product pack is shown as a black line in each graph. The values obtained for each time point are also shown, as well as the standard deviation values. MRS: MRS broth; VFS-OK: vaginal fluid simulant of Owen and Katz; MVFS: modified vaginal fluid simulant of Tomás and Nader-Maciás.

4. Discussion

The positive impact of the use of probiotic products, and their derivatives, such as prebiotics, postbiotics and others, on the improvement of human health is well documented [7,19,31–33]. Regarding probiotics, the selection of the probiotic strain is one of the key steps in product development. In general, strains belonging to the *L. acidophilus* phylogenetic group are commonly used in probiotics for vaginal application [7,33–35]. Studies

of the human vaginal flora using molecular methods have come to show that this is not, however, the most prevalent species in healthy vaginal flora (which is usually dominated by *L. crispatus*, *L. jensenii*, *L. iners* and *L. gasseri*) [2,36]. Nevertheless, they are still used on a large scale, probably because they have many of the ideal characteristics described for a good probiotic species. In particular, it has been demonstrated that this species has the ability to adhere to the vaginal and cervical epithelial cells, produce hydrogen peroxide and bacteriocins and resist spermicides and hygiene products [37,38]. In addition, studies of safety have also been performed with this species, to ensure a reduced pathogenic potential, low frequency of antibiotic resistance genes and the ability of the probiotic strain to coexist with the normal vaginal flora [37].

When we compared the overall profiles of survival of the probiotics over time, we found that not all products containing *L. acidophilus* (Baciginal IS®, Baciginal Activ®, Gynoflor® and Pregyn-S®) had the same profile. The two *L. acidophilus*-containing probiotics (Gynoflor® and Baciginal Activ®) had higher rates of recovery both from MRS and MVFS and the same recovery rate over time, indicating that the probiotic strain was robust and stable over time. On the other hand, the product Baciginal IS ® had a recovery logarithmic reduction of around 80% of the claimed value, after 48 h of dispersion, when MVFS was used. The product Pregyn® had the lowest recovery rate of all the enrolled mediums, and the viability of the cells decreased over time. These results emphasize the importance of selecting, besides the lactobacilli species, the most adequate strain profile and formulation, in order to assure the best survival rates for each specific clinical condition. Although the probiotic mechanisms and the ability to survive manufacturing processes are known to be strain-dependent, it has been found that the recovery of probiotics is also dependent on other factors, including the use of prebiotics in the formulation [39]. This observation is corroborated by this study, since the two products containing the same species, and probably the same strain (Baciginal Activ®, Baciginal Is®), still showed different variations and recovery profiles over time. Finally, another explanation for these differences could be the longevity of the strains, as both Baciginal IS ® and Pregyn®, despite using them before the expiration date and respecting the conditions of storage, were both closer to the expiration date than the other products. Thus, these results seem to indicate that there is a need for rigorous testing to ensure the quality of the batches and the stability of the product throughout its recommended period of use.

Overall, we found that the recovery of all probiotics decreased over time. In this study, the only probiotic in which the recovery increased over 48 h was the Isadin α barcilus®. The prolonged release profile is not described, so more research is needed regarding this product, because it is likely that the type of formulation (vaginal gelatin capsules with oil content) is the factor influencing this result. Moreover, one should also consider the fact that the probiotic strain in this product is *L. plantarum*, which has, in our experience, a good in vitro multiplication profile. Another product in which we noted an overall profile of stability over time, and in which we obtained recoveries above the alleged values indicated by the company (using both culture media and vaginal simulants), was Gynophilus®, in which the probiotics species is *L. rhamnosus*. Although neither *L. plantarum* nor *L. rhamnosus* are constituents of the healthy vagina [2,36], both species have been extensively used in probiotic products [40–43], probably due to their inherent characteristics as commensals and their enhanced adherence to the mucosa. However, one must also consider and discuss whether the observed results for these two products (increased recovery number of cells over time) are in accordance with the product information provided to the consumer and the possible consequences of these results.

Compared with previous studies [15–17], where most recoveries were lower than those claimed for probiotics marketed for non-vaginal applications, our study suggested that the products in test are more consistent. While maintaining the viability over time is an important factor, this is not an absolute measure of the effectiveness of the product. Thus, the ability of the strains to maintain the expression of their probiotic mechanisms after recovery is more important and a factor to be evaluated. Since, normally, these are single

daily dose products, their viability at 24 h is more crucial than the value retrieved at 48 h. However, we also intended to evaluate the influence of time to gain a better understanding of the robustness of the probiotic strain. A longer viability could modify the requirements of daily dosing to less frequent use. In our study, the logarithmic reduction of CFU per capsule varied depending on the product used. Therefore, we advocate that all products marketed as useful probiotics be tested by our or similar methods to assess survival in vaginal surroundings.

In this study, lactobacilli recovery was carried out, not only in a standard culture media served as a control for comparison (MRS), but also in two different vaginal simulants (VFS-OK and MVFS). MVFS is adapted from VFS-OK by adjusting its chemical composition to be even closer to the vaginal fluid characteristics found in healthy women of childbearing age [25]. By comparing the overall recoveries in the three solutions used in this study, we found that the recovery in MRS is, in most cases, superior to that obtained in VFS-OK and MVFS. This result was expected, since MRS is designed to optimize the growth of lactobacilli [44]. Still, when comparing the recoveries after 48 h of incubation in VFS-OK and MVFS to MRS, we found that the logarithmic decrease was lower in MVFS for most probiotics. Therefore, this study supports Nader-Macias's work [25], which proposed using a vaginal fluid simulant more similar to the real vaginal fluid, containing glycogen, mucin and higher concentrations of glucose, especially for studies covering 48 h incubation or longer. It seems that MVFS better simulates the human vaginal fluid and, therefore, the probiotic strain survival reflects the reality more than MRS or even VFS-OK. These results should be taken into account in the choice of a solution for the dispersion of probiotic products in future in vitro studies. Furthermore, our results emphasize the need for the pharmaceutical companies developing probiotic products for vaginal application to assess strain recovery in vaginal fluid simulants in order to mimic in vivo real conditions. On the other hand, the use of prebiotics or postbiotics can circumvent this need by working with metabolites and not living material. Previous studies have revealed the great potential of these alternatives for vaginal application [20,22] that, nonetheless, will still need to be tested for their safety and efficacy in order to be marketed as drugs.

In summary, commercial probiotic products for vaginal use were tested for survival capacities when dissolved in different dispersion media. In this study, we aimed to contribute to the state-of-the-art research regarding the quality control and in vitro prediction of the effectiveness of probiotic products for vaginal application, supporting the definition of a battery of in vitro testing to characterize the efficacy, safety and quality of these products. This strategy is urgent in order to support the commercialization of probiotic products for vaginal application that should be considered as drugs due to the effect of the probiotic strains.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol3040082/s1, Table S1: Main characteristics of the probiotic products studied.

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Data Availability Statement: The data presented in this study is available within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Petrova, M.I.; Lievens, E.; Malik, S.; Imholz, N.; Lebeer, S. Lactobacillus species as biomarkers and agents that can promote various aspects of vaginal health. *Front. Physiol.* **2015**, *6*, 81. [CrossRef] [PubMed]
2. Fettweis, J.M.; Serrano, M.G.; Girerd, P.H.; Jefferson, K.K.; Buck, G.A. A new era of the vaginal microbiome: Advances using next-generation sequencing. *Chem. Biodivers.* **2012**, *9*, 965–976. [CrossRef] [PubMed]
3. Miller, E.A.; Beasley, D.E.; Dunn, R.R.; Archie, E.A. Lactobacilli Dominance and Vaginal pH: Why Is the Human Vaginal Microbiome Unique? *Front. Microbiol.* **2016**, *7*, 1936. [CrossRef] [PubMed]
4. Borges, S.; Silva, J.; Teixeira, P. The role of lactobacilli and probiotics in maintaining vaginal health. *Arch. Gynecol. Obstet.* **2014**, *289*, 479–489. [CrossRef]
5. Boris, S.; Barbés, C. Role played by lactobacilli in controlling the population of vaginal pathogens. *Microbes Infect.* **2000**, *2*, 543–546. [CrossRef]
6. Mastromarino, P.; Vitali, B.; Mosca, L. Bacterial vaginosis: A review on clinical trials with probiotics. *N. Microbiol.* **2013**, *36*, 229–238.
7. Nader-Macías, M.E.; Tomás, M.S.J. Profiles and technological requirements of urogenital probiotics. *Adv. Drug Deliv. Rev.* **2015**, *92*, 84–104. [CrossRef]
8. McFarland, L.V.; Elmer, G.W. Pharmaceutical probiotics for the treatment of anaerobic and other infections. *Anaerobe* **1997**, *3*, 73–78. [CrossRef]
9. Reid, G.; Jass, J.; Sebulsky, M.T.; McCormick, J.K. Potential uses of probiotics in clinical practice. *Clin. Microbiol. Rev.* **2003**, *16*, 658–672. [CrossRef]
10. European Commission. Regulation (EU) 2017/745 of the European Parliament and of the Council of 5 April 2017 on Medical Devices, Amending Directive 2001/83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 and Repealing Council Directives 90/385/EEC and 93/42/EEC. 2017. Online Resource. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32017R0745 (accessed on 31 July 2023).
11. European Pharmacopeia, Live Biotherapeutic Products. *Monograph* **2017**, *3054*. Online Resource. Available online: https://www.gmp-compliance.org/gmp-news/revisions-of-ep-chapter-5-1-8-2-6-36-2-6-38-and-monograph-3053 (accessed on 31 July 2023).
12. Yunes, R.A.; Poluektova, E.U.; Belkina, T.V.; Danilenko, V.N. Lactobacilli: Legal Regulation and Prospects for New Generation Drugs. *Appl. Biochem. Microbiol.* **2022**, *58*, 652–664. [CrossRef] [PubMed]
13. Pendharkar, S.; Skafte-Holm, A.; Simsek, G.; Haahr, T. Lactobacilli and Their Probiotic Effects in the Vagina of Reproductive Age Women. *Microorganisms* **2023**, *11*, 636. [CrossRef] [PubMed]
14. World Health Organization. *Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation*. 2006. Online Resource. Available online: http://www.fao.org/3/a-a0512e.pdf (accessed on 31 July 2023).
15. Hughes, V.L.; Hillier, S.L. Microbiologic characteristics of Lactobacillus products used for colonization of the vagina. *Obstet. Gynecol.* **1990**, *75*, 244–248. [PubMed]
16. Goldstein, E.J.; Citron, D.M.; Claros, M.C.; Tyrrell, K.L. Bacterial counts from five over-the-counter probiotics: Are you getting what you paid for? *Anaerobe* **2014**, *25*, 1–4. [CrossRef]
17. Patrone, V.; Molinari, P.; Morelli, L. Microbiological and molecular characterization of commercially available probiotics containing Bacillus clausii from India and Pakistan. *Int. J. Food Microbiol.* **2016**, *237*, 92–97. [CrossRef]
18. Mugambi, M.N.; Musekiwa, A.; Lombard, M.; Young, T.; Blaauw, R. Synbiotics, probiotics or prebiotics in infant formula for full term infants: A systematic review. *Nutr. J.* **2012**, *11*, 81. [CrossRef]
19. Al-Ghazzewi, F.H.; Tester, R.F. Biotherapeutic agents and vaginal health. *J. Appl. Microbiol.* **2016**, *121*, 18–27. [CrossRef]
20. Collins, S.L.; McMillan, A.; Seney, S.; van der Veer, C.; Kort, R.; Sumarah, M.W.; Reid, G. Promising Prebiotic Candidate Established by Evaluation of Lactitol, Lactulose, Raffinose, and Oligofructose for Maintenance of a Lactobacillus-Dominated Vaginal Microbiota. *Appl. Environ. Microbiol.* **2018**, *84*, e02200-17. [CrossRef]
21. Salminen, S.; Collado, M.C.; Endo, A.; Hill, C.; Lebeer, S.; Quigley, E.M.M.; Sanders, M.E.; Shamir, R.; Swann, J.R.; Szajewska, H.; et al. The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. *Nat. Rev. Gastroenterol. Hepatol.* **2021**, *18*, 649–667. [CrossRef]
22. Shen, X.; Xu, L.; Zhang, Z.; Yang, Y.; Li, P.; Ma, T.; Guo, S.; Kwok, L.-Y.; Sun, Z. Postbiotic gel relieves clinical symptoms of bacterial vaginitis by regulating the vaginal microbiota. *Front. Cell Infect. Microbiol.* **2023**, *13*, 1114364. [CrossRef]
23. Migliore, A. On the new regulation of medical devices in Europe. *Expert. Rev. Med. Devices* **2017**, *14*, 921–923. [CrossRef] [PubMed]
24. Owen, D.H.; Katz, D.F. A vaginal fluid simulant. *Contraception* **1999**, *59*, 91–95. [CrossRef] [PubMed]
25. Tomás, M.S.J.; Nader-Macías, M.E. Effect of a medium simulating vaginal fluid on the growth and expression of beneficial characteristics of potentially probiotic lactobacilli. *Commun. Curr. Res. Educ. Top. Trends Appl. Microbiol.* **2007**, *2*, 732–739.
26. Valenta, C. The use of mucoadhesive polymers in vaginal delivery. *Adv. Drug Deliv. Rev.* **2005**, *57*, 1692–1712. [CrossRef] [PubMed]
27. Vermani, K.; Garg, S. The scope and potential of vaginal drug delivery. *Pharm. Sci. Technol. Today* **2000**, *3*, 359–364. [CrossRef] [PubMed]

28. Del Gaudio, G.; Lombardi, L.; Maisetta, G.; Esin, S.; Batoni, G.; Sanguinetti, M.; Senesi, S.; Tavanti, A. Antifungal activity of the non cytotoxic human peptide hepcidin 20 against fluconazole resistant Candida glabrata in human vaginal fluid. *Antimicrob. Agents Chemother.* **2013**, *57*, 4314–4321. [CrossRef]
29. Juárez Tomás, M.S.; De Gregorio, P.R.; Terraf, M.C.L.; Nader-Macías, M.E.F. Encapsulation and subsequent freeze-drying of Lactobacillus reuteri CRL 1324 for its potential inclusion in vaginal probiotic formulations. *Eur. J. Pharm. Sci.* **2015**, *79*, 87–95. [CrossRef]
30. Borges, S.; Costa, P.; Silva, J.; Teixeira, P. Effects of processing and storage on Pediococcus pentosaceus SB83 in vaginal formulations: Lyophilized powder and tablets. *Biomed. Res. Int.* **2013**, *2013*, 680767. [CrossRef]
31. Ventura, M.; Turroni, F.; van Sinderen, D. Probiogenomics as a tool to obtain genetic insights into adaptation of probiotic bacteria to the human gut. *Bioeng. Bugs.* **2012**, *3*, 73–79. [CrossRef]
32. Palmeira-de-Oliveira, R.; Palmeira-de-Oliveira, A.; Martinez-de-Oliveira, J. New strategies for local treatment of vaginal infections. *Adv. Drug Deliv. Rev.* **2015**, *92*, 105–122. [CrossRef]
33. Falagas, M.; Betsi, G.I.; Athanasiou, S. Probiotics for the treatment of women with bacterial vaginosis. *Clin. Microbiol. Infect.* **2007**, *13*, 657–664. [CrossRef] [PubMed]
34. Huang, H.; Song, L.; Zhao, W. Effects of probiotics for the treatment of bacterial vaginosis in adult women: A meta-analysis of randomized clinical trials. *Arch. Gynecol. Obstet.* **2014**, *289*, 1225–1234. [CrossRef] [PubMed]
35. Homayouni, A.; Bastani, P.; Ziyadi, S.; Mohammad-Alizadeh-Charandabi, S.; Ghalibaf, M.; Mortazavian, A.M.; Mehrabany, E.V. Effects of probiotics on the recurrence of bacterial vaginosis: A review. *J. Low. Genit. Tract Dis.* **2014**, *18*, 79–86. [CrossRef] [PubMed]
36. Lamont, R.F.; Sobel, J.D.; Akins, R.A.; Hassan, S.S.; Chaiworapongsa, T.; Kusanovic, J.P.; Romero, R. The vaginal microbiome: New information about genital tract flora using molecular based techniques. *BJOG* **2011**, *118*, 533–549. [CrossRef] [PubMed]
37. Harzallah, D.; Belhadj, H. Lactic Acid Bacteria as Probiotics: Characteristics, Selection Criteria and Role in Immunomodulation of Human GI Muccosal Barrier. In *Lactic Acid Bacteria–R & D for Food, Health and Livestock Purposes*; InTech Open: London, UK, 2013; pp. 197–217.
38. Klaenhammer, T.R.; Altermann, E.; Pfeiler, E.; Buck, B.L.; Goh, Y.-J.; O'Flaherty, S.; Barrangou, R.; Duong, T. Functional genomics of probiotic Lactobacilli. *J. Clin. Gastroenterol.* **2008**, *42 Pt 2*, S160–S162. [CrossRef]
39. de Vrese, M.; Schrezenmeir, J. Probiotics, prebiotics, and synbiotics. *Adv. Biochem. Eng. Biotechnol.* **2008**, *111*, 1–66.
40. Bharwani, A.; Mian, M.F.; Surette, M.G.; Bienenstock, J.; Forsythe, P. Oral treatment with Lactobacillus rhamnosus attenuates behavioural deficits and immune changes in chronic social stress. *BMC Med.* **2017**, *15*, 7. [CrossRef]
41. Grenov, B.; Namusoke, H.; Lanyero, B.; Nabukeera-Barungi, N.; Ritz, C.; Mølgaard, C.; Friis, H.; Michaelsen, K.F. Effect of Probiotics on Diarrhea in Children With Severe Acute Malnutrition: A Randomized Controlled Study in Uganda. *J. Pediatr. Gastroenterol. Nutr.* **2017**, *64*, 396–403. [CrossRef]
42. Chandel, D.S.; Perez-Munoz, M.E.; Yu, F.; Boissy, R.; Satpathy, R.; Misra, P.R.; Sharma, N.; Chaudhry, R.; Parida, S.; Peterson, D.A.; et al. Changes in the Gut Microbiota After Early Administration of Oral Synbiotics to Young Infants in India. *J. Pediatr. Gastroenterol. Nutr.* **2017**, *65*, 218–224. [CrossRef]
43. Andersson, H.; Tullberg, C.; Ahrné, S.; Hamberg, K.; Ahrén, I.L.; Molin, G.; Sonesson, M.; Håkansson, Å. Oral Administration of Lactobacillus plantarum 299v Reduces Cortisol Levels in Human Saliva during Examination Induced Stress: A Randomized, Double-Blind Controlled Trial. *Int. J. Microbiol.* **2016**, *2016*, 8469018. [CrossRef]
44. De Man, J.; Rogosa, M.; Sharpe, M.E. A medium for the cultivation of lactobacilli. *J. Appl. Microbiol.* **1960**, *23*, 130–135. [CrossRef]