Effect of a medium simulating vaginal fluid on the growth and expression of beneficial characteristics of potentially probiotic lactobacilli

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The effectiveness of a probiotic formulation applied in human vagina depends on the possibility of adaptation of the beneficial microorganisms in this environment. The understanding of the interactions of potentially probiotic microorganisms with the vaginal fluid, which shows some specific physical and chemical properties, can help to the design of improved vaginal probiotic formulations. The aims of this paper were to design a medium simulating vaginal fluid (MSVF), and to evaluate the growth, pH modifications and expression of beneficial characteristics of potentially probiotic vaginal lactobacilli in the growth medium designed. *Lactobacillus acidophilus* CRL 1251 and 1266, *L. gasseri* CRL 1259, *L. paracasei* CRL 1289, *L. johnsonii* CRL 1294, and *L. salivarius* CRL 1328 were able to grow or survive in the MSVF designed. The production of inhibitory substances (lactic acid, hydrogen peroxide or bacteriocine) varied at different extent, and *L. johnsonii* CRL 1294 showed auto-aggregation ability under the culture condition assayed.

Keywords vaginal fluid; culture media; probiotic lactobacilli; beneficial characteristics

1. Introduction

The secretions of the female genital tract maintain the moisture of the epithelial surface of vagina. The vaginal fluid is composed by plasma transudation through the vaginal wall, secretions of Bartholin's and Skenes's glands, cervical mucus, endometrial and tubal fluids, with additional contributions from residual urine and exfoliated epithelial cells [1]. The characteristics and composition of this fluid determine in a great extent the interactions between resident microbiota and urogenital pathogens [2].

The vaginal fluid contains water (90 to 95%), organic and inorganic salts, urea, carbohydrates, mucins, fatty acids, albumin, immunoglobulins, and others macro-molecules. Estrogens stimulate the deposit of glycogen in vaginal epithelia, which can be degraded by vaginal bacteria and/or by epithelial cells. The resulting glucose is metabolized to lactic acid, which is the main responsible of vaginal acidity (pH<4.5).

The carbohydrate concentrations of vaginal fluid are approximately 15 g/l of glycogen and 6.2 to 10 g/l of glucose. Mannose and glucosamine were determined in samples of vaginal fluid, but in very low concentrations, compared to glucose [3]. Different low molecular weight organic acids (lactic, acetic, formic, succinic, propionic and butyric acids) are present in vaginal environment, at a total concentration of around 3.1 g/l [4-6]. However, lactic and acetic acids are the major components.

The proteins of vaginal fluid derive mainly from plasma transudation and cervical mucus. In samples of vaginal fluid, Rajan *et al.* [3] determined the presence of albumin, transferrin, inmunoglobulins and glycoproteins that bind type I-piliated bacteria. Valore *et al.* [6] assayed the presence of antimicrobial peptides and proteins in vaginal fluids, finding lysozyme and lactoferrin, among others. The proteins in vaginal fluid are found in amounts from 0.018 to 3.75 g/l [7]. According to Geshnizgani and Onderdonk [2], the smaller value could be attributed to the detection of only a fraction of the total proteins present in vagina.

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Na^+, K^+ and Cl^- ions were detected in samples of vaginal fluid and seem to play an important role in the regulation of lubrication and transudation in the vaginal mucosa [1, 5]. On the other hand, Ca^{++} was detected after ovulation [1].

The effectiveness of a probiotic formulation applied in human vagina depends on the possibility of adaptation of beneficial microorganisms in this environment, where the vaginal fluid with specific physical and chemical properties is included. The understanding of the interactions of potentially probiotic microorganisms with some of the components of this ecological system could help to the design of improved vaginal probiotic formulations. For this purpose, *in vitro* conditions similar to those *in vivo* are needed. However, the harvesting of vaginal fluid is very difficult by the small amounts available (approximately 0.75 ml at certain time in vagina). Therefore, a synthetic medium with a similar composition to that of genital tract secretions is required.

There are some few proposed culture media that have a similar composition to the vaginal fluid [1, 2]. However, the media described in the literature are not completely appropriate to evaluate the growth of lactobacilli. Therefore, the aims of this paper were to design a growth medium simulating the vaginal fluid, and to evaluate *in vitro* the growth, pH modifications and expression of beneficial characteristics of potentially probiotic vaginal lactobacilli in the designed growth medium.

2. Materials and Methods

2.1 Microorganisms and culture conditions

The microorganisms employed were isolated from vaginal smears of healthy women of Tucumán, Argentina, and identified by phenotypic and genetic methodologies [8, 9]. *Lactobacillus gasseri* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1259 (producer of inhibitory amounts of lactic acid against urogenital pathogens) [10]; *L. acidophilus* CRL 1251 and 1266, and *L. paracasei* CRL 1289 (hydrogen peroxide producers) [11, 12]; *L. salivarius* CRL 1328 (bacteriocin producer) [13, 14], and *L. johnsonii* CRL 1394 (auto-aggregating) [15, 16] were previously characterized by their probiotic and technological properties.

Before the experiments, a 30 µL aliquot of each lactobacilli, stored in milk-yeast extract (g/l: 130, non-fat milk; 5, yeast extract; 10, glucose) at -20°C, was activated in 5 mL LAPTg broth (g/l: 15, peptone; 10, tryptone; 10, glucose; 10, yeast extract; 1, Tween 80) and incubated at 37°C for 24 h. Then, a 150 µL aliquot of each lactobacilli culture was sub-cultured twice at 37°C for 12 h in 5 mL of the Medium Simulating Vaginal Fluid (MSVF, composition detailed in item 2.2). The last cultures were centrifuged (10000 *g* for 10 min). The cell pellets were washed twice with saline solution (8.5 g/l NaCl) and re-suspended in 0.5 ml of the same solution. The lactobacilli were inoculated (2% v/v) into 100 ml of MSVF, and then incubated at 37°C, without agitation.

2.2 Formulation and preparation of MSVF

Table 1 shows the formulation of MSFV proposed in this paper, and also the chemical formulation of different media proposed by other authors, compared to the composition of the physiological vaginal fluid reported in different works [1-5, 7]. The MSFV was prepared in different steps:

Mixture 1: The amounts specified in Table 1 of glucose, lactic and acetic acids, NaCl, KCl, cystein and Tween 80 were disolved in 0.89 l of distilled water (pH=4.2-4.3). This mixture was sterilized in autoclave at 121°C for 15 min.

Mixture 2: The mixture 2 was prepared with the following concentrated solutions: 50 ml of 20% glycogen, 18.8 ml of 1.33% mucin, 1.25 ml of 40% urea (each solution was previously sterilized in autoclave), and 40 ml of 5% albumin (sterilized by filtration, membrane pore of 0.22 µm).

MSFV: Mixture 1 (0.89 l) was added to mixture 2 (0.11 l), in order to reach the final concentration of each component specified in Table 1. The MSVF was stored at refrigeration temperature.

Table 1. Comparative composition of different culture media simulating vaginal fluid.

Component	Vaginal fluid [a] (g/l)	CDM [b] (g/l)	VFS [c] (g/l)	MSVF [d] (g/l)
Glucose	6.2-10	10.80	5.00	10.00
Glycogen	15	1.00	-	10.00
Glycerol	0.16	-	0.16	-
Lactic acid	0.9-4.0	-	2.00	2.00
Acetic acid	0.52	-	1.00	1.00
Albumin	0.018-3.75	2.00	0.018	2.00
Mucin	0.25	0.25	-	0.25
Urea	0.49	0.50	0.40	0.50
NaCl	Na^+: 1.00-1.95; Cl^-: 2.20-2.94; K^+: 0.55-1.17; Ca^{2+}: 0.12	3.50 (Na^+: 1.38; Cl^-: 2.12)	3.51(Na^+: 1.38; Cl^-: 2.13)	3.50 (Na^+: 1.38; Cl^-: 2.12)
KCl		1.50 (K^+: 0.79; Cl^-: 0.71)	-	1.50 (K^+: 0.79; Cl^-: 0.71)
KOH		-	1.40 (K^+: 0.97)	-
$Ca(OH)_2$		-	0.222 (Ca^{2+}: 0.12)	-
K_2HPO_4	-	1.74	-	-
KH_2PO_4	-	1.36	-	-
$MgSO_4$	-	0.30	-	-
$NaHCO_3$	-	0.04	-	-
Tween 80	-	0.20	-	1.064
Cystein-HCl	-	0.50	-	0.50
Hemin	-	0.50	-	-
Vitamin K	-	0.10	-	-
Vitamin mix [e]	-	5 ml of 100X solution	-	-
pH	Normal: < 4.5	7.2	4.2	4.25 ± 0.05

[a] The vaginal fluid contains other components non detailed in this Table: others short chain aliphatic acids (formic, succinic, propionic, butyric acids), immunoglobulins, antimicrobial peptides and proteins, free amino acids, etc. [6, 7].
[b] CDM: Defined medium simulating the secretions of genital tract [2].
[c] VFS: Vaginal fluid simulant [1].
[d] MSVF: Medium simulating vaginal fluid proposed in this work.
[e] Mix of 14 vitamins from Sigma Chemical Co (K3129).

2.3. Evaluation of lactobacilli growth in MSFV

Samples were taken at different times (from 3 to 72 h) from cultures of different lactobacilli to evaluate the growth and expression of beneficial properties, by applying different analytical procedures. The determination of Colony Forming Units per milliliter (CFU/ml) was performed by using the plate dilution method, with peptone water (1 g/l peptone) as dilution medium, and LAPTg agar (LAPTg-15 g/l agar) as culture medium. The plates were incubated at 37°C for 48 h. The pH was determined with a pHmeter (Digimeter IV; Luftman; Argentina).

2.4. Evaluation of expression of potentially beneficial properties in MSFV

Hydrogen peroxide (H_2O_2) production in cultures of *L. paracasei* 1289, *L. acidophilus* CRL 1251 and CRL 1266 was tested by employing qualitative and quantitative methods previously published [9, 11, 12]. Briefly, the qualitative method uses horseradish peroxidase incorporated in tetramethyl-benzidine (TMB)-MRS agar, which catalyzes the oxidation of TMB (chromogenic substrate) to blue or brown color in those colonies able to produce H_2O_2. On the other hand, the quantitative spectrophotometric

assay involves the oxidation of *o*-dianisidine in presence of the H_2O_2 produced during the growth of microorganisms in broth media.

The lactic acid production was determined in the supernatant of *L. gasseri* CRL 1259 culture, by High Performance Liquid Chromatography (HPLC). An ISCO chromatograph (USA) with a column for organic acids (Bio-Rad HPX-87H 300 x 7.8 mm) was used (flow of 0.6ml/min, at 45°C). 5mM H_2SO_4 (pH 2) was employed as mobile phase. The organic acids were detected at 210 nm (ISCO V4 UV detector, USA), and the results were analyzed with software Peak Simple II (USA).

The bacteriocin levels in the supernatant of *L. salivarius* CRL 1328 culture were determined by employing the plate diffusion method previously described [13, 14]. Briefly, aliquots of different dilutions of neutralized supernatant were poured into holes of LAPTg agar plates (LAPTg-10 g/l agar), which contained vaginal 10^6-10^7 CFU/ml *Enterococcus faecalis* as indicator microorganism. The plates were incubated for 5 h at room temperature and then for 24 h at 37°C. The highest dilution that produced a clear inhibition zone was referred as Arbitrary Units per milliliter (AU/ml).

Finally, the extent of auto-aggregation of *L. johnsonii* 1294 was assessed according to the quantitative spectrophotometric method previously reported [15, 16]. In the present work, a modification was performed, by measuring the $OD_{600\ nm}$ variations of the cellular suspensions every 1 h during 4h, in both PBS buffer (g/l: NaCl, 8; KH_2PO_4, 0.34; K_2HPO_4, 1.21; pH 7) and acetate buffer (0.305 l of 0.2 M acetic acid, 0.195 l of 0.2 M sodium acetate, and 0.5 l distilled water, pH 4.5).

3. Results

3.1 Growth of vaginal lactobacilli in MSVF

Figure 1 shows the growth in MSVF of six strains of potentially probiotic vaginal lactobacilli. The results obtained indicate that each microorganism presents a different behaviour. Most of the microorganisms evaluated were able to grow in MSVF, except *L. gasseri* CRL 1259. The viability of this microorganism decreased progressively during the first 24 h of culture (around 2 log cycles), and then remained stable until 72 h of incubation. On the other hand, *L. acidophilus* CRL 1251 was markedly affected by the culture conditions, showing a decrease of viability between 3 and 12 h of culture (from 5 x 10^6 to 4 x 10^3 CFU/ml), and then a pronounced increase after 72 h of assay.

The biomass of *L. johnsonii* 1294 increased slightly during the culture in MSVF. However, *L. salivarius* CRL 1328, *L. acidophilus* CRL 1266 and *L. paracasei* CRL 1289 showed the highest difference between the initial number and the maximal number of viable cells under the culture conditions tested, with an increase in the number of CFU/ml of around one log cycle after 7 or 24 h of incubation. Then, the viability of these microorganisms slightly decreased until the end of experimental culture.

The pH modifications of MSVF were concordant with the growth pattern observed for the vaginal lactobacilli evaluated, since the three microorganisms that reached the highest growth (*L. acidophilus* CRL 1266, *L. paracasei* CRL 1289 and *L. salivarius* CRL 1328) also produced the highest pH decreases (pH=3.80 ± 0.10 at 72 h of culture). However, the final pH after the growth of the other microorganisms was around 4.1 (only 0.2 pH units lower than the initial pH of growth medium).

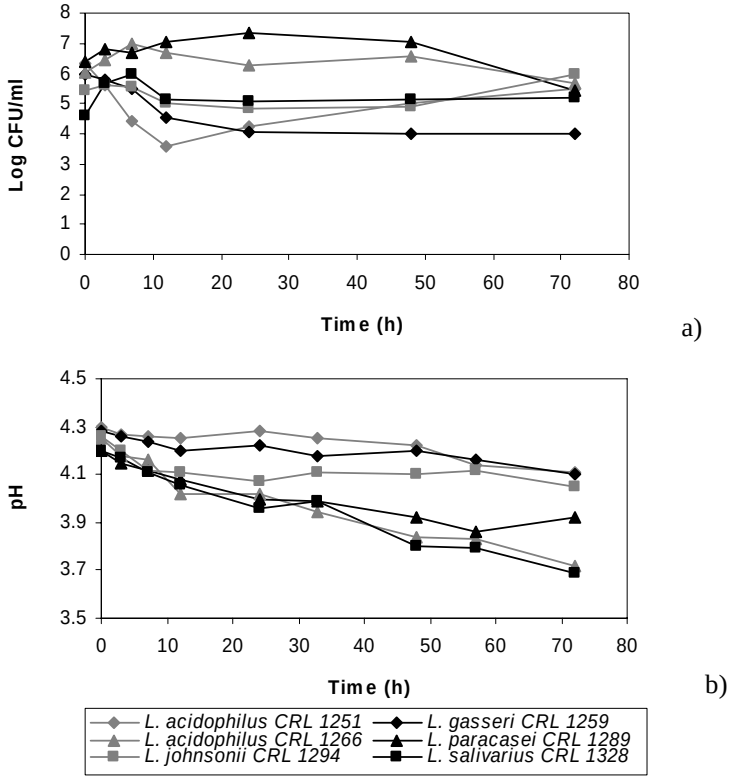

Fig. 1 Growth a) and pH modifications b) of vaginal lactobacilli in MSVF.

3.2 Expression of potentially beneficial properties in MSVF

The H_2O_2 production of *L. acidophilus* CRL 1251, CRL 1266, and *L. paracasei* 1289 in non-agitated cultures was not detected in MSVF. By applying the *o*-dianisidine horseradish peroxidase spectrophotometric assay, the H_2O_2 levels for the three strains tested were under the detection limits of this method. On the other hand, most of the isolated colonies of these microorganisms turned brown in the TMB-MRS plates, seeded with bacterial cells previously cultured in MSVF.

The lactic acid production in MSVF was only assayed during the growth of *L. gasseri* CRL 1259. This microorganism was previously selected by its capacity to inhibit the growth of different uropathogenic microorganisms by the lactic acid produced. Although the number of viable cells of this microorganism did not increased after 72 h of incubation, a small amount of lactic acid was detected, simultaneous to the pH decrease. Nevertheless, the levels of lactic acid produced were very low (approximately 0.2 g/l at end of culture).

Although *L. salivarius* CRL 1328 was able to grow in MSVF, the bacteriocin production was minimal (only 40 AU/ml in all the times assayed).

Figure 2 shows the auto-aggregation patterns of *L. johnsonii* CRL 1294 in acetate buffer, at different times of culture in MSVF. Auto-aggregation percentages in PBS buffer were slightly higher than those obtained in acetate buffer (data not shown). The auto-aggregation of cells harvested after 12 and 24 h of incubation were higher (average 52%) to that of cells harvested after 7 and 30 h (average 40%).

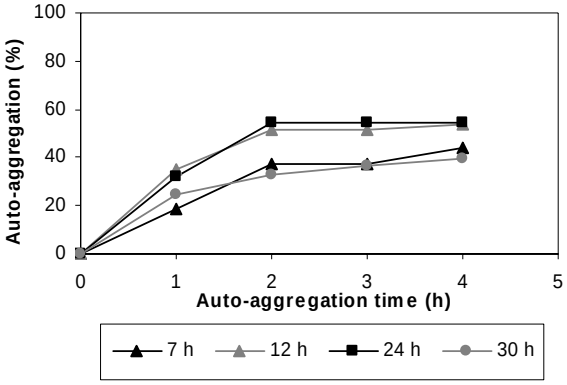

Fig. 2 Auto-aggregation of *L. johnsonii* CRL 1294 in acetate buffer, from cells harvested after different times of incubation in MSVF (7, 12, 24 and 30 h).

4. Discussion

Lactobacilli are the predominant microorganisms in the healthy human vaginal ecosystem. These microorganisms, as other members of the normal microbiota, are adapted to the environment in which they live, resisting their displacement by external factors that disturb the vaginal balance [7]. Different characteristics displayed by lactobacilli are determinant for their permanence and stability in the vaginal ecosystem. First, the capability to adhere to epithelial cells is decisive for the colonization of vaginal mucosa. Later, these microorganisms must be able to proliferate, and to produce antimicrobial substances that prevent the growth of uropathogenic microorganisms [17].

The lactobacillus strains used in the present work could potentially be used for the elaboration of probiotic products for vaginal application. Therefore, is important to know their technological and physiological properties. Within the last ones, their capacity to proliferate or to maintain their viability in a medium with similar characteristics to the vaginal medium must be determined.

Geshnizgani and Onderdonk [2] developed a defined medium simulating the secretions of genital tract (CDM) in order to evaluate the growth of different microorganisms isolated from normal vaginal microbiota (*Bacteroides fragilis*, *Gardnerella vaginalis*, *Lactobacillus acidophilus*, *Prevotella bivia*, *Propionibacterium jensenii*, *Candida albicans*, *Corynebacterium* sp., etc.). This medium has a high pH (7.2) compared to the normal vaginal pH (<4.5), and contains many components not assayed in vaginal secretions ($MgSO_4$, $NaHCO_3$, hemin, vitamin K and a mixture of 14 vitamins), but needed for the growth of nutritionally exigent microorganisms [6]. The medium does not include organic acids, and the glycogen concentration is too low referred to that quantified in the human vaginal fluid (1g/l versus 15 g/l).

On the other hand, Owen and Katz [1] formulated a vaginal fluid simulant (VFS), in order to *in vitro* evaluate their effect on the properties of topical contraceptives and prophylactic products. This formulation is simple, does not include glycogen nor mucin, and the glucose concentration is relatively low (5 g/l). The VFS was designed in order to simulate mainly the pH and the osmolarity of vaginal fluid, which are properties that affect the effectiveness of intra-vaginal gels. This medium also was employed by others researchers to characterize bioadhesive liposome gels for local therapy of vaginitis [18, 19]. Owen and Katz [1] suggested to include some nutrients excluded from the initial formulation to study the influence of vaginal environment on the growth of microorganisms.

Some other researchers have employed an artificial vaginal fluid (AVF) to determine the effect of different agents on the rheological property of cervical mucus, but to elucidate the mechanisms involved in the calcium-dependent fertility regulation process [20]. AVF is similar to VFS proposed by Owen and Katz [1], but also includes non specified amino acids and mucin. The effects of chelating agents (EDTA) and spermicidal agents (nonoxynol-9) were evaluated, principally from a physiological point of view.

The MSVF proposed in this work is more appropriate to evaluate the growth of vaginal lactobacilli, since it provides similar conditions (pH and most of the components in similar concentrations) to those of the healthy vagina. Most of the microorganisms tested were able to grow and modify the pH of MSVF. *L. acidophilus* CRL 1266 and *L. salivarius* CRL 1328, two of the three microorganisms with optimal growth in MSVF, are able to use both glucose and glycogen as carbon sources in fermentation-growth medium, according to our previous studies of carbohydrate fermentation [8]. However, the growth of *L. paracasei* CRL 1289 in MSVF could be sustained sufficiently by the glucose present, since this microorganisms does not ferments glycogen [8]. In the vaginal environment, those lactobacilli glycogen-negative can obtain energy from fermentable carbohydrates derived from the enzymatic degradation of this polysaccharide by other microorganisms [21].

The studies performed on the growth of vaginal microorganisms in the vaginal environment are not numerous. Geshnizgani and Onderdonk [2] evaluated the growth of different microbial species in CDM described before. For most of the microorganisms, clear phases of increase and decrease of biomass were observed. For example, the higher number of viable cells of vaginal *L. acidophilus* was reached after 24 h of culture at 36°C, and then a decrease of around 0.5 logarithmic units at 72 h of incubation was observed. Reid *et al.* [22] used the CDM proposed by Geshnizgani and Onderdonk (but at pH = 5) in order to compare the growth of lactobacilli (*L. rhamnosus* GR-1 and *L. fermentum* B-54) and uropathogenic microorganisms (*Proteus mirabilis*, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Candida albicans*). Most of the microorganisms assayed were able to grow in CDM, except *E. faecalis* and *S. aureus*. However, the increase of biomass of lactobacilli in CDM (determined by absorbance measures) was very low. Nevertheless, the addition of 10 g/l skim milk stimulated the growth of lactobacilli, on the detriment of pathogenic microorganisms.

In this work, the expression of beneficial characteristics of the strains cultured in MSVF was evaluated. The H_2O_2 production in MSVF by vaginal lactobacilli could not be determined by the quantitative method in similar conditions than in previous assays (non-agitated cultures in LAPTg and MRS broths) [12]. Agitated cultures in MSFV were not performed because the objective of this paper was to simulate the conditions of the human vagina. Anyway, the three H_2O_2-producing lactobacilli maintained their capability to produce the oxidative metabolite after growing in MSVF, qualitatively demonstrated by the pigmented colonies obtained in TMB-MRS plates.

L. johnsonii CRL 1294 showed auto-aggregation ability after grow in MSVF. It is important to emphasize that this property was determined also in buffer acetate, at a similar pH to that of vaginal medium.

L. salivarius CRL 1328 was able to use the nutrients available in MSVF, mainly for biomass formation but not for the bacteriocin production. Probably the MSFV does not provide the appropriate conditions (pH or nitrogen sources) for the biosynthesis of bacteriocin [23]. According to our previous studies, the optimal production of bacteriocin of *L. salivarius* CRL 1328 was obtained at an initial pH of 6.5 or 8, different to the pH 4.25 used in the MSVF [14]. Is important to remark that in the vaginal fluids are present free amino acids and other proteins different of albumin and mucin [3, 6, 7], which could be used by *L. salivarius* CRL 1328 to *in vivo* synthesize bacteriocin.

In spite of some limitations, the proposed culture medium turns out appropriate to study the effects of the conditions of vaginal environment on the growth of potentially probiotic lactobacilli. Nevertheless, the definitive behaviour needs to be evaluated, mainly by experimental models developed in animals, and later, through clinical assays with healthy women.

Acknowledgements. The support by CONICET (PIP 6248) is gratefully acknowledged. The strains evaluated were licensed to Probiotical-Anidral for their industrial production.

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