Strategies for the evaluation and selection of potential vaginal probiotics from human sources: an exemplary study

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RESEARCH ARTICLE

Abstract

During the last years, the application of probiotics in gynaecological clinical practice has gained increasing relevance regarding therapy and prevention. This trend has also provoked the need for having tailored pharmaceutical preparations containing powerful microbial strains with defined properties. For the development of such preparations, several factors and criteria have to be considered, thereby not only focusing on identity and safety aspects as well as individual properties of the bacterial strains, but also on technological issues, such as stability and targeted release from the preparation. Against this background, this report exemplarily addresses the development procedure of a probiotic bacterial formulation for gynaecological application, covering the search for suitable strains, assessing their microbiological, molecular biological and physiological characterisation, and the selection for their use in clinical trials. In detail, starting with 127 presumptive lactobacilli isolates of vaginal origin, a step-by-step selection of candidate strains meeting special criteria was thoroughly examined, finally leading to a preparation consisting of four individual *Lactobacillus* strains that possess particular significance in women´s urogenital health. Relevant issues and quality criteria of probiotic preparations used in gynecology are addressed and exemplarily introduced.

Keywords: probiotic, lactobacilli, gynaecology, development

1. Introduction

Infections of the urogenital tract in women represent a major burden on the quality of life of women and the health care system in many countries (Reid and Bocking, 2003). Complications arising from bacterial vaginosis (BV) include increased risk of sexually transmitted diseases, including human immunodeficiency virus and elevated risk of preterm birth (PTB). Pharmaceutical interventions, such as antibiotics, have been suboptimally effective and have failed to reduce the incidence of PTB. The absence of lactobacilli in the vagina, a specific feature of BV, raises the question as to whether restoration of lactobacilli, by probiotic therapy, can restore the normal flora and improve the chances of having a healthy term pregnancy (Li *et al.*, 2012; Reid, 2002).

The rationale for probiotic use in women´s health is quite strong (MacPhee *et al.*, 2010). Certain lactobacilli strains can safely colonise the vagina after oral and vaginal administration, displace and kill pathogens, including *Gardnerella vaginalis* and *Escherichia coli*, and modulate the immune response to interfere with the inflammatory cascade that leads to PTB (Spurbeck and Arvidson, 2011). Additional attributes of probiotics include their potential to degrade lipids and enhance cytokine levels, which promote embryo development. In a society that focuses on disease rather than health and drug therapy rather than natural preventive measures, it will take some effort to get remedies, such as probiotics, into mainstream care. Perhaps, the escalating health care budgets and emergence of ‘superbugs’ will provide the incentives to put in place clinical trials designed to evaluate how best to use the commensal organisms that, after all, make up more of

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our body than human cells, and without which none of us would survive (Reid and Bocking, 2003). Next to direct and local application, the oral application of vaginal probiotic preparations has gained considerable importance (Hemalatha *et al.*, 2012; Reid, 2002; Zuccotti *et al.*, 2008). This trend has further stimulated the interest in developing probiotic products for gynaecological applications. In this context, preparations with defined probiotic bacterial strains possessing tailored functionality have been preferred (Andreu, 2004; Spurbeck and Arvidson, 2011).

In general, probiotic strains have to fulfil a set of requirements, which, among several ones, relate to criteria, such as safety, bacterial identity, maintenance of viability and bacterial stability, in both the preparation and the final environment of application, and antagonistic properties against relevant pathogens (Zuccotti *et al.*, 2008). In the development of such preparations, it is of crucial relevance on one hand to consider the variety of individual properties associated with candidates being selected from a pool of pre-selected strains, on the other hand to provide tailored preparations possessing acceptable shelf-life. Last but not least, the impact of the preparation when applied either to patients or to healthy subjects in terms of preventive application is important (Andreu, 2004). In addition, strains contained in multi-strain preparations need to be compatible among each other or even possess synergistic potential. Thus the development of probiotic preparations for gynaecological purposes has to follow a stepwise process, in which decisions for selection, based on a series of scientific results concerning microbiology, molecular biology, pharmaceutical technology and medicinal functionality, play a considerable role.

The present study describes a practical example for such a development procedure, thereby considering a product that is aimed at oral application and already patented (Kiss *et al.*, 2013). Actually, the paper presenting the results of a clinical study conducted was accepted for publication (Kaufmann *et al.*, 2014). Based on a collection of 127 lactobacilli isolates, it will be demonstrated how such a decision tree is managed and how the most relevant strains are selected and then combined and put into practice by technological means. This paper, therefore, may serve as a useful roadmap for the development of such preparations, as it describes the different steps and milestones needed for this purpose.

2. Material and methods

Strain origin

The *Lactobacillus* isolates originate from a study conducted to characterise the dominant species of *Lactobacillus* colonizing the vagina of 126 healthy pregnant women and gain a better understanding of the potential role of species that might be associated with infection-free status (Kiss *et al.*, 2007). This study was approved by the Ethics Committee of the Medical University of Vienna (EK 80/2005) in accordance with the declaration of Helsinki and the guidelines for good clinical practice. In detail, lactobacilli isolates cultured on De Man, Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany) were purified by replicated loop-streaking, followed by subcultivation in MRS broth (Merck) under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2) for 24 h at 37 °C. Aliquots of 1.5 ml were centrifuged, washed twice with sterile sodium chloride solution (0.9%) and one time with sterile ethylendiamine tetra-acetic acid (50 mM, pH 8) and then stored at -20 °C (Kiss *et al.*, 2007). Starting from the frozen biomass, the isolates were resuscitated in MRS broth (anaerobic conditions, 37 °C, 48 h) and consequently loop-streaked on MRS agar in three replicates. From the last one, a single colony was Gram-stained, microscopically examined, then cultured in MRS broth and incubated under anaerobic conditions for 24 h at 37 °C. Thereof, master cell bank cryo-cultures were prepared by adding 20% (v/v) glycerol to the grown broth culture. The prepared aliquots were then stored at -80 °C. A further aliquot of 1.5 ml was subjected to DNA extraction for conduction of polymerase chaine reaction (PCR)-based analysis techniques.

Basic strain identification and characterisation

One ml of each MRS broth-cultured isolate was centrifuged and the biomass pellet subjected to catalase testing in 3% (v/v) hydrogen peroxide solution and oxidase testing by reacting the biomass with one drop of reagent (Biomérieux, Marcy l'Etoile, France) on a filter paper. In a next step, the growth behaviour at different atmospheric conditions was tested. The isolates were loop-streaked twice on MRS agar and incubated for 48 h at 37 °C under aerobic and anaerobic conditions. The growth of each isolate was benchmarked in three classes: +++ (≥3 mm), ++ (1-3 mm), and + (≤1 mm). Further, the acidification capacity was quantified by measuring the finally obtained pH after anaerobic incubation in MRS broth for 48 h at 37 °C. The acid resistance of the isolates was quantified by cultural enumeration after 3 h of incubation in MRS broths possessing defined pH values (pH 2, 2.5, 3, 3.5, and 4). The pH of the broths was adjusted by adding 1 M hydrochloric acid. In detail, the microbial count of a 1:100 diluted, 24 h incubated MRS broth culture was assessed using MRS agar. In parallel, 0.1 ml of the aforementioned MRS broth culture was added to 9.9 ml of each pH-adjusted MRS broth, mixed and anaerobically incubated for 3 h at 37 °C. Then, the microbial count of an aliquot was measured in duplicate. The production of extracellular hydrogen peroxide (H_2O_2) was quantified by two different methods. First, the attribute of H_2O_2 production was semiquantitatively measured by applying H_2O_2 test sticks (Merck) for assessing aerobically and anaerobically incubated strains according to Wilks *et al.* (2004). Further, a chromogenic agar medium-based

technique was applied to characterise the H_2O_2 production of the strains. The medium published by Eschenbach *et al.* (1989) was optimised regarding its peroxidase concentration. Hence, the finally applied medium consisted of MRS agar supplemented with 0.25 mg/ml 3,3´,5,5´tetramethylbenzidine (Sigma-Aldrich, Vienna, Austria) and 0.01 mg/ml horseradish peroxidase (Sigma-Aldrich). Isolates were loop-streaked on the chromogenic agar and incubated for five days at 37 °C under anaerobic conditions (GENbag anaer; Biomérieux). Then, each agar plate was subjected to atmospheric air for 30 min. The intensity of the resulting blue coloration of the colonies due to the production of extracellular H_2O_2 was optically assessed according to a five level scale (+++, ++, +, (+), and –). The bile salt resistance of the isolates was tested by loop-streaking each strain on a set of MRS agar plates containing increasing levels of bile salts (Sigma-Aldrich). The concentrations ranged from 0, 0.1, 0.2, 0.3 and 0.4 to 0.5% of bile salts. Plates were incubated for 48 h at 37 °C. The growth characteristics were quantified in four levels (+++, ++, +, and –). The commercially available test series API 50 CH-L and the API ZYM (Biomérieux) were applied for the characterisation of the carbohydrate metabolism and for the semiquantitative detection of enzymatic activities, respectively.

Molecular identification and typing

The molecular identification strategy of the isolates consisted of PCR-based tools for the identification at genus level followed by a multiplex PCR for the identification at species level. In the following step, the isolates were analysed by molecular fingerprinting on the strain level by random amplified polymorphic DNA (RAPD)-PCR, partly by repetitive (rep)-PCR and pulsed field gel electrophoresis (PFGE) and 16S rDNA sequencing. In detail, the genomic DNA of all isolates and selected reference strains (*Lactobacillus casei* DSM 20011, *Lactobacillus crispatus* DSM 20584, *Lactobacillus gasseri* DSM 20243, *Lactobacillus jensenii* DSM 20557, *Lactobacillus paracasei* subsp. *paracasei* DSM 5622, *L. paracasei* subsp. *tolerans* DSM 20258, *Lactobacillus rhamnosus* DSM 20247, and *Lactobacillus zeae* DSM 20178) analysed was isolated with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) based on the manufacturer's protocol for Gram-positive bacteria. The concentration and purity of the DNA was checked by agarose gel electrophoresis and photometric analysis. The PCR-based identification on *Lactobacillus* genus level was conducted based on the primers LbLMA1/R16-1 (Dubernet *et al.*, 2002) in a 25 µl format, including negative and positive controls. The PCR reaction was carried out in a Mastercycler (Eppendorf, Hamburg, Germany). Each PCR reaction was composed of 2.5 µl 10× PCR buffer (Finnzymes, Espoo, Finland), 18 µl sterile distilled water, 1 µl of each primer (10 pmol/µl; Eurofins MWG Operon, Ebersberg, Germany), 1 µl dNTPs (10 mM; Carl Roth, Karlsruhe, Germany), 0.5 µl Dynazyme DNA polymerase (2 U/µl; Finnzymes) and 1 µl DNA template. PCR was performed at 95 °C for 5 min, 34 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final elongation step at 72 °C for 8 min. The PCR products were analysed by agarose gel electrophoresis. The PCR-based identification on species level followed the strategy proposed by Song *et al.* (2000). Based on this multiplex PCR, the isolates were grouped into four different species groups. Each group was then further analysed with the considerable species-specific PCR primer sets according to Song *et al.* (2000) and Ward *et al.* (1999).

The molecular typing to identify all isolates on strain level was conducted by RAPD typing, by successively applying a set of eight different RAPD primers. The analysis was carried out in 25 µl volume in a Mastercycler. Each PCR reaction was composed of 2.5 µl 10× PCR buffer (Finnzymes), 17.5 µl sterile distilled water, 2 µl of primer (12.5 pmol/µl; Eurofins MWG Operon) 1 µl dNTPs (10 mM; Carl Roth), 1 µl Dynazyme DNA polymerase (2 U/µl; Finnzymes) and 1 µl DNA template. PCR was performed at 95 °C for 5 min, 45 cycles of 95 °C for 1 min, 36 °C for 1 min, 72 °C for 1 min, and a final elongation step at 72 °C for 8 min. The RAPD PCR products were separated with agarose (2%, w/v) electrophoresis. The molecular fingerprinting of the 12 final strains was also done by rep-typing with the primer $(GTG)_5$ (Versalovic *et al.*, 1994). PCR was carried out in a Mastercycler. Each PCR reaction was composed of 2.5 µl 10× PCR buffer (Finnzymes), 18,5 µl sterile distilled water, 1 µl of $(GTG)_5$ primer (50 pmol/µl; Eurofins MWG Operon) 1 µl dNTPs (10 mM; Carl Roth), 1 µl Dynazyme DNA polymerase (2 U/µl; Finnzymes) and 1 µl DNA template. PCR was performed at 95 °C for 7 min, 30 cycles of 90 °C for 30 s, 40 °C for 1 min, 65 °C for 8 min, and a final elongation step at 65 °C for 16 min. The rep-PCR products were separated with agarose gel (1.25%, w/v) electrophoresis. The remaining 41 strains after the first selection step were also analysed by PFGE typing. The preparation and analysis procedure corresponds to PFGE protocols described previously (Miranda *et al.*, 1991; Patterson and Kelly, 1998; Leuschner *et al.*, 2002). The macrorestriction of DNA, necessary for PFGE typing was conducted by the enzymes *Apa*I, *Sfi*I, *Sma*I and *Not*I. 16S rDNA sequencing was conducted for selected strains by applying the primer set and PCR protocol published by Di Cello *et al.* (1997). The PCR products were purified applying the PCRExtract Mini Kit (5Prime, Hamburg, Germany) and sequenced (Eurofins MWG Operon). The received sequences were analysed with the BLASTn tool (http://blast.ncbi.nlm.nih.gov) and a minimum sequence identity of 98% was chosen as a criterion for species identification.

Strain safety assessment

The haemolytic capacity of the 41 lactobacilli strains selected was analysed on Columbia blood agar (Biomérieux), *Bacillus cereus* DSM 31 served as a positive control. For this purpose, aliquots from a 24 h MRS broth culture were loop-streaked on Columbia blood agar and incubated anaerobically at 37 °C for 48 h. Then, the type and intensity of haemolysis were evaluated. The antibiotic susceptibility of 41 strains against selected antibiotics was screened by broth microdilution and the E-test technique. In detail, the VETMIC ACE-ART microdilution plates containing tetracycline, clindamycin, streptomycin erythromycin, gentamycin and ampicillin and the E-test technique (clindamycin, metronidazole) were applied according to Mayrhofer *et al.* (2008). Further, the antibiotic susceptibility of the 12 shortlisted *Lactobacillus* strains was analyzed with an extended spectrum of 20 antibiotic substances (ampicillin, ampicillin/sulbactam, cefazolin, vancomycin, chloramphenicol, erythromycin, clindamycin, tetracycline, gentamicin, kanamycin, neomycin, streptomycin, metronidazole, ciprofloxacin, trimethoprim, sulfamethoxazole/trimethoprim, fusidic acid, rifampicin, linezolid, quinupristin/dalfopristin) by a broth microdilution technique according to ISO 10932/IDF 223 (ISO, 2010).

Screening for specific virulence factors in *Lactobacillus rhamnosus* strains

The ability to degrade mucin is seen as a relevant safety risk. Therefore, potential probiotic strains should be tested for this attribute (Salminen *et al.*, 1998; Zhou *et al.*, 2001). Scientific studies have shown that about 1% of the human intestinal microbiota is able to degrade mucin. To assess this property, two faecal samples of healthy volunteers were cultured in brain heart infusion broth (Oxoid, Wesel, Germany) and LB broth (Sigma-Aldrich) under anaerobic conditions for 24 h at 37 °C. These samples served as positive controls. Nine potentially probiotic *L. rhamnosus* strains were loop-streaked on MRS agar, and after anaerobic incubation for 24 h at 37 °C one colony was transferred into 10 ml MRS broth and further incubated under the same conditions. Ten µl of each incubated MRS broth culture were dropped onto the agar test medium according to Zhou *et al.* (2001) containing 0.5% (w/v) hog gastric mucin (Sigma-Aldrich) as exclusive carbohydrate source. For growth control purposes, a set of these agar plates was enriched with 3% (w/v) glucose. All test plates were anaerobically incubated for 72 h at 37 °C. Then, the agar plates were stained by flooding the agar surface with Amido black staining solution (0.1% Amido black, 3.5 M acetic acid) for 30 min and destained with 1.2 M acetic acid (three repetitions). Then, mucin decay became visible on the agar plate as a partly decolourised halo around the colony, while the other part of the agar plate remained dark (for details, see Zhou *et al.*, 2001).

The possible presence of glycosidases was tested with the synthetic substrate analogue 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma-Aldrich) and 4-methylumbelliferyl α-D-galactopyranoside (Sigma-Aldrich). Both substances were dissolved at a concentration of 100 µg/ml in Tris-HCl buffer (50 mmol/l). The fluorescence intensity was measured in an UV cabinet set at 365 nm and compared with a negative control. In detail, the strains were cultured in 10 ml MRS broth under anaerobic conditions for 18 h at 37 °C. The cells were harvested by centrifugation and washed twice with sterile physiological NaCl solution. Then, the cells were suspended in 5 ml of the same solution. Thereof, 50 µl were pipetted into the wells of a microtiter plate and mixed with 10 µl substrate. As a negative control, 20 µl substrate were mixed with 50 µl sterile NaCl solution. The microtiter plate was sealed and incubated for 24 h at 37 °C. Thereafter, the fluorescence intensity of each test was compared with the negative control.

The presence of arylamidases was tested according to Oakey *et al.* (1995) and Fernandez *et al.* (2005) by applying the substrates Boc-Val-Pro-Arg-7-AMC (Sigma-Aldrich), simulating thrombin; Z-Phe-Arg-7-AMC (Sigma-Aldrich), simulating kallikrein; and *N*-succinyl-Ala-Ala-Pro-Phe-7-AMC (S 9761; Sigma-Aldrich), simulating chymotrypsin. Each substrate was diluted in 50 mmol/l Tris-HCl buffer yielding a concentration of 100 µg/ml. The increase of the fluorescence intensity was measured in an UV cabinet at a wavelength of 365 nm and compared with a negative control.

Antimicrobial activity of lactobacilli strains against vaginal pathogens

The antimicrobial activity against selected vaginal pathogens was tested by a modified agar streak-spot technique according to Jin *et al.* (2007). First, a 24 h old MRS broth culture of the lactobacilli strains was streaked with a sterile cotton swab in two parallel lines about 20 mm apart on the centre of an MRS agar plate for testing against *E. coli* and *Candida* spp., and on neutralised MRS-starch agar (pH 6.7; 1% soluble starch) for testing against *G. vaginalis*. These plates were then incubated anaerobically for 24 h at 37 °C. The preculturing of the pathogenic test strains was carried out in MRS broth for *Candida albicans* IHEM 9863, *C. albicans* IHEM 3243, *Candida glabrata* IHEM 4210 and *C. glabrata* IHEM 19237. Brain heart infusion broth (Oxoid) was applied for preculturing *E. coli* LMG 9007, *E. coli* LMG 10266, *G. vaginalis* LMG 7832, *G. vaginalis* LMG 14325, *Candida krusei* Cd25 and *C. krusei* Cd 26. 5 µl of the fresh cultured test strains were dropped between the two pre-incubated lactobacilli streaks and separately

dropped near the margin of the MRS or neutralized MRS-starch agar plate as a growth control. All test plates, except the ones containing *C. krusei* strains (aerobic conditions), were incubated anaerobically for 48 h at 37 °C. If any antimicrobial substance is secreted by the tested lactobacilli strain, the substance will diffuse into the agar adjacent to the bacterial streaks. Two parallel streaks of bacterial culture on the agar plate create a strip of an antimicrobial zone. Failed growth of the spotted bacterial and fungal strains between the two bacterial streaks indicates the presence of antimicrobial substance(s) secreted by the tested bacterium into the agar (Jin *et al.*, 2007).

The antimicrobial interactions between the finally selected 12 lactobacilli isolates were tested using the agar spot-overlay technique. Aliquots of 10 µl of 18 h incubated MRS broth cultures of the eleven strains compared were spotted onto an MRS agar plate and incubated under anaerobic conditions for 24 h at 37 °C. Then, 200 µl of an 18 h incubated MRS culture of the remaining strain were mixed with 4.5 ml molten soft agar and applied as agar overlay onto the incubated agar spot plate. The agar plate was again incubated under anaerobic conditions for 48 h at 37 °C. The influence of the strains in the spots on the test strain growth was semiquantitatively evaluated after 24 and 48 h of incubation.

3. Results and discussion

In general, the selection of potential vaginal probiotics followed the strategy shown in Figure 1. Out of a pool of 168 presumptive lactobacilli isolates originating from a former project (Kiss *et al.*, 2007), 127 isolates could be successfully resuscitated in MRS broth. The purity of the isolates was checked by repeated loop-streaking. Based on this culturing, the so-called master cell bank was established in form of multiple glycerol stocks of each isolate and stored at -80 °C. Moreover, the biomass of each isolate was harvested, washed and subjected to DNA extraction. All 127 isolates were applied to a set of phenotypic and genotypic tests, including Gram-staining, catalase and oxidase activity, growth behaviour under aerobic and anaerobic atmospheres, acidification capacity, production of extracellular hydrogen peroxide, and bile salt tolerance (data not shown).

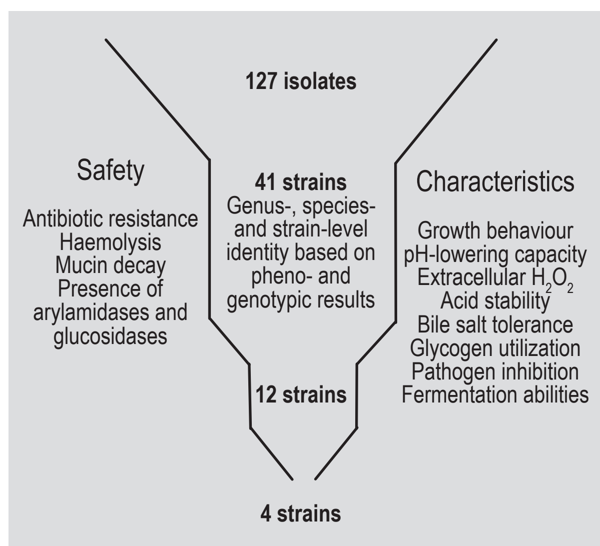

Figure 1. Strategy for selection of potential vaginal probiotic strains.

The results of the genus-specific PCR showed that 99 out of the 127 isolates were members of the genus *Lactobacillus*. Thereof, 68 isolates belong to the following species: 26 *L. gasseri*, 25 *L. rhamnosus*, 9 *L. crispatus*, and 8 *L. jensenii*. The first selection of strains was based on the similarity of the RAPD fingerprints; where possible, duplicates were excluded and a set of 41 strains was determined for further characterisation. These strains belong to the species *L. gasseri* (18), *L. crispatus* (8), *L. jensenii* (6) and *L. rhamnosus* (9). These strains were further investigated with the API 50 CHL test kit, basic PFGE-typing, and applying antibiotics resistance screening by broth microdilution and the E-test technique. Mucin degradation and the presence of glycosidases and arylamidases were tested in all 9 *L. rhamnosus* strains (data not shown).

The second selection of strains was based on the results of the individual growth intensity (under aerobic and anaerobic conditions), the acidification, stability against bile salts, extracellular H_2O_2 production, glycogen utilization (APH 50 CH-L), enzymatic profile (ApiZym), absence of β-haemolysis, and growth inhibition of pathogens (*E. coli*, *G. vaginalis*, *C. krusei*, *C. albicans* and *C. glabrata* strains) (Table 1 and Figure 2). Based on this selection procedure, 12 strains (3 of each species) were selected for further analysis: *L. jensenii* (LbV 8, LbV 110, LbV 116), *L. rhamnosus* (LbV 69, LbV 96, LbV 136), *L. crispatus* (LbV 10, LbV 61, LbV 88) and *L. gasseri* (LbV 62, LbV 150N, LbV 162).

The third selection resulting in 4 final candidate strains was based on their stability assessment under acidic conditions, detailed antibiotic sensitivity profiles as demonstrated by the broth microdilution technique, rep and RAPD PCR fingerprints, antagonisms against the other 11 *Lactobacillus* candidate strains as tested by agar dot-overlay procedure (for exemplary results, see Table 2 and Figure 3), safety aspects (mucin-decay, presence of arylamidases and glucosidases), and technological properties, such as fermentation yield. Undergoing these test criteria, the strains *L. crispatus* LbV 88 (DSM 22566), *L. gasseri* LbV 150N (DSM 22583), *L. jensenii* LbV 116 (DSM 22567) and *L. rhamnosus* LbV96 (DSM 22560) were selected for the final product and deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) strain database for patenting purposes according to the Budapest

Table 1. Antimicrobial activity of the finally selected *Lactobacillus* strains against vaginal pathogens.[1]

Strain	*Candida krusei* (48 h)		*Candida albicans* (48 h)		*Candida glabrata* (48 h)		*Escherichia coli* (48 h)		*Gardnerella vaginalis* (72 h)	
	Cd 25	Cd 26	Cd 30	Cd 31	Cd 33	Cd 34	Ec 5	Ec 6	Ga 1	Ga 3
L. crispatus LBV 88	+	-	+/++	+	+	+	-	-/+	+++	+++
L. rhamnosus LBV 96	+++	+++	+++	+++	++	+	-/+	-/+	+++	+++
L. jensenii LBV 116	+/++	-/+	++	+/++	+	+	-	+	+++	+++
L. gasseri LBV 150N	+++	+++	+++	+/++	+	-	-	-/+	+++	+++

[1] +++ = pronounced inhibition of the pathogen (no colonies growing in-between the test lines); ++ = inhibition (only small single colonies visible in-between the test lines); + = weak inhibition (grown colonies are smaller compared to the growth control); - = no inhibition.

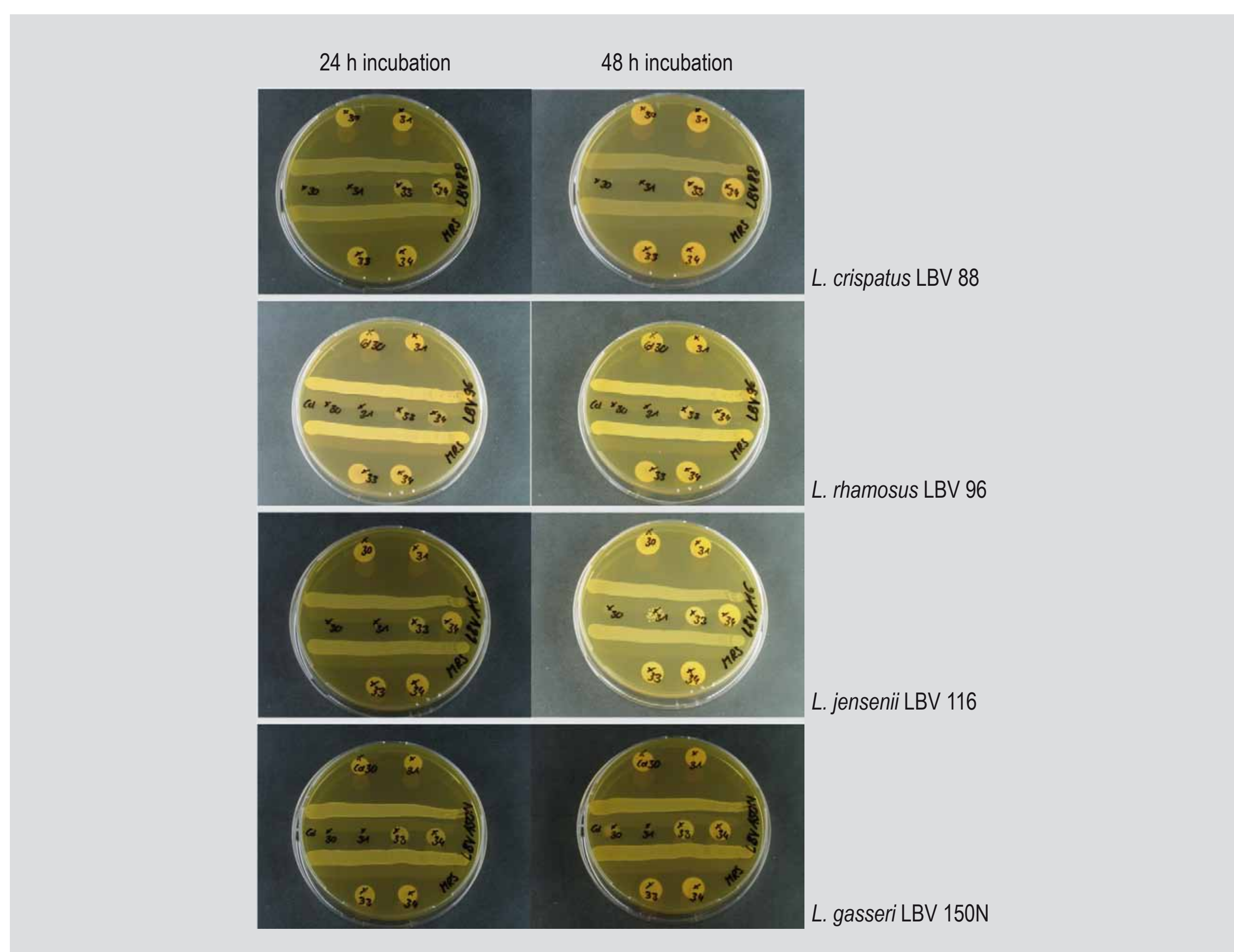

Figure 2. Antimicrobial activity of selected *Lactobacillus* strains against *Candida albicans* (30, 31) and *Candida glabrata* (33, 34) after 48 h incubation at 37 °C under aerobic conditions. The inhibition of the spotted test strains is displayed by poor or even no growth in the area between the two lines of *Lactobacillus* colonies. The corresponding growth controls are suited near the margin of the agar plate.

treaty and the international recognition of the deposit of microorganisms. The identity check of the deposited strains again included the following assessments: physiological screening by API 50 CH-L, molecular typing by $(GTG)_5$ rep-PCR, RAPD-PCR, and PFGE as well as 16S rDNA sequencing. Further investigations clearly displayed the unique molecular strain identity of the finally selected strains (Figures 4 and 5), also when compared to the

Table 2. Results of the agar spot overlay method for testing antimicrobial interactions of the finally selected *Lactobacillus* strains after 48 h incubation.

Strain	Inhibited strains[1]			
	LbV 96	**LbV 88**	**LbV 116**	**LbV 150N**
L. rhamnosus LbV 96		+++	+	+
L. crispatus LbV 88	-/+		-/+	-/+
L. jensenii LbV 116	-/+	-/+		-
L. gasseri LbV 150N	-/+	+/++	-	

[1] +++ = strong inhibition (distinctive inhibition zone); ++ = medium inhibition (small inhibition zone without growth); + = weak inhibition (reduced growth near the colony of the tested strain); - = no inhibition.

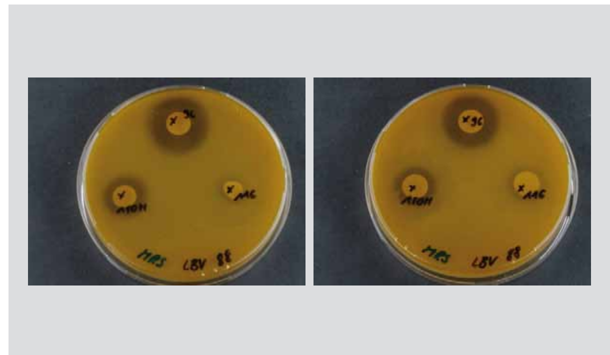

Figure 3. Inhibition of *Lactobacillus crispatus* LBV88 by *Lactobacillus rhamnosus* LBV 96, *Lactobacillus jensenii* LBV 116 and *Lactobacillus gasseri* LBV 150N. The formation of an inhibition halo displays the antimicrobial activity against the tested *Lactobacillus* strain.

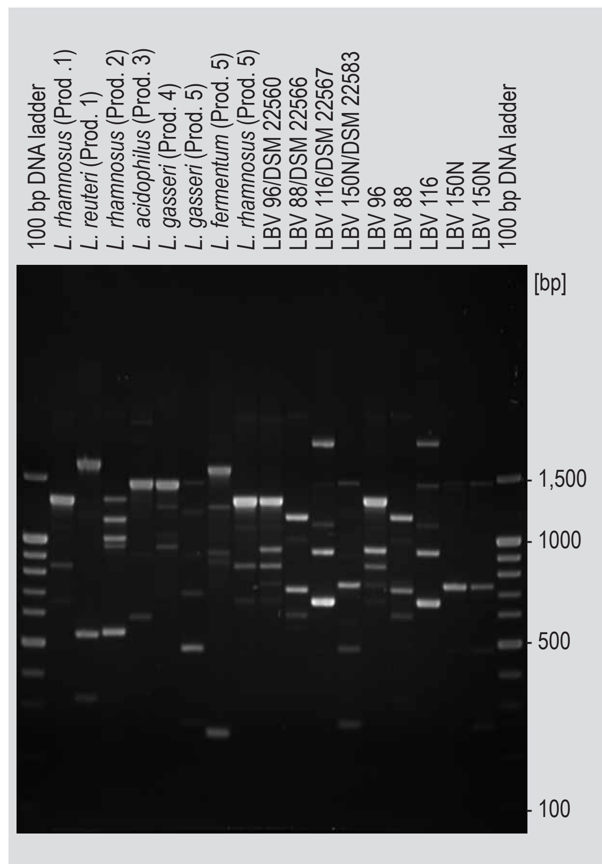

Figure 4. Random amplified polymorphic DNA PCR based molecular typing with Primer 4. The strain specific fingerprints of the four final (LBV) strains allow the differentiation from strains isolated from five different commercial probiotic *Lactobacillus* products for vaginal health. In addition, the strain conformity of the aliquots deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen is shown.

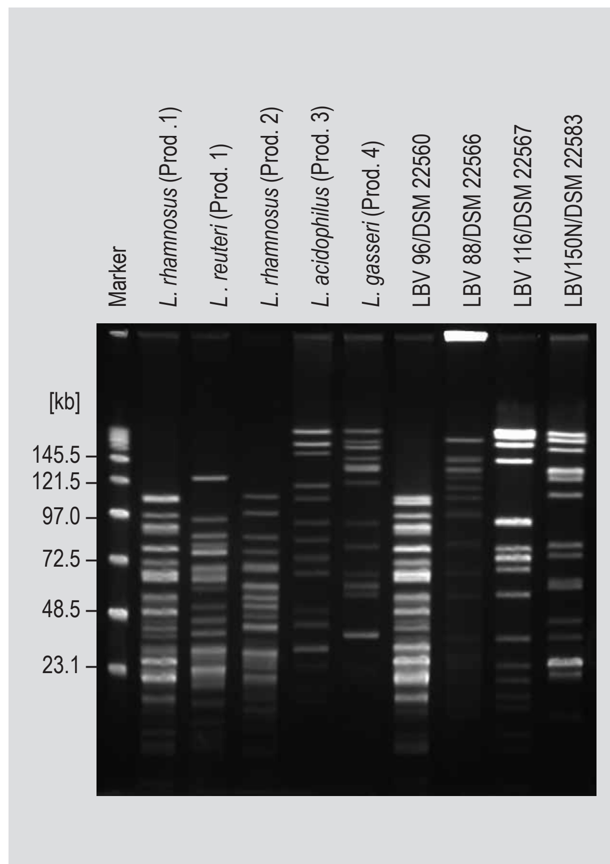

Figure 5. Pulsed field gel electrophoresis fingerprint (Apal, 21 h, pulse time gradient 8-2 s). The strain specific fingerprints of the four final (LBV) strains allow the differentiation from strains isolated from four different commercial probiotic *Lactobacillus* products for vaginal health.

molecular fingerprints of *Lactobacillus* strains originating from five commercially available probiotic products used for vaginal health (*L. rhamnosus* and *L. reuteri* from product 1; *L.* rhamnosus from product 2; *L.* acidophilus from product 3; *L. gasseri* from product 4 and *L. gasseri, L. fermentum*, and *L. rhamnosus* from product 5).

Targeted formulation of the selected strains

To achieve high levels of viable lactic acid bacteria in the vagina, two possible pathways of technology may be chosen. One is a local application of lyophilized strains in capsules, the other one an orally applicable formulation that has to guarantee a high bacterial survival rate during the passage through the gastrointestinal tract and allow the transit of some cells capable of colonising the vaginal area.

During the last two decades, several groups of scientists focused their investigations on innovative lactic acid bacterial formulations ensuring a high protective effect against gastric juice, which usually is a natural barrier against microorganisms. In addition, application forms need to exhibit sufficient shelf life stability (Miller and Reid, 2012; Stadler and Viernstein, 2003). Most manufacturing processes for dry formulations involve freeze- or spray-dried bacteria, depending on their individual heat resistance. The increasing quest for systems directly applicable for the production with dried lactic acid bacteria stimulated the use and adaptation of mainly pharmaceutical technologies, but also the search for new coating materials and some basic changes in fermentation and drying technologies. Pharmaceutical powder formulation with the additional use of enteric coating techniques afford end products, such as capsules, tablets, suppositories or tampons. Other methods, such as the encapsulation into biological matrices (fats or gelatine) or hot melt coating processes, have so far not attained a sufficient level of confidence for use in routine production. Table 3 gives a survey of currently commercially available pharmaceutical preparations, including their specific properties.

The four selected strains were produced in separated fermentations, freeze-dried with cryo-protectants and encapsulated. The capsules displayed a good microbiological stability, even at room temperature. The detailed composition of the capsules, including the viable cell numbers of each strain, are reported by Kaufmann *et al.* (2014).

4. Conclusions

The scientific development of probiotic formulations for gynaecological purposes is based on a series of selection steps being first carried out at laboratory scale. Starting with a collection of possible candidates originating from human isolates, these strains have to undergo several assessments in terms of different criteria before they can be subjected to technological formulations and, finally, to clinical investigations. Owing to the individual capacity of each selected strain, different beneficial effects of such products have been demonstrated so far ,leading to some proven evidence about their efficacy. Therefore, the present study may give some guidance in assisting scientists on their way to develop such preparations.

Table 3. Probiotic formulations used in gynaecology and their corresponding characteristics.

Cell-protecting concepts/excipients		Application route	Commercial products
Capsules	Gelatine capsules	local	Döderlein vaginal capsules; HÄLSA Pharma GmbH, Lübeck, Germany
	HPMC-capsules[1]	local	Vagisan lactic acid bacteria; HÄLSA Pharma GmbH, Lübeck Germany
	Gastric juice resistant capsules	local	Gynophilus vaginal capsules; Probionov, Aurillac, France
		oral	Astarte; Pizeta Pharma S.p.a., Ponte San Giovanni, Italy
		oral	LaciBios Femina; ASA Sp. z o.o., Głubczyce, Poland
		oral	fem dophilus; Jarrow Formulas, Inc., Los Angeles, CA, USA
		oral	Ombe; HSO Pharma GmbH, Gerasdorf Austria
		oral	Combi Flora; Effective Natural Products (www.effectivenp.com), USA
Tablets	Gastric juice resistant tablets	local	Hylaktiv Vagilact; Ratiopharm, Ulm, Germany
		local	Gynolact; Vitabalans oy, Hämeenlinna, Finland
		oral	Lacto Lady; Vitabalans oy, Hämeenlinna, Finland
		oral	Gynoflor vaginal tablets; Pharmacia & Upjohn, Vienna, Austria
Suppositories	Adeps solidus	local	Vagiflor vaginal suppositories; Chiesi GmbH, Hamburg, Germany
	Oleum cacao	local	SymbioVag; SymbioPharma GmbH, Herborn, Germany
		local	4Vag vaginal suppositories; Hypo-A GmbH, Lübeck, Germany
Tampons	Hydrogenated vegetable fats	local	Ellen probiotic tampon; Ellen AB, Spain

[1] HPMC = hydroxypropyl methyl cellulose.

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