UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

Padronização do co-cultivo de *Trichomonas vaginalis*, *Candida albicans* e *Lactobacillus crispatus*: um novo método para caracterização da atividade antimicrobiana

Fernanda Gomes Cardoso

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Trabalho de Conclusão de Curso apresentado ao Curso de Farmácia da Universidade Federal do Rio Grande do Sul como requisito à obtenção do título de grau de Farmacêutico.

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Coorientador: Drª. Graziela de Vargas Rigo

Porto Alegre, 2022.

Dedico este trabalho à minha orientadora Tiana Tasca e à coorientadora Graziela de Vargas Rigo, as quais são minha inspiração.

AGRADECIMENTOS

Esse é um momento muito especial para mim, pois sempre foi algo muito sonhado e idealizado e finalmente esse dia chegou. A todos os leitores desse trabalho, espero que sintam todo o amor que eu tenho por esse tema.

Primeiramente, quero agradecer minha mãe e meu pai, Ana e Fernando, por me apoiarem em todas minhas decisões e escolhas, pelo carinho, educação, dedicação em me dar tudo o que eu preciso, as caronas até a faculdade ou estágio, as esperas para almoços e as conversas que me incentivavam a ser cada vez melhor. Desculpem pela falta de atenção desses últimos meses. Eu sei que esse momento é muito importante para vocês, amo vocês demais.

Agradeço também ao Gabriel, meu namorado, por me apoiar nesse momento tão desafiador para mim, pela compreensão, amor e carinho ao longo de quase todos os anos de faculdade. Obrigada por me mostrar que nunca devemos ficar acomodados no que trabalhamos e sempre devemos buscar inovação e desafios, você é inspirador. Agradeço ao César e a Cristina, meus sogros, a Anne e o Rodrigo, meus cunhados, por todas as conversas acolhedoras e todo carinho neste tempo. Agradeço também a minha afilhada, Manuela que me traz muita alegria, calmaria e amor nesses momentos tão corridos e estressantes.

Agradeço a Tiana, minha orientadora, por ser essa mulher inspiradora, sendo apaixonada pelo que faz, é impossível não ser `'contaminada" pelo amor aos tricos. Lembro do primeiro dia que a vi, no meu antigo estágio, me encantei com o jeito e a felicidade dela em ver e falar sobre *Trichomonas*. Agradeço também a Grazi, essa mulher que encanta e ajuda todo mundo que está à sua volta, fico muito feliz em te ter não só como coorientadora, mas também como amiga. Obrigada pela ajuda nesse trabalho, pelos conselhos, ideias e por sempre me ouvir e acreditar no meu potencial. Eu dedico esse trabalho às duas, pois vocês me incentivam a ser uma pesquisadora com pensamento crítico e uma pessoa melhor. Agradeço a todos integrantes do GPTrico, Giulia, Saulo, Corina, Julia, Caroline e Ágata. Agradeço em especial a Giulia, minha dupla inseparável desde o primeiro semestre, pelo apoio, almoços e sorvetes pós faculdade, conselhos, risadas, por me trazer para esse grupo de pesquisa e por estar presente (mesmo que do outro lado do continente) nesse momento tão especial para mim. Também agradeço ao Saulo, por toda ajuda nesses últimos meses, não só nos experimentos, mas por ser meu ouvinte de reclamações diárias e pelos docinhos com café para amenizar o estresse e cansaço. Também agradeço a todos integrantes do LABDIM, Alexandre, Rodrigo, Dayse, Deisiane, Ana Luiza e Flávia, por me receberem no vale com braços abertos, vocês também fazem parte desse trabalho e me inspiram.

Agradeço a Chris, minha dupla no estágio, por ter se tornado uma amiga que eu sei que posso contar para tudo. Fico muito feliz em dividir esse momento contigo tão difícil, mas também tão cheio de alegria que é a formatura.

Agradeço a todos os meus familiares, amigos e professores, pois todos têm um papel enorme na minha formação.

APRESENTAÇÃO

Esse Trabalho de Conclusão de Curso foi redigido sob a forma de artigo ao qual foi elaborado segundo as normas da revista *Microbes and Infection*, apresentadas em anexo.

Standardization of *Trichomonas vaginalis*, *Candida albicans* and *Lactobacillus crispatus* co-culture: a new method for characterizing the antimicrobial activity

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Abstract:

Trichomonas vaginalis is an extracellular flagellated protozoan that causes trichomoniasis, the most common nonviral sexually transmitted infection, associated to several complications mainly in asymptomatic carriers. Another vaginitis causative agent is Candida albicans, a member of normal vaginal microbiota that due an immune imbalance should cause vulvovaginal candidiasis, infection associated with recurrent cases. Causative vaginitis agents are associated to resistant-treatment isolates. Lactobacillus crispatus is a predominant member of normal vaginal microbiota responsible for antimicrobial substance production associated to protection of vaginal mucosa. In this study it was standardized the in vitro co-culture of T. vaginalis, C. albicans and L. crispatus, to mimetize in vivo polymicrobial vaginal environment in the infection site. Preliminary tests showed that MRS was the optime medium for co-culture, density of L. crispatus and C. albicans was 1x108 and 1x105 CFU/ml, respectively; T. vaginalis density was 1x105 trophozoites/ml. Biofilm formation and yeast-hyphae transition was not impacted by co-culture. Flow cytometry showed the presence of L. crispatus, C. albicans and T. vaginalis when all microorganisms were in co-culture. Furthers studies are necessary for validation of this platform, which may be used to accesses the interaction of co-culture members and to detect the antimicrobial activity of new molecules.

Keywords: *Trichomonas vaginalis*; *Candida albicans*; *Lactobacillus crispatus*; co-culture.

1. INTRODUCTION

Trichomoniasis is the most common nonviral sexual transmitted infection worldwide, caused by the flagellated protozoan *Trichomonas vaginalis*, which is responsible for 156 million new cases, annually [1]. The clinical manifestations are variable, wherein infected patients can be asymptomatic or symptomatic. Also, symptoms and severity of infection among women and men are different. In view of this, infection in men is in general self-limited and transitory, however when symptoms manifest is linked to purulent urethritis [2]. By contrast, infected women present vaginitis and related symptoms are itching, irritation and a frothy malodorous discharge [3]. Pharmacologic treatment of trichomoniasis is centered on 'Food and Drug Administration' (FDA) approved drugs belonging to 5-nitroimidazole, such as metronidazole, tinidazole and secnidazole [4-5]. Therapeutic failures have been linked to decrease in drug sensitivity through mechanisms involving aerobic and anaerobic resistance. Clinical resistance is associated with oxygen presence, occurring reoxidation of active groups, while in vitro resistance is the laboratory induced anaerobic type, which is linked to reduction of activity enzymes responsible for drug activation [6-7]. Gastrointestinal adverse events, such as nausea, abdominal pain and diarrhea, represent other limitations of treatment by reducing patient adherence to drugs [8]. In addition, *T. vaginalis* facilitates transmission and acquisition of HIV/AIDS which supports the HIV epidemic in endemic regions for trichomoniasis. [9]. Other complications in women include infertility, bacterial vaginosis, cervical cancer, pelvic inflammatory disease and pregnancy complications [10].

Vaginal eubiosis is characterized by the prevalence of several species of *Lactobacillus,* classified into five community-state types [11]. The domain of *Lactobacillus* spp. at the vaginal microbiota, mainly *L. crispatus*, provides more protection against infections caused by several microorganisms due the production of antimicrobial substances such as lactic acid, bacteriocins

and hydrogen peroxide [12]. Homeostasis perturbation in the vaginal microbiome leads to an increase of opportunist infections and it facilitates the establishment of sexually transmitted infections, such as trichomoniasis [13]. Furthermore, the presence of *Candida* spp. in the vaginal microbiome represents another risk factor to infectious vaginitis development. Due to immunity imbalance, this opportunistic pathogen can proliferate, leading to vulvovaginal candidiasis (VVC), the second most common cause of vaginitis [14]. Symptoms including vaginal itching, pain during sexual intercourse, discomfort when urinating and abnormal vaginal discharge [15]. Is estimated 138 million women annually are affected by recurrent VVC, which is characterized by four or more episodes of the infection per year [16]. Several species of *Candida* are responsible for VVC, however, *C. albicans* is the most predominant species related to vaginitis [17]. As observed in trichomoniasis, candidiasis also showed an increase in drug resistance against the main therapeutic choice, fluconazole [18]. Candidiasis and trichomoniasis are not notifiable, and do not exist in surveillance systems neither the detection of treatment-resistant isolates is stimulated. It is worth mentioning that cases of co-infection among *T. vaginalis* and *C. albicans* are common, presenting an occurrence rate of 14.28%, associated with local discomfort and increase of morbidity [19]. However, there are few studies that demonstrate the relationship among these microorganisms.

In this regard, development of new molecules is highly important for treatment of trichomoniasis and vulvovaginal candidiasis. Several steps of research are necessary to approve a drug for therapeutic use. First steps are preclinical trials, which are realized in vitro and in vivo tests. Therefore, there is a lack of standardized methodology to evaluate in vitro *T. vaginalis*, *C. albicans* and *L. crispatus* co-culture, to mimetize the in vivo polymicrobial vaginal environment that the vulvovaginitis agents confront in the infection site. The aim of this study was to develop a new method for evaluation of polymicrobial interaction through co-culture of vaginitis causative agents and vaginal microbiota *L. crispatus*, to characterize new drugs with

antimicrobial activity. The application of reliable methodology that replicates the host's polymicrobial environment to perform in vitro-screening of active compounds would direct to in vivo and preclinical studies the most promising molecules for antimicrobial development.

2. MATERIALS AND METHODS

2.1 *Trichomonas vaginalis* culture and trophozoite densities

Protozoan *T. vaginalis* (ATCC 30236) was cultured in TYM medium (trypticase-yeast extract-maltose) [20] supplemented with 10% adult bovine serum (ABS) and 5 mg/ml streptomycin or streptomycin/ampicillin for 24 h and under microaerophilic conditions at 37 ºC. On the co-culture, suspension was centrifuged and resuspended in MRS broth (Sigma-Aldrich) [21] supplemented with 10% ABS. Different densities were utilized at assays, ranging $1x10^6$ to $1x10^2$ trophozoites/ml, measured with Trypan-blue exclusion dye (0.2%) on an optical microscope (Nikon TE 2000-U Eclipse).

2.2 Fungal and bacterial cultures and densities

Yeast *C. albicans* (ATCC 24433) was cultured in Sabouraud dextrose agar, for 24 h at 37 ºC. *L. crispatus* (CCT 7554), acquired from the tropical culture collection of André Tosello foundation, was grown in MRS broth for 72 h at 37 ºC, under microaerophilic conditions. The maximum concentration utilized, on co-culture assay, were $1x10^6$ CFU/ml for *C. albicans* and $1x10^8$ CFU/ml for *L. crispatus*, which corresponds to 0.5 McFarland. These suspensions were adjusted in OD 600 nm (Spectramax M2e Multimode Microplate Reader, Molecular Devices, USA) and each suspension was serially diluted (1:10) in MRS supplemented with 10% ABS.

2.3 Culture medium for co-culture assay

TYM, MRS, Sabouraud dextrose broth and RPMI-1640 medium (Sigma-Aldrich) culture media were utilized to evaluate the capacity for support growth of all microorganisms in the co-culture during 24h of incubation. Although, MRS supplemented with 10% ABS was chosen for use in next experiments.

2.4. Concentration determination in co-culture

In order to determine the better concentration of *T. vaginalis*, *C. albicans* and *L. crispatus* for co-culture, a checkerboard on 96-well culture plates was created, where one microorganism species was evaluated in fixed concentration while the other was tested in ranges of densities. When *C. albicans* remains constant in the concentration of $1x10^5$ CFU/ml, *T. vaginalis* range $1x10^6$ - $1x10^2$ trophozoites/ml (identified by TV 1 - TV 5, respectively) and *L. crispatus* $1x10^8$ - $1x10^4$ CFU/ml (identified by, LC 1 - LC 5, respectively). Checkerboard with *L. crispatus* constant used fixed concentration of $1x10^8$ CFU/ml (LC1) while both *T. vagi*nalis and *C. albicans* (CA 1 to CA 5) range $1x10^6$ - $1x10^2$ trophozoites/ml and CFU/ml, respectively. The polymicrobial condition within the well was created by addition of 50 µl of each microorganism, to a final volume of 200 µl, adjusted with medium culture. Plates were incubated at 37 ºC for 24 and 48 h, under microaerophilic conditions. Figure 1 demonstrate an example of all conditions created when *C. albicans* remains at the $1x10^5$ CFU/ml.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CA 2	LC 1	LC 2	LC 3	LC 4	LC 5	TV 1	TV 2	TV 3	TV 4	TV 5	MC
B	CA 2	LC 1	LC 2	LC 3	LC 4	LC 5	TV 1	TV 2	TV 3	TV 4	TV 5	MC
C	CA 2 + TV 1	CA 2 + TV 2	CA 2 + TV 3	CA 2 + TV 4	CA 2 + TV 5	CA 2 + LC 1	CA 2 + LC 2	CA 2 + LC 3	CA 2 + LC 4	CA 2 + LC 5	TV 1+ CA 2 + LC 1	TV 2 CA 2 + LC 1
D	CA 2 + TV 1	CA 2+ TV 2	CA 2 + TV 3	CA 2 + TV 4	CA 2 + TV 5	CA 2 + LC 1	CA 2 + LC 2	CA 2 + LC 3	CA 2 + LC 4	CA 2 + LC 5	TV 1+ CA 2 + LC 1	TV 2 CA 2 + LC 1
E	TV 3 CA 2 + LC 1	TV 4 + CA 2 + LC 1	TV 5 + CA 2 + LC 1	TV 1+ CA 2 + LC 2	TV 2 CA 2 + LC 2	TV 3 + CA 2+ LC 2	TV 4 + CA 2 + LC 2	TV 5 + CA 2 + LC 2	TV 1 CA 2 + LC 3	TV 2 CA 2 + LC 3	TV 3 + CA 2 + LC 3	TV 4 + CA 2 + LC 3
F	TV 3 CA 2 + LC 1	TV 4 + CA 2 + LC 1	TV 5 + CA 2 + LC 1	TV 1+ CA 2 + LC 2	TV 2 CA 2 + LC 2	TV 3 + CA 2 + LC 2	TV 4 + CA 2+ LC 2	TV 5 + CA 2 + LC 2	TV 1 CA 2 + LC 3	TV 2 CA 2+ LC 3	TV 3 + CA 2 + LC 3	TV 3 + CA 2 + LC 3
G	TV 5 + CA 2 + LC 3	TV 1 + CA 2 + LC 4	TV 2 + CA 2+ LC 4	TV 3 + CA 2 + LC 4	TV 4 + CA 2 + LC 4	TV 5 + CA 2 + LC 4	TV 1+ CA 2 + LC 5	TV 2 + CA 2 + LC 5	TV 3 + CA 2 + LC 5	TV 4 + CA 2 + LC 5	TV 5 + CA 2 + LC 5	MC
H	TV 5 + CA 2 + LC 3	TV 1 + CA 2 + LC 4	TV 2 + CA 2 + LC 4	TV 3 + CA 2 + LC 4	TV 4 + CA 2 + LC 4	TV 5 + CA 2 + LC 4	TV 1+ CA 2 + LC 5	TV 2 + CA 2 + LC 5	TV 3 + CA 2 + LC 5	TV 4 + CA 2 + LC 5	TV 5 + CA 2 + LC 5	MC

Figure supplementary 1: All combinations of each microorganism on plate, herein *Candida albicans* (CA 2) was maintained in concentration $1x10^5$ CFU/mL, while *Trichomonas vaginalis* and *Lactobacillus crispatus* range $1x10^6$ - $1x10^2$ trophozoites/ml (TV 1 - TV 5) and $1x10^8$ - $1x10^4$ CFU/ml (LC 1 - LC5), respectively. MC is a culture medium, for sterility control.

2.5 Determination of cells viability

After 24 h of co-culture incubation at 37°C 5% CO_2, each well was diluted to 1:1000 and inoculated in agar Sabouraud to determine and quantify (CFU/mL) *C. albicans* colonies formation, or in MRS agar for *L. crispatus.* LBS agar also was utilized [21], which is more selective for isolation of *L. crispatus* than MRS agar. Then, these agar plates were incubated for 24 - 48 h at 37 °C under microaerophilic conditions. Trypan blue exclusion dye (0.2%) was utilized to analyze under optical microscope (Nikon TE 2000-U Eclipse) *T. vaginalis* viability. In addition, we used absorbance at 600 nm to measure microbial growth.

2.6 Flow cytometry

The viability of each microorganism in co-culture was also evaluated by flow cytometry in a combination 1:1:1 of the cultures adjusted according to the better concentration in co-culture. *T. vaginalis* was counted in Neubauer Hemocytometer with Trypan-blue exclusion dye (0.2%) on optical microscope (Nikon TE 2000-U Eclipse) and adjusted to the concentration of 1 x10^5 trophozoites/ml, *C. albicans* and *L. crispatus* were adjusted to the final concentration at 0.5 McFarland, also another concentration of *C. albicans* was utilized, 10^5 CFU/ml. The assay was measured using the BD FACS Verse flow cytometer and the data analyzed with the BD CellQuesT program. The population was analyzed in 30 seconds with one air-cooled blue laser at 488 nm using a two-parameter histogram of forward angle light scatter (FSC) versus side scatter (SSC), respectively, to evaluate cellular size and granularity.

2.6 Yeast to hyphal transition

The morphological transformation of *C. albicans* yeast to hyphae in co-culture was realized according to Suchodolski et al. with modifications [23]. Briefly, 250 µl yeast suspension at the concentration 1x10^5 and 1x10^6 CFU/ml was incubated alone or in combination with *L. Crispatus* (1x10^6 CFU/ml) and *T. vaginalis* (1x10^6 trophozoite/ml), and the final volume completed with MRS with/without 10% fetal bovine serum (FBS). This assay was performed on microtubes, incubated under microaerophilic (5% CO_2) and aerobic conditions for 6 at 37 °C. After incubation, all combinations were analyzed under the microscope (Nikon TE 2000-U Eclipse) in the Neubauer hemocytometer. The results were expressed by hyphae formation rate regarding the total *Candida* cells.

2.8 Biofilm biomass evaluation

The biofilm biomass of *C. albicans* was compared with the biomass of *C. albicans* in co-culture, this evaluation was performed utilizing violet crystal assay, accordingly Trentin et al., with modifications [24]. Briefly, *L. crispatus* suspension with 72-h growth was adjusted in 0.9 % of sterile saline solution at 0.5 of the MacFarland scale. *T. vaginalis* and *C. albicans* were adjusted to the concentration of $1x10^6$ trophozoites/ml and CFU/ml, respectively. Evaluated conditions were only *C. albicans, C. albicans* with *T. vaginalis, C. albicans* with *L. crispatus,* all microorganisms co-incubated and sterility control (only culture medium), where 50 µl of each microorganism was added on well and final volume was 200 µl. Then, this plate was incubated for 24 h at 37º C under microaerophilic conditions. After 24 h, well contents are removed and washed with sterile saline solution, to obtain microorganism fixation heat at 60º C was necessary. Then, crystal violet (0.4%) on each well and remained at room temperature for 15 min. After this time, dye was removed by several washes and ethanol was added for 30 min and absorbance was measured at 570 nm (Spectramax M2e Multimode Microplate Reader, Molecular Devices, USA). The value obtained for sterility control was discounted in all readings and the results were expressed as a percentage of biofilm formation, where only *C. albicans* represented 100% of biofilm.

2.9 Statistical analyses

All experiments were performed at two independent times at least (two different cultures, n = 2), in duplicate. Results were expressed as means ± SD. Statistical analysis was conducted using the student's t test for comparison between two groups. Statistical significance was considered at p-value < 0.05.

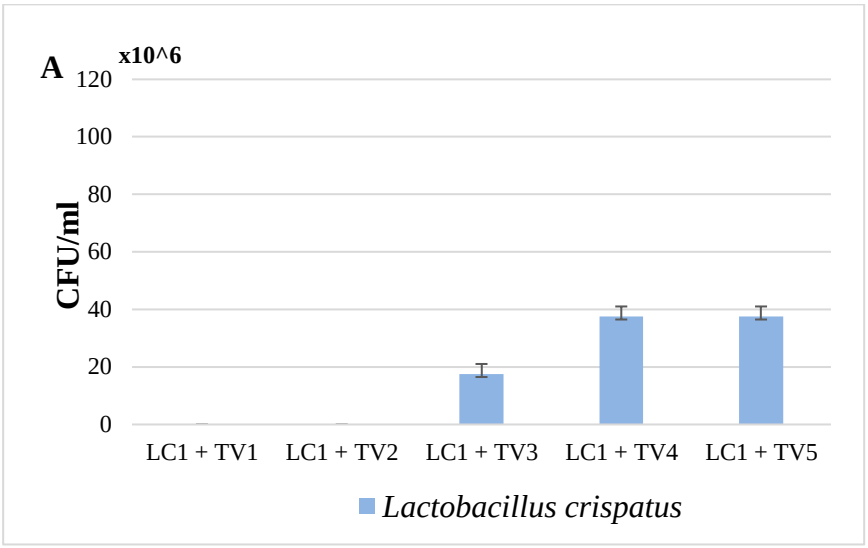
3. RESULTS

3.1 MRS was the optimal culture medium suitable for all members in co-culture

In the co-culture standardization, it was necessary to evaluate several parameters, such as the determination of the optimum culture medium, concentration of *T. vaginalis*, *C. albicans* and *L. crispatus*, time of incubation and determination of cell viability after co-culture. The cell viability was conducted through exclusion Trypan-blue assay for *T. vaginalis*, absorbance reading in 600 nm and quantification of colonies on agar to determine CFU/ml for *C. albicans* and *L. crispatus*. MRS was the culture medium chose which enabled the viability of all microorganisms. This finding was mainly due to *L. crispatus* being not viable in the other culture media tested.

3.2 *L. crispatus* at $1x10^8$ CFU/ml proliferated in lower *T. vaginalis* and *C. albicans* densities

Checkerboard was performed with *L. crispatus* at $1x10^8$ UFC/ml (LC1); *T. vaginalis* at $1x10^6$ (TV1), $1x10^5$ (TV2), $1x10^4$ (TV3), $1x10^3$ (TV4), $1x10^2$ trophozoites/ml (TV5) and *C. albicans* at 1×10^6 (CA1), $1x10^5$ (CA2), $1x10^4$ (CA3), $1x10^3$ (CA4), 1×10^2 (CA5) CFU/ml. *L. crispatus* was not recovered on MRS agar or LBS agar after co-culture with TV 1 e TV 2 (Fig. 1 A). In the presence of *L. crispatus* only, *T. vaginalis* remained viable until TV3, however in the presence of all microorganisms, *T. vaginalis* maintained its viability until TV2 (Fig 1 B and D). In co-culture of *L. crispatus* and *C. albicans*, lactobacilli were recovered from condition LC1+CA2 (Fig. 1 C). *C. albicans* at all densities remains viable in all combinations (Fig 1 C and D). Therefore, the in vitro growth of *L. crispatus* was inversely proportional to higher densities of *T. vaginalis* and *C. albicans*.


A
x10^6
120
100
80
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CFU/ml
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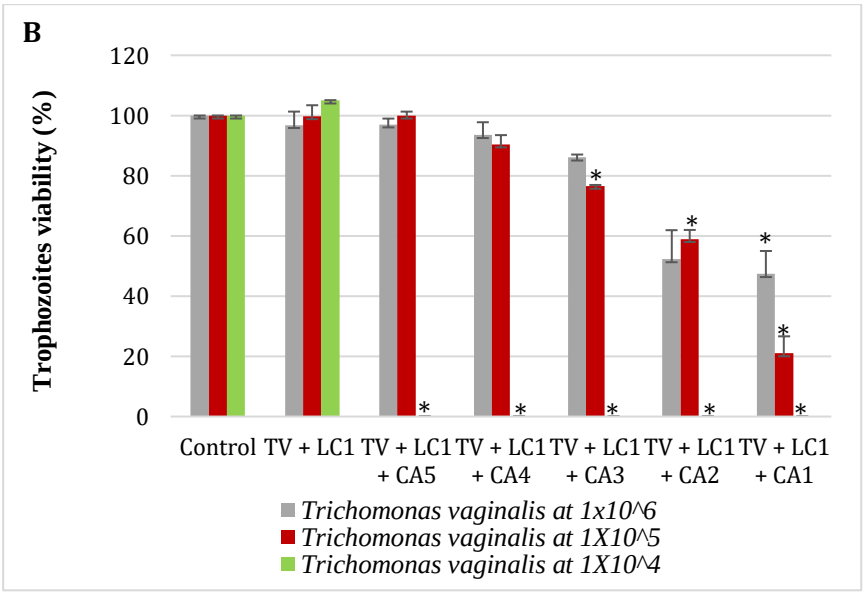
Lactobacillus crispatus


B
120
100
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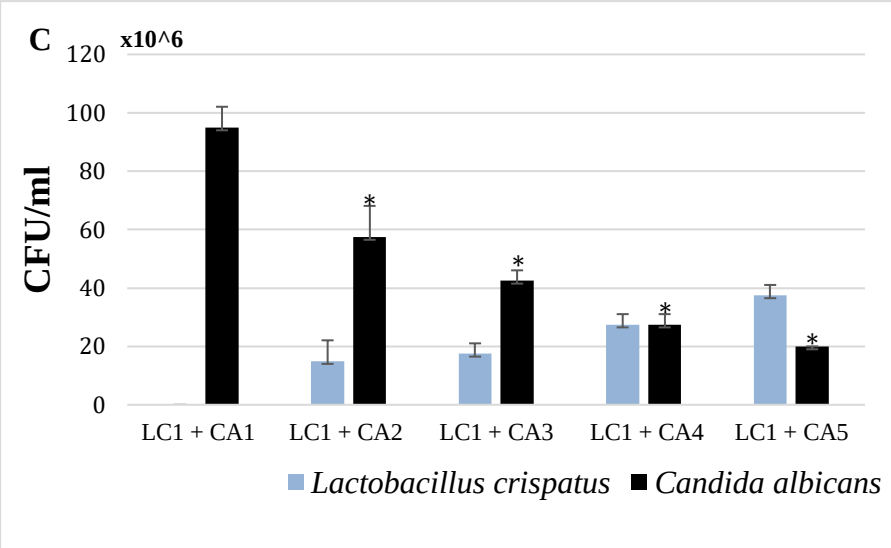
20
0
Trophozoites viability (%)
Control
TV + LC1
TV + LC1 + CA5
TV + LC1 + CA4
TV + LC1 + CA3
TV + LC1 + CA2
TV + LC1 + CA1
Trichomonas vaginalis at 1x10^6
Trichomonas vaginalis at 1X10^5
Trichomonas vaginalis at 1X10^4



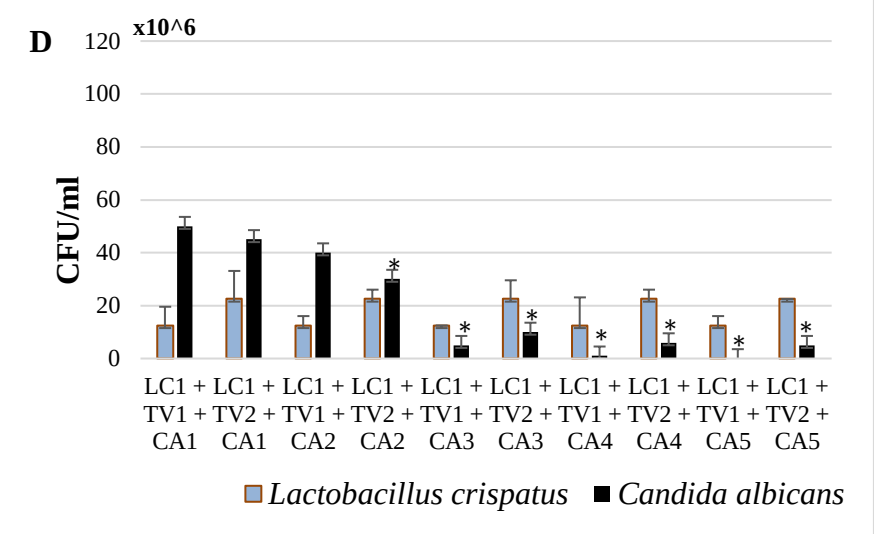
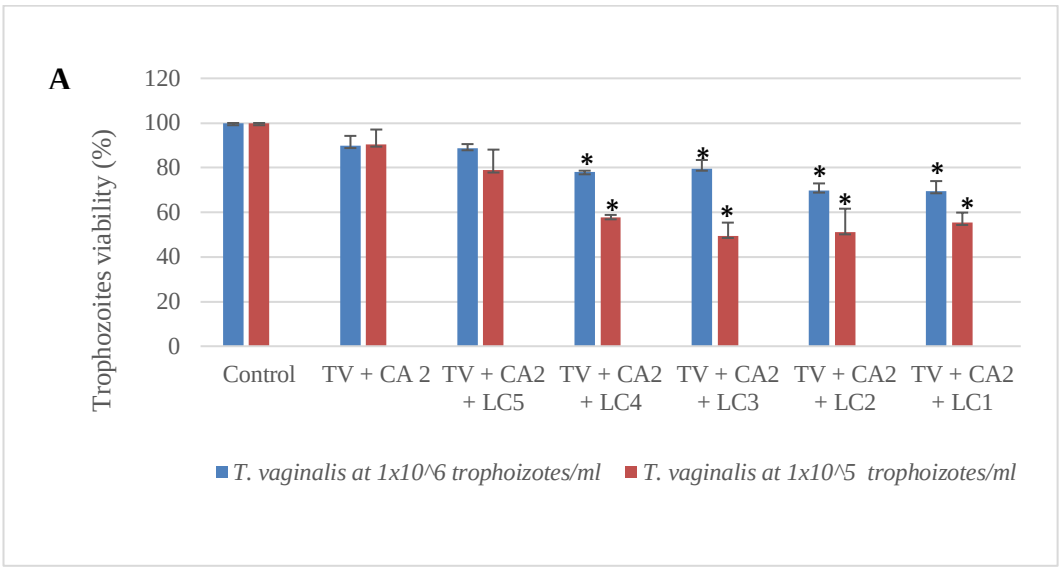


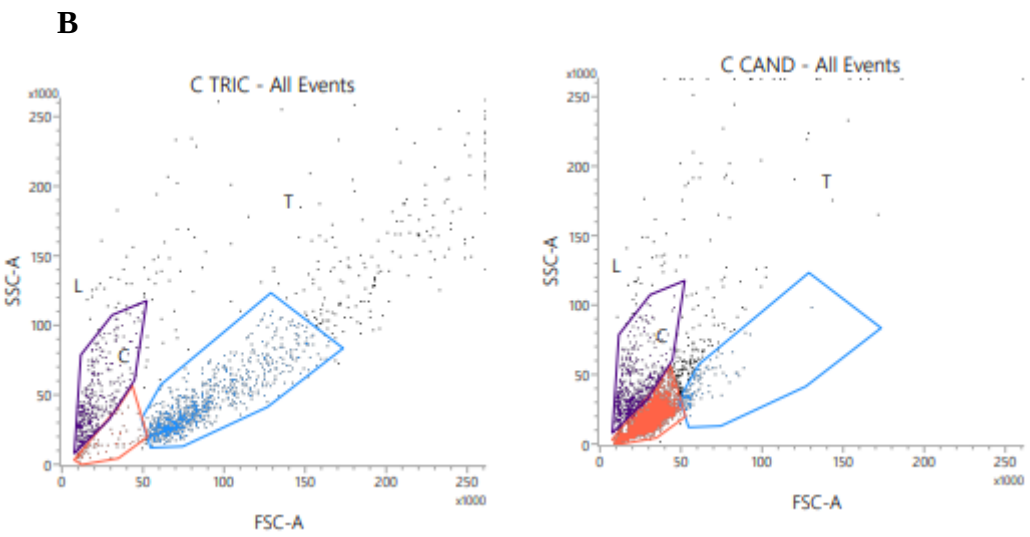
Figure 1: (A) CFU/ml of *L. crispatus* (LC1) at $1x10^8$ when is with *T. vaginalis* ranging of $1x10^2$ to $1x10^6$ trophozoites/ml (TV 1-TV 5). **(**B) Trophozoites viability when concentration of *T. vaginalis* ranges $1x10^6$, $1x10^5$ and $1x10^4$ trophozoites/ml (TV 1 - TV 3) when in co-culture with *L. crispatus* at 10^8 UFC/mL (LC1) and *C. albicans* ranging $1x10^6$ and $1X10^5$ CFU/ml. (C) CFU/ml of *L. crispatus* (LC1) at $1x10^8$ and *C. albicans* ranging of $1x10^6$ to $1x10^2$ CFU/ml in co-culture. (D) CFU/ml of *L. crispatus* (LC1) at $1x10^8$ and *C. albicans* ranging of $1x10^6$ to $1x10^2$ CFU/ml with *T. vaginalis* at $1x10^6$ and $1X10^5$. Bars mean media ± standard deviation compared to control. Results are representative of two independent experiments performed in duplicate assays. (*) Statistically significant difference ($p < 0.05$).

3.3 *C. albicans* at $1x10^5$ proliferated in a constant concentration when in presence of *T. vaginalis* and *L. crispatus*

Checkerboard was performed with *C. albicans* at $1x10^5$ UFC/ml (LC1); *T. vaginalis* at $1x10^6$ (TV1), $1x10^5$ (TV2), $1x10^4$ (TV3), $1x10^3$ (TV4), $1x10^2$

trophozoites/ml (TV5) and *L. crispatus* at 1×10^8 (LC1), $1x10^7$ (LC2), $1x10^6$ (LC3), $1x10^5$ (LC5), 1×10^4 (LCA) CFU/ml. *C. albicans* was recovered in Sabouraud agar in all created conditions at $1x10^6$ CFU/ml. In the presence of just *C. albicans*, *T. vaginalis* remained viable until TV3, however in the presence of all microorganisms, *T. vaginalis* maintained its viability until TV2 (Fig 2 A). *L. crispatus* was also observed in Gram stain, which showed a gram-positive bacillus on co-culture. Moreover, on flow cytometry the presence of *L, crispatus* was showed when in co-culture with *C. albicans* and *T. vaginalis* (Fig 2 B).





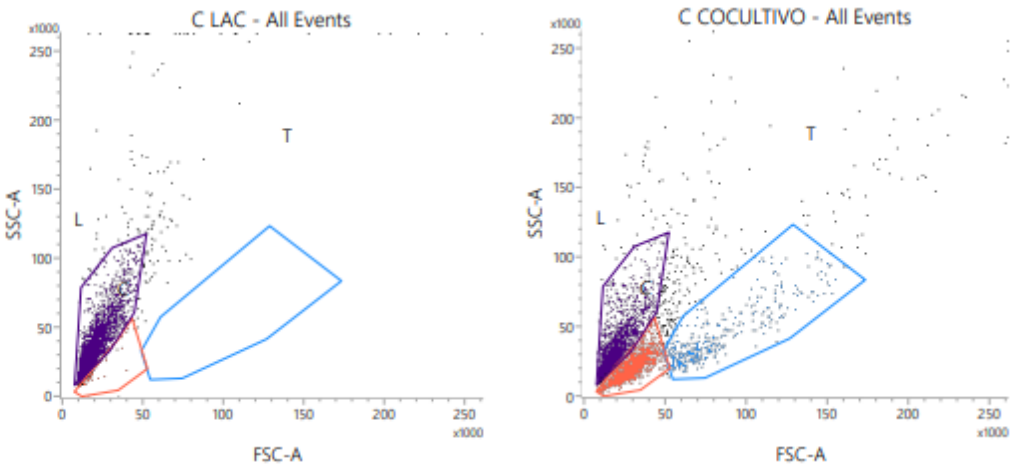


Figure 2: (A) Trophozoites viability when concentration of *T. vaginalis* ranges $1x10^6$, $1x10^5$ and $1x10^4$ trophozoites/ml (TV 1 - TV 3) when in co-culture with *C. albicans* at 10^6 UFC/mL (CA2) and *L. crispatus ranging* $1x10^8$ and $1X10^4$ CFU/ml. Bars mean media ± standard deviation compared to control. Results are representative of two independent experiments performed in duplicate assays. (*) Statistically significant difference ($p < 0.05$). **(B)** Flow cytometric properties of *T. vaginalis*, *C. albicans*, *L. crispatus* and co-culture of all microorganisms at $1x10^5$ trophozoites/ml, $1x10^5$ CFU/ml and $1x10^8$ CFU/ml, respectively.

3.4 Co-culture did not impact the biofilm formation and yeast-hyphae transition of *C. albicans*

CV staining method unveiled the co-culture was performed with CA2 and TV2; CA2 and LC1 because these densities demonstrated to be the best conditions for evaluating these microorganisms. The combination of CA2, TV2 and LC1 did not cause significant impact on the biofilm formation of *C. albicans* (fig. 4). Co-culture of CA 2 + TV2, CA2 + LC1 and CA2+ TV2 + LC1 maintained in 98%, 97% and 86% of biofilm formation, respectively. The yeast to hyphal transition was evaluated under optical microscope and expressed as a percentage, considering that 100% was related to maximum hyphae production observed in CA1 supplemented with 10% FBS in aerobic or 5% CO_2 incubation conditions (Fig 5). The presence of FBS both under aerobic and microaerophilic conditions induced the yeast-hyphae transition, although co-culture of TV1 with CA1 and TV1, CA1 with LC1 did not influence this mechanism of *C. albicans*. When CA1 was with LC1 in presence of FBS under microaerophilic condition,

yeast-hyphae transition was hampered in 40%. The absence of FBS under microaerophilic condition showed that hyphae formation was induced in co-culture.

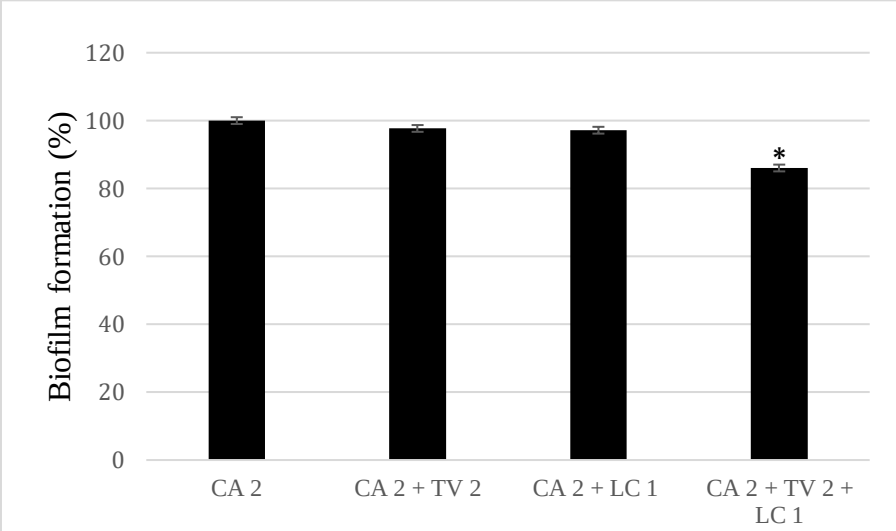


Figure 4: Impact of co-culture at the biofilm formation of *C. albicans* at $1x10^5$ CFU/ml (CA2). *T. vaginalis* at $1x10^5$ trophozoites/ml (TV2) and *L. crispatus* at $1X10^8$ CFU/ml (LC1). Bars mean media ± standard deviation compared to control. Results are representative of one experiment performed in triplicate assays. (*) Statistically significant difference ($p < 0.05$).

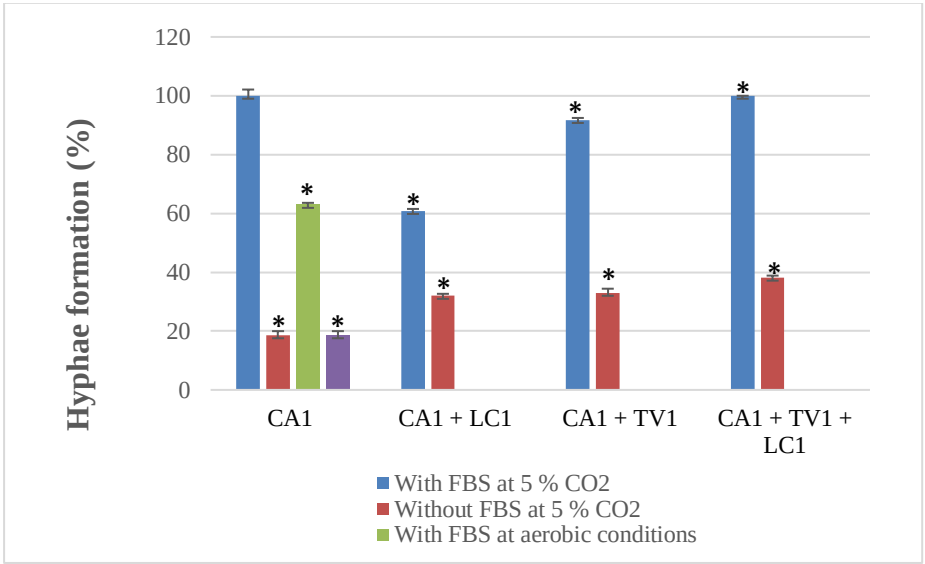


Figure 5: Impact of co-culture at the yeast-hyphae transition of C. albicans at 1x105 CFU/ml (CA2), T. vaginalis at 1x105 trophozoites/ml (TV2) and L. crispatus at 1X108 CFU/ml (LC1). Bars mean media ± standard deviation compared to control CA1 with FBS at 5% CO2. Results are representative of one experiment performed in duplicate assay. (*) Statistically significant difference ($p < 0.05$).

4. DISCUSSION

The flagellated protozoan *T. vaginalis* causes trichomoniasis, a vaginal infection that is accompanied by a dysbiotic microbiome composed principally of anaerobic bacteria and low densities of species of *Lactobacillus* [25-26]. Species of *Candida* are recovered in 20% of asymptomatic women [27], and *C. albicans* is the predominant member of mycobiota of healthy vagina, however due to an immunity imbalance and normal microbiota modifications, yeast modify their morphology to hyphal form which is linked to the VVC [28]. These vaginal infections are related to resistant-treatment isolates and high expenses in public health, for this reason discovery of alternative drugs are extremely necessary. Moreover, an in vitro test that mimetize the real condition in infection on the host and detects antimicrobial activity also has major importance. Drugs discovery and their developing lead 12-15 years and has a cost above 1 billion dollars, wherein basic research has an important role on target identification and molecule selection [29]. For this reason, in this current study, co-culture of *T. vaginalis, C. albicans* and *L. crispatus* was standardized to be use as new platform that evaluates a promise of synthesized or natural molecules.

Co-culture is an experimental microenvironment in which *T. vaginalis, C. albicans* and *L. crispatus* interact with competition for nutrients from the environment, for this reason the fastidious bacteria is the most prejudiced. The protozoan has the capability to phagocyte several cells, including species of lactobacilli [30], which may be a justification for the absence of *L. crispatus* recovered in MRS or LBS agar when it was in combination with *T. vaginalis* at higher densities (TV 1 and TV 2). On other hand, when *L. crispatus* is at $1x10^8$ CFU/ml, C. *albicans* at the $1x10^5$ CFU/ml and *T. vaginalis* at $1x10^5$ trophozoites/ml, the presence of bacteria was detected through Gram staining and flow cytometry.

Among the advantages of co-culture methodology, several studies indicated that the interaction of bacteria and fungi can induce metabolic expression [31-32], which are not

expressed in laboratory monoculture. As an example, Bertrand S. *et al*. [33] reported that interaction of bacteria of genus *Streptomyces* and Basidiomycete fungi can active metabolites production in the laboratory, being a potential way for the development of new molecules for treatment of several diseases. Indeed, metabolites produced by *T. vaginalis*, *C. albicans* and *L. crispatus* in standardized co-culture described in this present study, can be evaluated and discussed in further studies. Another advantage of co-culture is the possibility of evaluating the microorganism interaction, whether when causative vaginitis agents are interacting induces the production of virulence traces and decreases the susceptibility for choice treatment. Moreover, *L. crispatus* represents the protection of vaginal bacteriobiota; thus, in further studies our group will evaluate whether the interaction with *T. vaginalis* and *C. albicans* inhibits the microbial resistance and the production of virulence traces by employing this platform.

Limitations of this study were appearing along their development, such as the *L. crispatus* culture establishment. This fastidious bacterium needs a specific culture medium (MRS), and incubation period is 72 h, whereas *T. vaginalis* e *C. albicans* has optimal growth in 24 h. Also, other two microorganisms compete for the same nutrients source with this bacterium, which turns its growth injured. Another limitation is related to determining the growth for *L. crispatus* by UFC/ml in an agar non-selective. Even though MRS agar is a suitable culture medium for this bacteria, other microorganisms can grow into, as the case for *C. albicans*, which promotes the count unfeasible of bacteria colonies, principally. Furthermore, the lack of specific dyes for each one member of co-culture prejudices a specific marking for flow cytometry.

Overall, we believe that the in vitro co-culture could be a new tool for antimicrobial activity assay of promising molecules against causative vaginitis agents. The perspectives to evaluate the validation of this novel platform are: to detect the antimicrobial activity of new molecules in this co-culture system, comparing with a single microorganism culture; to evaluate

whether cell toxicity is influenced when *T. vaginalis* and *C. albicans* are in co-culture; and to perform flow cytometry analysis with the combination of TV1, CA2 and LC1 to stablish feasible profile markers.

Acknowledgments

This study was supported by grants from the following Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Universal grant 428538/2018-5), Fundação de Apoio à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) PPSUS grant 21/2551-0000128-3).

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