Molecular epidemiology and evaluation of *Candida* spp. persistence in the vaginal mucosa, in chronic cases of vulvovaginal candidosis

Paula Cristina Gonçalves Faria Gonçalves

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Supervisor: Prof. Doctor Ana Palmeira de Oliveira

Co-supervisor: Doctor Joana Rolo

Júri:

Prof. Doctor Joaquim Paulo Mateus Serra

Prof. Doctor Filomena da Luz Martins Pereira

Prof. Doctor Teresa Maria Fonseca de Oliveita Gonçalves

Prof. Doctor Carmen Maria Lisboa da Silva

Covilhã, 7 june 2022

Declaração de Integridade

Eu, Paula Cristina Gonçalves Faria Gonçalves, que abaixo assino, estudante com o número de inscrição D2008 de Biomedicina da Faculdade de Ciências da Saúde, declaro ter desenvolvido o presente trabalho e elaborado o presente texto em total consonância com **o Código de Integridades da Universidade da Beira Interior**.

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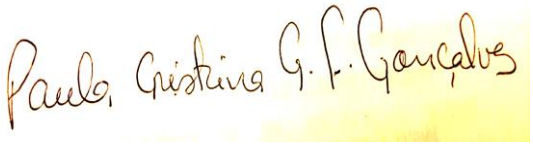

Dedication

To God, he knows well why…

To my husband Francisco M.P. Gonçalves and children Rafael, Anayeli and Svetlana Gonçalves for various sacrifices made to achieve this thesis.

To my parents Paulo de Faria and Deolinda de Faria and to my brothers Tita, Dilson, Décio and José Edmir de Faria for strength.

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Resumo

A candidose vulvovaginal é uma infeção que afeta a maioria das mulheres em idade fértil; contudo dados epidemiológicos sobre a sua incidência e risco de cronicização ainda carecem de esclarecimento, sabendo-se que varia de região para região. Este estudo visou avaliar em 93 isolados vaginais de 36 mulheres em idade fértil com candidose vulvovaginal episódica (16) e/ou crónica (20), fatores de virulência, características genotípicas e a relação destas com cronicização da candidose vulvovaginal, e comparar as características dos fungos provenientes dos dois grupos clínicos de modo a obter diferenças que permitam perceber as causas da cronicização. Identificamos as estirpes fenotipicamente, utilizando o meio cromogénico CHROMagar e análise dos perfis bioquímicos. Avaliamos a concentração mínima inibitória dos compostos azólicos (fluconazol e clotrimazol) utilizando o método de microdiluição *in vitro* segundo a norma CLSI em dois diferentes pHs (pH4.5 e pH7) para o fluconazol, a capacidade de: a) formar biofilmes utilizando o método de cristal violeta segundo Sherry *et al*, 2017, b) formar tubo germinativo nas estirpes *Candida albicans* segundo a metodologia descrita por Ellepola e Samaranayake, 2008, c) produzir fosfolipase utilizando o método adaptado Price *et al*, 1982, e) produzir proteinase descrito por Vinodhini et al, 2016 e a f) hidrofobicidade da parede celular e a capacidade de resistir a pHs altos. Adesão a células HeLa (linha de células epiteliais de câncer cervical humano) e a resistência à fagocitose por células RAW 264.7 (macrófagos murinos), foram testadas *in vitro*, a sua semelhança genética foi avaliada através da análise dos perfis de ADN (tipagem molecular por RAPD). Também foi determinada a atividade antifúngica de $NaHCO_3$ contra *C. albicans* ATCC 10231 e 12 estirpes clínicas VVC usando o método de microdiluição, a formação de biofilme e do tubo germinativo (hifa) em presença do $NaHCO_3$. Para comparação dos dados dos dois grupos clínicos utilizamos o programa *graphpad prism 7* para análise estatística (teste do $\chi 2$). **Resultados** *C. albicans* foi a espécie mais frequente. Quando comparamos os resultados da determinação da concentração mínima inibitória dos isolados dos dois grupos clínicos, não encontramos diferenças significativas entre eles. Verificamos que não houve diferenças significativas entre os grupos, relativamente à distribuição do número de estirpes formadoras de biofilme em casos esporádicos de candidose vulvovaginal ou de candidose vulvovaginal crónica. Na candidose vulvovaginal esporádica (considerando apenas estirpes com capacidade de formar tubo germinativo moderada) verificou-se uma maior ($p < 0.05$) capacidade de produzir tubos germinativos em relação a candidose vulvovaginal crónica. Em relação à proporção de isolados fortes em produzir fosfolipase, não foram encontradas diferenças significativas

entre os isolados esporádicos e crónicos. A avaliação dos perfis de DNA de isolados consecutivos de candidose vulvovaginal cronica revelou existirem diferenças. Os isolados de levedura envolvidos na infeção esporádica aderiram em maior proporção às células HeLa (relacionada com a sua capacidade de produzir biofilme) ($p < 0,05$), enquanto que a capacidade de evadir a fagocitose (relacionada com produção elevada de protéases) ($p < 0,05$) foi mais evidente nos isolados crónicos; sendo a resistência dos casos esporádicos à fagocitose relacionada com uma maior hidrofobicidade da parede celular ($p < 0,05$). Verificámos que *C. albicans* tinha a capacidade de proliferar em caldos numa ampla faixa de valores de pH (de 7,40 a 9,77) e que a concentração inibitória mínima de $NaHCO_3$ contra *C. albicans* foi de 12,5 mg / ml (pH 8,97). Não foi possível determinar a concentração letal mínima, o que sugere tratar-se de um mecanismo de ação fungistático. As células de *C. albicans* expostas ao $NaHCO_3$ (em concentrações pelo menos duas vezes a mínima inibitória), apresentaram redução de 1,5 vezes na taxa de crescimento normal e redução de 93% nas células produtoras de hifas, quando comparadas às células não expostas ao $NaHCO_3$. Além disso, houve redução de 50% na massa do biofilme, quando as células de *C. albicans* foram expostas a quatro vezes mais do que a concentração mínima inibitória. **Conclusões**: Os nossos resultados mostram que, curiosamente, uma das estratégias para *Candida* spp persistirem no hospedeiro e expressarem poucos fatores de virulência, e assim desencadearem menor reação de defesa, e que os isolados de casos episódicos são fortes produtores de biofilmes e de tubos germinativos, o que ajuda a aderir a células Hela (e, portanto, ao epitélio vaginal), provavelmente relacionado de alguma forma com a hidrofobicidade da parede. Mais ainda, quando em contacto com os macrófagos estes isolados persistem, não formam tanto biofilme nem aderem tao bem ao epitélio, mas resistem melhor à fagocitose porque produzem mais protéases e são mais resistentes a variações de pH. As estirpes provenientes de casos crónicos têm também a capacidade de modelar os fatores de virulência ao longo do tempo, o que lhes permite melhor persistir no hospedeiro. Concluímos que em presença de $NaHCO_3$ o crescimento dos isolados clínicos e de coleção (ATCC 10231) tiveram independentemente do pH, crescimento reduzido, atenuação da formação de biofilmes e hifas, pelo que se perfila este composto como um adjuvante para terapia com vista ao controle da cronicização da candidose vulvovaginal.

Palavras-chave

Fatores de virulência; Candidose vulvovaginal; cronicização; recorrência; persistência; resistência aos antifúngicos.

Abstract

Vulvovaginal candidosis is an infection that affects most women of childbearing age; however epidemiological data on its incidence and risk of chronicity still need clarification, knowing that it varies from region to region. This study aimed to evaluate, in 93 vaginal isolates from 36 women of childbearing age with episodic (16) and/or chronic (20) vulvovaginal candidosis, virulence factors, genotypic characteristics and their relationship with chronicization of vulvovaginal candidosis, and to compare the characteristics of fungi from the two clinical groups in order to obtain differences that allow us to understand the causes of chronicity. We identified the strains phenotypically using the chromogenic medium CHROMagar and analysis of biochemical profiles. We evaluated the minimum inhibitory concentration of azole compounds (fluconazole and clotrimazole) using the in vitro microdilution method according to the CLSI standard at two different pHs (pH4.5 and pH7) for fluconazole, the ability to form biofilms using the crystal violet method according to Sherry et al, 2017, the ability to: a) form germ tube in *Candida albicans* strains according to the methodology described by Ellepola and Samaranayake, 2008, b) produce phospholipase using the adapted method Price et al, 1982, c) produce proteinase described by Vinodhini et al, 2016, d) cell wall hydrophobicity and e) ability to resist high pH. Adhesion to HeLa cells (human cervical cancer epithelial cell line) and resistance to phagocytosis by RAW 264.7 cells (murine macrophages) were tested in vitro, as well as evaluating their genetic similarity through the analysis of DNA profiles (molecular typing by RAPD). The antifungal activity of $NaHCO_3$ against *C. albicans* ATCC 10231 and 12 clinical strains VVC was also determined using the microdilution method, biofilm formation and germ tube (hypha) formation in the presence of $NaHCO_3$. For analysis and comparison of data from the two clinical groups, we used the graphpad prism 7 program for statistical analysis ($\chi 2$ test). **Results**: *C. albicans* was the most frequent species. When we compared the results of the determination of the minimum inhibitory concentration of the isolates from the two clinical groups, we did not find significant differences between them. We found that there were no significant differences between groups regarding the distribution of the number of biofilm-forming strains in sporadic cases of vulvovaginal candidosis or chronic vulvovaginal candidosis. In sporadic vulvovaginal candidosis (considering only strains with moderate germ tube capacity) there was a greater ($p < 0.05$) capacity to produce germ tubes in relation to chronic vulvovaginal candidosis. Regarding the proportion of isolates strong in producing phospholipase, no significant differences were found between sporadic and chronic isolates. The evaluation of the DNA profiles of consecutive isolates of chronic vulvovaginal candidosis revealed differences. Yeast isolates involved

in sporadic infection adhered in greater proportion to HeLa cells (related to their ability to produce biofilm) ($p < 0.05$), while the ability to evade phagocytosis (related to high protein production) ($p < 0.05$) was more evident in the chronic isolates; the resistance of sporadic cases to phagocytosis related to a greater hydrophobicity of the cell wall ($p < 0.05$). We found that *C. albicans* had the ability to proliferate in broths over a wide range of pH values (7.40 to 9.77) and that the minimum inhibitory concentration of $NaHCO_3$ against *C. albicans* was 12.5 mg/ml (pH 8.97). It was not possible to determine the minimum lethal concentration, which suggests that it is a fungistatic mechanism of action. *C. albicans* cells exposed to $NaHCO_3$ (at concentrations at least twice the minimum inhibitory) showed a 1.5-fold reduction in the normal growth rate and a 93% reduction in hyphae-producing cells, when compared to non-exposed cells to $NaHCO_3$. Furthermore, there was a 50% reduction in biofilm mass when *C. albicans* cells were exposed to four times the minimum inhibitory concentration. **Conclusions:** Our results show that, interestingly, one of the strategies for *Candida* spp persists in the host and express few virulence factors, thus triggering a lower defense reaction, and that isolates from episodic cases are strong producers of biofilms and germ tubes, which helps to adhere to Hela cells (and therefore to the vaginal epithelium), probably related in some way to the hydrophobicity of the wall. Furthermore, when in contact with macrophages these isolates persist, do not form as much biofilm or adhere as well to the epithelium, but are better resistant to phagocytosis because they produce more proteases and are more resistant to pH variations. The strains originating from chronic cases also have the ability to model virulence factors over time, which allows them to better persist in the host. We conclude that in the presence of $NaHCO_3$ the growth of clinical and collection isolates (ATCC 10231) had, regardless of pH, reduced growth, attenuation of the formation of biofilms and hyphae, so this compound is profiled as an adjuvant for therapy with a view to controlling the chronicization of vulvovaginal candidosis.

Keywords

Virulence factors, vulvovaginal candidosis, recurrence, persistence, antifungal resistance.

Índice

List of Figures

List of tables

Chapter 3. SPECIES DISTRIBUTION AND ANTIFUNGAL SUSCEPTIBILITY PROFILES OF ISOLATES FROM WOMEN WITH NON-RECURRENT AND RECURRENT VULVOVAGINAL CANDIDIASIS

Chapter 4. RECURRENT VULVOVAGINAL *CANDIDA* SPP ISOLATES PHENOTYPICALLY EXPRESS LESS VIRULENCE TRAITS

Chapter 5. EVALUATION OF OVERTIME PHENOTYPIC VARIATION OF YEASTS IN CHRONIC VULVOVAGINAL CANDIDOSIS CASES

Acronym list

ATCC – American Type Culture Collection

CSH- cell surface hidrofobicity

CLSI- Clinical Laboratory Standards Institute

cVVC- chronic vulvovaginal candidosis

DNA- Deoxyribonucleic acid

DMSO- Dimethylsulfoxide

sVVC- episodic or sporadic vulvovaginal candidosis

EUCAST-The European Committee on Antimicrobial Susceptibility Testing

GT- germ tube

MIC-minimum inhibitory concentration

MOPS- ácido 3- (*N*- morfolino) propanossulfônico

PBS – Phosphate Buffer Saline

PCR – Polymerase Chain Reaction

pH - Hydrogen potential

Pz – precipitation zone

RAPD – Randomly Amplified Polymorphic

RPMI1640- Roswell Park Memorial Institute medium 1640

SDA – Saboraud Dextrose Ágar

VVC- Vulvovaginal candidosis

YPD -Yeast Peptone Dextrose medium

Chapter 1. Introduction

Vaginal Microbiome

The set of all microorganisms present in the human body and the products of its metabolism forms the microbiome [1], alternatively defined as "the sum of genome and genes carried by microorganisms species existing in specific environmental of biological nice" [2]. Due to controversies in the definition of microbiome, a workshop was held and in the same context, an online survey, in which several experts in this area participated. The definition of microbiome adopted was "The microbiome is defined as a characteristic microbial community occupying a reasonable well-defined habitat which has distinct physio-chemical properties" based on the first definition of microbiome elaborated by Whipps *et al*. cited for [3]. These microorganisms present in the microbiome, are predominantly bacteria and fungi. The principal ecological niches of the human microbiome are intestinal, oral, vaginal and skin; they differ according to the type of microorganisms they specifically present [4]. In the vaginal environment various microorganisms can be found which can change, during the different age group of women and under behavioural influences. According to hormonal changes, menstrual cycle and the characteristics of vagina (as described by Farage *et al*, 1981 cited by Farage and Maibach 2006), six phases of vaginal variation and major microbiome variation can be described, such as: Phase 1. Newborn (in this phase there is a transfer of transplacental estrogens residues from mother to daughter, high glycogen content, production of lactic acid, microbial colonization by *Lactobacillus* spp.); Phase 2. Childhood (this phase is characterized by neutral or alkaline vaginal pH as the number of acid lactic producing microorganisms decreases), 3. Pubescence (in this phase there is menstruation and an increase of acid lactic producing microorganisms), 4. Reproductive age (in this phase the vaginal pH becomes acidic and there is production of glycogen due to the secretion of ovarian follicles), 5. Pregnancy (in this stage there is an increased risk of infection and acidic pH), 6. Menopause (stage in which menstruation does not occur, an increase in colonization of enteric microflora, there is in an atrophic vagina and an increase of vaginal pH) [5].

The most common bacteria of this microflora are *Lactobacillus*, being related to the maintenance of normal equilibrium of vagina. There are about 10^8 to 10^9 colonies forming units per gram of bacteria in the vaginal fluid of a healthy woman, where lactobacilli dominate in number of 10^7 to 10^8 colonies forming units per gram [6]. The *Lactobacillus* are related with the absence of infection by various genera of bacteria and fungi [7]. The mechanism used by *Lactobacillus* to maintain an healthy microbiome is the production by these bacteria of acid lactic, bacterioncins, peroxide of hydrogen and

other antibacterial compounds, as well as their ability to adhere to specific places in the vagina [7]. Hormonal imbalances, certain woman behaviour, such as sexual lifestyle, personal hygiene, diseases, food and other intrinsic and extrinsic factors may cause alteration in microbiome that, may result in the development of an infection [6]. Among the microorganisms that form the vaginal microbiome, there are fungi that make up a subset of this ecosystem. The predominant fungi in this microbiome are of the genus *Candida* spp. mainly of the species *Candida albicans*. Larsen and Galask 1984 in their work - Influence of estrogen and normal flora on vaginal candidosis in the rat, (cited by: Bradford and Ravel 2017) treated rats with estrogen in order to be able to colonize them with *C. albicans* and to be able to verify a possible interaction between bacterium and fungi, verifying that there was no interference in the existence of fungi, with only a limitation of proliferation of this fungi [8].

Vaginal colonization by fungi of the genus *Candida* spp.

The rate of asymptomatic vaginal colonization by *Candida* spp., varies from 10 to 30% in healthy women and in reproductive age around the world [9]. In Portugal, the prevalence of *Candida albicans* in the vagina of 1004 normal, asymptomatic women was 10.4%, being lower (6.8%) in those taking combined oral contraceptives and higher (13.0%) in those using intrauterine devices [10].

The vaginal colonization by *Candida* spp, occurs when these species are present in the vagina and the woman has no symptoms or signs of disease [9] and this transition from colonizer to pathogenic agent requires changes in the vaginal environment that favour this pathogenicity [11]. As for the development of a fungal infection by the genus *Candida* spp. the presence or colonization by these fungi is associated with predisposition. The predisposition factors for the two cases (infection and colonization) may be different [11].

Candida spp.

It was only after 1980, in the 20th century, that fungi began to considered possible causes of diseases [9]. It started with their association to stomachs aftosa still in the first half of 19th century, but only in 1953 Robin, described the biology of an etiologic agent called *Oidium albicans*. In the 20th century another Dutch researcher, Berkhout, gave a great advance to the work initiated by Robin, regarding the finished description of the fungi of the genus *Candida* spp., denomination attributed by her. C*andida* has its origin from the latin word term “Candidus” which meand white-bright. This name is still used for the genus that causes candidosis [9].

From the term *Candida* the word candidiasis or candidosis emerged, being this the disease caused by fungi colonizing the human body, which can be found in the gastrointestinal mucosa, oral mucosa, vaginal mucosa and in the skin of healthy individuals (20%). When there is an imbalance in the normal microflora of organism and/or a change in the immune system occurs, an infection with this microorganism can be developed [12].

Visually, the colonies of different species of the genus *Candida* cannot be distinguished, but routine methods are used to distinguish them. The culture medium CHROMagar *Candida* mediumis a selective culture medium, in which chromogenic subtracts are present to differentiate the colonies of *Candida* spp. and is used to identify them [9]. Another method used for species identification consists of evaluating the fermentation and assimilation capacity of various carbohydrates, which is made by an automated method called VITEK 2, that shows good precision [12].

Another way to differentiate species among this genus is based on the ability of *Candida albicans* and a few non-*albicans* species, such as *Candida dubliniensis* and *Candida stellatoidea*, to change morphology during their life cycle (dimorphism), presenting as yeast form, pseudohyphae and hyphae forms. In true hyphae, there is first the prolongation of the hyphae, before the formation of septa, which are called germ tube (hyphae without septa), an exclusive characteristic of *Candida albicans* species [13]. *Candida albicans* differs from other dimorphic fungi in that it is able to change to hyphal form, which is related to pathogenicity, contrary to the remaining fungi with dimorphic characteristic [14]. Except for *C. glabrata*, yeasts among *Candida* usually present as blastoconidia that elongate and remain connected, giving rise to a linear structure, without separation called pseudohifa [13].

The morphological alteration of *Candida* spp. can be induced by changes in pH (up or equal to 7), the percentage of CO_2 (10% above the environmental concentration) and temperature (37ºC) , and by the presence of N-acetilglucosamine, [15][14].

Candida albicans can also undergo morphological transition from colonies, ranging from white colonies to opaque colonies reversibly. This transition from white colonies to opaque colonies is influenced by an high level of CO2, by genotoxic and oxidative stress metabolism of white blood cells and sources of sugar (for example, inside a macrophage or neutrophil, however, the nutritional environment completely changes for the fungus)[15][16].

Candida albicans, which is the most studied species of this genus, has mannan and β- (1 → 3) - D-glucan on its cell wall. These components can be used for the diagnosis of invasive fungal infections, as these molecules can be detected in the blood at an early stage of the infection and before the onset of clinical symptoms. The detection of these antigens is a faster procedure than traditional methods, performed by histological studies or cell culture [17]. It has been shown cell wall mannoproteins of *Candida dubliniensis* are related to adhesion between cells, immunomodulatory abilities, and antigenic variability [17].

Fungi of this genus have a resistant cell wall, consisting of N-Acetylglucosamine under a polymeric form called chitin which is also important for modification cell surface proteins (it forms part of the N-linked polysaccharide chain that is added to glycosylated proteins and is a building block in the synthesis of GPI- anchor that maintain certain proteins in the plasma membrane)[18].

Resistance to azole antifungals by *Candida albicans* species and non-*albicans* species has become common, with evidence of increasing [19]. Assessing the susceptibility of yeasts to antifungal agents is a work of great importance because it allows the determination of antifungal resistance patterns, even if their results do not directly correlate with the expected clinical response [19].

Bitew and Abebaw assessed the prevalence of strains related to vulvovaginal candidiasis and found an increase in the incidence of *non-albicans* strains and also higher rate of resistance to fluconazole from *non-albicans* species [20].

Candidosis

Humans are colonized by fungi of the genus *Candida* spp., the species *Candida albicans* being the most frequent. When there is an imbalance in the body, such as changes in the immune system, these fungi are likely to cause various types of infections [21]. *Candida* spp commonly causes several types of infections in humans, most species of this genus can cause different types of candidosis [22].

Candidosis is classified according to the organ that is infected by fungi of the genus *Candida* spp. Mucocutaneous candidosis (oral, cutaneous, intestinal, and vulvovaginal) and systemic candidosis are the two main groups of existing candidosis. In the first group of mucocutaneous candidosis, the tissues most affected are those of the genital organs and the digestive tract [23]. In cutaneous candidosis, the groins, armpits, and skin folds

in general, interdigital spaces of the hands, feet and nails are often affected by infection [17].

The second group of systemic candidosis, occurs through the entry of yeast through the epithelial barriers and affecting various organs of the body, for example, pulmonary candidosis, endocarditis, nephritis, among others, thus becoming a serious disease, which can lead to death [23]. Candidemia is cited in studies carried out in intensive care as the fourth most common cause of infection in the bloodstream. In population studies, most of them report candidemia as the seventh to tenth most common infection in the bloodstream [23]. The occurrence of candidemia was related to the ability of *Candida* spp to develop biofilms on various surfaces, such as biomaterials and host tissues. In many hospitals, broad spectrum antifungals are used prophylactically in patients with some type of implanted biomaterial, which has resulted in saving lives [23].

Oral candidosis is part of the group of mucocutaneous candidosis, commonly referred to as thrush, is caused mainly by the species *Candida albicans,* but *non-albicans* species like *Candida glabrata, C. guillermondii, C. krusei, C. lusitaniae, C. parapsilosis, C. pseudotropicalis, C. stellatoidea and C. tropicalis* area also involved in this infection [24].

This disease can manifest itself in one or two ways, erythematous or white. The white form characterized by white lesions which in turn comes in two forms, the pseudomembranous and the form of hyperplastic candidosis. Oral erythematous candidosis manifests itself with reddish lesions and in turn presents itself in several forms: acute atrophic candidosis, chronic atrophic candidosis, median rhomboid glossitis, angular and linear cheilitis. Three more types of oral candidosis are also described, which do not fall into the above two groups: chronic mucocutaneous candidosis, cheilocandidosis and chronic multifocal candidosis [25].

Cutaneous candidosis (affecting the interdigital space, fingers, feet, hands, nails, buttocks and other parts of the skin less frequently), although less frequent than other mucocutaneous candidosis, can affect individuals of all age groups, being the fungus of the genus *Candida* spp., the most common cause of skin infections [24].

Vulvovaginal candidosis is one of the main mucocutaneous infections, being the second major type of vaginal infections, hence the relevance of its study [20].

Vulvovaginal Candidosis

Candidosis caused by non-albicans species is clinically indistinguishable from that caused by *C. albicans*; However, such species are often more resistant to treatment, especially *C. glabrata*, which often cause chronic vulvovaginal candidosis (cVVC) [26].

Medicines for the treatment of vulvovaginal candidosis fall into two groups, oral and topical. And based on their appearance and form of use, topical drugs are distributed in various formulations, which do not influence the effectiveness of the treatment and include creams, pills and vaginal suppositories [27].

Jack Sobel (2016) gathered criteria to take into account when choosing anti-candida therapy and pointed to ten important factors to take into account for this choice. These are: the type of formulation, the antifungal efficacy time, the type of effect (fungicide or fungistatic), the time to symptom relief, the possible side effects, the ability to prevent recurrence, the impact in microbiota, the cost of the medication and spectrum [28].

Chronic vulvovaginal candidosis

The recurrent vulvovaginal candidosis corresponds to a type of chronic candidosis defined in terms of frequency by the occurrence of four or more episodes within the period of one year (RVVC) [29]. Some factors that can lead to the clinical chronic of vulvovaginal candidiasis are lack of response to the antifungal therapy used (resistance), genetic predisposition, factors inherent to lifestyle and the virulence of the strains involved. In recurrent cases non-*albicans* species seem to assume particular relevance [4][27].

Some authors relate chronic vulvovaginal candidosis to be caused by non-*albicans* species, as they are less studied and less frequent than *Candida albicans*. However, there are studies with considerable numbers of *non-albicans* species, which may point to an increase in the prevalence of these species in vulvovaginal candidosis and its chronicity[29].

Vaginal immune response in vulvovaginal candidiasis.

The vagina also contains immune cells, microbial stimulation through ligands such as lipopolysaccharide (LPS) that stimulate TLR-4 triggers the release of cytokines. The

production of the cytokines results in a barrier made up of epithelial cells. Epithelial cells produce various antimicrobial peptides, essences in the constitution of mucous membranes and respond to stimuli quickly [30].

These cells express immunity receptors, capable of detecting microorganisms and sending activation signals for secretion of immune mediators [31][32]. Exposure to stimuli (microorganisms, for example), triggers a rapid response by the constitution of mucosal surfaces by antimicrobial peptides. Soluble mediators are secreted as signals (antimicrobial peptides, chemokines and cytokines) [33].

Epithelial cells have the ability to distinguish the different forms in which *C. albicans* (yeast, hyphae and pseudohypha), so the immune system produces a response before the yeast starts the inflammatory response [33].

Signal production by epithelial cells contributes to the appearance of symptoms in vulvovaginal candidosis, and in the absence of an inflammatory response, asymptomatic colonization is considered. Studies on the inflammatory response indicates that susceptibility to infection is associated with an aggressive inflammatory response, while resistance is associated with the activation of an anti-inflammatory profile [33][31].

Objectives

The difficulty of clinical resolution in a considerable number of cases of vulvovaginal candidosis and in as well as number of patients who have cVVC (chronic) has preoccupied the scientific community, which leads to the incentive to search for their genotypic and phenotypic causes. These circumstances led us to outline this project, aiming to contribute or even discover some new characteristics in these strains of *Candida* spp. by means of making a comparison between the two groups of strains from VVC and cVVC.

The questions we set out to answer is: What are the genotypic and phenotypic differences between the strains of *Candida* spp., that lead to the chronicity of the infection in the vaginal mucosa?

Our main approach is to study, in a collection of *Candida* spp. obtained from women suffering from VVC and cVVC, the molecular epidemiology, comparing several isolates of the same patient with chronic vulvovaginal candidosis (3 or more samples); verify the genotypic differences between them; assess the presence of various virulence factors of

the strains, such as the formation of biofilms, the production of extracellular enzymes, their polymorphism specifically the formation of a germ tube, the hydrophobicity of cell walls, the resistance to pH, the infectious ability using cell lines. The comparison of the results obtained between the two clinical groups will allow to identify which mechanisms are used by *Candida* spp. to persist in the vaginal mucosa.

For the purposes of this thesis, we have divided into nine main sections: chapter 1) Background, chapter 2) virulence factors as promoters of chronic vulvovaginal candidosis : a review, chapter 3) Species distribution and antifungal susceptibility profiles of isolates from women with non-recurrent (sporadic) and recurrent (chronic) vulvovaginal candidosis, chapter 4) Recurrent (chronic) vulvovaginal *Candida* spp isolates phenotypically express less virulence traits; chapter 5) Evaluation of overtime phenotypic variation of yeasts in chronic vulvovaginal candidosis cases, chapter 6) Chronic vulvovaginal *Candida* spp. clinical isolates are more resistant to phagocytosis in-vitro; chapter 7) Sodium bicarbonate is an antifungal agent independently of pH: effect against vulvovaginal *Candida* spp.; 8) General discussion and 9) General conclusion.

The six chapters that we now describe in terms of contextualization are research articles included in this thesis, starting with the literature review in chapter 2 to chapter 7, the result of bibliographical research and laboratory investigation carried out within the scope of this thesis.

The second chapter consists of a review of the literature in which the objective was to gather scientific evidence that linked the virulence factors of *Candida* spp and chronic vulvovaginal candidosis. Also addressed in this article were themes about alternative treatment therapies and prophylactic measures that could be used in the prevention of chronic vulvovaginal candidosis.

The third chapter consists of an experimental work, where the strains were identified at the species level, as well as the susceptibility of all strains to two azoles that are commonly used in the treatment of this infection was evaluated. Following the objective of the work, which is to compare the strains of the two clinical groups, hoping to obtain differences.

The fourth chapter consists of the experimental evaluation of virulence factors of strains of *Candida* spp, classified into two clinical groups (sporadic vulvovaginal candidosis and chronic vulvovaginal candidosis). The virulence factors evaluated were the ability to: form biofilms, produce phospholipases and form germ tube. For this work we expected

to obtain greater virulence in the group of strains obtained from women with chronic vulvovaginal candidosis, to justify the chronicity of these strains.

The fifth chapter consists of a genotypic evaluation of the strains of the cVVC group, of the behavior of the strains in the different stages of chronicity, taking into account whether the same strain caused the chronicity or not. The behavior of the ability to form biofilms in related strains was also evaluated in several episodes of cVVC and at different pHs (acid and neutral).

The sixth chapter consists of evaluating the adhesion of clinical isolates of *Candida albicans* and *Candida glabrata* from two groups (sVVC and cVVC) to HeLa cells ((human cervical cancer epithelial cell line) and the resistance to phagocytosis of the same isolates to RAW 264.7 (murine) cells macrophages).

The seventh chapter consists of evaluating the action of NaHCO2 at 3 different pHs on clinical isolates of *Candida albicans* and a collection strain (ATCC 10231), evaluating the ability to form biofilms and the ability to form germ tubes, with a view to an alternative therapy for the treatment of vulvovaginal candidosis

Note: At the end of this project and the preparation of the literature review, we started to adopt a new terminology, described in detail in chapter 2 (virulence factors as promoters of chronic vulvovaginal candidosis: a review). As until then, some articles were already published, in their transcription we kept the published designation with the addition of the one adopted in parentheses. For example, RVVC (chronic vulvovaginal candidosis, cVVC).

Chapter 2. Virulence factors as promoters of Chronic Vulvovaginal Candidosis: a review

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In this chapter, the article referenced here is transcribed with annotations referring to the nomenclature adopted in the meantime.

Virulence factors as promoters of Chronic Vulvovaginal Candidosis: a review

Abstract

The vast majority of the species of the genus Candida is commensal in humans; however, some are opportunistic pathogens that can cause infection, called candidosis. Among the different types of candidosis, we highlight the vulvovaginal (VVC) which can occur in two main clinical variants: chronic (cVVC) and episodic or sporadic. The incidence of cVVC has been worrying the scientific community, promoting the research on genotypic and phenotypic causes of its occurrence. We summarize important findings on factors that favour chronic vulvovaginal candidosis with respect to molecular epidemiology and the expression of various virulence factors, while clarifying the terminology involving these infections.

The aim of this review was to gather research that linked virulence factors to VVC and its persistence and recurrence, using two databases (Pubmed and Google Scholar). Predisposing factors in women for the occurrence of cVVC and some studies that refer new preventive and alternative therapies were also included, where appropriate.

Several studies have been shedding light on the increasing number of persistence and recurrences of VVC. The expression of virulence factors has been related to both chronic forms of VVC and antifungal resistance. Other studies report mutations occurring in the genome of Candida during the infection phase which may be important indications for new therapies. The introduction of preventive therapies and new therapies have revealed great importance and are also highlighted here.

Keywords: Persistence, chronic candidosis, vaginal infection, *Candida albicans*

Adopted Terminology

While performing this review it turned evident for us the different meanings and uses of medical terms described in the literature, rending the understanding of the global problem unnecessarily difficult. Unfortunately, scientific literature is filled with misuses, misnomers and misinterpretations that create additional barriers to the communication of information concerning important points and, worst, may induce the reader in error.

Mary Beth Saffo stated in 1993, more than a century after de Bary (1879) adopted the term symbiosis: "biologists still disagree about the word's meaning. Many researchers define symbiosis in the sense of de Bary, as an intimate, outcome-independent interaction between species; others use symbiosis as a synonym for mutualistic or non-parasitic associations"[34].

So, it takes many years up to consensual definitions are adopted, if ever; the same problem applies to the ecology of *Candida* spp and its relation to the human being in health and disease.

In order to effectively arrive to practical conclusions, we reviewed some definitions required to be clarified, and summarized a comprehensive proposal based on the terminology we adopted. With the advances in microbiome research new concepts arose. It is now clear that the human being harbours on its surface and in its inner cavities different kinds of microorganisms. This means that it has been colonised by them, representing a kind of life sharing called symbiosis.

Symbiotic in biology refers to an ecological relationship in which one organism (the symbiont) lives in a long-term relationship (**symbiosis**) in a natural ecosystem with another organism (the host), without no further implications on the final result of their relationship. This can be beneficial only to the symbiont (**commensalism**) or also to the host (mutualism) but in other cases it is harmful to the later (**parasitism**) [34].

Colonization means the settlement of a microorganism on an epithelium, establishing a stable, although sometimes transient, stay excluding contamination. Colonization is required for commensal or mutualistic symbiosis but also represents the first stage of disease (parasitism). Under this perspective the first two shall be considered to be **asymptomatic carriage**, while the later is best recognized as **prodromic stage of infection.**

So, we do only partially agree with Chatzivasileiou & Vyzantiadis (2019) when stating that "Colonisation does not necessitate therapeutic intervention when asymptomatic"

but clearly this applies only to women with non-recurrent disease. In fact, intermitent long-standing prophylatic therapy is recommended for women with RVVC, thus making suppression of asymptomatic carriage desirable [9].

While until now and to our knowledge there is not a single description of a mutualistic relationship between *Candida* and humans, due to evolutionary adaptation. *Candida* is consensually accepted to behave as commensal, at the points of their frequent entrance in the body (respiratory and digestive tracts) and contact (skin), although in particular circumstances may change to a pathogenic role (parasitic). This is not the case for the vagina where ecologically the access is limited to sexual intercourse. So, there is no reason to view *Candida* simply as a true vaginal commensal. Instead, its presence in the vagina, supposedly temporary although for different periods of time, may be clinically considered to be a reversible colonization state (normal women) or a prodromic one of *Candida* spp non-obligatory parasitism (in susceptible women), sooner or later inducing harm (symptoms and/or signs). To treat or not to treat is then the result of defining it as asymptomatic carriage or prodromal colonization.

Symbiotic shall not to be confused with synbiotic whose updated definition is that of "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" [35].

From the above, it is also clear that carriage means not a **disease** (or having a microbiologic cause an **infection**) that requires objective or subjective harm either structural or functional of the hole body or of one of its parts.

The name for the disease is not in nowadays practice a cause of confusion. In fact, while almost no author does still use the word moniliasis, many do call either candidiasis or candidosis for infections caused by *Candida* spp. When reviewing the literature on fungi nomenclature a conclusion is not clearly drawn, due to divergence in proposals launched by different scientific societies [36].

We chose to call the disease as Candidosis for being in accordance with the use of the suffix osis as a generic for all mycoses [37].

We also prefer to call "chronic" for the *Candida* infections that do show a prolonged course as in practical terms a **recurrence** (**latu sensu**) is the reappearance of the clinical picture but caused by the same group of microorganisms.

In clinical research and epidemiological studies more quantifiable definition is desirable. In this context, recurrent VVC is defined by the occurrence of more than two confirmed

(three or more) episodes within a twelve-month period. Because the frequency is not consensual as there had been proposals of three or five, although four being the most reported, this must be specified in each clinical study on this subject.

So, assuming that there are differences in their pathophysiology that represent different clinical approaches, concerning frequency we adopted the following Clinical **Classification of VVC** and their precedent stages:

Asymptomatic carriage: *Candida* colonization in women with no problems or history of significant disease.

Sporadic or Episodic Candidosis: infrequent, meaning not more than two episodes in a year (12 calendar months) period.

Prodromic carriage: *Candida* colonization in women with no problems presently but having history of frequent episodes and being expected to sooner or later will turn symptomatic.

Chronic, Recalcitrant or **Refractory Candidosis**: of long-term duration, being long-lasting and characterized by long suffering, they can assume two different clinical profiles:

Persistent or **Resistant Candidosis** being continuous, never ceasing, with no remissions, although with fluctuations in intensity.

Recurrent or **Relapsing Candidosis** those that reappear after an objective confirmed (clinical and culture) remission, and that can be subclassified as **reinfection,** when caused by the same agent as the previous episode and as **new infection** if by a different strain.

To easily define a specific case adequate tools are required. Knowing that the syndrome diagnosis is insufficient, and even microscopy misses many cases, and that, in contrast, molecular biology techniques like polymerase chain reaction (PCR) may lead to overdiagnosis, the gold standard method to choose is culture. If these are performed and the diagnosis is so confirmed the presented classification is easily applied. If the diagnosis has been syndromic no distinction can be made other than a clinical relapse, with suspected etiology, and here persistence or relapse will not be differentiable. In other words, for defining correctly a resistant or persistent case culture is mandatory.

Unfortunately, in most of the reviewed papers it is not possible to identify which clinical presentations were included, other than being of long duration (chronic). So, we kept the

original description: recurrent vulvovaginal candidosis (RVVC) or vulvovaginal candidosis (VVC).

Introduction

Candida is a genus of fungi belonging to the phylum Deuteromycota, class Blastomycetes, order Cryptococeales and family Cryptococaceae [38][39]. They are yeast-like fungi, composed of oval or spherical, unpigmented cells (Figure 1) that form pseudomycelia or a mycelium. They present asexual reproduction by multipolar budding [15] [38]. Within this genus, *Candida albicans* and other related Candida spp, such as *Candida dubliniensis* and *Candida stellatoidea*, exhibit variations in their life cycle (dimorphism), or transitioning between the yeast, pseudohyphae and hyphae shape [40]. In general, about 200 species of this genus have been described; among them 17 are human commensals. Opportunistically they can cause various candidoses, from superficial to systemic [41]. Some species of this genus are part of the human saprophytic flora and can be found in the oral mucosa (53%), digestive tract/ intestine, vagina (75%) and skin (53%) [15]. It is not wise, however, to consider these body parts equally functional prone; since *Candida* spp are ubiquitous and in healthy conditions frequently come in contact with the skin, the digestive tract, and even the respiratory one, but not the vagina. Most infections are caused by *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*. These fungi are able to infect humans because they have accommodated to human body temperatures, to survive on its surfaces or to penetrate them, and resist immune defences [40][42][11][43][44]. But the most relevant species of this genus is *Candida* (C.) *albicans*, which causes about 90% of mucosal infections and over 50% occurrences of candidemia. However, recent studies on candidemia in several countries, such as Denmark, North Korea, Kuwait, and Portugal report an increase in the number of candidemia caused by *C. albicans* and other non-*albicans Candida* spp, but mainly of the later ones [42]. This fungus can also cause vaginal infections which are called VVC [9], and the recurrence of these infections is the object of this review.

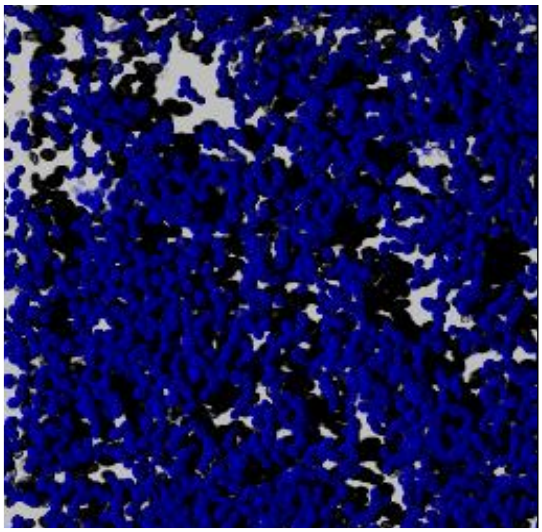

Figure 1. *Candida albicans* ATCC10231 cells stained with 2% calcofluor (Sigma-Aldrich, United States of America) and observed under fluorescence confocal microscopy. [45].

Methods

The aim of this review was to gather research that linked virulence factors to VVC and its persistence and recurrence, using two databases (Pubmed and Google Scholar). Therefore, we searched the literature using the keywords: virulence factors, VVC, alternative therapies, biofilm, cell wall hydrophobicity, *C. albicans*. Studies focusing on predisposing factors in women for the occurrence of cVVC and some studies that refer new preventive and alternative therapies were also included, when appropriate.

With these keywords we obtained 1147 titles. The period initially stipulated for this review was 10 years (from January 2010 to August 2020). After assessing the content of the abstracts, 115 publications were selected according with the aims of this review. In addition to this search, and by means of an additional consultation, we also included 31 articles with publication dates previous to this period, assessing their relevance on the theme and objectives and/or because they presented data that we were unable to obtain in more recent articles, obtaining a total of 146 articles.

Vulvovaginal Candidosis

Despite therapeutic advances, VVC continues to be a common concern worldwide, affecting mainly women of childbearing age [46][47][48]. VVC is an important clinical problem among those caused by *Candida* spp. It is the second most prevalent vaginal

infection in women of childbearing age, preceded by bacterial vaginosis. Its importance is based on the fact that it affects a woman's social life because of varied and disturbing symptoms and high incidence [49][50]. The main symptoms are irritation of the vulva, itching, burning, redness in the vulvovaginal area, vulvovaginal oedema rather than dryness, vaginal pain and pain during sexual intercourse frequently associated to a thick, lumpy discharge [50][51].

Several *Candida* spp. may cause VVC, including *C. glabrata*, *C. tropicalis, C. krusei*, *C. lipolytica*, *C. haemulonii* and *C. parapsilosis*, but the most prevalent species is *C. albicans*, according to epidemiologic studies conducted in various places throughout the world [17][48][49][50][52][51][53][54][55]. This preponderance may be related to its polymorphic behavior and its vaginotropism [40]. Additionally, the reason why *C. albicans* is the predominant may well be the consequence of its particular adaptation to the human being, mainly the gut, which constitutes its main reservoir. Women under chronic use of corticosteroid not only have more VVC episodes related to non-*albicans Candida* spp than the overall group but also show more in vitro resistant strains to common antifungal agents especially azoles [53]. For the majority of infections, only one species is present, but in some cases two or more are involved. When infections are caused by more than one species, the more common combination is *C. albicans* and *C. glabrata* [56][57]. Retrospective studies have reported an increased incidence of non-albicans VVC, with relevance to *C. glabrata*. It is important to mention the emergence of 10 new cases of *C. glabrata* vaginal infection per year in a referral clinic in the state of Detroit, USA [57].

Chronic vulvovaginal candidosis

Using an online survey, submitted to 6010 women 18 years or older in five countries in Europe (France, the United Kingdom, Germany, Italy and Spain) and in the United States of America, Foxman *et al* concluded that the overall prevalence of recurrent cases among VVC patients was 9% [32]. Blostein et al sought to ascertain the prevalence of VVC and RVVC in 50-year-old women using data from the National Ambulatory Medical Care Survey (NAMCS) and data from a 2011 multi-country internet panel survey of vulvovaginal candidosis conducted by Ipsos Health (Ipsos.org) and obtained a prevalence rate of RVVC of 23% in this age group. These results point to a global prevalence of 2-23-% for RVVC/cVVC, depending on the age range [58]. In fact, recurrence of VVC is more prevalent during the reproductive years, and associated with host predisposition [59][60].

Notably, several host genetic mutations have already been associated with a potential risk factor for relapsing, such as mutations in the NLRP3 gene, which enable activation of caspase-1 and mutations in the TLR2 gene, which are related to increase or decrease of host organism response / defense [61]. Another possible factor of VVC chronicity was associated with high *Candida* spp resistance rates to treatment [62][63]. VVC and cVVC therapies are based on topical application of azole agents, and their abuse can cause resistance and consequently persistence [59]. To Lockhart *et al,* cVVC can occur in three different ways: with the same strain that over time will show no genetic variation, with the same strain with some genetic variation from one episode to another or with a different strain. These authors sequentially analysed the DNA of strains causing RVVC *C. albicans* infections, and found that in 18 patients the strains isolated from different episodes were the same with little or no genetic variation over time [60]. Mayer *et al* related the pathophysiologic process of candidosis with the expression of virulence factors of strains involved in cVVC and with host predisposition factors, which may contribute to chronicity of the disease, and found to be the ability to form biofilms one of the characteristics of *Candida* spp, more related to virulence [10]. Despite the clinical importance of cVVC, due to its high incidence and symptoms associated with this infection that compromise women's quality of life [64][65], little is known about the characteristics of strains related to the persistence of *Candida* spp. in the vaginal mucosa [26]. The current review summarizes the search by several researchers to uncover molecular and phenotypic data from *Candida* spp., which may be useful in designing new strategies for controlling and treating cVVC, and the current status, and challenges in the treatment and prevention of the occurrence of new episodes of VVC. Specifically, it was intended to summarize results on factors that favour the occurrence of cVVC with respect to molecular epidemiology and antifungal resistance, as well as the expression of several strain virulence factors, such as biofilm formation, extracellular enzyme production, hydrophobicity of *Candida* spp. cell wall, its polymorphism specifically the formation of a germ tube, ability to withstand low pH (pH of the vagina), along with some strategies of *Candida* to cope with host defences.

Resistance to antifungals

It is important to evaluate the yeasts susceptibility to antifungals to determine patterns of antifungal resistance, even if its results do not directly correlate with the clinical expected response.

For this purpose it is convenient to enumerate the two methods that are mainly used: the Clinical and Laboratory Standards Institute (CLSI) protocol and the one from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [66][67], Disk diffusion and E-test have also been used to determine fungal susceptibility to antifungals [68][69]. When comparing liquid medium, E-test and disk diffusion tests used to test sensitivity of *Candida* spp to posaconazole, fluconazole and voriconazole. with disk diffusion as proven to be a good alternative to the liquid-based microdilution method [70][71]. Pina-Vaz and collaborators were one of the first groups to used flow cytometry to assess susceptibility to three antifungals: amphotericin B, fluconazole and 5-fluorocytosine. They concluded that flow cytometry is a good method because it has a strong correlation with the conventional method and reduces the time involved in the laboratory procedure[72].

In the treatment of VVC two main families of antifungal drugs are used presently as first-line options and often also as a preventive strategy: azoles and echinocandins. Two other families of drugs are used as a second line and in combination therapy which are fluoropyrimidines and polyenes [73].

Interestingly, cases caused by non-*albicans Candida* spp do present with milder symptoms but more chronic in evolution. Several authors have evaluated antifungal susceptibility to several species of the genus *Candida* [74][75][76][77]. *Candida albicans* which is still the most common species involved, showed high susceptibility to vacorinazole (100%) and to fluconazole (75%) [78][79][80][81]. Several non-*albicans Candida* spp have been described by several authors as being less susceptible to azoles, such as fluconazole, mainly *Candida glabrata*, *C haemulonii* [73][81][82][83], and *C. krusei* [84].

Mechanisms of antifungal resistance

Antifungal resistance may be species dependent, as is the case for *C. krusei* in relation to fluconazole, or acquired, as has been pointed out for the recent identified *C. auris* [79].

Antifungal resistance mechanisms are related to those involved in the mode of action of each group. Azoles, such as fluconazole, voriconazole, itraconazole, posaconazole, triazole and isavuconazole, disintegrate the fungal cell membrane by inhibiting the activity of the enzyme lanosterol 14-α-demethylase responsible for ergosterol biosynthesis leading to accumulation of a toxic sterol [85]. To resist azoles, the Candida spp. yeast can activate efflux pumps, promoting a decrease in drug concentration within the cell and consequently decreasing its action on the target enzyme (Figure 2).

Bhattacharya *et al* using reverse transcriptase real time PCR and two fluorescence methods studied fluconazole resistant and susceptible *Candida albicans* clinical isolates, and found changes in efflux pump genes in both groups [86]. *Candida* spp., also developed the ability to mutate the ERG11 gene which causes the sensitivity of the enzyme lanosterol 14-α-demethylase to be reduced, preventing its binding to triazole derivatives [87]. Some antifungals such as fluconazole, voriconazole, itraconazole, clotrimazole and amphotericin B promote in vivo resistance when administered for a long time [88]. Branco *et al* described a missense mutation in the ERG gene in fluconazole and voriconazole resistant *C. parapsilosis* strains; the mutation inhibits Erg3 gene function, causing a failure in the conversion of sterol intermediates to ergosterol [85]. Previous and similar work has also been performed with *C. parapsilosis* by Pinto e Silva *et al*, who also associate mutations in the same gene with azole resistance [88].

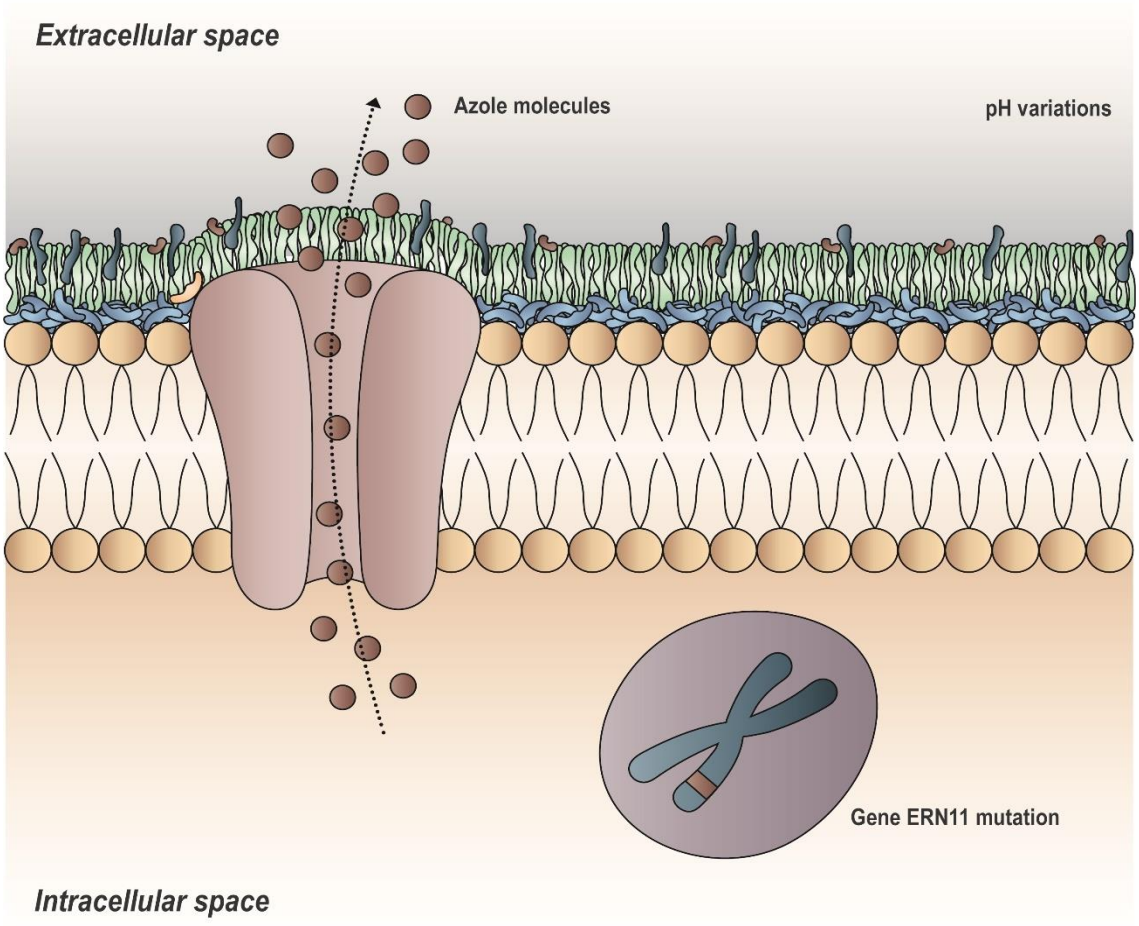

Figure 2 *Candida* spp mechanism involved in resistance to azoles, by activation of the efflux pumps, removing the antifungal from the intracellular space to the extracellular.[45].

variations on pH may also influence the resistance to azole compounds. Danby et al studied in vitro the influence of neutral and acidic pH (7 to 4) on the expression of resistance in *C. albicans* and *C. glabrata* strains. They found that at lower pH *C. glabrata* strains may evidence greater resistance to azole compounds and amphotericin B, but not to caspofungin and flucytosine. For *C. albicans*, no significant changes were observed with pH variation[86]. Results obtained by Gilbert Donders *et al* contradict the above, because the pH remained low during the 1.5-year treatment with fluconazole [88].

Virulence potential

The present review concerns *Candida* spp virulence factors. Even considering the wisdom of integrating both the microorganism and the host under the damage response framework perspective, to limit the approach to the role of the first is important not only because it has practical relevance in current laboratory diagnosis methodology, but also by taking into account that most of the damage produced by the host self-cells, mainly neutrophils, is induced by *Candida* spp virulence factors [89][90]. Candida has acquired evolutionary virulence factor expression over time. These factors proved to be a preponderant condition for the onset of infection and development of candidosis, which contribute significantly to the recurrence of the disease [14]. The concomitant expression of multiple virulence factors within the genus *Candida* spp may favour the occurrence of VVC [62].

In addition and of relevance, El-Houssaini *et al* conducted a study to assess the correlation between virulence factors and antifungal resistance in VVC, and obtained significant positive correlations ($p < 0.05$) between resistance profiles and virulence patterns of *C. albicans* isolates recovered from vulvovaginal samples [91]

Adhesion to the vaginal mucosa and biofilm formation

Calderone,1993 (cited by [92] and [93]) already considered adhesion to be a prerequisite for colonization, and an essential step in the establishment of infection. *C. albicans* adhere to epithelial cells, endothelial cells, extracellular matrix and inert materials implanted in the host's body. Various mechanisms of adhesion appear to be used by *C. albicans´* cells.

Consolaro *et al* point to a possible relationship between the production of proteolytic enzymes and adhesion of cells to the mucosa: a reduction of the production of these enzymes, by action of the inhibitor Pepstantina Sap, reduces the adhesion of fungal cells [94]. During the process of adhesion, the adhesins recognise the host binders, as proteins

and serum components from the extracellular matrix of the tissues of host, or promote the linkage to abiotic surfaces by mean of hydrophobic interactions [93].

The ability to form biofilms by *Candida* spp. is believed to be one of the most important virulence factors in cVVC, as it is correlated with in vivo resistance to antifungals [95][26]. Biofilms are formed by a complex process (Figure 3), which includes cell adhesion to the mucosa (vaginal, in VVC), followed by cell proliferation and hyphal-like cell formation at the top of the biofilm; and then by the accumulation of extracellular polysaccharide matrix material surrounding the biofilm and finally cell dispersion within biofilm [15] (Figure 3).

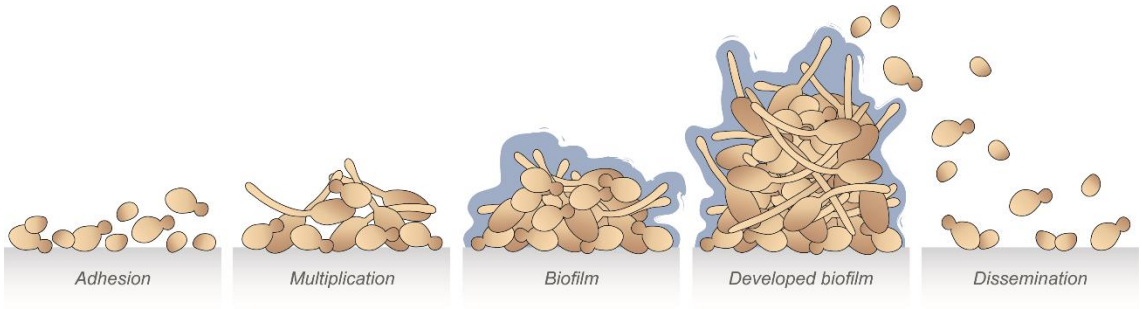

Figure 3. Biofilm formation steps of *Candida albicans*.[45].

Candida spp in biofilms are more resistant to antifungals because they are protected by a complex extracellular matrix that creates a microenvironment [15]. The formation of biofilms also increases the expression of efflux pumps (removing antifungals out of the intracellular medium), as well as metabolic plasticity [15] [96][97], and the ability to resist the host immune response; and so contribute to cVVC [17]. Ballie and Douglas evaluated whether the susceptibility of *C. albicans* biofilms to some antifungal agents (amphotericin B, fluconazole and ketoconazole) is dependent on growth rate. For this purpose adherent *C. albicans* were grown under glucose limiting conditions and in the presence of high concentration of each of the three selected antifungals, and showed that amphotericin B other than inducing an higher rate of reduction of biofilm cells than the other antifungal agents, it was not dependant of the growth rate [98]. Sherry, et al showed that the ability to form heterogeneous biofilms by *Candida* spp strains causing VVC and particularly that of *C. albicans* correlated negatively with the results of treatment [99].

Interestingly, by comparatively evaluating isolates obtained from two clinical groups of VVC and cVVC, Faria-Gonçalves *et al*, found that virulence factors (biofilm formation,

germ tube formation and phospholipase production), were more expressed in the VVC group in relation to to the cVVR group. The hypothesis was raised that these strains by reducing the expression of virulence factors, may more easily escape the immune system and may persist [100].

Production of extracellular enzymes

The production of extracellular enzymes, such as phospholipases, proteinases, hemolytic enzymes and entereases, do facilitate nutrient supply and promote fungal access to host tissues, being so considered as an important virulence factor for infection establishment [15][101][102].

C. albicans invasion of epithelial cells has been reported to be mediated by phospholipases, which cleave cell membrane phospholipids and so induce cell membrane disruption, allowing for host cell invasion [103]. Phospholipase production capacity evaluated in a group of 156 symptomatic women with VVC showed that *C. albicans* strains were found to be the strongest producer of this virulence factor [104].

When comparing the prevalence of VVC and the ability to produce phospholipases and aspartyl proteinase in women with type 2 diabetes and in non-diabetic women, Bassyouni, *et al*, observed a strong correlation between the occurrence of VVC and the increase in aspartyl proteinase production [105]. Ghannom, in a review article, gathered evidence from in vitro and in vivo studies that phospholipase production by *Candida* spp. acts in adhesion, lysis and damage of epithelial and endothelial cells [103]. In contrast, Seifi *et al*, in 2015 found no correlation between the production of extracellular enzymes (phospholipases, proteinases and coagulase) and resistance to fluconazole by strains obtained from patients with VVC and strains isolated from healthy individuals [106]. Most importantly, it was found that the production of phospholipases seems to be equally important for RVVC and sporadic VVC infections. Hence, phospholipase production seems to be an important virulence factor for *Candida* spp. mucosal infection in general [100].

To evaluate the involvement of catalases in the process of drug resistance, virulence and immunogenicity, Miyasaka *et al*, found that the immune response was observed when mouse antiserum immunized with *C. albicans* was incubated with catalase of the same species. This happened for all *Candida* spp. The results of this study demonstrate that

the antisera tested should probably be specific to counteract the action of catalases [107]. The production of proteolytic enzymes was evaluated in several species of *Candida* spp, and it was found that some non-albicans *Candida* species were also able to produce extracellular enzymes, but in a smaller percentage, when compared with *C. albicans* [108].

Morphological switch

Within the genus *Candida*, some species do show morphologic variations during their life cycle (polymorphism). *C. albicans* changes from yeast form to hyphal form when induced by conditions such as pH above 7, presence of N-acetylglucosamine [64], increase in CO2 concentration and temperature equal to 37ºC [15][109]. As hyphae the fungus is considered to be more invasive since at this stage the expression of a set of genes that favour virulence has been activated [15]. Apart from *C. albicans* within the genus *Candida*, only *C. dubliniensis* is capable of forming chlamydospores. The role of chlamydospores in the pathogenesis of *Candida* spp has not yet been clarified, but genes associated with the development of chlamydospores in *C. albicans* are associated with several vital functions of this species including virulence [110].

The hyphal form creates a barrier to phagocytosis, as this voluminous form of *Candida* spp. may not be able to be taken by macrophages [111]. Germ tube formation in *C. albicans* and *C. dubliniensis* is regulated by the NRG1 gene, which is expressed differently in both species and under different conditions [110]. Medeiros *et al*, concluded that germ tube formation is of great importance in the development of VVC, as they found in *Candida* spp. obtained from the vagina and anus of 62 women with VVC a correlation between germination and the presence of symptoms, and that germ tube formation is relevant during infection but not in colonization [112].

The association of the expression of immuno-inflammatory factors of *C. albicans* with the genes that encode virulence, was evidenced from the study conducted by Rossetti *et al*. They showed a correlation between the expression of genes SAP2, SAP5, SAP6, ECE1 and HWP1(from *Candida* strains) with imuno-inflamatory factors, which are related to the formation of hyphae and pseudo-hyphae, possibly due to the activity of multiple inductors of the inflammasome [113].

Cell surface hydrophobicity

Some authors suggested that there is a relationship between adhesion, cell surface hydrophobicity (CSH) and VVC symptoms [114]. Adhesion, CSH and resistance to antifungals such as fluconazole may also be related, and increased CSH may lead to increased adherence and resistance to fluconazole in Candida spp [115]. However, contrary to the foregoing, some authors have not found a significant relationship between CSH and fluconazole resistance [116]. Adhesion of microorganisms to host mucosal surfaces is a vital prerequisite for successful microbial colonization and infection, and so has an important role in the pathogenesis of *Candida* spp. infection. It is well recognized that CSH is linked to adhesion and pathogenicity of *C. albicans* [117], since hydrophobic cells are more adherent than hydrophilic cells to epithelial and endothelial tissues, hence the ease of infection in these tissues [118]. CSH and adhesion in Candida spp. are influenced by microbiome characteristics, considering that temperature of 37°C, neutral pH and low hydrostatic pressure decreases hydrophobicity and adhesion [116].

Other virulence factors

Anti-Als3 immunoglobulin G (IgG), being a ferritin receptor, also assures adequate iron availability during *C. albicans* hyphae growth, by increasing its expression [119]. Interestingly, Als3 sequence turned to be the target for vaccines, that in clinical trials has already shown very interesting clinical results [120]. As stated by Casadevall and colleagues, this is the first vaccine composed of a purified recombinant fungal antigen tested in humans, and also the first targeting a commensal organism inducing significant reduction of symptoms without eradicating the microbe. In addition, it is a therapeutic vaccine, because its target population is women already infected with *C. albicans* [121].

Candidalysin (Ece1-III62–92K) recent identified by Moyes and colleagues is a factor required for *C. albicans* for epithelial invasion but also a relevant mediator of neutrophils chemotaxis [122][123], both mechanisms explaining the symptoms related to acute VVC.

Under a clinical perspective the role of quorum-sensing molecules, particularly farnesol [124] and tyrosol [125], produced by *Candida* spp in virulence is still confusing. They have different roles with apparently opposing effects, suggesting that they act mainly as communication mediators, although some specific pathogenetic actions are clear. For example, farnesol induces masking of one of its cell wall pathogen-associated molecular patterns (PAMPs), β -glucan, in the presence of an acidic pH and lactate, so protecting

the yeast from being phagocyted [126], and activates Neutrophil Extracellular Traps (NETs) production, actions that farnesolic acid and tyrosol do not [125].

Immunopathogenesis of VVC

Other than the evolutionary response of the microorganism to adapt, resist and persist, some characteristics of the woman's body may induce changes in the normal behavior of *Candida* spp., inducing their evolution from commensal state to a causal agent of disease (vulvovaginal, recurrent or not) [8].

Among the most common predisposing factors identified in relation to cVVC are immunosuppressive diseases, type 2 diabetes [127], excessive antibiotic consumption and some hormonal profiles and dysregulations (due to pregnancy, menstrual cycle, chronic stress or other physiological event) [34]. In particular, pregnancy was appointed as risk factor for VVC, because a significant higher prevalence was found in pregnant women when compared to non-pregnant ones. Under these conditions there is greater expression of virulence factors of *Candida* spp. [9][35].

A comparative study between women with HIV + under antiretroviral therapy (HAART) and women with HIV-, focusing on the expression of *Candida* spp. virulence factors, described a lesser expression in most virulence factors in women HIV + [36].

The consumption of sodium glucose co-transporter 2 (SGLT2) inhibiting antidiabetics is also related to the appearance of VVC, as they favour the appearance of fungal infections of the urinary tract due to the mode of action, causing a high concentration of glucose in the urine (renal glycosuria), providing thus a substrate to the development of pathogens and consequently the appearance of infection [37][38].

Other known host predisposing factors are genetic variation and inflammation in the vaginal mucosa [81][128]; it is also important to add that the immune response is deficient in some cases of *Candida* infection [39]. Akimoto *et al*, conducted a study to evaluate the relationship of various factors with the occurrence of cVVC. They evaluated blood glucose, insulin resistance, chronic stress, antioxidant capacity, general immune status, local inflammation, and vaginal microbiota in symptomatic and asymptomatic groups both with positive cultures. They found that chronic stress (decreased cortisol levels in the early morning) and antioxidant reduced capacity may be host predisposing factors to cVVC [81].

Some studies address the genetic predisposition of some women to cVVC, namely regarding the variation in the NLRP3 gene, which allows the activation of caspase-1. Caspase-1 is responsible for cleavage of the inactive precursors of interleukin-1 beta (IL-1β) and interleukin-18 (IL-18) into biologically active cytokines. A mutation in the NLRP3 gene could cause a decrease in the inflammatory response of these cytokines (IL-18 and IL-1β), hence the supposed relationship to chronic inflammation caused by cVVC. The objective of Jaeger et al was to establish a correlation between the VNTR (number of repeated tandem variables) in the NLRP3 gene and susceptibility to cVVC. Their results suggest that IL-1β-mediated inflammation associated with NLRP3 gene expression may be a cause in the pathogenesis of cVVC and envisage the use of this pathway as a potential therapeutic target [17].

Mannose binding lectin (MBL) is an important component of the innate immune system. MBL is capable of binding to mannan fraction of Candida cell wall, activating complement pathway. The variant of the B allele of the MBL2 genetic polymorphism, is a predisposing factor for women with this genetic variation [41].

In another study, Rosentul *et al*, sought to relate single nucleotide polymorphism in some genes, such as TLR1, TLR2, TLR4, CLEC7A and CARD9, to cVVC susceptibility in a group of 119 women with cVVC and a control group of 263 healthy women with no history of vaginal infection by Candida spp. In their results, only the TLR2 gene showed a polymorphism that increases susceptibility to cVVC. These authors supported this hypothesis with results obtained from two individuals with polymorphism, in which there was a decrease in the production of T-cell-derived cytokines IFNy and IL-17, in relation to the other elements of the sample [128].

Moshfeghy *et al.,* searched for depression, anxiety and stress in women with cVVC and found a positive relationship between their presence in women with cVVC in comparison of that in healthy ones indicating their role as predisposing factors for cVVC [129].

A possible link between the genetic makeup of the host with regard to the release of cytokines in response to stimulation of *C. albicans* and the potential risk of cVVC, was demonstrated by Jaeger et al when they conducted a study, using two groups with cVVC and different genetic factors and their respective controls. They analyzed the production of cytokines in peripheral blood cells of the two groups, stimulating them in vitro with *C. albicans*, for subsequent genomic evaluation, concluding that SIGLEC15 is a risk factor for cVVC. Therefore, they concluded that SIGLEC15, a lectin expressed by various immune cells that binds sialic acid–containing structures is a candidate gene involved in cVVC susceptibility [130].

The immune response during cVVC caused by *C. albicans* can be compromised because these isolates induce the migration of neutrophils, induce low production of HOCl by neutrophils, induce oxygen consumption by neutrophils, present high levels of reduced thiol, induce low intracellular oxidant species production by neutrophils and do not induce microbicidal activity of neutrophils [131]. In addition, *C. albicans* do also induce vaginal epithelial cells to release matrix metalloproteinase inducer [132].

As being the concentrations of immunoglobulins too low in the vaginal fluid to be effective, it is believed that the main defensive barrier is that of the innate immune system. In fact, it is logical that being the vagina invaded by foreign microorganisms, like yeast, the phagocytic system should destroy them immediately [133]. Interestingly, yeast can reduce hosts ability to do so, and the protection by vaginal lactic acid can work as a protector by promoting antigen masking [133]. In addition, other than reducing cell wall antigen exposure [134] and inactivating complement proteins, through proteinases production or blockade [135], yeast that are phagocyted by macrophages at the front line may be protected inside them, if not lysed. It has been shown that dysfunctional phagolysosomes are unable to destroy their contained yeast, and so, when they rupture, in a process mediated by candidolysin [136], [137], they liberate living yeast capable of inducing disease. These findings have also been reported in relation to recurrent urinary tract infections, that shows *Candida* spp of an intracellular life phase [138].

Treatment strategies for cVVC

The possibility of preventing the occurrence of cVVC has been taken into account, other than the described long-term prophylactic treatment. After six months fluconazole prophylaxis long-term follow-up shows that three out of five patients do present ongoing disease, only one get the problem solved, and the last one converted to sporadic infection [139]. And once again stress the importance of confirming the cause as only 37.82% (59) of 159 women with curdy white discharge had the diagnosis of VVC established [140]. Tietz studied the possibility of preventing acute episodes in cVVC patients whose isolated causative agent was *C. glabrata*. This strain proved to be resistant to fluconazole and clotrimazole, leading this researcher to assume that a combination therapy of a systemic antifungal with other of vaginal application would result in an excellent strategy. The combination used was posaconazole (as a systemic antifungal) and cyclopyroxolamine

(intravaginal antifungal cream) and after 30 days of treatment with this therapy, 93.3% of the 15 treated patients had mycological and clinical cure, but one year later only 26.7% of patients remained without infection [141].

Another study supporting combined therapy (one local antifungal and other with probiotics) was conducted by S. Palacios *et al.*, in this case associating vaginal therapy with clotrimazole with *Lactobacillus plantarum* I1001 vaginal tablets for a period of six months [142]. *L. plantarum* I1001 It is a probiotic created to combat and prevent cVVC, with very good characteristics for this purpose (good adhesion to vagina epithelial cells, resistance to high concentrations of antimicrobials and antifungals). The results were statistically significant in relation to the reduction in the occurrence of cVVC by 63% [142].

Oral capsules containing *L. plantarum* P17630 were tested in a randomized, double-blind, placebo-controlled study in women with a history of cVVC. There was an increase in the colonization of lactobacilli, as well as an improvement in the symptoms of VVC, in contrast to the control group [143]. Although these studies do not provide conclusive evidence, they suggest the possibility of cVVC prevention strategy with combined therapies [141], [142].

Another strategy under investigation is the administration of vaccines. Bernardis *et al.* in 2015 developed a vaccine (PEV7), consisting of virosomes and secretory aspartyl proteinase 2 (Sap2) as antigen of *C. albicans* and showed therapeutic potential for the treatment of cVVC [144]. Pericolini *et al*, also looked for antibodies (anti-Sap) induced by vaccine or passively administered to contribute to prevent *C. albicans* vaginitis, by injecting two recombinant forms of Sap2 directly in the vaginal cavity of mice. Sap2 with complete enzymatic activity preserved, was not able to cause a significative increase in the number of polymorphonuclear cells of non-treated mice, on the contrary to what occurred in the assays with the protein enzymatically inactive. This highlights the role of Sap2 as promoter of inflammation in *C. albicans* vaginitis [145]. Regarding the immunization for prevention, Bernardis *et al* gathered various works that evidence the possibility of immunization. Immunization with monoclonal antibodies (anti-Sap2) in animal models increased the antibodies against immunoglobulin G (IgG) and immunoglobulin A (IgA) and there was protection against *C. albicans* [55]. In a randomized, double-blind, placebo-controlled clinical trial to evaluate an immunotherapeutic vaccine (NDV-3A) containing a recombinant *C. albicans* adhesive / invasin protein, patients involved in the study showed rapid and robust immune responses, and a longer period without symptoms of the disease. The researchers

demonstrated that NDV-3A showed strong indicators of being a possible prevention method for the appearance of RVVC [120].

Alternative therapies for cVVC

The search for alternative therapies due to resistance to common antifungal agents has been the subject of several investigations [104]. Therapies with plant extracts and essential oils obtained from various parts of a variety of plants is one on going line of research [146], [147]. The effect of *Hippophae rhamnoides* plant extracts, leaves and stem on the reduction of virulence, as well as the effect of plant extracts in synergy with some antifungals, is an example of alternative therapies developed for the treatment of VVC [147]. Sadowska *et al,* evaluated the effect of these compounds using two ATCC *Candida albicans* and *Candida glabrata* strains. The minimum inhibitory concentration (MIC) for both strains of fluconazole and their synergism with plant extracts was also determined. A considerable decrease in the expression of virulence (ability to form biofilms and germ tubes), was found in the assays with extracts obtained in various parts of the plant [147].

The effect of *Thymbra capitata* on *Candida* spp. biofilms was evaluated, and the MIC of strains obtained from women with cVVC was determined. This essential oil with antiseptic properties showed to have relevant antifungal capabilities as it considerably reduces the ability of Candida spp. biofilms alone and in synergy with other antifungals (fluconazole in this case) and a reduction in their metabolic capacity [146].

The MIC of essential oils extracted from *Thymus pulegioides* and main components (thymol, carvacrol, p-cymene and c-terpinene) were determined against *Candida* spp. from women in clinical cases of chronic vulvovaginal candidosis and other fungi [148]. Propidium iodide was used to analyze the effect of the compounds on the fungal cytoplasmic membrane by flow cytometry and showed that the essential oils obtained from this plant hold antifungal capacity [148].

Third-generation antidepressants, namely selective serotonin reuptake inhibitors, such as sertraline and fluoxetine, have also been the subject of *in vitro* studies, showing inhibitory activity on formation of biofilms from *Candida* spp. [149]. Fluoxetine was evaluated alone and in synergy with fluconazole on resistant strains obtained from women with vulvovaginal candidosis and showed not only that fluoxetine is effective but

also that it works in synergy with fluconazole and that shall be considered as antifungal therapy in resistant cases [150].

Hydroalcohoolic extracts of *Platonia insignis* and fractions of it (fraction of dicloromethan and ethyl acetate), were studied by da Silva *et al.* 2020 by determining the MIC of these extract and its fractions, their effect on the virulence factors expression and also their cytotoxity in macrophage cells cultures. They found positive results in low concentration, revealing good therapeutic capacities for treatment of vulvovaginal candidosis [151].

A mixture of lactobacilli (*Lactobacillus acidophilus* GLA-14 (BCCM / LMG Bacteria Collection, LMG S-29159) and *Lactobacillus rhamnosus* HN001) investigated in combination with lactoferrin has shown positive results (safe and effective) when compared to placebo in reducing RVVC symptoms and recurrences [152]. The effectiveness of the vaginal gel containing extracts of *Thymus vulgaris* and *Eugenia caryophyllus* and a capsule for vaginal use containing the probiotic *Lactobacillus fermentum* LF10, the probiotic *Lactobacillus plantarum* LP02 (EPB) was tested in 3 groups of women with bacterial vaginosis, vulvovaginal candidiasis and RVVC (cVVC). The results suggest future evaluations in controlled studies [77].

Resistant strains may be treated in the future with new drugs as is the case for ceragenins [153]. Ibrexafungerp is a substance with superior characteristics compared to normal antifungals, with very favorable clinical results in women with VVC [154].

Antimicrobial peptides have been shown to be safe and effective in the treatment of vulvovaginal candidosis and its recurrence [155].

Conclusions

We consider for the future to not use the word recurrence for the overall chronic cases, leaving room for more specific identification of persistent and relapsing clinical presentations, and the method used to differentiate them, that is still culturing.

It will also be useful not to use the term “colonization” in clinical studies but preferably “carriage”, asymptomatic in women without complains, particularly if not at particular risk of developing VVC.

Given the clinical importance of recurrence of vulvovaginal candidosis, and due to its increased incidence, concern is growing and the cause shall be looked both at the pathogen *Candida* spp., and the host, seeking to find prevention strategies.

Recurrence of vulvovaginal candidosis may be associated with a set of virulence factors expressed by *Candida* spp. that result in antifungal resistance. On the other hand, the expression of virulence factors of microorganisms may inhibit the performance of the host immune system. Many epidemiological data and experimental studies justify the assumption of implication of virulence factors in antifungal resistance and consequent occurrence of cVVC. An example is the ability to form biofilms, which is considered one of the most important virulence factors, due to *Candida* spp. 's ability to resist antifungals. Additionally, the virulence factors of the microorganism alone would not be sufficient for the occurrence of cVVC; indeed, host pre-availability is also important to ensure the success of the infection.

In the area of prevention, studies are also underway that are based on a combination therapy including a systemic and a local antifungal. Although inconclusive, some studies point to new therapies for the treatment of recurrent vulvovaginal candidosis.

It can be concluded that there is still much to be clarified to address the causes of recurrence of vulvovaginal candidosis, regarding the expression of virulence factors (Figure 4) and subsistence mechanisms in the vaginal mucosa of women. On the other hand, the detailed study of these mechanisms may lead to the establishment of new therapeutic and prevention strategies.

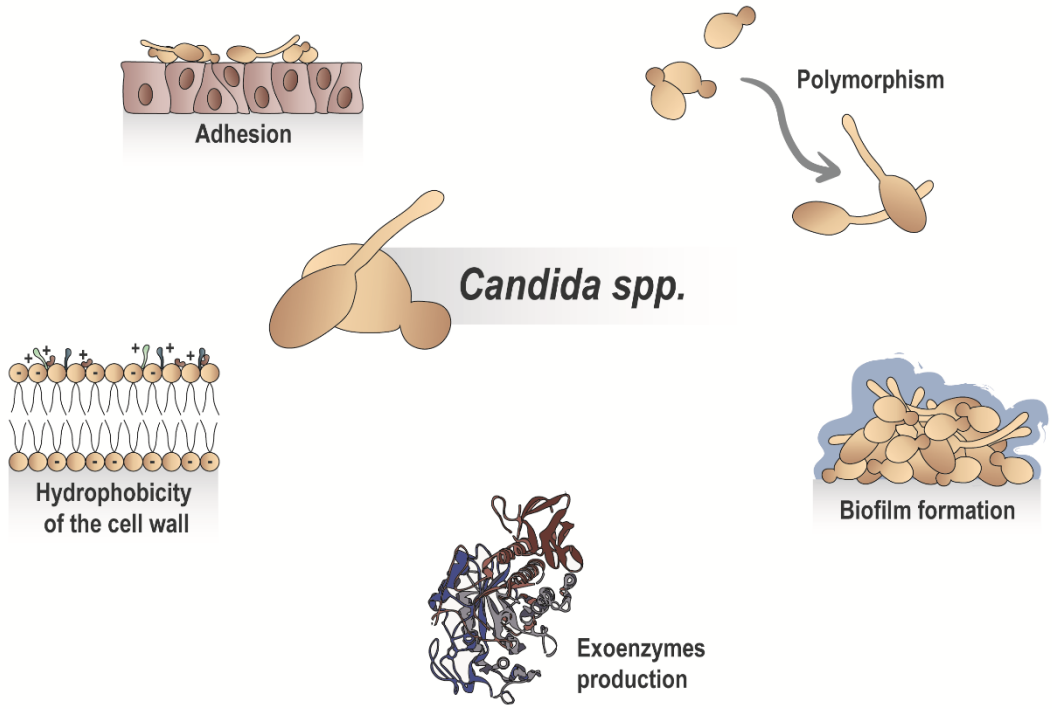

Figure 4. Main virulence factors involved in VVC/RVVC (cVVC). [45]

Chapter 3. Species distribution and antifungal susceptibility profiles of isolates from women with non-recurrent (sporadic) and recurrent (chronic) vulvovaginal candidosis

Rolo J, **Faria-Gonçalves P**, Barata T, Oliveira AS, Gaspar C, Ferreira SS, Palmeira-de-Oliveira R, Martinez-de-Oliveira J, Costa-de-Oliveira S, Palmeira-de-Oliveira A, "Species distribution and antifungal susceptibility profiles of isolates from women with non-recurrent (NR-VVC) and recurrent vulvovaginal candidosis (RVVC). 2021. in https://doi.org/10.1089/mdr.2020.0139

In this chapter, the article referenced here is transcribed with annotations referring to the nomenclature adopted in the meantime.

Species distribution and antifungal susceptibility profiles of isolates from women with non-recurrent (sporadic) and recurrent (chronic) vulvovaginal candidosis

Abstract

Recurrent vulvovaginal candidiasis (RVVC) (chronic vulvovaginal candidosis, cVVC) is caused by Candida spp., a vaginal colonizer. Despite the clinical importance of RVVC (chronic vulvovaginal candidosis, cVVC), little is known regarding the characteristics of the disease in Portugal.

Thirty-six clinical cases were analyzed, comprising 93 yeast vulvovaginal isolates obtained from women attending a gynecologic consultation at a private clinic. Of these, 18 women were diagnosed with RVVC (chronic vulvovaginal candidosis, cVVC) while other 18 women had a sporadic episode of infection (non-recurrent vulvovaginal candidiasis, NR-VVC) (sporadic vulvovaginal candidosis, sVVC). Species identification was performed with CHROMagar chromogenic medium and by analysis of biochemical profiles. In addition, antifungal susceptibility testing for two azole compounds was performed by broth microdilution.

We found that *C. albicans* was isolated from both NR-VVC (sporadic vulvovaginal candidosis) and RVVC (chronic vulvovaginal candidosis, cVVC) cases, being highly predominant; *C. glabrata*, and *C. tropicalis* were also isolated. Resistance to at least one antifungal was detected in up to 65% of the isolates and resistance to both antifungals reached a frequency of 25%. Moreover, azole-resistant isolates were distributed among all species identified.

We conclude that in the studied group of patients, *C. albicans* is in fact the major player both in NR-VVC (sporadic vulvovaginal candidosis, sVVC) and in RVVC (chronic vulvovaginal candidosis, cVVC), being *C. glabrata* more frequently associated with recurrence ($p<0.05$). In addition, we found a high proportion of azole-resistant strains.

Keywords: Antifungal resistance, Vulvovaginal candidiasis, Persistence, Women's health, *Candida albicans*

Introduction

Candida albicans is an opportunistic pathogen, although considered to be a commensal of mucosa such as those of the mouth and the vagina [156][9]. In the vaginal environment, it may be present in 10-30% of asymptomatic healthy women [9][10][157][158], but it can also cause vulvovaginal candidiasis (VVC), which is the second most common vaginal infection[156][9]. Clinically, VVC is characterized by an overgrowth of yeast cells, involving changes in behavior leading to invasion of the vaginal mucosa [11]. The symptoms are discomfort, itching and pain. Around 5 to 10% of VVC infections re-occur (recurrent vulvovaginal candidiasis, RVVC) (chronic vulvovaginal candidosis, cVVC), causing high morbidity with a significant decrease in the quality of life of women [156]. RVVC is defined as the occurrence of at least four episodes of VVC per year [159]. Several risk factors have been implied in RVVC, such as sexual activity, pregnancy, poorly controlled diabetes, prolonged use of antibiotics and oral contraceptive use [160],[161]. Nonetheless, comprehensive studies focusing on the epidemiological differences between RVVC (cVVC) and non-recurrrent VVC (NR-VVC (sporadic vulvovaginal candidosis, sVVC)) are scarce.

In most cases, VVC is caused by *C. albicans*, but other *Candida* spp. may be the etiological agent, such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*, in decreasing order of frequency. Recently, an increasing trend in the number of non-*C. albicans* VVC infections has been reported, and the frequency of these species in RVVC (chronic vulvovaginal candidosis, cVVC) infections is even higher [160]. Such an increase is worrisome, since it has been associated with treatment failure in complicated cases of VVC and RVVC (chronic vulvovaginal candidosis, cVVC). Non- *C. albicans* species show in general lower susceptibility to these drugs [162][48]. For this reason, understanding the characteristics of these infections, regarding the frequency of the different species as well as the overall rates of resistance to azole compounds is crucial to assure the efficacy of prescribed treatment. A Portuguese study that analyzed blood samples from patients with fungaemia reported that *C. albicans* was the most prevalent *Candida* species, followed by *C. parapsilosis* and *C. glabrata* [163]. A relatively low antifungal resistance rate was reported (84 to 98 % overall susceptibility rates for azoles) [163]. However, studies focusing on vulvovaginal candidiasis in Portugal remain unreported.

In this study, we aimed to better understanding NR-VVC (sVVC) and RVVC (cVVC) in a sample of Portuguese women, focusing on species distribution and azole resistance rates.

Materials and Methods

Biological samples.

Vaginal swabs were collected between 2008 and 2014 and inoculated in Sabouraud Dextrose Agar (VWR, Avantor, Pennsylvania, United States of America) as a standard procedure to confirm the diagnosis of vulvovaginal candidiasis, of women in a private clinic located in Northwest Portugal (Póvoa de Varzim). The cultures were kept at –80ºC in Brain Heart Infusion broth (BHI, VWR, Avantor, Pennsylvania, United States of America) supplemented with 20% glycerol (VWR, Avantor, Pennsylvania, United States of America). Cultures were kept frozen for further study, after anonymizing codification. Patients' clinical data were obtained from the practitioner (Table 1). For this work, 93 cultures were analyzed, one from each of 18 sporadic cases, non-recurrent vulvovaginal candidosis isolates (NR-VVC (sVVC), collected from 18 different women, one isolate each); and 75 from different episodes of recurrency, recurrent vulvovaginal candidiasis isolates (RVVC (cVVC), collected from 18 different women, in which 2-12 isolates per women were collected, Table 2). All women were symptomatic at time of sampling. The classification of the clinical cases as sporadic (NR-VVC) (cVVC) or chronic/recurrent (RVVC)(cVVC) was performed by the attending practitioner, following clinical and microbiological evidence at time of sampling as previously suggested [164]. The patients were not recruited or particularly selected for inclusion in this study: the samples constitute a convenience sample, since these were collected for diagnostic purposes only. The study has been approved by the Ethics committee of Universidade da Beira Interior (CE-UBI-Pj-2018-022). This sampling procedure has been described before [100].

Table 1. Epidemiological data of non-recurrent candidiasis (NR-VVC) clinical cases enrolled in the study from whom *Candida* spp. were isolated.

Non-recurrent vulvovaginal candidiasis (NR-VVC) (sVVC) **cases**					
Case code	**Age (years)**	**Year of isolation**	**Treatment prescribed**	**Risk factors**	**No of isolates**
A	45	2010	Itraconazole	None at sampling	1
B	35	2010	Fluconazole	None at sampling	1
C	26	2013	Fluconazole	None at sampling	1
D	39	2012	Benzydamine hydrochloride	Intrauterine device	1
E	29	2010	Not treated	Hepatic lesion	1
F	44	2012	Itraconazole	Antidepressants therapy	1
G	29	2010	Itraconazole	None at sampling	1
H	32	2008	Fluconazole	None at sampling	1
I	24	2012	Fluconazole	None at sampling	1
J	47	2013	Fluconazole	Intrauterine device	1
K	62	2014	Clotrimazole	None at sampling	1
L	60	2013	Fluconazole	Type II diabetes	1
M	48	2010	Fluconazole	Antibiotic therapy	1
N	48	2010	Fluconazole	Antibiotic therapy	1
O	25	2013	Fluconazole	Antidepressants therapy	1
P	40	2008	Fluconazole	Type II diabetes	1
Q	26	2010	Fluconazole	None at sampling	1
R	36	2013	Fluconazole	Antibiotic therapy	1
				Total no. of isolates	**18**

*Age at first sampling; **The same patient suffered from two series of episodes of RVVC (chronic vulvovaginal candidosis, cVVC); ***Other: gentian violet; essential oils; boric acid; sodium bicarbonate

Table 2. Epidemiological data of recurrent vulvovaginal candidiasis (RVVC) (chronic vulvovaginal candidosis, cVVC) clinical cases enrolled in the study from whom *Candida* spp. were isolated.

Recurrent vulvovaginal candidiasis (RVVC) (chronic vulvovaginal candidosis, cVVC) **cases**					
Case code	**Age* (years)**	**Year of isolation**	**Treatment prescribed*** **	**Risk factors**	**No of isolates**
A1**	38	2008-2009; 2012	Fluconazole-Itraconazole-Probiotics	Antibiotic therapy	5
B1	50	2009-2010	Fluconazole	None at sampling	2
C1	34	2010	Fluconazole-Probiotics	None at sampling	3
D1	64	2013-2014	Clotrimazole-Fluconazol	None at sampling	2
E1	43	2010	Itraconazole	Antidepressants therapy	2
F1	37	2010	Fluconazole/Itraconazole-Other	None at sampling	4
G1	46	2013-2014	Fluconazole-Itraconazole-Other	Antidepressants therapy	12
H1	51	2012-2013	Fluconazole/Itraconazole-Other	Antibiotic therapy	4
I1	56	2010	Fluconazole/Itraconazole-Other	Antidepressants therapy	4
J1	49	2010	Fluconazole-Probiotics-Other	None at sampling	8
K1	33	2010	Fluconazole-Other	None at sampling	7
L1	45	2013	Fluconazole	None at sampling	2
M1	31	2010	Itraconazole	None at sampling	2
N1	46	2010	Fluconazole	None at sampling	2
O1	70	2013	Not treated	None at sampling	2
P1	44	2009-2010	Fluconazole-Other	Antidepressants therapy	10
Q1	27	2010	Itraconazole	None at sampling	2
R1	32	2009	Benzydamine hydrochloride	None at sampling	2
				Total no. of isolates	**75**

*Age at first sampling; **The same patient suffered from two series of episodes of RVVC (cVVC); ***Other: gentian violet; essential oils; boric acid; sodium bicarbonate

Species identification.

Phenotypic identification of *Candida* species was performed with CHROMagar (Biomérieux, Paris, France), after sub-culturing the clinical isolate twice in Sabouraud Dextrose Agar (VWR, Avantor, Pennsylvania, United States of America). When the phenotypic result was dubious or when a non-*C. albicans* was apparently present, the result was confirmed with automated analysis of 46 biochemical reactions with Vitek-2 (Biomerieux, Marcy-l'Étoile, France).

Determination of antifungal susceptibility profiles.

Susceptibility to a triazole compound, fluconazole (Sigma-Aldrich, Germany) and an imidazole compound, clotrimazole (Sigma-Aldrich, Germany) was assessed for all *Candida* isolates, by the microdilution assay, according with the Clinical and Laboratory Standards Institute (CLSI) protocol CLSI M27-A3—Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts standards [66]. Absorbances were read at 600 nm in a microplate reader (Anthos 2020 microplate reader, Bio-Rad, California, United States of America). According with CLSI M27-A3, the MIC was defined as the concentration of fluconazole, that inhibited 50% of yeast growth, in comparison with a growth control that consisted of culture media only (MIC50). *C. albicans* ATCC90028 was used in all assays to assess quality control. Yeast isolates were considered susceptible to fluconazole when the minimum inhibitory concentration (MIC) was ≤ 2 μg/ml (*C. albicans*, *C. tropicalis*) and susceptible dose-dependent when MIC ≤ 32 μg/ml (*C. glabrata*). Since no guidelines are available to assess susceptibility to clotrimazole in the CLSI guideline 16 or EUCAST (http://www.eucast.org/clinical_breakpoints/), it was defined by a MIC value ≤ 1 μg/ml according with previous studies focusing on vulvovaginal isolates [162].

Determination of the ability to form biofilm.

The ability to form biofilms in vitro was evaluated for all isolates as previously described [100][165][166]. *Candida albicans* ATCC10231 was included as a positive control in each assay, which has been previously described as an adherent yeast isolate [167]. As previously described, isolates were classified as “moderately adherent” if the Abs595nm of their biofilm was in the range of the one obtained for ATCC10231 (0.62-0.82); and “strongly adherent” if the Abs595nm of their biofilm was superior to 0.82. If the strains could form biofilm that corresponded to at least 50% of the ability of ATCC10231 to form biofilm (Abs595nm 0.3-0.62), then they were classified as “weakly adherent”. Finally, “non-adherent” isolates were considered when Abs595nm was lower than 0.3. Two

independent assays were performed for each isolate; the result was validated only when the standard deviation between the Abs595nm obtained was less than 0.15 or when both assays gave a consistent result regarding the classifiers described above.

Statistical analysis.

Differences in proportions observed between the two clinical groups (NR-VVC) (sVVC) and RVVC (cVVC)) were analysed with the two-sided Fisher's exact test, with 95% confidence interval, using Graphpad Prism 7 (San Diego, United States of America). The impact of the differences obtained for the frequencies found between the two clinical groups were assessed using power calculation tools (ClinCalc LLC, United States of America).

Results

Yeast species distribution among vulvovaginal candidosis (sporadic vulvovaginal candidosis, sVVC) cases

In 70 (75%) of the 93 samples, the yeast isolates recovered were found to be *Candida albicans*. The remaining 23 (25%) isolates belonged to non-*C. albicans* species, of which *C. glabrata* was the most prevalent one. The species distribution was the following: *C. albicans* (70/93, 75%), *C. glabrata* (19/93, 20%) and *C. tropicalis* (4/93,5%).

Recurrent clinical cases were caused in their great majority by the same *Candida* species (13 out of 18 clinical cases). In the remaining five clinical cases (G1; H1; J1; K1; P1), in one of the episodes, a different *Candida* species was recovered. Regarding the association between species distribution and recurrence, we found that *C. albicans* was the most prevalent *Candida* species among both NR-VVC (sVVC) cases (17/18, 94%) and RVVC (cVVC) cases (53/75, 71%)(Figure 1). The difference in frequency observed was statistically significant ($p<0.05$). In RVVC (cVVC) cases, *C. glabrata* was the second most frequent species detected (19/75, 25%), which was exclusively associated with recurrence (chronicity) ($p<0.05$). *C. tropicalis* was isolated in both clinical groups, with similar frequency (NR-VVC (sVVC):6%; RVVC (cVVC): 4%).

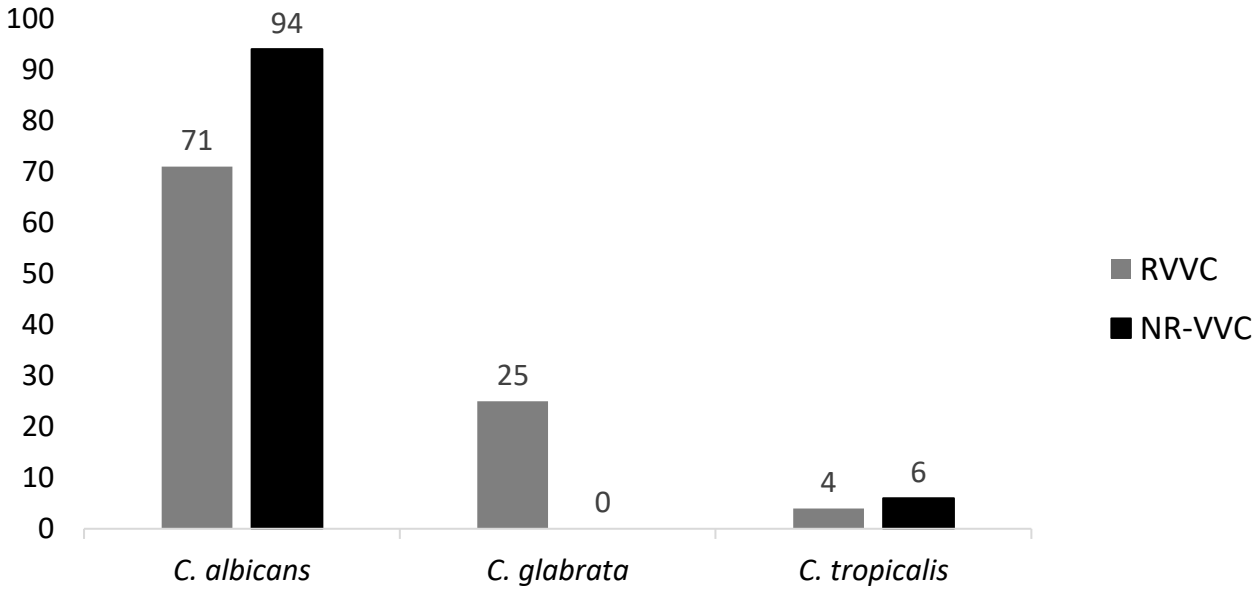

Figure 1. Frequency (%) of *Candida* species among non-recurrent (NR-VVC) (sVVC) and recurrent cases (RVVC) (cVVC). The values above each bar indicate specific value of frequency (%) for each species.[168].

Distribution of antifungal resistance among vaginal *Candida* spp. isolates obtained from sporadic and recurrent (chronic) cases.

We found 32% (30/93) of sensitive isolates to the two antifungals tested. Overall, in this study we found 65% (60/93) resistant isolates to at least one of the antifungals tested and 25% (23/93) were resistant to the two antifungals. If we consider the isolation of only one azole-resistant isolate per recurrent case, and one azole-resistant isolate per sporadic case (chosen randomly) then the resistance of fluconazole reached 61% for *C. albicans* (19 out of 36 cases) and 3% for *C. glabrata* (1 out of 36 cases). The resistance to clotrimazole reached 45% for *C. albicans* (14 out of 36 cases), 8% for *C. glabrata* (3 out of 36 cases) and 5% for *C. tropicalis* (2 out of 36 cases).

Table 3. Distribution of *Candida* spp. isolates recovered from non-recurrent vulvovaginal cases (NR-VVC) (sVVC) and recurrent vulvovaginal (RVVC) (cVVC) candidosis cases included in the study. FLU: Fluconazole; CLO: Clotrimazole; R: resistant; SDD: susceptible dose-dependent; S: susceptible

Species (%)	**Recurrent vulvovaginal candidiasis (RVVC)** (chronic vulvovaginal candidosis, cVVC) **isolates (75)**					
	FLU (%)	**MIC µg/mL (%)**	**Treatment (no isolates, %)**	**CLO (%)**	**MIC µg/mL (%)**	**Treatment (no isolates, %)**
Candida albicans (53/75, 71%)	R (31/53, 58%)	>512 (3/31, 10%) 256 (2/31, 6%) 64 (7/31, 22%) 32 (3/31, 10%) 16 (13/31, 42%) 8 (3/31, 10%)	Itraconazole (9/31, 29%) Fluconazole (8/31, 26%) Probiotics (2/31, 6%) Clotrimazole (1/31, 3%) Fluconazole/Itraconazole (1/31, 3%) Other (10/31, 32%)	R (23/53, 43%)	8 (3/23, 13%) 4 (11/23, 48%) 2 (9/23, 39%)	Itraconazole (5/23, 22%) Fluconazole (5/23, 22%) Fluconazole/Itraconazole (2/23, 8%) Other (11/23, 48%)
	SDD (2/53, 4%)	4 (2/2, 100%)	Fluconazole (1/2, 50%) Benzydamine hydrochloride (1/2, 50%)	-	-	-
	S (20/53, 38%)	2 (6/20, 30%) <2 (14/20, 70%)	Fluconazole (4/20, 20%) Benzydamine hydrochloride (1/20, 5%) Fluconazole/Itraconazole (1/20, 5%) Probiotics (1/20, 5%) Other (8/20, 40%) Not treated (5/20, 25%)	S (30/53, 57%)	1 (14/30, 47%) 0.5 (9/30, 30%) <0.5 (7/30, 23%)	Fluconazole (8/30, 27%) Itraconazole (4/30, 13%) Probiotics (3/30, 10%) Benzydamine hydrochloride (2/30, 7%) Clotrimazole (1/30, 3%) Other (7/30, 23%) Not treated (5/30, 17%)
Candida glabrata (19/75, 25%)	R (1/19, 5%)	64 (1/1, 100%)	Itraconazole (1/1, 100%)	R (9/19, 47%)	16 (1/9, 11%) 8 (1/9, 11%) 4 (5/9, 56%) 2 (2/9, 22%)	Itraconazole (4/9, 45%) Fluconazole (2/9, 22%) Probiotics (1/9, 11%) Other (2/9, 22%)
	SDD (18/19, 95%)	32 (1/18, 5%) 16 (3/18, 17%) 8 (5/18, 28%) 4 (5/18, 28%) <2 (4/18, 22%)	Fluconazole (5/18, 28%) Itraconazole (5/18, 28%) Probiotics (1/18, 5%) Other (6/18, 34%) Not treated (1/18, 5%)	S (10/19, 53%)	1 (2/10, 20%) 0.5 (7/10, 70%) <0.5 (1/10, 10%)	Fluconazole (3/10, 30%) Itraconazole (2/10, 20%) Other (4/10, 40%) Not treated (1/10, 10%)
Candida tropicalis (3/75, 4%)	R (1/3, 33%)	16 (1/1, 100%)	Other (1/1, 100%)	R (2/3, 67%)	4 (1/2, 50%) 2 (1/2, 50%)	Other (1/2, 50%) Not treated (1/2, 50%)
	S (2/3, 67%)	<2 (2/2, 100%)	Other (1/2, 50%) Not treated (1/2, 50%)	S (1/3, 33%)	<0.5 (1/1, 100%)	Other (1/1, 100%)
Non-recurrent vulvovaginal candidiasis (NR-VVC) (sporadic vulvovaginal candidosis, sVVC) **isolates (18)**						

Species (%)	**FLU (%)**	**MIC µg/mL (%)**	**Treatment (no isolates, %)**	**CLO (%)**	**MIC µg/mL (%)**	**Treatment (no isolates, %)**
Candida albicans (17/18, 94%)	R (9/17, 53%)	512 (1/9, 11%) 64 (4/9, 44%) 16 (3/9, 34%) 8 (1/9, 11%)	Fluconazole (5/9, 56%) Itraconazole (2/9, 22%) Clotrimazole (1/9, 11%) Benzydamine hydrochloride (1/9, 11%)	R (7/17, 42%)	8 (1/7, 14%) 4 (1/7, 14%) 2 (5/7, 72%)	Fluconazole (5/7, 72%) Benzydamine hydrochloride (1/7, 14%) Other (1/7, 14%)
	SDD (2/17, 12%)	4 (2/2, 100%)	Fluconazole (2/2, 100%)			
	S (6/17, 35%)	2 (2/6, 33%) <2 (4/6, 67%)	Fluconazole (4/6, 67%) Itraconazole (1/6, 16.5%) Other (1/6, 16.5%)	S (10/17, 58%)	1 (4/10, 40%) 0.5 (3/10, 30%) <0.5 (3/10, 30%)	Fluconazole (6/10, 60%) Itraconazole (3/10, 30%) Clotrimazole (1/10, 10%)
Candida tropicalis (1/18, 6%)	R (1/1, 100%)	64 (1/1, 100%)	Fluconazole (1/1, 100%)	R (1/1, 100%)	4 (1/1, 100%)	Fluconazole (1/1, 100%)

Other: gentian violet; essential oils; boric acid; sodium bicarbonate

Regarding the correlation between recurrence of the infection and rates of resistance, we found that 12 out of the 18 (67%) *Candida* spp isolates that were recovered from NR-VVC (sVVC) cases were resistant to at least one of the antifungals tested and six out of the 18 (33%) were resistant to both antifungals. The great majority of isolates recovered from RVVC (cVVC) cases, 64% (48/75) were resistant at least to one of the antifungals and 23% (17/75) were resistant to both antifungals. When we analyzed the frequencies described above in each clinical group and compared them, we found that there was no significant association between rates of resistance and recurrence of the infection ($p>0.05$).

Resistance to fluconazole was detected in ten out of 18 sporadic vulvovaginal cases (55%), while resistance to clotrimazole was observed in eight out of the 18 (44%) In the RVVC (cVVC) cases, resistance to fluconazole was detected in 12 out of 18 cases (67%), and resistance to clotrimazole was found in 12 out of 18 cases (67%). The differences observed between the two clinical groups were found to be non-significant ($p>0.05$). The distribution of the frequencies among the two clinical groups was analyzed using a power calculation tool that estimated that the sample size should be equal between the two clinical groups; the inclusion of the exact number of clinical cases (18) in each clinical group strengthens this analysis.

The rates of resistance to azole compounds found in this study were compared with the ability of the strains to form biofilms (Table 4). The great majority of the NR-VVC (sVVC) (64%; 48/75) and RVVC (cVVC) (83%; 15/18) strains had the ability to form in-vitro biofilms (adherent/strongly adherent phenotypes). We found that most of the strains that were resistant to either fluconazole or clotrimazole had a higher ability to form biofilms, among the two clinical groups (NR-VVC (sVVC): 62%; RVVC (cVVC): 74%). The difference between the two clinical groups was not statistically significant ($p>0.05$). However, the difference between the distribution of the isolates able to form biofilms (strongly adherent phenotype) between azole-resistant and azole-susceptible isolates inside each clinical group was statistically significant ($p<0.05$, Table 4).

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Table 4. In-vitro biofilm phenotypes of *Candida* spp. isolates recovered from non-recurrent vulvovaginal cases (NR-VVC) (sporadic vulvovaginal candidosis, sVVC) and recurrent vulvovaginal (RVVC) (chronic vulvovaginal candidosis, cVVC) candidosis cases included in the study. Comparison with resistance to either fluconazole or clotrimazole is showed. R: resistant; S: susceptible

Biofilm phenotype	RVVC (cVVC) isolates		NR-VVC (sVVC) isolates	
	Azole S	Azole R	Azole S	Azole R
Not adherent	23 % (2/9)	77 % (7/9)	-	-
Weakly adherent	56 % (10/18)	44 % (8/18)	0 % (0/3)	100 % (3/3)
Adherent	67 % (6/9)	33 % (3/9)	100 % (2/2)	0 % (0/2)
Strongly adherent	26 % (10/39)	74 % (29/39)	38 % (5/13)	62% (8/13)

Susceptibility of vaginal Candida spp. isolates to most prescribed antifungals and relation with course of treatment.

In general, we found that 46% (43/93) of vaginal *Candida* spp. isolates were resistant to fluconazole. The distribution of the isolates by species was the following: 43% (40/93) resistant *C. albicans* isolates, 1% (1/93) resistant *C. glabrata* and 2% (2/93) resistant *C. tropicalis*. Resistance to fluconazole ranged from 8 to 512 μg/mL. Regarding distribution of fluconazole-resistant isolates per clinical groups, we found that the proportion of fluconazole-resistant *C. albicans* (50%; fluconazole-resistant RVVC (cVVC) *C. albicans* 41%, Figure 2) and fluconazole-resistant *C. tropicalis* was higher among NR-VVC (sVVC) isolates (5%; fluconazole-resistant RVVC (cVVC) *C. tropicalis* 1%, Figure 2). The differences observed between the two groups were non-statistically significant ($p>0.05$).

With respect to clotrimazole resistance, overall we found that 42 out of 93 *Candida* spp. isolates (45%) were resistant to this antifungal agent. Of these 42, 32% (30/42) were resistant *C. albicans*, 10% (9/93) resistant *C. glabrata*, and 3% (3/93) resistant *C. tropicalis*. Resistance to clotrimazole ranged from 2 to 16 μg/mL. Regarding distribution of clotrimazole-resistant isolates per clinical groups, and similarly with the observations regarding fluconazole resistance, the proportion of clotrimazole-resistant *C. albicans* (39%; clotrimazole-resistant RVVC *C. albicans* 31%, Figure 2) and clotrimazole-resistant *C. tropicalis* was higher among NR-VVC (sVVC) isolates (5%; clotrimazole-resistant RVVC (cVVC) *C. tropicalis* 3%, Figure 2). The differences observed between the two groups were, as reported for fluconazole resistance, non-statistically significant ($p>0.05$).

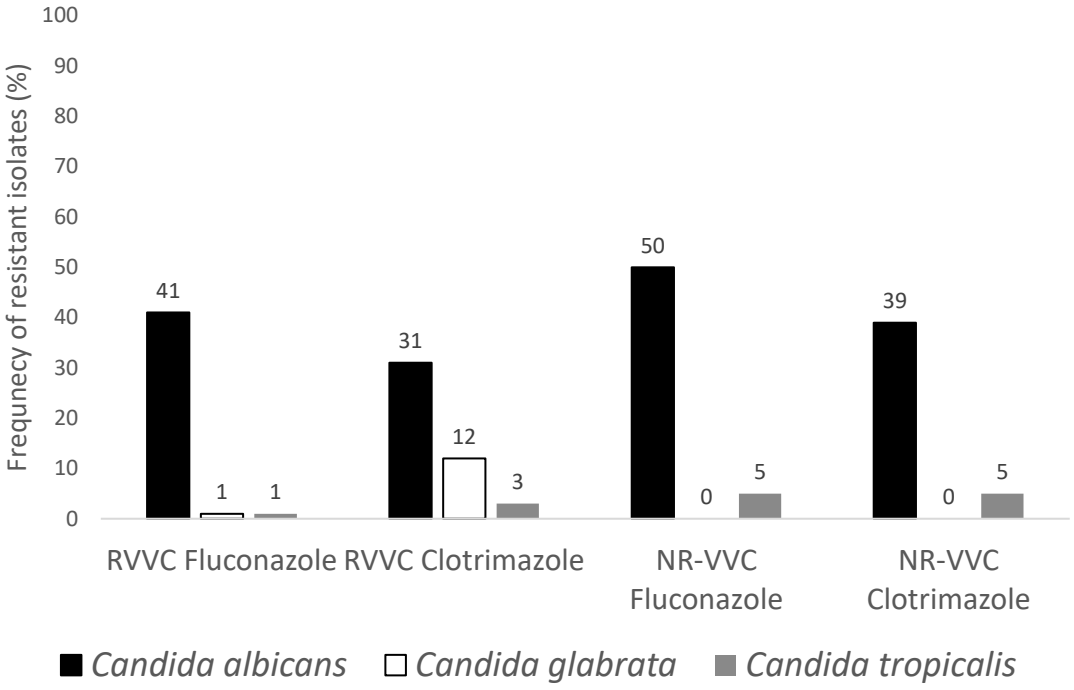

Figure 2. Distribution of antifungal-resistant isolates recovered from recurrent vulvovaginal candidiasis cases (RVVC) (cVVC) and non-recurrent vulvovaginal cases (NR-VVC) (sVVC). The values above each bar indicate specific value of frequency (%) for each species. Frequencies (%) take into account the total number of RVVC (cVVC) (75) or NR-VVC (sVVC) (18) *Candida* isolates. .[168].

The effect of previous treatment and possible risk factors for VVC are presented in Table 1. For 18 of these cases, the infection did not re-occur in a period of one year and were so considered to be sporadic vulvovaginal cases (NR-VVC) (sVVC). Regarding these cases, in which one *Candida* spp isolate was recovered per case, twelve were treated with fluconazole, three were treated with itraconazole, one with clotrimazole and one with benzydamine hydrochloride. For the remaining case, no course of treatment was applied. Amongst NR-VVC (sVVC) cases, the median age of the patients was 38 ± 12 years. The ones treated with fluconazole yielded isolates resistant to fluconazole (6/12, 50%); and/or were resistant to clotrimazole (6/12, 50%) (Table 2). The isolate obtained from the single case treated with clotrimazole was susceptible to this antifungal; however, it was resistant to fluconazole. Two isolates out of the three isolates treated to itraconazole were resistant to fluconazole (2/3, 67%); all three isolates were susceptible to clotrimazole. The single case treated with benzydamine hydrochloride was resistant to

fluconazole and clotrimazole; and the single case that received no treatment was susceptible to fluconazole but resistant to clotrimazole.

The RVVC (cVVC) cases corresponded to 18 clinical cases, of which 75 *Candida* spp. isolates were recovered. Of these clinical episodes, sequential isolates were obtained ranging from 2 to 12 (Table 1). Ten of these cases were treated with fluconazole at least once, seven with alternative treatments (washes with gentian violet, essential oils, boric acid or sodium bicarbonate), five with itraconazole, three with a combination of flucanozole with itraconazole, three with probiotics, one with clotrimazole and one with benzydamine hydrochloride. No course of treatment was applied regarding one clinical case (two episodes). The median age of the patients suffering recurrence was 45 ± 11 years. We found that the isolates recovered from the patients with recurrence that were treated with fluconazole were in their great majority susceptible to this drug (10/18; 56%); resistance to fluconazole was observed in eight isolates (8/18, 44%). Only one was treated with clotrimazole, and the isolates recovered were in fact susceptible to this drug. The 15 isolates recovered from patients that were treated with itraconazole were resistant to fluconazole (10/15, 67%) and/or clotrimazole (9/15, 60%). Regarding the 39 isolates recovered from patients that received alternative treatments, 33% (13/39) were resistant to fluconazole and/or clotrimazole (16/39, 41%).

There was no statistically significant association in the proportions observed regarding the rates of resistance and course of treatment, in each clinical group and between these ($p>0.05$).

Discussion

Candida albicans can be asymptomatically carried (10-30%) in the vagina of healthy individuals [9][169]; but it can also turn into a pathogenic state and cause vulvovaginal candidosis (VVC). This infection can re-occur, and when it does at least four times in a 12 month period [6], it is considered to be recurrent (RVVC) (chronic vulvovaginal candidosis, cVVC). Clinical cases in which *Candida* spp is isolated only once in a period 12 months are considered sporadic or non-recurrent (NR-VVC) sVVC).

The most prevalent Candida species obtained in the present study was *C. albicans;* nevertheless, an increasing trend of non-*C. albicans* species was observed in both clinical groups, which is in agreement with the literature concerning vulvovaginal colonization and infection [162][170][171]. In the case of the non-*C. albicans*, in this investigation *C. glabrata* was predominant, followed by *C. tropicalis*, which agrees with previously reported data by other authors in studies performed worldwide 23. *C. glabrata* is the second most common isolated *Candida* species in vulvovaginal specimens [171][172]. *C. krusei* can also be frequently isolates from the vaginal cavity, but in our study, we did not find this species. Its frequency can vary between 3-15% on vaginal samples depending on geographic location and methodology used for its identification [173][20]. The same can be said for *C. tropicalis* [173], that we detected in our study, but in a small proportion (5%).

There are not to our knowledge many studies comparing species distribution between NR-VVC (sVVC) and RVVC (cVVC) clinical cases. In our study we found that *C. albicans* was more frequently associated with NR-VVC (cVVC) cases (sporadic, $p<0.05$) and *C. glabrata* was more prevalent among RVVC cases (chronic, $p<0.05$). Persistence in the vaginal cavity, therefore, seems to be more associated with infection by *C. glabrata*. Previous studies have also reported that non-*C. albicans* species, such as *C. glabrata*, are more frequently isolated in RVVC (cVVC) clinical cases [48]. One explanation could be due to the finding that *C. glabrata* has innate resistance to azoles, being therefore able to persist despite treatment [172]. *C. glabrata* has also an enhanced ability to form biofilms [174] and can be highly resistant to fluconazole under certain environmental conditions [175]. Other hypothesis could be due to mixed colonization of the vaginal mucosa, which could possibly occur yielding concomitantly present *Candida* species. After the first round of treatment, the resistant population would rise, probably composed by intrinsically resistant or less susceptible species such as *C. krusei* or *C. glabrata* [160]. Mixed vaginal infections have been described and previously studied, regarding mixed isolation of *C. albicans* and *Gardnerella vaginallis* [176], one of the

pathogens associated with bacterial vaginosis, the most common vaginal infection [177]. Studies focusing on the vaginal mycobiome are scarce; but co-carriage of multiple Candida species in the vaginal cavity have been already reported. However, these studies are hampered by the methodology used to generally diagnose VVC, that is based on symptomatology and phenotypic testing, leading to misidentification of the pathogenic Candida species [173].

In fact, the increase of the frequency of vulvovaginal candidiasis cases has been accompanied with a steady growth of persistence of azole-resistant isolates. In our study, resistance to one antifungal reached 65% and resistance to both antifungals reached a frequency of 25%. In addition, azole-resistant isolates were found among all Candida species recovered. A frequency of 43% fluconazole resistant - *C. albicans* was recovered, which is worrisome comparing with the literature that reports low rates of fluconazole resistance always with values close to 20% [79][178][179]. However, recent studies have highlighted the decreased susceptibility to azoles among vulvovaginal *C. albicans* isolates [180][181][182][91], following the trend that we describe here. This data may be indicative that prevention and care measures in the administration of antimycotics are not being considered or adequately taken in consideration. However, this should be a matter of caution and of a wider and deeper investigation.

On the other hand, and considering especially recurrent isolates, one must consider that some isolates obtained in our study might be isogenic isolates. However, the rates of resistance considering the recovery of only one resistant isolate per case were similar, indicating that the bias introduced by the occurrence of isogenic strains is low. In the non – *C. albicans* species, high rates of resistance are to be expected due to their intrinsic resistance to the azoles, as already mentioned [48][172]. Regarding the frequency of resistance to clotrimazole, it was somewhat lower in *C. albicans* and *C. glabrata*, but higher in *C. tropicalis*. The differences observed could be due to intrinsic resistance ability of *C. tropicalis* to azoles [171], that can occur through an increased expression of efflux transporters upon exposure to azole compounds [183].

We found no relation regarding the distribution of azole-resistant isolates and the distribution of the isolates by the clinical groups ($p>0.05$). The same absence of relation was found regarding the frequency of azole resistance, course of treatment and distribution by each of the clinical groups ($p>0.05$). One could expect that fluconazole resistance would be higher among RVVC (cVVC) isolates, since a direct implication with treatment failure would be implied. Previous studies have reported a higher frequency of non-*C. albicans* species among RVVC (cVVC) cases, that have an intrinsic lower

susceptibility to azole compounds, hence hindering efficient treatment with these drugs [48]. Treatment is usually prescribed empirically, because antifungal susceptibility testing can take 3-5 days. In addition, many women start self-medication with over-the-counter antifungals before attending a gynaecologist. In addition, it has been described that exposure to azoles can lead to the rapid emergence of azole resistance in *C. albicans* [184]. We found that the azole-resistant isolates were equally distributed between the two groups. These results are related with the high frequency of azole-resistant *C. albicans* that we found in our study. Recent studies report that azole resistance is emerging in vulvovaginal *C. albicans* [185][186], in line with the results obtained by us. All these findings could lead to a selection of the less susceptible isolates among the colonising population.

We have also assessed the contribution of the ability to form biofilm for azole resistance. The ability to form biofilms by *Candida* spp. on the vaginal mucosa has been extensively studied [17]. Previous studies have associated the increased capacity to form biofilms with resistance to azole compounds, probably due to a lower diffusion of the drugs [99]. However, recent reports have failed to associate these [79][91]. In our study, most of the azole-resistant isolates had an increased ability to form biofilm, supporting previous studies [99], but these isolates were equally distributed between the two clinical groups, and therefore the formation of biofilm does not seem to be particularly relevant for persistence in the vaginal cavity in our study collection.

The lack of correlation in our data can probably be explained by the high azole resistance rates, and azole-resistant isolates equally distributed in both clinical groups, not contributing specially to persistence. Therefore, one can hypothesize that recurrence might be related to other factors, such as the ones related to host adaptation, risk factors related with host characteristics or the differential expression of virulence factors. Further studies are needed in order to elucidate the cues used by *Candida* spp. to persist in the vaginal mucosa and cause recurrence (chronicity). Nevertheless, the finding of a high azole-resistance rate among vulvovaginal isolates is worrisome, and supports further investigation. Limited treatment options for VVC are available, and recent studies have focused on the use of probiotics, often in in combination with azoles [187]. Thus, it can be concluded from the outset that a large study is needed to provide an overview of the situation of vaginal fungal infections in Portugal. Research in a clinical context may prove to be an ally in the reduction of resistance rates by gaining insights for new therapies and in the long run might be responsible for a reduction in associated costs.

Chapter 4. Recurrent (chronic) vulvovaginal *Candida* spp isolates phenotypically express less virulence traits

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In this chapter, the article referenced here is transcribed with annotations referring to the nomenclature adopted in the meantime.

Recurrent (chronic) vulvovaginal *Candida* spp isolates phenotypically express less virulence traits

Abstract

Objective: Vulvovaginal candidosis (VVC) is a condition that impacts the quality of life of women worldwide. At least 5-8% of all VVC cases re-occur. Recurrent vulvovaginal candidosis (RVVC) (chronic vulvovaginal candidosis, cVVC) can be defined as the occurrence of a VVC episode at least four times per year. The reasons for recurrence to occur are poorly understood. This work aims to identify key phenotypic traits associated with RVVC (chronic vulvovaginal candidosis, cVVC) Candida spp. isolates that might be used to plan strategies to control RVVC (chronic vulvovaginal candidosis, cVVC).

Methods: The capacity to form biofilms (with the microtitration plate assay), to develop germinative tube in the presence of fetal bovine serum and to produce phospholipase (in the egg-yolk plate assay) was assessed for a collection of Candida spp. isolates obtained from 17 women diagnosed with RVVC (cVVC) and 16 women with non-recurrent VVC (VVC) (sporadic vulvovaginal candidosis, sVVC). The differences obtained regarding the proportion of isolates expressing each virulence factor was assessed by statistical analysis (χ^2).

Results: We found that *C. albicans* isolates had a higher ability to form germinative tubes than RVVC (chronic vulvovaginal candidosis, cVVC) isolates (29% vs 4%, $p<0.05$). In addition, the ability of *Candida* spp. isolates to form biofilm (63% vs 51%) and to produce phospholipase (13% vs 11%) was also higher, though not statistically different ($p>0.05$).

Conclusions: We conclude that biofilm formation and phenotypic switching associated with germinative tube production are particularly important *C. albicans* virulence factors for acute, sporadic VVC (sVVC) cases.

Keywords: *Candida* spp., persistence, vaginal, pathogenicity, biofilm, phospholipase.

Introduction

Vulvovaginal candidosis is the second most common vaginal infection (20-25%) [156][33]. It affects millions of women worldwide, particularly women of child-bearing age [11],[170],. The infection is caused by Candida spp, which are recognized vaginal opportunistic pathogens. These can be present in 10 to 20% of asymptomatic women of fertile age [156] [188], and these frequencies increase during pregnancy [156]. Alterations of the vaginal ecosystem can modify the balance between different populations that constitute the microbiome, with the appearance of an infective *Candida* spp. population that leads to vulvovaginal candidosis [189]. About 90% of *Candida* spp. vaginal strains are described to be *Candida albicans*, but other *Candida* species can also cause colonization and/or infection (*Candida glabrata*, *Candida tropicalis*, *Candida krusei, Candida guilliermondii* and *Candida parapsilosis)* [11][20][190].

Among women of fertile age, about 75% have at least one episode of vulvovaginal candidosis and of these, 40% have a second event [11]. Recurrent vulvovaginal candidosis (RVVC or chronic vulvovaginal candidosis, cVVC) is defined by the occurrence of more than four episodes per year; the prevalence of this condition is 5-8% in adult women [11],[191]; 10% to 33% of RVVC (cVVC) cases are caused by non-*albicans Candida* species. Nevertheless, in acute and chronic states, *C. albicans* remains as the species most frequently isolated [11].

Many studies have attempted to clarify the process of recurrence (chronicity) and the factors involved in RVVC (cVVC). The use of antibiotics, immunosuppressants, genetic and environmental factors have been described to influence RVVC (cVVC) [11]. The persistence of *C. albicans* strains in the vagina due to ineffective eradication related to resistance to antifungals is also a possible explanation for the occurrence of RVVC (cVVC) [192]. The impact of phenotypic expression of virulence factors has also been previously reviewed [164]. In relation with this, the finding that vaginal Candida spp. isolates have the ability to produce biofilms [17] has been associated with its ability to persist in the vaginal mucosa due to its biofilm biochemical properties that might be difficult to eradicate. Besides the formation of biofilms, vaginal Candida spp. can express other virulence factors such as the formation of germinative tubes (exclusively observed for *C. albicans*, phenotypic switching) [193] and production of phospholipases [194]. The relation between the expression of these relevant virulence factors and recurrence (chronic), through studies that compare isolates obtained from chronic and acute clinical cases, however, has not been yet explored.

In this study, we aim to shed light on the hallmarks of recurrent vulvovaginal candidosis (cVVC), particularly on the expression of virulence factors and its relationship with persistence in the vaginal mucosa.

Methods

Biological samples.

The samples were obtained from a total of 33 women attending the gynaecological consultation of a private clinic in the Oporto region, in Portugal. Of this group, 17 women were diagnosed with RVVC (cVVC), according with symptoms, clinical history and frequency of recurrence as previously suggested [164] while 16 women only had a single episode of vulvovaginal candidosis infection (non-recurrent VVC, VVC) (sVVC) in a period of one year (Table 1). Other causes for symptoms were excluded by the attending gynaecologist. The patients were enrolled independently of having taken antifungals previously or during sampling; and were included blindly, without registry of any possible risk factors. There were no particular criteria for selecting the patients for inclusion in this study: these were part of a convenience sample, since samples were collected for diagnostic purposes only. The collection of samples was performed with the aid of a cotton swab and speculum (obtaining the sample from the vaginal canal) and subsequently inoculated in Sabouraud Dextrose Agar (VWR, Avantor, Pennsylvania, United States of America), according to routine procedure. After incubation and registration of results for diagnostic purposes, the cultures, instead of being discarded for incineration, were kept in the research laboratory for further studies. The cultures were kept at −80ºC in Brain Heart Infusion broth (BHI, VWR, Avantor, Pennsylvania, United States of America) supplemented with 20% glycerol (VWR, Avantor, Pennsylvania, United States of America). Any identification of patients was removed and replaced with a code assigned to them, being accessible only to the clinicians involved in the study, without disclosure to the researchers or third parties. The study has been approved by the Ethics Committee of Universidade da Beira Interior (CE-UBI-Pj-2018-022).

Table 1. Epidemiological data of clinical cases enrolled in the study. VVC: acute vulvovaginal candidosis cases, only one isolate was recovered. RVVC: recurrent vulvovaginal candidosis cases (chronic vulvovaginal candidosis, cVVC), more than one isolate was recovered according with recurrent episodes of infection.

Clinical case	**Age**	**Year of isolation**	**No of isolates**	**Clinical case**	**Age***	**Year of isolation**	**No of isolates**
VVC1	45	2010	1	**RVVC1**	38	2009-2010; 2012**	4
VVC2	35	2010	1	**RVVC2**	50	2009-2010	2
VVC3	26	2013	1	**RVVC3**	34	2010	3
VVC4	39	2012	1	**RVVC4**	64	2013-2014	2
VVC5	29	2010	1	**RVVC5**	43	2010	2
VVC6	32	2014	1	**RVVC6**	37	2010	4
VVC7	44	2012	1	**RVVC7**	46	2013-2014	12
VVC8	29	2010	1	**RVVC8**	51	2010; 2013**	5
VVC9	61	2013	1	**RVVC9**	56	2010	3
VVC10	35	2009	1	**RVVC10**	49	2010	7
VVC11	47	2013	1	**RVVC11**	33	2010	2
VVC12	62	2014	1	**RVVC12**	46	2009-2010	2
VVC13	52	2013	1	**RVVC13**	45	2013	2
VVC14	25	2013	1	**RVVC14**	31	2010; 2016 **	3

VVC15	26	2010	1	**RVVC15**	70	2013	2
VVC16	36	2013	1	**RVVC16**	44	2009-2010	6
				RVVC17	32	2009	2
Total number of isolates			16	Total number of isolates			63

*Age at first sampling

**The same patient suffered from two episodes of RVVC (chronic vulvovaginal candidosis, cVVC)

Species identification.

for phenotypic identification of Candida species, automated analysis of 46 biochemical reactions was performed with Vitek-2 (Biomerieux, Marcy-l'Étoile, France). Following previously described results from the literature, Vitek-2 identifications as "*Candida famata*" and "*Candida famata/Candida guilliermondii*" were considered *Candida guilliermondii* [195].

Determination of the ability to form biofilm.

the ability to adhere to a polysterene surface and form a biofilm was evaluated for all isolates according to adapted methodology from Leighann Sherry *et al* [165]. Yeast cultures were subcultured twice on Sabouraud Dextrose Agar (SDA, VWR, Avantor, Pennsylvania, United States of America) and inoculated in Yeast Peptone Dextrose broth (YPD, Fisher Scientific, New Hampshire, United States of America). After incubation for 24 h at 37°C in an orbital shaker (Argitob 200 Aralab, Sintra, Portugal), the cells were collected by centrifugation (Hettich Zentrifugen, MiKRO 200R, Sigma-Aldrich, Missouri, United States of America) at 5,000 g for 5 minutes, and washed twice with sterile Phosphate Buffer Saline (PBS: 1.37 M NaCl, Fisher Scientific, New Hampshire, United States of America; 27mM KCl, ChemLab, Zedelgem, Belgium; 100 mM Na_2HPO_4, Fisher Scientific, New Hampshire, United States of America; 20 mM KH_2PO_4, ChemLab, Zedelgem, Belgium). The cells were then resuspended in Roswell Park Memorial Institute culture medium (RPMI-1640, Sigma-Aldrich, Missouri, United States of America]) at 0.5 MacFarland, using a densitometer (Grant-bio, DEN-1, Grant Instruments, Fisher Scientific, New Hampshire, United States of America), that corresponds to approximately 1-5 x 10^6 cells/mL. The suspension was transferred to 96 wells microplates and incubated for 24h at 37°C (Binder GmbH, Tuttlingen, Germany). After the period of incubation, the culture medium was carefully removed from each well, avoiding touching the bottom of the well, so that biofilm formed is not removed, and washed three times with sterile PBS to remove planktonic cells. The biofilm was fixed with methanol (Fisher Scientific, New Hampshire, United States of America) and stained with a 0.02 % (v/v) crystal violet solution (VWR, Avantor, Pennsylvania, United States of America). The stained biofilm was resuspended with an 33 % (v/v) acetic acid solution (Fisher Scientific, New Hampshire, United States of America). Absorbance was obtained by reading the plates on an Anthos 2020 microplate reader (Bio-Rad, California, United States of America) at 595 nm (Abs_{595nm}). A positive control was included in each assay: *Candida albicans* ATCC10231, which has been previously described as an adherent yeast isolate [167]. The results obtained for each isolate were classified according with the

results obtained using this methodology with the positive control in three independent assays (results not shown). Therefore, isolates were classified as "moderately adherent" if the Abs_{595nm} of their biofilm was in the range of the one obtained for ATCC10231 (0.62-0.82); and "strongly adherent" if the Abs_{595nm} of their biofilm was superior to 0.82. If the strains could form biofilm that corresponded to at least 50% of the ability of ATCC10231 to form biofilm (Abs_{595nm} 0.3-0.62), then they were classified as "weakly adherent". Finally, "non-adherent" isolates were considered when Abs_{595nm} was lower than 0.3. Two independent assays were performed for each isolate; the result was validated only when the standard deviation between the Abs_{595nm} obtained was less than 0.15 or when both assays gave a consistent result regarding the classifiers described above.

Determination of the ability to form germinative tubes.

The formation of germinative tube (GT) by the yeast isolates was evaluated according to the adapted methodology proposed by Ellepola and Samaranayake [196], only for the *C. albicans* strains. Suspension of yeast cells were prepared in approximately 2 ml of sterile PBS with the pH adjusted to 7.2 (pH meter ORION STAR A211, Fisher Scientific, New Hampshire, United States of America), at 0.5 MacFarland as described above. The suspension (250 µL) was transferred to 1 mL of YPD, enriched with 10 % (v/v) fetal bovine serum (Biochrom GmbH, Berlin, Germany), and incubated in an orbital shaker (Argitob 200 Aralab, Sintra, Portugal) at 37º for 3 hours at 180 rpm. After incubation, 10 µL of the culture was collected and observed at an optical microscope (YS100, Nikon, Tokyo, Japan), with 40x magnification, in a Neubauer chamber (Optik-Labor, Görlitz, Germany). The number of cells expressing the germinative tube was visually counted out of the total number of cells. *C. albicans* ATCC 10231 was the positive control (the proportion of cells forming germinative tube using this methodology was 27 % (27/100), obtained in three independent tests). The results obtained for the isolates were classified by comparing the proportion (%) of cells with germ tubes out of the total of cells, with the result obtained using the control (*C. albicans* ATCC 10231, 27 %). The results were classified as following: "strong germinative tube producers" if the proportion of cells forming germinative tube was 27 % or higher; "moderate germinative tube producers" if the proportion of cells forming germinative tube was between 13.5 % and 27 % (corresponding to at least 50 % of the ability of ATCC 10231 to produce germinative tubes); "weak germinative tube producers" if the proportion of cells forming germinative tube was between 13.5 % and 1 %; "negative germinative tube producers" if no cells forming germinative tubes were observed. Two independent assays were performed for

each isolate; the result was validated only when the standard deviation between the proportions obtained was less than 10 %.

Determination of the ability to produce phospholipase.

The preparation of cell suspension followed the procedure used in the determination of the capacity to form a germinative tube. The production of phospholipase production was evaluated with the procedure described in Raja Vinodhini et al, 2016 [197]. A cell suspension was prepared in sterile PBS at 0.5 MacFarland as described above, and inoculated (10 µl) in Sabouraud Dextrose egg yolk agar (13 g SDA (VWR, Avantor, Pennsylvania, United States of America), 11.7 g NaCl (Fisher Scientific, New Hampshire, United States of America), 0.11 g $CaCl_2$ (Fisher Scientific, New Hampshire, United States of America) in 200 mL distilled water supplemented with 10% (v/v) of egg yolk emulsion, prepared at 50% (v/v) in 0.85% NaCl (Fisher Scientific, New Hampshire, United States of America) solution. The plates were incubated at 37°C (Binder GmbH, Tuttlingen, Germany) for 5 days. After incubation, the plates were visually inspected, being considered as positive for phospholipase production when an opaque zone of precipitation around the colony was observed. The enzymatic activity (Pz) was determined by calculating the ratio between the diameters of colony versus the precipitation zone. According with the literature [197], the lower Pz value, the higher the enzymatic activity. When Pz = 1.0: absence of enzymatic activity; when $0.64<Pz<1.0$: positive enzyme activity; and when $Pz \leq 0.63$: strongly positive enzymatic activity. *C. albicans* ATCC 10231 was used in each assay as the positive control ($Pz \leq 0.63$). Two independent assays were performed for each isolate, being the result only validated when the standard deviation between the results obtained in each assay was less than 0.1.

Analysis of results.

The results for each isolate was expressed as the average of two independent assays. Sstatistical analysis of the proportions of isolates expressing each virulence factor or other characteristic of interest in each clinical group (RVVC) (cVVC) vs VVC (sVVC) was performed using the two-sided Fisher independence test (Software GraphPad Prism 7, San Diego, United States of America), considering that the variables under evaluation were significantly dependent when the test value was $p < 0.05$. In addition, the median of the results obtained for each test in each clinical group was also assessed using the t-student test (Software GraphPad Prism 7, San Diego, United States of America).

Results

Candida species are differently distributed among VVC (sVVC) and RVVC (cVVC) clinical cases.

This research included 79 yeast isolates, obtained from 33 women, with clinical symptoms associated with vulvovaginal candidosis. For 16 of these cases, the infection was diagnosed as sporadic (acute); for the purpose of this study, these were considered non-recurrent VVC (sVVC) cases (VVC1-VVC16, Table 1). A single isolate per patient was obtained. The median age of the patients was 36±12 years. The remaining clinical cases (17) were diagnosed as recurrent (cVVC) (RVVC1-RVVC17, Table 1). At least two isolates were obtained from each patient; the maximum number of isolates obtained from a patient was 12. Overall, 63 isolates were included in this clinical group. The median age of the patients was 45±11 years.

Regarding the distribution of vulvovaginal clinical isolates by species, as determined by automated analysis of biochemical profiles by Vitek-2 (BioMérieux), overall, we found that *Candida albicans* was the most prevalent species (40 %, 32/79), followed by *Candida glabrata* (33 %, 26/79) and *Candida guilliermondii* (27 %, 21/79). When considering the distribution of species according with each clinical group (VVC vs. RVVC), we found that the frequency of *C. albicans* was higher in acute cases (44 % vs 40 %, Table 2), but this difference was non-significant ($p>0.05$). Interestingly, we found that *C. glabrata* was more frequently associated with recurrent (chronic) cases (36% vs 18%), $p<0.05$, and that *C. guilliermondii* was more frequently associated with acute cases (38% vs 24%), $p<0.05$ (Table 2).

Table 2. Distribution of the isolates according with the phenotypic expression of the virulence factors studied in this research. VVC: vulvovaginal candidosis (episodic vulvovaginal candidosis, eVVC); RVVC: recurrent vulvovaginal candidosis (chronic vulvovaginal candidosis, cVVC). The number indicate the number of isolates in each category.

Phenotypic expression of virulence traits	***C. albicans***		***C. glabrata***		***C. guilliermondii***	
Biofilm formation	**VVC**	**RVVC**	**VVC**	**RVVC**	**VVC**	**RVVC**
Strongly adherent	43% (3/7)	64% (16/25)	67% (2/3)	39% (9/23)	83% (5/6)	47% (7/15)

Weakly adherent	29% (2/7)	12% (3/25)	(-)	30% (7/23)	17% (1/6)	27% (4/15)
Moderately adherent	14% (1/7)	4% (1/25)	(-)	17% (4/23)	(-)	13% (2/15)
Non-adherent	14% (1/7)	20% (5/25)	33% (1/3)	13% (3/23)	(-)	13% (2/15)
Germinative tube formation	**VVC**	**RVVC**	**VVC**	**RVVC**	**VVC**	**RVVC**
Moderate producer	29% (2/7)	4% (1/25)	-	-	-	-
Weak producer	71% (5/7)	96% (24/25)	-	-	-	-
Phospholipase production	**VVC**	**RVVC**	**VVC**	**RVVC**	**VVC**	**RVVC**
Strong producer	29% (2/7)	24% (6/25)	(-)	4% (1/23)	(-)	(-)
Moderate producer	43% (3/7)	26% (9/25)	(-)	4% (1/23)	33% (2/6)	13% (2/15)
Negative	29% (2/7)	40% (10/25)	100% (3/3)	92% (21/23)	37% (4/6)	87% (13/15)

Phenotypic expression of virulence traits among VVC (sVVC) and RVVC (cVVC) isolates

Of the 79 *Candida* sp. isolates tested, 67 (85 %) produced biofilm and 26 (33%) were phospholipase producers (Table 2). In addition, all *C. albicans* isolates formed germinative tubes under the conditions tested. Of these, only a few were classified as strong producers, since the results regarding the phenotypic expression of each virulence factor was similar to the results obtained for the positive control: 42 (53%, 42/79) were strongly adherent, 9 (11%, 9/79) were strong phospholipase producers (Pz<0.63), and only three *C. albicans* (9%, 3/32) isolates formed germinative tubes in the same frequency as the control. We did not obtain any isolate that achieved the classification of "strong producer" for all the virulence traits tested. Two *C. albicans* isolates (2/79, 3%) were both strong biofilm and germinative tube producers; and seven (7/79, 9%), six *C. albicans* and one *C. glabrata*, were both strong biofilm and phospholipase producers. We did not find any strong phospholipase producer among germinative tube producers.

The great majority of *C. albicans* isolates were able to produce biofilm (26/32, 81%), and were strongly adherent (19/26, 73%) (Table 2). The formation of germinative tubes was observed also for all strains (32/32, 100%), although only three strains (3/32, 9%) had a frequency of germinating cells similar to that observed in the control. Most isolates also produced phospholipase (20/32, 63%), but of these, only eight (8/20, 40%) were considered strong producers (Table 2). *C. glabrata* isolates were also able to produce biofilm (22/26, 85%); of these, 11 (11/22, 50%) were found to be strongly adherent (Table 2). Only two isolates (2/26, 8%) produced phospholipase, and of these, only one was a strong producer. Regarding *C. guilliermondii*, the great majority of isolates was also biofilm-producers (19/21, 90%) (Table 2). Strong biofilm producers accounted for the majority (12/19, 63%). There were only four isolates able to produce phospholipase (4/21, 19%), and were classified as moderate producers (0.64 <Pz> 1.0).

The results were analysed taking in account if the strains were recovered from a sporadic acute vulvovaginitis (VVC) (sVVC) or from a recurrent (chronic) vulvovaginitis (RVVC or cVVC) (Figure 1). The median of the results obtained for each phenotypical evaluation of virulence was different for each clinical group. Specifically, the median of the absorbance obtained in the microtiter plate assay for the determination of the ability to form biofilm for the isolates recovered from VVC cases, was 0.96, which corresponded to strongly adherent biofilms; isolates recovered from RVVC cases showed a median of 0.80 that corresponded to moderately adherent biofilms (Figure 1). The same type of result was observed when the median of the proportion of *C. albicans* cells producing germinative tubes was compared between the two groups: 2.2% (VVC) (sVVC) vs 0% (RVVC) (cVVC). Regarding the median of the Pz values obtained for the determination of phospholipase production, it was the same for both clinical groups (Pz=1, negative producers). The statistical significance of the results described above was further analysed. No statistical meaning ($p<0.05$) was attributed to the differences observed between the two clinical groups (Table 3).

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Table 3. p-values regarding the statistic analysis of the distribution of biofilm, germinative tube and phospholipase producers among RVVC (cVVC) and VVC (sVVC) isolates.

Statistical test (VVC vs RVVC)	**Frequency of *C. albicans***	**Frequency of *C. glabrata***	**Frequency of *C. guilliermondii***	**Median absorbance (biofilms)**	**% germinative tube formation***	**Median Pz vales (phospholipase)**	**% Strong biofilm producers**	**% Strong germinative tube producers***	**% Strong phospholipase producers**
p-value	0.3875	**0.0100**	**0.0311**	0.7024	0.4940	0.2981	0.1159	**<0.0001**	0.8282

* *C. albicans* only

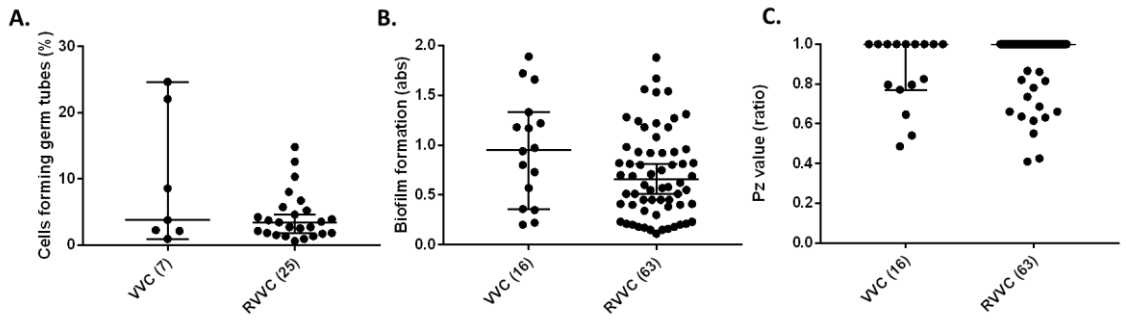

Figure 1. Average of the results obtained for each isolate, regarding the determination of the phenotypic expression of each virulence factor, according with each clinical group. VVC: non-recurrent vulvovaginal candidosis (sporadic vulvovaginal candidosis, eVVC); RVVC: recurrent vulvovaginal candidosis (chronic vulvovaginal candidosis, cVVC). The median (with the respective 95% confidence interval) of the results obtained considering all isolates of each group is also showed. A. Formation of the germinative tube. B. Biofilm formation. C. Phospholipase production. [100].

The phenotypic expression of strong virulence traits is more frequent among VVC (sVVC) isolates.

Finally, the proportion of strong phenotypic producers in each clinical group for each virulence trait studied was compared between the two clinical groups (Figure 2). We found that the difference observed between the proportion of *C. albicans*' germinative tube producers that behaved similarly to the control was statistically significant (VVC (sVVC): 2/7, 29%; RVVC (cVVC): 1/25, 4%; $p<0.05$; Table 3). Regarding the proportion of strongly adherent biofilm producers among VVC (sVVC) and RVVC (cVVC) isolates (VVC (eVVC):10/16, 63% vs RVVC (cVVC): 32/63, 51%), as well as the strong phospholipase producers (VVC (sVVC): 2/16, 13% vs RVVC (cVVC): 7/63, 11%), it was higher among VVC (sVVC) comparing with RVVC (cVVC) isolates but the difference was not statistically significant ($p>0.05$, Table 3).

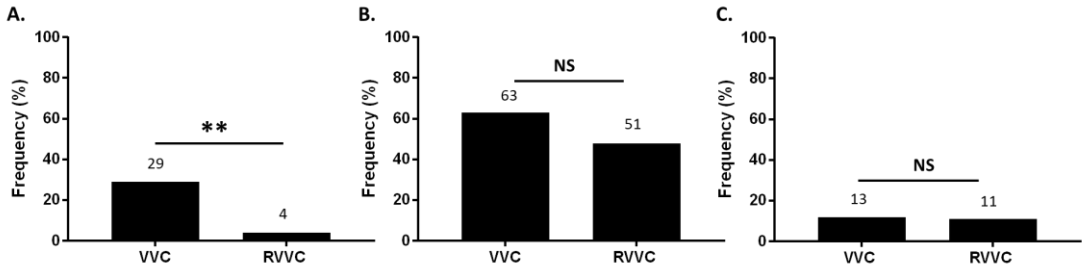

Figure 2. Frequency (%) of isolates strongly expressing each virulence factor, in each clinical group. Numbers above bars indicate frequency (%). VVC (eVVC): non-recurrent vulvovaginal candidosis; RVVC

(cVVC): recurrent vulvovaginal candidosis. A. Formation of the germinative tube. B. Biofilm formation. C. Phospholipase production. **p<0.01; NS: non-significant. [100].

We have also hypothesized if recurrent isolates altered the expression of virulence traits overtime (Table 4). Focusing on the clinical cases with more isolates (RVVC7, RVVC10 and RVVC16), we found that was a lot of variation regarding the expression of the virulence traits overtime. However, we did not find any particular trend, since in some clinical cases the strength of the phenotypic expression increased overtime, while for some cases the opposite was found (Table 4). The same type of results was obtained considering the clinical cases with fewer isolates per case (Table 1).

Table 4. Phenotypic expression of virulence traits overtime, considering date of isolation of each isolate for the same patient. SA: strongly adherent. WA: weakly adherent. A: adherent. NA: not adherent. MP: moderate producer. SP: strong producer. N: negative.

Clinical case	**Dominant *Candida* species**	**Biofilm formation**	**Germinative tube formation**	**Phospholipase production**
RVVC7	*C. glabrata*	SA-WA-NA-WA-A	-	N
RVVC10	*C. albicans*	WA-SA	Weak producer	MP-SP-N-MP-SP
RVVC16	*C. guilliermondii*	SA-WA	-	N

Discussion

Vulvovaginal candidosis is a recognized illness affecting millions of women worldwide [11]. Its frequent re-occurrence is a cause for high morbidity [11], and strongly impacts in the women's quality of life. Recurrence (chronicity) can be related to a myriad of different aspects related with the physiology of the vaginal mucosa, such as host risk factors, genetic determinants, immune system response, among others [198]. The relevance of the local distribution of *Candida* species and their phenotype on the onset of vulvovaginal candidosis has been recently highlighted [198]. In this study, we aimed to characterize phenotypically the isolates obtained in recurrent cases and compare these with isolates obtained from acute cases. The main objective was to find differences in the phenotypic expression of virulence factors that would be related with the ability to persist in the vaginal mucosa.

In our collection, *Candida albicans* was the most prevalent *Candida* spp, both in acute and chronic cases. This result is in accordance with the literature, that describes *C. albicans* as the most prevalent vaginal *Candida* species [11], [20]. Regarding the distribution of the remaining species, it was different ($p<0.05$) between the two clinical groups. While in acute cases, *C. guilliermondii* was the second most prevalent, in the recurrent cases *C. glabrata* was the second more prevalent *Candida* species. *C. guilliermondii* has been reported as a rare fungal pathogen[190]. However, it has recently been isolated as the etiological agent of mucocutaneous [199] and vulvovaginal infections [200]. We hypothesize if acute infections could be provoked by opportunistic pathogens, that are often temporary colonizers of the skin. Due to its intrinsic characteristics, *C. guilliermondii* might be more associated with vulvar infection, and this hypothesis should be further studied and supported. Regarding *C. glabrata*, its role in RVVC (cVVC) is well documented [11] [20][201], and is thought to occur due to its high frequency of resistance to antifungals and ability to form biofilm [114].

We found also that the ability to form biofilm was detected in the great majority of the isolates, regardless of the species (81-90%). However, strongly adherent biofilm formers were more frequently found among *C. albicans*. Biofilm formation has been studied as the main *Candida* spp virulence factor, since has been associated with adherence to the mucosae and intrinsic resistance to antifungal treatment [17]. Surprisingly, in this study the occurrence of strongly adherent isolates was higher among isolates recovered from acute cases than those obtained from recurrent cases, though the difference observed was not statistically significant ($p>0.05$). Nonetheless, this result might indicate that biofilm formation is not a relevant virulence factor for recurrence, at least in its most

strongly adherent form. Formation of biofilms on the vaginal mucosa have been described to be associated to *Candida* spp. pathogenesis on this particular niche [17]. However, the contribution of *Candida* spp. biofilms for recurrence is still not clear. A recent study focusing on recurrent (chronic) *Candida* isolates has described a heterogeneity in their ability to form biofilms [99], which could be an indication as well that the formation of biofilms is not exclusively necessary for recurrence (chonicity).

Considering the results for germinative tube formation, we found that all *C. albicans* isolates presented this virulence factor, as expected [202]. The formation of the germinative tube has been associated with increased tissue damage, being considered as the switch from colonizing to pathogenic state of *C. albicans* [193]. It has also been associated with a high stimulation of the innate immune response [203]. In our study, we found that recurrent isolates exhibited less this phenotype, considered a virulence trait, than the isolates obtained from acute cases ($p<0.05$). These results indicate that the expression of genes coding for this virulence factor might be downregulated in recurrent isolates, possibly hindering the immune response. One important factor that is associated with the frequency of RVVC (cVVC)is a deficiency on the innate immune response [198]. Previous studies have described that a mutation in the NLRP3 gene could cause a decrease in the inflammatory response of cytokines IL-18 and IL-1β, leading to chronic inflammation caused by RVVC (cVVC)[204]. Therefore, we hypothesize if downregulating the formation of germinative tubes could be a strategy to improve the persistence of isolates in the vaginal mucosa, leading to RVVC (cVVC). This hypothesis needs further study and clarification.

Interestingly, we found that the production of phospholipase was almost exclusively detected in *C. albicans* and *C. guilliermondii*. A small proportion of *C. glabrata* was also able to phenotypically express this virulent factor. The production of phospholipases has been observed in a wide array of different *Candida* species [205]. Phospholipases are enzymes associated with infection processes, since they facilitate adhesion and invasion due to their digestion ability [206]. Similarly, to the remaining results already discussed, the frequency of strong producers of phospholipases was higher among isolates obtained from acute cases, though the difference observed in this case was non-significant; and the frequencies observed between the two clinical groups were similar (13% vs 11%). Therefore, we conclude that the production of phospholipase might be an important virulence factor for the isolates involved in both acute and chronic disease.

Overall, RVVC (cVVC) isolates were not extremely virulent; in fact, it seemed to be the opposite. Since the virulence factors that were studied in this work are associated with

adherence, invasion and immune modulation, we might hypothesize that persistence in the vaginal mucosa might be achieved by downregulating these processes (although not fully). This downregulation would probably cause a dormancy in the innate immune system, since inflammatory processes would be practically non-existent. Previously, there was a single study focusing in this subject where it was described no statistically significant differences between the virulent phenotypes of VVC (eVVC) and RVVC (cVVC) isolates [112]. In fact, considering the median of the results obtained, we found no statistically significant differences as well; only when we focused our analysis on the stronger phenotypic expression of virulence, we were able to find significant differences. This finding supports the strength of our analysis, although more studies are needed to understand the apparent dormant state of yeast isolates in the vaginal mucosa, which is possibly associated with its persistence.

Our study presents some limitations. The associated risk factors were not addressed, since the women were included blindly, without special regard to antifungal therapy, diabetes, hormonal-replacement therapy or other known risk factors as recently reviewed [198]. We therefore expect that the distribution of women with each risk factor is equal in both groups. Another limitation is related with the culture time, since the isolates have been collected in different time-points, according with their isolation. Expression of virulence traits in *Candida albicans* post-infection occurs in a timely manner [11]], and therefore culture time could hypothetically influence the expression of virulence. However, analysis of our results of recurrent cases considering the timeline of infection has revealed that isolates recovered at first sampling express the phenotype in a strongly manner in comparison with latter isolated strains. The reverse situation was also observed. Therefore, we conclude that time of isolation did not introduce a bias in our results. These results also indicate that modulation of expression occur along time in recurrent cases, justifying this study and the results herein described. Finally, the expression of other virulence traits, such as protease production, haemolysis, ability to resist to low pH among others has not been addressed in this manuscript. We hope that more studies will follow that will further explore the role of expression of virulence in recurrence of vulvovaginal candidosis (chronic vulvovaginal candidosis, cVVC).

Conclusion

The results described in this study indicate that recurrent vulvovaginal isolates isolated from chronic cases show a lower phenotypical expression of virulence traits when

compared with isolates obtained from acute, sporadic cases. We hypothesize that persistence in the vaginal mucosa might be achieved through a phenotypic arrest of virulence, which would in turn be non-stimulant for the innate immune system, rendering chronic persistence in the vaginal mucosa achievable.

Chapter 5. Evaluation of overtime phenotypic variation of yeasts in chronic vulvovaginal candidosis cases

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In this chapter, the article referenced here is transcribed with annotations referring to the nomenclature adopted in the meantime.

Evaluation of overtime phenotypic variation of yeasts in chronic vulvovaginal candidosis cases

Abstract

Chronic vulvovaginal candidosis results either from reinfection or results from the ability of *Candida* spp. to persist in the vulva and/or vagina. Persistence is usually associated with increased antifungal (mainly azoles) resistance rates, which can explain treatment failure, and/or increased expression of virulence factors by *Candida* spp. The aim of this study was to assess the mechanisms leading to *Candida* spp persistence, by studying sequential isolates from women with chronic vulvovaginal candidosis, focusing on strains genotypes, azole resistance and ability to form biofilms along the period of clinical evaluation.

The strains were identified at species level by automated analysis of biochemical profiles and molecular typing evaluated by polymorphic DNA analysis. The capacity to form biofilm was assessed with a microtiter plate assay. Fluconazole susceptibility was determined by the microdilution broth assay at both pH 7 (following the recommended guideline) and pH 4 (as representative of vaginal pH).

We studied samples from 29 clinically recurrent cases. In 53% of the chronic cases there were two or more isolates that had a phylogenetic relationship while the remaining (47%) were caused by different species. In those cases where related strains were involved in recurrence, we verified an increase in MIC at pH 7 and also an increased capacity to form biofilms over time. Significant correlation between these two parameters was observed only in cases caused by *C. glabrata*, evidencing the importance of these two factors to enhance persistence in the vaginal mucosa for this particular species.

Keywords: chronic, recurrence, vulvovaginal candidosis, molecular typing, antifungal resistance, biofilm

Introduction

Despite therapeutic advances, vulvovaginal candidosis (VVC) continues to be a common concern worldwide, affecting mainly women of childbearing age [11][43]. *Candida albicans* is generally accepted as being the predominant species involved in VVC [207], a preponderance that may be related to its dimorphic behaviour. But there is a tendency to increase the incidence of non-*Candida albicans* [208][209]. VVC is often difficult to treat and the infection frequently clinically reappears. In research, recurrent vulvovaginal candidosis (RVVC) (cVVC) is defined as the occurrence of at least three episodes within an one year period [44]. Clinically, any reappearance of the disease may be the result of persistence or reinfection. The former represents an antifungal treatment failure while the later may be more related to host immune system failure or to massive inoculation.

Recurrence (chronicity) is a condition that causes significant discomfort, pain and significantly affects life quality of the affected women (and their partners). Despite the clinical importance of RVVC (cVVC), the knowledge regarding the characteristics of strains related to its persistence within the vaginal mucosa [210] or vulvar skin [211] is scarce. In fact, case reports of recurrences have been frequently associated to antifungals resistance, immunocompromised patients and host genetic factors [212] [43] [210]. As mentioned above VVC is caused mainly by *C. albicans*, even considering that the pattern distribution of infections by *Candida* spp has changed, with an increase in the relative frequency of non-albicans species, such as C. *glabrata*, *C. parapsilosis*, *C. guillermondii* or even S. cerevisiae [213][48][207][214]. Candida species vary in their susceptibility to antifungal agents, so persistence can be associated with treatment failure. Therefore, susceptibility tests have considerable clinical significance, since they can determine the choice for appropriate therapy [212], although direct correlations cannot be assumed.

Persistence of *Candida* spp. in the vaginal mucosa is also related to the expression of virulence factors by the fungus. This allows, for instance, invasion of vaginal and/or vulvar epithelium and deep-seated infection, leading to consequent recurrence (chronicity) [215]. Virulence factors of *Candida* spp., are closely related to their ability to adhere, penetrate and cause lysis in mucosal cells; specifically their ability to form biofilms, to form germinative tubes, to produce extracellular enzymes, and even to adapt to vaginal pH [15]. Biofilms of *Candida* spp., are formed when cells adhere to the epithelium, and stick together by means of a polymeric matrix. This confers to *Candida* cells resistance to antifungal agents, and due to this importance, it is considered a relevant virulence factor related to recurrence [216]. However, studies that relate the

expression of virulence factors and antifungal resistance in sequential isolates collected from recurrent cases are scarce, and therefore the impact of these issues on the ability of *Candida* to persist in the vulvar and/or vaginal epithelium is difficult to assess. Thus, the aim of this work was to evaluate overtime the behaviour of VVC isolates from recurrent episodes, regarding antifungal resistance at acidic and neutral pH, and the ability to produce biofilms.

Materials and methods

Culture collection

We used samples collected from November 2009 to December 2013 from women with a clinical history of vulvovaginal candidosis (Table 1). The patients were diagnosed with vulvovaginal candidosis, based on clinical symptoms and signs with microbiological confirmation (microscopy and culture). We evaluated samples from 17 clinical cases of which 5 were considered statistically recurrent (RVVC) (stastistically chronic cVVC) and 12 only clinically recurrent cases (C-RVVC) (clinically cVVC) (infections occurring frequently but less than 4 episodes in an year period). This collection features chronic clinical cases that fall into the two groups. For this study clinical cases included in the first group (RVVC) (stastistically cVVC) had at least four episodes in one year, although only isolates from a few episodes were available for this study (at least two per case). The remaining were classified in the second group (C-RVVC) (clinically cVVC), since the number of episodes was less than four in one year, not reaching the number of four episodes that characterize the statistical definition of recurrent vulvovaginal candidosis for many researchers.

Sixty-two Candida spp. strains were obtained from a total of 17 women who attended a gynaecological consultation at a private clinic located at the northwestern part of Portugal (Póvoa do Varzim). The samples were collected with the aid of a cotton swab from the right high vaginal wall and subsequently inoculated in Sabouraud Dextrose Agar (VWR, Avantor, Pennsylvania, United States of America) plates, which is the routine procedure of the physician. After registration, the plates, were incubated and evaluated but, instead of being discarded through incineration, they were

kept in the laboratory for additional studies, as fungus identification or antifungal sensitivity testing, if/when necessary. Patient's confidentiality was protected since, at the lab the identification of patients was not available as it has been replaced by codes assigned by the practitioner. The study has been approved by the Ethics Committee of University of Beira Interior, Covilhã, Portugal (CE-UBI-Pj-2018-022).

Table 1. Epidemiological data of clinical cases enrolled in the study in which *Candida* spp. were isolated from statistical recurrent vulvovaginal candidosis (RVVC) (stastistically cVVC) and clinically recurrent vulvovaginal candidosis (C-RVVC) (clinically cVVC).

Clinical diagnostic	Year of isolation	Treatment outcome	No of isolates	Observations
RVVC1	2009; 2010- 2012	Fluconazole-Itraconazole-Probiotics	4	Other episodes
RVVC5	2009-2010	Fluconazole	2	Colonization after treatment, shows previous *Candida* spp. isolates
C-RVVC9	2010	Boric acid-Probiotics	3	Previous episodes without isolation of *Candida* spp.
C-RVVC2	2013-2014	Clotrimazole-Fluconazole	2	
C-RVVC3	2010	Not treated	2	
C-RVVC4	2010	Fluconazole/Itraconazole-Other	4	Previous and later episodes
RVVC3	2013-2014	Fluconazole-Itraconale-Other	12	
C-RVVC5	2010-2013	Fluconazole/Itraconazole-Other	5	Episodes from March to November
RVVC4	2010	Fluconazole/Itraconazole-Other	3	
C-RVVC6	2010	Fluconazole-Probiotics-Other	7	
C-RVVC1	2010	Fluconazole-Other	2	3 episodes in less than a year
RVVC2	2019-2010	Fluconazole	2	Presents *Candida* spp. isolates prior to these
C-RVVC7	2013	Itraconazole	2	
C-RVVC8	2010	Fluconazole	2	Episodes from 05/2010 to 11/2011
C-RVVC10	2013	Not treated	2	Other episodes from 06/2012 to 09/2012
C-RVVC11	2009-2010	Fluconazole-Other	6	
C-RVVC12	2009	Itraconazole	2	Two episodes in a year
	62			

Other: gentian violet; essential oils; boric acid; sodium bicarbonate

Preparation of samples

The isolates were sub-cultured twice at 37ºC in Sabouraud Dextrose Agar (VWR Avantor, United States of America) before each experiment.

Species Identification

The species identification of clinical isolates was performed by automated analysis of their biochemical profiles using Vitek® (Biomerieux, Marcy-l'Étoile, France).

Molecular typing by amplification of polymorphic DNA

DNA extraction

A cellular suspension was prepared in Phosphate Buffer Saline (PBS: 1.37M NaCl, Fisher Scientific, New Hampshire, United States of America; 27mM KCl, ChemLab, Zedelgem, Belgium; 100 mM Na2HPO4, Fisher Scientific, New Hampshire, United States of America; 20 mM KH2PO4, ChemLab, Zedelgem, Belgium) at 5 McFarland using a densitometer (Grant-bio, DEN-1); hereafter the suspension was centrifuged for 5 minutes at 1300 rpm (Hettich Zentrifugen, MiKRO 200R, Germany). The extraction was made from the pellet obtained, using the Kit Omega bio-tek (E.Z.N.A. ® Tssue DNA kit, D3396-01, United States of America), according with the manufacturer´s instructions.

RAPD/PCR conditions

The PCR reaction was performed with primer M13 with the following sequence 5′ GAGGGTGGCGGTTCT 3′[16][51]; the primer was synthesized by Stabvida (Caparica, Portugal) and provided desalted and lyophilized. Primers were reconstituted with TE Buffer (Life Technologies, Carlsbad, California, USA) to a concentration of 50 µM (stock solution) and stored at -20 ºC until its use. Briefly the reaction was performed in 13.625 µl total volume containing 1.25 µl of DNA template, 6.875 µl of NZYTaq 2× Green Master Mix (Nzytech, Lisboa, Portugal), 0.275 µl of primer (1.01 µM), and 5.225 µl nuclease-free water. The mixture was amplified in T100TM Thermal Cycler (Biorad, Hercules, Califórnia, USA) with the following conditions: initial denaturation at 94ºC for 5 minutes, followed by 40 cycles (denaturation at 94º C for 1 minute, annealing at 45 ºC for 20 s, elongation at 72ºC for 2 minutes) and a final elongation step at 72ºC for 7 minutes. Amplification

products were loaded in a 1% agarose (agarose for gel electrophoresis LONZA Rockland, ME USA) gel, supplemented with 0.03 µl/mL Midori Green Advanced DNA Stain (Grisp, Porto, Portugal) and horizontal electrophoresis performed in 1× TAE buffer (Thermo fisher scientific) at 120 V for 60 minutes. DNA fragments were then visualized at UV Transiluminator (UVITEC Cambridge, England). Automated analysis of the obtained genetic profiles was performed with program InfoQuest FP (Bio-Rad, California, USA). Dendrograms were performed using the UPGMA algorithm, with 1% optimization and 1,5% tolerance. Strains were considered related if they shared more than 80% similarity between their profiles and the same profile if they shared more than 90%.

Determination of fluconazole susceptibility profiles

The strains were subjected to fluconazole susceptibility testing by the broth dilution method, as recommended by the Clinical Laboratory Standards Institute (CLSI) [66], using fluconazole purchased from Sigma-Aldrich (Missouri, United States of America). The final concentration of drug in the plate varied between 0.125 and 512 µg/mL, and the experiments were performed as previously described [100]. The test was performed at pH 7-7.5 (following the recommended guideline) and pH 3.8-4.5 (considered the pH in mucosal vagina) [217]. Yeast isolates were considered susceptible to fluconazole when the minimum inhibitory concentration (MIC) was ≤ 2 µg/ml (*C. albicans*, *C. guilliermondii*) and susceptible dose-dependent when MIC ≤ 32 µg/ml (*C. glabrata*) (http://www.eucast.org/clinical_breakpoints/)[66].

Determination of the ability to form biofilms

The ability to adhere to a polystyrene surface and to form a biofilm was evaluated for all the isolates, according to adapted methodology from Leighann Sherry et al [99], and as previously described [100]. Two independent assays were performed for each isolate; the result was validated only when the standard deviation between the obtained Abs595nm was less than 0.15. By observing the isolates over time, according to the isolation period in each clinical case, we were able to evaluate whether there was an increase in absorbance values, a decrease in absorbance values or no variation (W / V). *Candida albicans* ATCC10231 was included as a positive

control in each assay, which has been previously described as an adherent yeast isolate [167]. The isolates were classified as “moderately adherent” if the Abs595nm of their biofilm was in the range of the one obtained for ATCC10231 (0.62-0.82); and “strongly adherent” if the Abs595nm of their biofilm was superior to 0.82. If the strains could form biofilm that corresponded to at least 50% of the ability of ATCC10231 to form biofilm (Abs595nm 0.3-0.62), then they were classified as “weakly adherent”. Finally, “non-adherent” isolates were considered when Abs595nm was lower than 0.3. Two independent assays were performed for each isolate; the result was validated only when the standard deviation between the Abs595nm obtained was less than 0.15 or when both assays gave a consistent result regarding the classifiers described above.

Data analysis

The statistical analysis was performed in GraphPad Prism 7.0 Software (San Diego, United States of America), in which the MIC values obtained for each pH were compared and assessed for significative difference using Chi-square Independent Test, being considered the variables significantly dependent, assuming p values < 0.05. Regarding the results of biofilms, these were correlated with MIC and considered a significant correlation for p values below 0.05.

RESULTS

Species identification and molecular typing of the strains

In statistically recurrent vulvovaginal candidosis cases (RVVC) (Statistically cVVC), our sample was composed by 24 isolates obtained from 5 clinical cases and identified as follows: 25% *Candida albicans* (6 isolates), 41.6% *Candida glabrata* (10 isolates) and 33.3% *Candida guilliermondii* (8 isolates). In these 5 clinical cases, recurrence was caused by unrelated strains in various cases 3/5 (29.4%), while more than one species were involved in some of infection episodes (Figure 1). By analyzing the molecular types of the isolates involved in the infection for each clinical case, we verified that 3/5 cases of statistical recurrence were caused by strains of completely different isolates,

and 2/3 of these cases had at least two related isolates during the recurrent episode (Table 2).

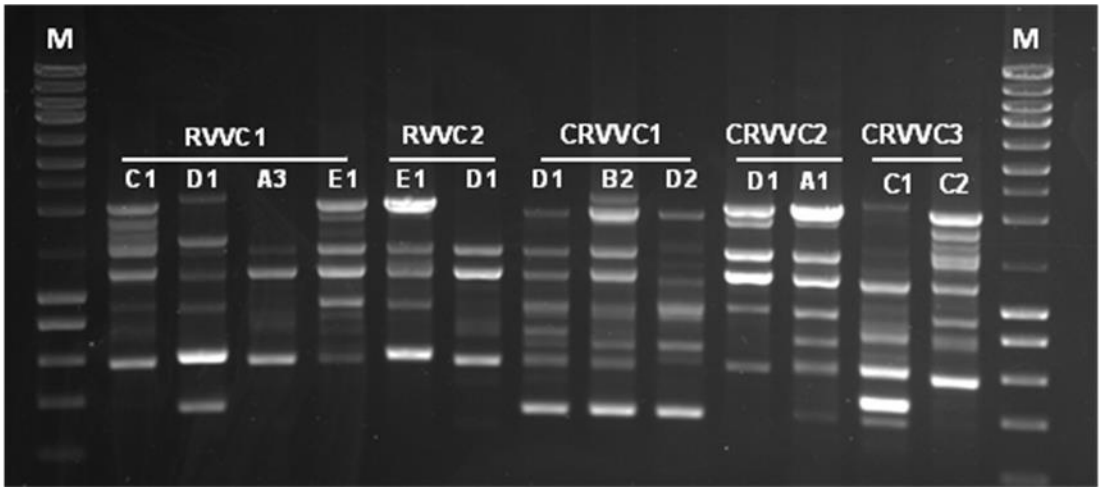


Figure 1. Example of result from Polymorphic DNA Amplification. RVVC1-2: statistically recurrent VVC (stastistically chronic cVVC); CRVVC1-3: clinically recurrent VVC (clinically cVVC)[218].

The clinically recurrent vulvovaginal candidosis cases group (C-RVVC) (Clinically cVVC) comprised 38 isolates obtained from 12 clinical cases and identified as follows: 47.4 % *Candida albicans* (18 isolates), 31.6% *Candida glabrata* (12 isolates) and 21.1% *Candida guilliermondii* (8 isolates). By polymorphic analysis of the DNA of isolates involved in the infection for each clinical case, we found that 6/12 cases of clinical recurrence were caused by strains of completely different isolates while the remaining 6 of those cases had at least two related isolates during recurrence (Table 2).

Table 2. Relationship between the isolates of *Candida* spp. in cases of statistical vulvovaginal candidosis (RVVC) (stastistically chronic cVVC) and clinically recurrent vulvovaginal candidosis (C-RVVC) (clinically chronic cVVC)

Code	N /I	Species involved	Dominant species	Relationship of isolates from same species
RVVC1	4	*C. albicans, C. glabrata, C. guilliermondii*	*C. albicans*	Non-related isolates
RVVC2	2	*C. albicans, C. guilliermondii*	-	Different species
RVVC3	12	*C. albicans, C. glabrata, C. guilliermondii*	*C. glabrata*	6 isolates of same individuals of *C. glabrata* subdivided in 3/3
RVVC4	3	*C. albicans, C. guilliermondii*	*C. albicans*	Non-related isolates
RVVC5	2	*C. guilliermondii,*	*C. guilliermondii*	2 related isolates

Code	N /I	Species involved	Dominant species	Relationship of isolates from same species
C-RVVC1	3	*C. glabrata, C. guillermondii*	*C. guilliermondii*	Related isolates
C-RVVC2	2	*C. albicans*	*C. albicans*	Non-related isolates
C-RVVC3	2	*C. albicans, C. glabrata*	-	Different species
C-RVVC4	4	*C. albicans, C. glabrata*	*C. albicans*	2 isolates of same strain
C-RVVC5	5	*C. albicans, C. glabrata,*	*C. glabrata*	4 related isolates
C-RVVC6	7	*C. albicans, C. glabrata*	*C. albicans*	2 isolates of same strain and two related
C-RVVC7	2	*C. albicans*	-	Diferent species
C-RVVC8	2	*C. albicans*	*C. albicans*	Non-related isolates
C-RVVC9	3	*C. albicans, C. glabrata, C. guillermondii*	-	Diferent species
C-RVVC10	2	*C. albicans, C. glabrata*	-	Different spcies
C-RVVC11	6	*C. albicans, C. glabrata, C. guillermondii*	*C. guillermondii*	2 isolates of same strain of *C. guilliermondii*
C-RVVC12	2	*C. guillermondii*	*C. guillermondii*	2 isolates of same strain

N/I: number of isolates

Determination of antifungal susceptibility profiles and ability to form biofilm overtime

Globally, the determination of the Minimum Inhibitory Concentration (MIC) revealed that 38.70% of isolates were resistant to fluconazole at pH 7, while at pH 4.5 the number of isolates resistant to fluconazole increased to 59.67%. The species *C. glabrata* showed the highest resistance capacity at pH 4.5 (reaching MIC values between 64 - 512µg/mL), but with a considerable variation according to the pH value, showing a significant increase in the MIC value for the level of resistance at pH 7 (resistance between concentrations 8-64 µg/mL); *C. guilliermondii* was also more resistant at pH 4.5, (with MIC values between 16-32 µg/mL for a pH 7 and between 128-512

µg/mL for pH 4.5) while *C. albicans* was more susceptible at this pH (MICs mostly between 8 - 16 µg/mL for pH 4.5 and for pH 7 the MICs were between 8 - 512 µg/mL).

The evaluation of the expression of virulence factors in *Candida* species is important, as they contribute to the antifungal resistance of these microorganisms. We assessed changes in susceptibility and in the ability to form biofilms over time, using clinical cases in which we found at least two equal or related isolates (8/17 cases: 2 RVVC; 6 C-RVVC), but restricting the analysis to only these same or related isolates (24 isolates) (Figures 2 and 3).

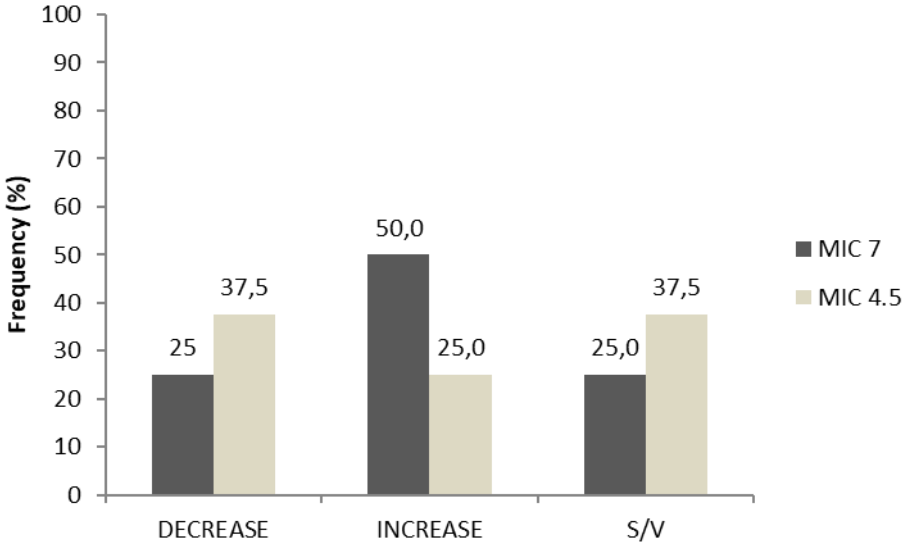


Figure 2. Variation of MIC over time in chronic cases of vulvovaginal candidosis (VVC) in 8 cases with related or equal isolates. Numbers indicate percentage of strains. W/V: without variation. RVVC (statistically recurrent VVC) (stastistically cVVC), C-RVVC (clinically recurrent VVC) (clinically cVVC). RVVC3; RVVC5. C-RVVC1; C-RVVC4; C-RVVC5; C-RVVC6; C-RVVC11; C-RVVC12. [218]

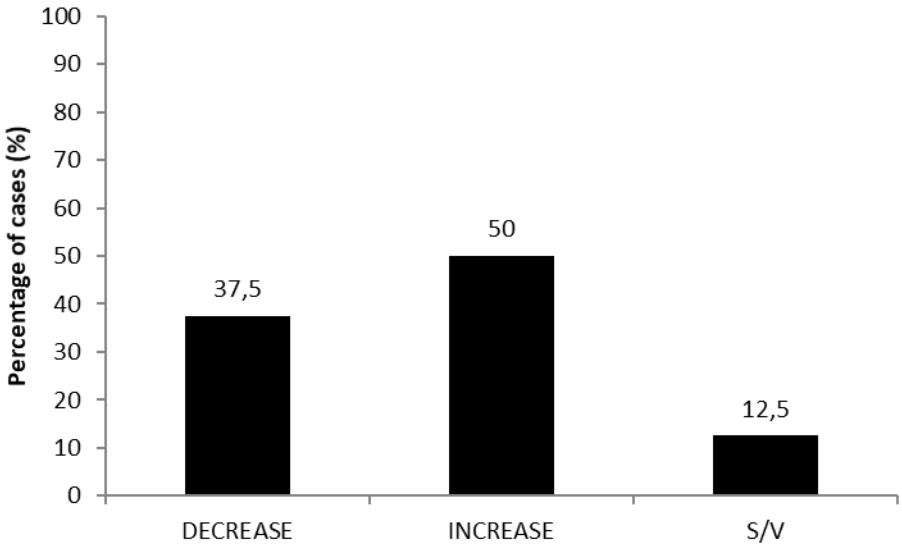


Figure 3. Trends in the capacity to form biofilms (%) for the equal or related isolates of 8 clinical cases. W/V: without variation. RVVC (statistically recurrent VVC) (stastistically cVVC), C-RVVC (clinically recurrent VVC) (clinically chronic cVVC). RVVC3; RVVC5. C-RVVC1; C-RVVC4; C-RVVC5; C-RVVC6; C-RVVC11; C-RVVC12.[218]

For this, we evaluated the averages of the results obtained from two independent assays, where there was an increase as follows. At pH 7 there was an increase registered in 2/8 (25% of cases); at pH 4.5 there was an increase in 2/8 (25%); in respect to the ability to form biofilms there was an increase in 4/8 cases corresponding to 50%. Otherwise, when there was a decrease, for pH 7 in 2/8 (25% of the cases) and for pH 4.5 in 3/8 (37.5%), the ability to form biofilms decreased in 3/8 (37.5%). In those cases where we did not see changes, at pH 7, 2/8 (25%) cases and at pH 4.5, 3/8 (37.5%) cases; the ability to form biofilms in 1/8 (12.5%) cases.

The strains sensitivity to fluconazole showed that there was significant differences between the MIC value at pH 7 when compared to that at pH 4.5, using Qui square (p-value<0,0001). We correlated susceptibility to antifungal agents with the ability to form biofilms, using the values of observations from 24 isolates from 8 clinical cases with the same or related isolates. We obtained a proof value of -0.277 and -0.322 correspondent to the correlation of mean observances of the capacity to form biofilms for MIC 4.5 and 7, respectively. Then, we concluded that there is a non-significant correlation between resistance to antifungal agents and the ability to form

biofilms by the yeasts recovered from the studied specimens. However, when these values are categorized by species, we observed a significant correlation between MIC at pH 7 and the ability to form biofilm, obtaining a proof value equal to 0.002 for *C. glabrata*. We also observed an increase in the ability to form biofilms at pH 7, implying an increase in resistance to fluconazole (Table 3).

Table 3. Correlation of MICs and the capacity to form biofilms by species (p values)

	Culture conditions	**Correlation between MIC & Biofilms (P value)**
Candida albicans	MIC pH =4.5	0.3667
	MIC pH =7	0.6667
Candida glabrata	MIC pH =4.5	0.2182
	MIC pH =7	**0.002**
Candida guilliermondii	MIC pH =4.5	0.4702
	MIC pH =7	0.7611

For the 8 cases where we verified sequential strains, in Supplementary Tables 1 and 2 we see that *Candida albicans* presented low average MICs. We can also see little fluctuation between strains. Regarding to biofilm formation, we observed a fluctuation over time, between strains, with a tendency to increase over time specifically in the case with a greater number of strains – C-RVVC4 (supplementary Table 1). The non- *C. albicans* strains presented an increase in the mean MIC50 at pH 4.5, when compared with pH 7, which reinforces the idea that at acidic pH there is a tendency towards increasing resistance (Supplementary Table 1 and 2). Regarding biofilm formation, it was observed for the two non- *C. albicans* species, a trend towards fluctuations over time, with a trend towards an increase in the biofilm production capacity (Supplementary Table 1).

Supplemental Table 1. Summary data on assessment over time (biofilms and MICs).

Code	Species	Sequence	Biofilms Abs)	MIC at pH 7.0 (µg/mL)	MIC at pH 4.5 (µg/mL)
C-RVVC4	*C. albicans*	1	0.57	*2*	1
		2	1.22	*4*	1
		3	1.67	*2*	1
		4	0.98	*16*	0.5
RVVC5	*C. guilliermondii*	1	1.08	*32*	128
		2	0.21	2	8
C-RVVC1	*C. guilliermondii*	1	1.24	2	1
		2	0.55	16	8
C-RVVC11	*C. guilliermondii*	1	0.16	*2*	128
		2	0.34	*32*	8
C-RVVC12	*C. guilliermondii*	1	0.82	*16*	128
		2	0.72	*8*	512
C-RVVC6	*C. albicans*	1	1.35	*64*	8
		2	1.34	*64*	8
RVVC3	*C. glabrata*	1	0.21	*8*	128
		2	0.75	*16*	512
		3	0.92	*4*	64
		4	0.51	1	8
		5	0.45	*4*	128
		6	0.69	2	2
C-RVVC5	*C. glabrata*	1	0.23	2	0.5
		2	0.40	4	64
		3	0.81	*8*	64
		4	0.41	*8*	2

Supplemental Table 2. Geometric mean MIC, MIC range and MIC_{50} (µg/ml) of fluconazole for 24 *Candida* strains tested at pH 4.5 and pH 7.

	Fluconazole pH 7			Fluconazole pH 4.5 (µg/ml)		
Species (no isolates)	**GM**	**MIC Range (µg/ml)**	**MIC_{50}**	**GM**	**MIC Range (µg/ml)**	**MIC_{50}**
***Candida albicans*(6)**	10	1-64	25	2.3	0.5-8	3.8
***Candida glabrata*(10)**	4.3	2-16	5.7	29	0.5-512	115
***Candida guilliermondii* (8)**	8	2-32	13.75	35	1-512	108
Total 24						

Discussion

Vulvovaginal candidosis has a high prevalence in women of childbearing age and half of these women are susceptible to develop recurrence [164]. This infection is usually caused by a single species at a time, but two or more species may be involved simultaneously [164]. In this work, we do not focus on colonization, because the isolates were obtained during periods of infection, with symptoms and clinical evidence that led us to classify the cases as candidosis. Chatzivasileiou P. and Vyzantiadis TA, gathered, in a literature review, several opinions on the definition of colonization, which was settled by the asymptomatic presence of species of Candida spp. in the vaginal tract[9]. We, instead, focus on chronic cases of vulvovaginal candidosis, considered to be clinically active. However, these definitions have not been used systematically and therefore the true prevalence of chronic vulvovaginal candidosis and its main characteristics are difficult to assess.

In our study, many of the isolates involved in the infection, were *C. albicans*; in a total of 62 *Candida* isolates we identified 24 isolates as *C. albicans*, about one third of samples. Globally it has been described that vulvovaginal candidosis, in general as well as when recurrent, is caused by this species in more than half of cases, being the most frequently isolated in this type of infection and also the best studied and categorized [186][99]. In this series the most prevalent is *C. albicans*, followed by non-albicans species such *as C. glabrata*, as described by various others authors [211][160][219].

L. Latey Bradford in 2017, investigated the genomic variations of *Candida* spp., over the course of an infection, based on the principle of the importance of knowing the role of strains variability in the persistence of the infection. There were few variations among *C. albicans* isolates collected at two times of the infection [220]. Amouri I *et al*, found also that recurrent *C. albicans* isolates showed little genetic variation [221]. As the present study was based on symptomatic clinically recurrences the isolates were not collected for all women at standardized periods, thus probably allowing to identify species and strain variations along time. Nonetheless, our study may probably better reflect what ecologically and clinically happens in recurrent vulvovaginal candidosis. In fact, in the majority of cases, both in the statistically recurrent group and in the clinically recurrent group, more than one species of *Candida*

was involved, being *C. albicans* the most frequent in number and distribution over the clinical cases. On the other hand, the non-*Candida albicans* species showed to be more related to recurrence and were the most persistent. Specifically, *C. guillermondii* was responsible for 4 out of the 8 cases of recurrence were there was persistence, in our study. Interestingly, the incidence of this species has been described as low in vaginal infections in northern Portugal (2.8%) [214] and as been reported as being rarely involved in VVC in Portugal [222][223]. Still, data regarding the prevalence of this species is scarce, and the existing studies remote to 2004-2009, therefore the possibility for an increase in the prevalence of this species could not be excluded.

During a vaginal infection with *Candida* spp., the vaginal pH is acidic, approximately equal to 4; in order to maintain the existing conditions during the infection, the same pH value should be adjusted in in vitro assays[224]. It was in this context that we performed a comparison between MIC values by performing the determinations using the current culture medium at two different pHs (neutral = 7 and acid = 4.5) for the reference antifungal (fluconazole) used for vulvovaginal candidosis [225]. The variability of activity of antimicrobials at different pH has already been evaluated for different microorganisms. Results previously obtained in bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, indicate their greater resistance to antimicrobials at acidic pH [226]. Claire S. Danby *et al*, assessed the effect of pH in antifungals susceptibility of *C. albicans* and *C. glabrata*, verifying a significant difference in susceptibility of *Candida glabrata* when testing at neutral and acidic pH, concluding that it is important to evaluate the susceptibility in vitro also in acidic pH for this species [76]. These results coincide with the ones we obtained for non-albicans *Candida* species when determining the susceptibility to fluconazole at acidic pH, since there were significant differences when we evaluated the fluconazole susceptibility patterns of these species at neutral and acidic pH, presenting a higher level of resistance at acidic pH.

In this particular study, we verified that cases of persistence of the strains in the vaginal mucosa is mostly associated to non-*albicans Candida* species, which also showed major variability in resistance to fluconazole, at both the different levels of adjusted mean pH used, what goes in accordance the study

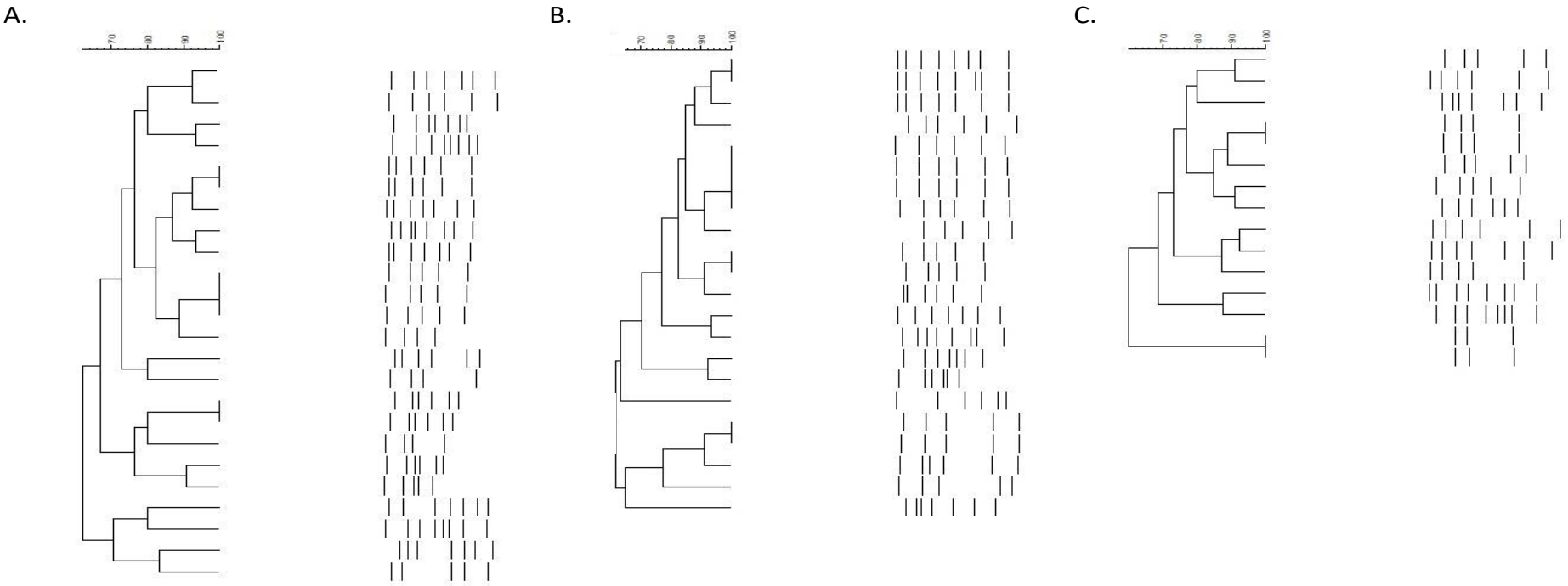
conducted by Mark Spitzer *et al*,[224]. Contrary to what Houdaii H. El-Houssaini, *et al*, in our study we did not observed significant relationship between MICs and the capacity to form biofilms, what makes us to hypothesize that the resistance to antifungal agents is not dependent directly to the capacity to form biofilms in these *C. albicans* strains, under these conditions[91]. With non-albicans *Candida* species, we only found significant differences in the correlation between the ability to form biofilms and the MIC value for *C. glabrata* species. By considering related strains only by characterizing polymorphic DNA using RAPD/PCR, the results showed that the majority of the strains tend to increasingly express biofilm over time. Correlating this virulence factor with resistance to fluconazole from the 24 related strains, we did not find any significant relationship, allowing us to conclude that the increase of expression of biofilm is not a determinant factor for the increase of resistance in this group, except for *C. glabrata*. Nevertheless, the increased expression of biofilm could be a cause for persistence in these clinical cases.

Our results indicate a variation in the behavior of the strains, when we take into account the treatment used in each clinical case, with an increase in MIC in some cases and a decrease in others, after treatment with azoles. We can also hypothesize, based on these results and the consulted bibliography that topical or oral treatment with specific antifungals could induce some resistance in the collected isolates, that could afterwards be observed in the performed in vitro susceptibility tests. In particular, Sofia Costa de Oliveira (2020) has described the acquired (or secondary) resistance after antifungal treatment, along with two other types of resistance, as one of the three mechanisms of resistance to azoles [227].

The modulation of the parameters evaluated along time was probably greatly influenced in this work, due to the small number of related or equal isolates in each clinical case. Our results were obtained from a small collection of isolates of women attending a private practice. Probably extending the methodology to a larger collection, other outcomes could be obtained. Nevertheless, we believe that changes in the expression of virulence factors such as biofilm production and azole resistance of the isolates along time may be important to understand the cause of the recurrence. In short, in spite of being a small sample, we suggest that by evaluating genotypic evaluation,

expression of virulence factors and MICs along time, important initial results can be obtained that can provide evidence for a possible indicator of recurrence (chronicity).

SUPPLEMENTAL MATERIAL



Supplemental Figure 1. Dendrogram depicting phylogenetic relationships between *Candida* spp. isolates (A: *Candida albicans*; B: *Candida glabrata*; C: *Candida guilliermondii*) in this study, by RAPD fingerprinting. [218].

Chapter 6. Chronic vulvovaginal *Candida* spp. clinical isolates are more resistant to phagocytosis *in-vitro*

Paula Faria-Gonçalves1,2,3, Ana Sofia Oliveira1,2, Carlos Gaspar1,2,4, Rita Palmeira-de-Oliveira1,2,4,5, José Marti-nez-de-Oliveira1, Teresa Gonçalves5, Ana Palmeira-de-Oliveira1,2,4 and Joana Rolo1*

(Submitted for publication)

Chronic vulvovaginal *Candida* spp. clinical isolates are more resistant to phagocytosis in-vitro

Abstract

Previous studies have revealed that *Candida albicans* isolates involved in chronic vulvovaginal candidosis (cVVC) phenotypically express less virulent traits than clinical isolates involved in sporadic infections. In this study, we aimed to further explore this finding by studying the behaviour of those same clinical isolates in in-vitro models of infection. Eighteen clinical *Candida albicans* isolates were collected from women suffering sporadic (eight isolates) or chronic infections (ten isolates). Adhesion to HeLa cells (human cervical cancer epithelial cell line) and resistance to phagocytosis by RAW 264.7 cells (murine macrophages cell line) were tested in-vitro. In addition, phenotypic expression of virulence factors related with either adhesion or resistance to phagocytosis was tested in-vitro. Results indicated that yeast isolates involved in sporadic infection adhered in a higher proportion of HeLa cells than those of chronic infections, which was related with their ability to produce biofilm ($p < 0.05$). The ability to evade phagocytosis was related to an elevated production of proteases ($p < 0.05$) by chronic isolates, while sporadic isolates' resistance to phagocytosis was related to a higher hydrophobicity of cell walls ($p < 0.05$). We conclude that the evasion of macrophage-mediated phagocytosis related to the production of proteases might be an important factor involved in the recurrence of vulvovaginal candidosis infection.

Keywords: adhesion; macrophages; virulence; vulvovaginal; yeast

Introduction

Vulvovaginal candidosis (VVC) is an affliction affecting millions of women worldwide [228]. It is most frequently caused by *Candida albicans*, a dimorphic yeast [57]. Other species of *Candida* can also cause this affliction, namely *Candida glabrata* [168]. VVC is characterized by an overgrowth of yeast in the vaginal mucosa, leading to swelling, irritation and excessive vaginal discharge. *Candida* spp.' infection of the vaginal mucosa has been related with its capacity to form biofilms [17], to produce germ tubes (exclusively for *C. albicans*) and to produce extracellular enzymes such as phospholipase and proteases [45]. In addition, evasion of the host immune system and consequent inflammation has also been identified as a virulence factor contributing to Candida spp.

pathogenesis in this specific niche. Particularly, epithelial invasion and immune cell infiltration have been found to be key developments for the progression of the disease. Neutrophil recruitment has been related with the onset of symptomatology [229], particularly for Candida albicans, which appears to be especially vaginopathogenic [230], due in part to its ability to produce hyphae and to express candidalysin, a potent exotoxin [230] [193].

Re-infection of the vaginal mucosa can occur, a condition commonly known as recurrent vulvovaginal candidosis or chronic candidosis (cVVC). cVVC is characterized by the occurrence of 3 or more episodes in the period of one year [159], causing high morbidity and burden to infected patients. Several risk factors have been implied as contributors to recurrence like pregnancy and diabetes mellitus. However, other than the increase on immune cell recruitment already reported [229], the cues used by Candida spp. to persist in the mucosa are largely elusive. Previous studies by our research group have identified that chronic *Candida* isolates are weak biofilm and germ tube producers, when compared with isolates involved in sporadic infections [100]. A recent study has revealed that vaginal isolates show adaptative behavior in their interaction with macrophages in-vitro, reflective of their infective ability [231]. In this work, we aim to further explore differences in the immune pathogenicity of *Candida* spp. isolates involved in cVVC by studying their interaction with epithelial and macrophage cell lines.

Materials and Methods

Yeast isolates

Twenty-two vulvovaginal candidosis isolates were included in the study. The isolates were obtained from 16 women attending a gynaecological consultation at a private clinic. The samples used for diagnosis that were going to be disposed were included in this study. The study was approved by the Ethics Commitee of the research centre where the experiments were conducted (CE-UBI-Pj-2018-022). The strains have been fully characterized in previous studies, including determination of their susceptibility to fluconazole and phenotypic expression of virulence factors [168][100] (Table 1). A *Candida albicans* type strain, ATCC 10231 was used in all experiments as internal control. The isolates were sub-cultured twice at 37ºC in Sabouraud Dextrose Agar (VWR Avantor, United States of America) before each experiment to access viability.

Table 1. Characterization of the collection of strains used, as reported in [168].

Isolate	Patient	Species	cVVC/sVVC	Fluconazole Susceptibility	Biofilm Formation
cVVC1	P1	*C. albicans*	cVVC	Resistant	Strongly adherent
cVVC2	P1	*C. albicans*	cVVC	Resistant	Strongly adherent
cVVC3	P1	*C. albicans*	cVVC	Resistant	Strongly adherent
cVVC4	P2	*C. albicans*	cVVC	Resistant	Strongly adherent
cVVC5	P3	*C. albicans*	cVVC	Resistant	Strongly adherent
cVVC6	P3	*C. albicans*	cVVC	Resistant	Non adherent
cVVC7	P4	*C. albicans*	cVVC	Susceptible	Strongly adherent
cVVC8	P5	*C. albicans*	cVVC	Susceptible	Weakly adherent
cVVC9	P6	*C. albicans*	cVVC	Resistant	Weakly adherent
cVVC10	P7	*C. albicans*	cVVC	Susceptible	Strongly adherent
sVVC1	P8	*C. albicans*	sVVC	Susceptible	Strongly adherent
sVVC2	P9	*C. albicans*	sVVC	Susceptible	Adherent
sVVC3	P10	*C. albicans*	sVVC	Resistant	Strongly adherent
sVVC4	P11	*C. albicans*	sVVC	Susceptible	Weakly adherent
sVVC5	P12	*C. albicans*	sVVC	Resistant	Strongly adherent
sVVC6	P13	*C. albicans*	sVVC	Resistant	Strongly adherent
sVVC7	P14	*C. albicans*	sVVC	Resistant	Strongly adherent
sVVC8	P15	*C. albicans*	sVVC	SDD	Strongly adherent

cVVC: chronic vulvovaginal candidosis; sVVC: sporadic vulvovaginal candidosis. SDD: susceptible dose-dependent.

Determination of the ability to produce proteinase

The production of phospholipase production was evaluated with the procedure described in Raja Vinodhini et al, 2016 [197]. A cell suspension was prepared in sterile PBS at 0.5 MacFarland as described above and inoculated (10 µl) in proteinase agar plates containing 2% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 2 % agar and 1% BSA solution in distilled water. The plates were incubated for 10 days at 37° C and the reading of enzyme activity (Pz) was determined by calculating the ratio between the diameters of colony versus the precipitation zone. According with the literature [13], the lower Pz value, the higher the enzymatic activity. When Pz = 1.0: means the absence of enzy-matic activity; when $0.64<Pz<1.0$: there is a positive enzyme activity; and when $Pz \leq 0.63$: there is a strongly positive enzymatic activity. C. albicans ATCC 10231 was used in each assay as the positive control ($Pz \leq 0.63$). Two independent assays were performed for each isolate,

being the result only validated when the standard deviation between the results obtained in each assay was less than 0.1.

Cellular surface hydrophobicity

The comparative analysis of cell surface hydrophobicity was determined as previously described [232]. A suspension of yeast cells was prepared in 10 mL of sterile PBS at 0.5 MacFarland that was distributed into three test tubes (1.3 mL each). Afterward, 100 µL from each of these test tubes were distributed into the wells of 96 well microtiter plates and initial OD was read at 620 nm using in a microplate reader (Anthos 2020 microplate reader, Bio-Rad, California, United States of America). Then, 0.3 mL of xilene (VWR Chemicals) was added to the remaining cell suspension (1.2 mL) in each tube, mixed vigorously for 3 min and allowed to separate for 15 min. 100 µL of the lower aqueous phase was carefully added to the wells of the microtiter plate and final OD was recorded at 620 nm. Tubes without cells were served as control. Triplicates were used for each sample and the experiment was repeated thrice. The percentage cell surface hydrophobicity (CSH) was calculated by using the following formula: Percentage CSH = (1 – final OD of aqueous phase/initial OD of cell suspension) × 100 and results were presented as percentage of CSH ± SD (standard deviation).

pH survival test

Cellular suspensions at 0.5 McFarland were prepared in 2 mL YPD (Fisher Bioreagent) adjusted at pH 5, pH 7 and pH9 at 0.5 McFarland. 20 µl was deposited in triplicate to 3three wells of a 96 well plate (initial suspension), serial diluted and plated in SDA to CFU count. The remaining volume of the suspension was placed in a 10 mL sterile glass tube and incubated in the orbital shaker (Argitob 200 Aralab, Sintra, Portugal) at 37º for 24 hours at 180 rpm. After 24 hours, the cultures at different pHs were re-moved from the orbital shaker and the number of surviving yeast was estimated by plating aliquots of serially diluted cultures in SDA, and incubating at 37ºC for 24h. A comparison was made of the result of CFU/mL obtained at different pH with the initial suspension.

Cell culture and co-culture assays

RAW 264.7 cells, a mouse leukaemic monocyte macrophage cell line from the European Collection of Authenticated Cell Cultures (ECACC, UK; Catalogue number 91062702), was included in this study. Cells were cultured in Dulbecco′ s Modified Eagle′ s Medium-DMEM (Gibco, Alfagene, Lisboa, Portugal) with 4.5 g/L of glucose (Sigma-Aldrich, St. Louis, MO, USA), and supplemented with 10% (*v*/*v*) of non-inactivated Fetal

Bovine Serum-FBS (Gibco, Alfagene, Lisboa, Portugal), 100 U/mL penicillin, 100 μ g/mL streptomycin (Gibco, Alfagene, Lisboa, Portugal), and 1.5 g/L of sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Sub-confluent cultures (~70–80%), were split using cell scrappers to remove attached cells, according to ECACC recommendations. RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% inactivated FBS, 23.8 mM sodium bicarbonate and 50 mM glucose was used for the experimental assays. For the co-culture assays with RAW 264.7 cells, a previously optimized procedure was performed [233][234]. RAW 264.7 cells, a mouse leukaemic monocyte macrophage cell line from the European Collection of Authenticated Cell Cultures (ECACC, UK; Catalogue number 91062702), was included in this study. Cells were cultured in Dulbecco′ s Modified Eagle′ s Medium-DMEM (Gibco, Alfagene, Lisboa, Portugal) with 4.5 g/L of glucose (Sigma-Aldrich, St. Louis, MO, USA), and supplemented with 10% (v/v) of non-inactivated Fetal Bovine Serum-FBS (Gibco, Alfagene, Lisboa, Portugal), 100 U/mL penicillin, 100 μ g/mL streptomycin (Gibco, Alfagene, Lisboa, Portugal), and 1.5 g/L of sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Sub-confluent cultures (~70–80%), were split using cell scrappers to remove attached cells, according to ECACC recommendations. RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% inactivated FBS, 23.8 mM sodium bicarbonate and 50 mM glucose was used for the experimental assays. For the co-culture assays with RAW 264.7 cells, a previously optimized procedure was performed [235]. Briefly, a cellular suspension of *Candida* spp. prepared in DMEM-F12 culture medium was incubated with a layer of HeLa cells with a multiplicity of infection (MOI) of 10, during 2 h. After removal of the non-adherent yeast cells with PBS, the adherent yeast cells were estimated by plating an aliquot of Trypsin-EDTA-treated supernatants on SDA. Colonies were counted after 48 h of incubation.

Statistical analysis

Differences between clinical groups (cVVC vs. sVVC) were assessed with the non-parametric Mann-Whitney test. In addition, the correlation between the parameters observed within each clinical group was determined using the Spearman test (one-tailed *p*-value). Differences were considered statistically significant when the *p*-value was below 0.05 (95% confidence interval).

Results

One of the main objectives of this work was to tackle differences between cVVC and sVVC in what regards phenotypes specifically described as determinants for virulence in *Candida albicans*. In this work, we studied the proteolytic activity, the cell wall hydrophobicity, and the ability to survive at different pH values. Overall, we found that cVVC isolates seemed to be more virulent than sVVC isolates as they produced more proteases (as noted by lower Pz values) and the hydrophobicity of their cell wall showed to be slightly higher (Figure 1A,B and Supplementary Figure S1). Regarding the pH survival test, we found that, at pH 9, cVVC isolates were more resistant, since cellular multiplication was not impaired at this condition (Figure 1C). The isolates belonging to the two clinical groups also behaved differently when in contact with cell lines. Specifically, sVVC isolates adhered better to Hela epithelial cells, as indicated by the higher number of CFU/mL that was recovered after the co-culture (Figure 2). Regarding the interaction with RAW 264.7 macrophages, we found that both cVVC isolates and sVVC isolates were able to escape phagocytosis, as evidenced by the high number of non-internalized yeasts (when compared with the initial suspension) and to remain viable even after internalization, although not to a great extent (Figure 2). However, we found that there were no statistical significant differences between the phenotypic expression of virulence factors among the two clinical groups (cVVC and sVVC; $p > 0.05$) (Figure 1), as well as the interaction with cell lines ($p > 0.05$).

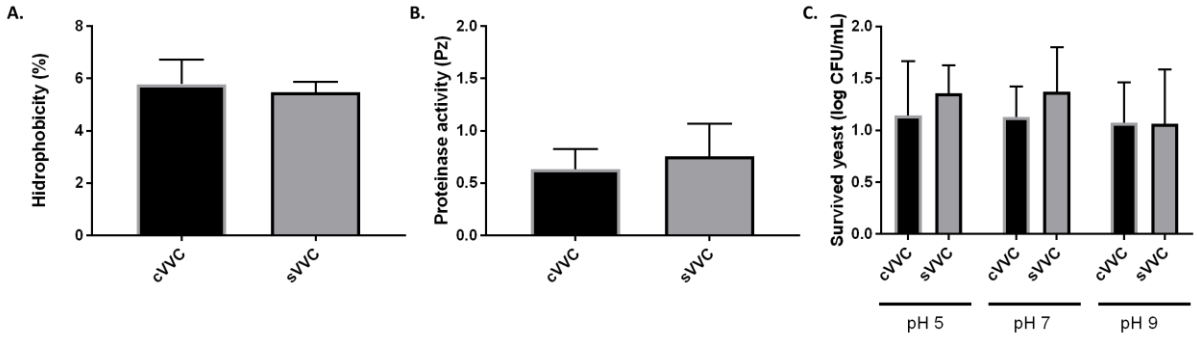


Figure 1. Phenotypic expression of virulence factors of the clinical isolates. (**A**) Hydrophobicity (%) of cell walls. (**B**) Proteinase activity (Pz value). (**C**) pH survival assay; the log difference (log CFU/mL) regarding the initial suspension is shown. In all assays, differences between clinical groups were found to be non-significant ($p > 0.05$). cVVC: chronic vulvovaginal candidosis isolates; sVVC: sporadic vulvovaginal candidosis isolates.

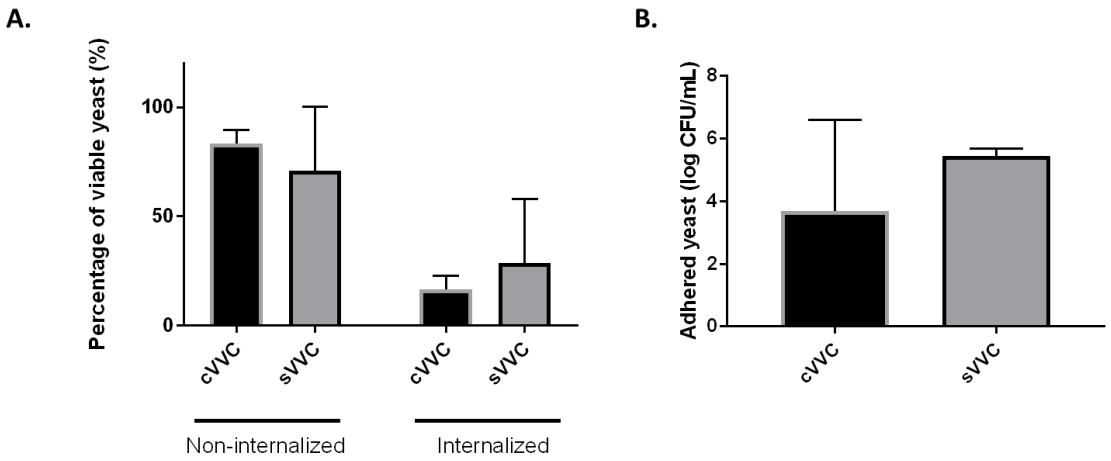


Figure 2. Interaction of clinical vulvovaginal isolates with cell lines. (**A**) Percentage (%) of non-internalized and internalized viable yeast cells, compared with the initial suspension, after infection of RAW 264.7 cells with an MOI of 1 during 3 h. For each isolate, three infections assays were performed (n = 3), and each in triplicate. (**B**) Number of adhered yeast cells (log CFU/mL) after infection of HeLa cells with an MOI of 10. In both RAW264.7 and HeLa assays, differences between the two clinical groups were found to be non-significant ($p > 0.05$). cVVC: chronic vulvovaginal candidosis isolates; sVVC: sporadic vulvovaginal candidosis isolates.

The correlation, for each clinical group, between the results obtained with the co-culture assays and the phenotypic expression of virulence factors was assessed using the Spearman test (Table 2). In regards to the assays with macrophages, we found that for the cVVC isolates there was a significant correlation ($p < 0.05$) between the percentage of viable internalized yeasts and proteinase activity. On the other hand, in the sVVC group, a significant correlation ($p < 0.05$) was observed between the proportion of viable internalized yeasts and the level of hydrophobicity of yeast cell walls. In the epithelial-adherence test (co-culture with HeLa cells), we found a significant correlation ($p < 0.05$) between the number of CFU/mL recovered after incubation and the ability to form biofilms in-vitro (as determined in Table 1 [100]) only for the sVVC clinical group (Table 2).

Table 2. Correlation of the results obtained in infection assays (RAW: macrophages, RAW 264.7 cells; Hela: epithelial, HeLa cells) with the results obtained for phenotypic expression of virulence factors. *p*-value according to the Spearman test is shown. PP: proteinase production; CWH: cell wall hydrophobicity; BF: biofilm formation. Adapted with permission from [100]. Copyright license 5320780581818, June 2022. cVVC: chronic vulvovaginal candidosis isolates; sVVC: sporadic vulvovaginal candidosis isolates.

	Internalized (RAW)		**Adhered (HeLa)**	
	cVVC	**sVVC**	**cVVC**	**sVVC**
Parameter (*p*-value)	PP (0.0458)	CWH (0.0216)	No correlation	BF (0.033)

Discussion

We found that cVVC isolates are more homogeneous in their interaction with response to cell lines. However, the results obtained with these isolates were not statistically significant in comparison with the results obtained with sVVC isolates, which illustrates the importance of these virulence factors to the immunopathogene-city of vulvovaginal Candida spp. isolates as a whole, as already described [45]. Recent reports focusing on isolates from different clinical origins involved in vulvovaginal candidosis reached the same conclusions [231][236].

One of the processes involved in phagocytosis mediated by macrophages are abrupt pH differences [237]. Resistance to pH differences can also be related with the increased ability of the strains to invade the vaginal mucosa. Therefore, we sought to determine if differences in the pH survival would be correlated with the ability to resist macrophages. We found no significant differences between the two clinical groups, or no relevant correlation ($p > 0.05$). Therefore, resistance to pH could be a virulence characteristic that is expressed by vulvovaginal *C. albicans* isolates as a whole and not particularly important for chronicity.

The ability to produce proteases by both *C. albicans* and *C. glabrata* has been linked with their virulence potential [238][108]. The secretion of aspartatic proteases (SAP) has been implicated as a virulence factor leading to not only epithelial damage but also to inflammation [238][239]. Moreover, SAP have been also described as strong immunomodulators, independently of their proteolytic activity [238]. Therefore, to colonize and infect the vaginal niche, these strains would benefit from the phenotypic expression of this virulence factor [240], as illustrated by the high frequency of SAP genes found among isolates recovered from vaginal infections [241]. We did find statistical significant correlation between the ability to evade phagocytosis with elevated

production of proteases of cVVC isolates. SAP have been also described as strong immunomodulators, independently of their activity [238]. We hypothesize that strong protease producers such as cVVC isolates also resist better macrophage attacks, as described early on [242].

On the other hand, sVVC isolates' resistance to phagocytosis was related with a higher hydrophobicity of cell walls. The hydrophobicity of the cell wall is largely influenced by its composition. The composition of *Candida* cell wall has been also studied as a virulence factor. The cell wall is composed of glucose polymers 1,3 and 1,6 β-glucans, chitin, and mannosylated proteins. The differential expression and exposure of cell wall components are thought to be a major virulence factor influencing the pathogenicity of the yeast isolate [243][244]. In fact, hydrophobic cells are more adherent to epithelial tissues than hydrophilic cells [118]. Therefore, this result is related with our finding that sVVC isolates adhered in a higher proportion to HeLa cells, which was al-so related with their ability to produce biofilm ($p<0.05$). *Candida* biofilms and their relationship with vulvovaginal candidosis have been extensively studied [17]. Previous results obtained by us indicated that sVVC isolates were strong biofilm producers, comparing with cVVC isolates [10], which is in accordance with these results.

In this study, we did not find a statistically significant difference between the two clinical groups concerning yeast-macrophage interaction, in accordance with a recent report [231] describing that resistance to phagocytosis is highly strain-specific. However, these same authors describe that sVVC isolates change the composition of their cell walls when grown in a vaginal simulating medium containing lactate We hypothesize that isolates involved in sporadic infection have a more hydrophobic cell wall and produce a stronger biofilm, in order to adhere to epithelial cells and escape phagocytosis. On the other hand, cVVC are less adherent but produce a larger amount of proteases, that would help to invade the tissue and lead to inflammation, a major factor contributing to the progression of the disease [229].

Previous studies have highlighted differences related to the ability to switch to the filamentous form observed in *C. albicans* and not other *Candida* species, a characteristic that has been linked with the high virulence of *C. albicans* [245]. In fact, evasion of phagocytosis mediated by macrophages has been linked with the formation of hyphae [246], although other reports have identified macrophage evasion independently of filamentation [247]. Other studies have highlighted the importance of the pseudohyphae in the stimulation of the inflammasome [248]. The persistence of *C. albicans* in the mucosa has been mostly linked with its ability to form biofilms and to resist azole

treatment [249], but other unknown virulence factors could also be implicated. It would be interesting to test other virulence factors like the expression of candidalysin that have been implicated in the immunopathogenesis of VVC/cVVC [45]. Another important key aspect to test would be the quantification of cytokines by the cell lines that would lead to neutrophil infiltration [250] a key aspect for the progression of the disease.

We conclude that yeast strains involved in vulvovaginal candidosis showed an equal ability to evade macrophage-mediated phagocytosis and to adhere to epithelial cells. We also found that cVVC isolates´ability to evade macrophages might be related to the production of proteases, although further studies are needed to understand the role of this virulence factor in the recurrence of vulvovaginal candidosis

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Chapter 7. Sodium bicarbonate is an antifungal agent independently of pH: effect against vulvovaginal *Candida* spp.

Paula Faria-Gonçalves, Carlos Gaspar, Ana Sofia Oliveira, Rita Palmeira-de-Oliveira, José Martinez-de-Oliveira[1], Teresa Gonçalves[5,6], Ana Palmeira-de-Oliveira[1,2,4], Joana Rolo[1*]

(submitted for publication)

Sodium bicarbonate is an antifungal agent independently of pH: effect against vulvovaginal *Candida* spp.

Abstract

Candida albicans is an opportunistic pathogen involved in many infectious diseases, including vulvovaginal candidosis (VVC). VVC is the second most frequent vaginal infection. About 5-8% of all VVC cases reoccur, which is a condition that has been associated with a negative impact in the quality of life of women worldwide. Sodium bicarbonate ($NaHCO_3$) has been empirically used in the form of vaginal douches in chronic VVC cases. In this study we aim to evaluate the potential of using sodium bicarbonate in the control of VVC chronicity.The activity of $NaHCO_3$ against *C albicans* ATCC 10231 and 12 VVC clinical strains was determined using microdilution method. Controls were included to test the viability of *C. albicans* in the pH range of the of the $NaHCO_3$ microdilutions tested. This was tested in parallel by ascertain the culture medium pH with NaOH in order to exclude from the results analysis, the effect of the pH in the outcomes observed in the study. Yeast cell morphology was observed using scanning electron microscopy. The expression of phenotypic traits associated with recurrence has been assessed in the presence of increasing amounts of $NaHCO_3$. Specifically, biofilm formation, formation of the germinative tube (hypha) and in-vitro growth rates have been determined for a range of NaHCO3 concentrations. We found that *C. albicans* had the ability to proliferate in a wide range of pH-adjusted broth (from 7.40 to 9.77). Interestingly, and despite this result, we also found that the minimum inhibitory concentration of $NaHCO_3$ against *C. albicans* was 12.5 mg/ml (pH 8.97). The minimum lethal concentration was not possible to determine, which suggests a fungistatic mechanism of action. *C. albicans* cells exposed to $NaHCO_3$ (at least two times the MIC), presented a reduction of 1.5 times of the normal growth rate, and a reduction of 93% in the cells producing hyphae, when compared with cells not exposed to $NaHCO_3$. In addition, there was a 50% reduction in biofilm mass, when *C. albicans* cells were exposed to 15 times the MIC.We conclude that $NaHCO_3$ is a pH-independent antifungal, and that *C. albicans's* virulence is attenuated in its presence. This compound might be an excellent adjuvant for therapy targeting control of chronicity of VVC.

Keywords: antifungal; virulence; vulvovaginal candidosis; *Candida*; biofilm; pH

Introduction

Candida spp is an opportunistic pathogen involved in many infectious diseases, including vulvovaginal candidosis (VVC)[11]. VVC is characterized by an overgrowth of *Candida* spp in the vaginal mucosa, which is associated with increased vaginal discharge, vulvar/vaginal pain and pruritus [11]. About 5-8% of all VVC cases reoccur, which is a condition that has been associated with a negative impact in the quality of life of women worldwide [11] [170]. Recurrent vulvovaginal candidosis (RVVC) is defined by the occurrence of at least three VVC episodes in 12 months [11]. The great majority of VVC/cVVC cases worldwide are caused by *Candida albicans* [251]. *C. albicans* is a dimorphic yeast, and the formation of germinative tubes [193], as well as the formation of biofilms in the vaginal mucosa [17], have been considered their main virulence factors. The standard treatment for VVC is based on topical or oral azole agents [170]. However, azole-resistance represents a challenge to VVC treatment, due to the paucity of alternative drugs. Our own results revealed high rates of azole resistant vulvovaginal isolates [168]. Several researches suggest the potential of alternative strategies to treat VVC [102], such as sodium bicarbonate. Sodium bicarbonate ($NaHCO_3$) is a crystalline salt obtained by combining the Na+ and HCO_3- ions. It is used widely in several applications, in food, industry and medical areas [252], being considered generally safe. The antimicrobial effect of sodium bicarbonate has been previously reported [253][254]]. The use of sodium bicarbonate either in a bath or as a vaginal irrigation for the empiric treatment of vaginal infections has been described [170][255]. However, its effect in-vitro against *Candida* spp. has been seldom reported. In this study, we aim to evaluate the potential of $NaHCO_3$ as an antifungal agent to treat *Candida* spp. VVC infections.

Materials and Methods

Candida spp isolates

A *Candida albicans* type strain, ATCC 10231 was used in all experiments. Furthermore, to demonstrate the effect of $NaHCO_3$ in the inhibition of the growth of clinical isolates, 12 vulvovaginal candidosis isolates were included in the study. The isolates were obtained from 12 women attending a gynaecological consultation at a private clinic. The samples used for diagnosis that were going to be disposed were included in this study. The study was approved by the Ethics Committee of the research centre where the experiments were conducted (CE-UBI-Pj-2018-022). The strains have been fully

characterized at the species level and fluconazole resistance status. These results have been already published in another study [168].

Substance in test

Sodium bicarbonate (NaHCO3), analytical grade, 99.99% purity, was purchased from JMGS, Portugal.

Determination of the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of sodium bicarbonate against *Candida* spp isolates

The activity of $NaHCO_3$ against *C albicans* ATCC 10231 and 12 vulvovaginal *Candida* spp. clinical isolates was determined using CLSI M27-A3 microdilution method [168][66], in two independent assays, using two different culture media: RPMI broth (Sigma, United States of America) and YPD (VWR Chemicals, United States of America). The pH value of the microdilutions of $NaHCO_3$ used in the assay was measured. In addition, the viability of one *C. albicans*, one *C. glabrata* and one *C. guilliermondii* clinical isolates, as well as ATCC 10231, in culture media (RPMI broth) in the same pH value (ascertained with NaOH, Sigma, USA) as the microdilutions tested was assessed in parallel, as a positive growth control. The determination of the MIC was also assessed in an additional experiment performed in parallel using a rich non-buffered culture medium (YPD broth, VWR Chemicals, United States of America). The minimum lethal concentration was assessed by plating in solid agar media (Sabouraud Dextrose Agar, VWR Chemicals, United States of America) an aliquot of 10 µl of each microdilution of $NaHCO_3$ after 24h of incubation with each *Candida* spp. strain tested. The minimum lethal concentration was defined as the minimum concentration in which no growth was observed (no observable colonies).

Assessment of growth rates in the presence of sodium bicarbonate

YPD broth supplemented with different concentrations (1.56, 3.13, 6.25 and 12.50 mg/mL) of $NaHCO_3$ was inoculated with a fresh culture of *C. albicans* ATCC 10231 at an $OD_{600\ nm}$ of 0.05. The cultures were incubated aerobically, in an orbital shaker, for 12 hours at 37ºC. Hourly aliquots were taken to measure OD. A calibrated growth curve was estimated for each experiment; the curve was used to estimate the culture doubling time in each condition.

Assessment of the ability to form germ tubes in the presence of sodium bicarbonate

The ability to form germ tubes was assessed in-vitro as previously described [100], using YPD broth supplemented with 1.56, 3.13, 6.25 and 12.50 mg/mL of $NaHCO_3$. The results were expressed regarding the proportion of hyphae cells in the total number of cells (%) and compared with untreated controls.

Assessment of the ability of sodium bicarbonate to disperse *Candida* spp. biofilms

The ability to disperse pre-formed *C. albicans´* biofilms was assessed in-vitro as previously described [100][149]. The treatment was performed with YPD broth supplement with 1.56, 3.13, 6.25, 12.50, 25 and 50 mg/mL of $NaHCO_3$.

Scanning electron microscopy (SEM)

Approximately $1x10^6$ cells of *C. albicans* ATCC 10231 were incubated at 37ºC in RPMI supplemented with $NaHCO_3$ (2x MIC: 25 mg/ml; MIC: 12.50 mg/ml; ½ MIC : 6.25 mg/ml; control without $NaHCO_3$). After incubation, the cells were washed once with PBS (phosphate-buffered saline), and fixed with 2% gluteraldehyde (Sigma-Aldrich, United States of America) for 30 minutes. Post-fixation was performed with osmium tetroxide (Sigma-Aldrich, United States of America). The samples were dehydrated in a graded ethanol series and freeze-dried in an automated freeze-dryer. SEM images were acquired in a scanning electronic microscope Hitachi S-2700, with a magnification of 3000x.

Results

Sodium bicarbonate is an antifungal agent independent of pH

We found that sodium bicarbonate had the ability to inhibit the growth of all *Candida* spp. isolates in test. The minimum inhibitory concentration (MIC) of $NaHCO_3$ against the yeast strains tested ranged between 12.5 mg/ml (pH 8.97) and 25 mg/ml (pH 9.45) in RPMI broth and between 1.56 mg/ml (pH 7.66) and 6.25 mg/ml (pH 8.02) in YPD broth (Table 1). The inhibitory effect was equally observed among fluconazole-resistant and fluconazole-susceptible isolates (Table 1). The minimum lethal concentration (MLC) of $NaHCO_3$ against the tested yeast was higher than the observed MIC, which is an indication that the inhibitory effect was fungistatic.

We hypothesized as well that the inhibitory effect might be pH-dependent. Therefore, we assessed the viability of *C. albicans* ATCC 10231, as well as one clinical isolate of each tested *Candida* species in RPMI broth adjusted to pH, the same pH values tested, but using NaOH to ascertain the pH value instead of $NaHCO_3$ (Table 2). The results revealed that there was no inhibitory effect using NaOH, indicating that $NaHCO_3$ has the ability to inhibit the growth of *Candida* spp., in a pH-independent manner.

Table 1. Mininium inhibitory concentration (MIC) and minimum lethal concentration (MLC) of sodium bicarbonate in RPMI and YPD. S: susceptible; R: resistant.

Strain	**Fluconazole susceptibility/ resistance**	**RPMI**		**YPD**	
		MIC	**MLC**	**MIC**	**MLC**
Candida albicans ATCC 10231	S	12.50	>25	6.25	>25
Candida albicans 1	R	12.50	>25	3.13	25
Candida albicans 2	R	25	>25	6.25	>25
Candida albicans 3	R	25	>25	6.25	>25
Candida albicans 4	R	6.25	>25	1.56	12.5
Candida glabrata 1	R	6.25	>25	1.56	25
Candida glabrata 2	R	12.50	>25	3.13	25
Candida glabrata 3	S	12.50	>25	3.13	>25
Candida glabrata 4	S	12.50	>25	3.13	>25
Candida guilliermondii 1	S	25	>25	6.25	>25
Candida guilliermondii 2	R	25	>25	6.25	>25
Candida guilliermondii 3	R	25	>25	6.25	>25
Candida guilliermondii 4	S	25	>25	6.25	>25

Table 2. Viability of *Candida* spp in RPMI at different pH values, by adding NaOH, correspondent to the pH values of the microdilutions of RPMI supplemented with $NaHCO_3$.+: positive growth. One clinical isolate of each species, as well as *C. albicans* ATCC 10231 was used in this assay.

pH value; $NaHCO_3$ (mg/ml)	*C. albicans* ATCC 10231	*C.albicans*	*C.glabrata*	*C.guilliermondii*
9.53; 50	+	+	+	+
9.45; 25	+	+*	+	+*
8.97; 12.5	+*	+	+*	+
8.02; 6.25	+	+	+	+
7.66; 3.13	+	+	+	+
7.52; 1.56	+	+	+	+
7.46; 0.78	+	+	+	+

*Correspondent $NaHCO_3$ MIC

Sodium bicarbonate affects *C. albicans* ATCC 10231 virulence.

Downregulating *C. albicans'* virulence in the vaginal tract could be a promising strategy to control recurrence. In this study we aim to evaluate the potential of using $NaHCO_3$ in the control of VVC recurrence (chronic).

We found that *C. albicans* ATCC 10231' biofilms are dispersed in the presence of $NaHCO_3$ (Figure 1). The effect is visible both on the reduction of biomass and metabolic activity, particularly by treating the biofilms with 50 mg/mL (4xMIC in RPMI).

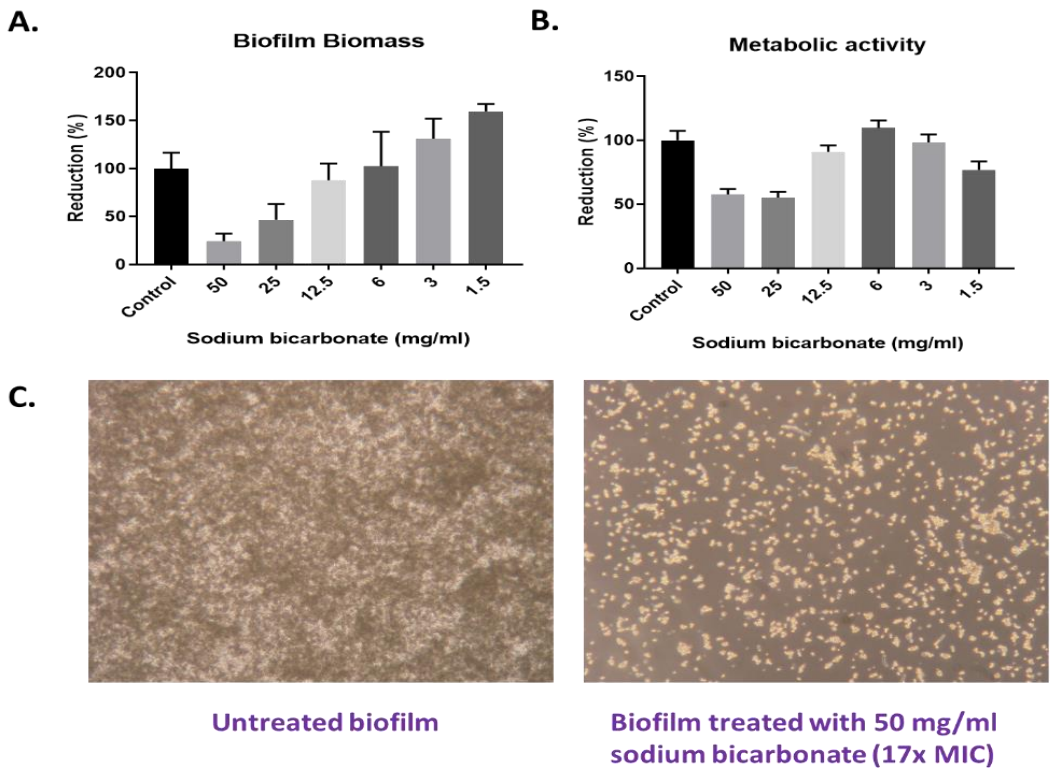

Figure 1. Effect of $NaHCO_3$ on *Candida albicans* ATCC 10231 biofilms**. A.** Reduction on biofilm biomass (%). **B**. Reduction on metabolic activity (%). **C.** Optical microscopic image of *C. albicans* ATCC 10231 biofilm.

In order to rule out structural effects caused by exposure to $NaHCO_3$, such as cell wall damage, we observed treated *C. albicans* ATCC 10231 cells with SEM (Figure 2). We conclude that cells showed no significant structural damage; however, we found evidence of disturbed hyphae (Figure 2). Therefore, we also assessed if $NaHCO_3$could inhibit the in-vitro formation of germinative tube. We found that the formation of germ tubes was inhibited in the presence of $NaHCO_3$, in a dose-dependent manner (Table 2). We have also tested if the presence of $NaHCO_3$ impaired *C. albicans* ATCC10231 growth rate. We found that at higher concentrations, the growth rate was at least twice diminished in the presence of $NaHCO_3$ (Table 2), suggesting that the effect of $NaHCO_3$ is fungistatic. These results are in accordance to the ones obtained in the antifungal test performed in this study.

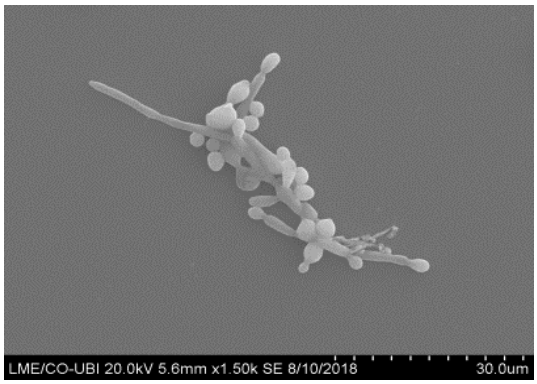

Figure 2. SEM photograph depicting disturbed hypha (arrow) of *C. albicans* ATCC 10231 exposed to 12.5 mg/ml $NaHCO_3$.

Table 3. Effect of of $NaHCO_3$ on C. albicans ATCC10231 growth rate and germ tube formation in the presence of sodium bicarbonate.

Sodium bicarbonate (mg/ml)	**Growth rate (OD/hour)**	**Doubling times / hour**	**Reduction (x)**	**Hyphae cells / total (%)**	**Reduction (%)**
0	3.912	0.92	-	10.70	-
1.56	3.234	0.74	**1.26**	11.81	**10.38**
3.13	2.814	0.76	**1.21**	5.81	**45.70**
6.25	2.358	0.62	**1.50**	0.73	**93.18**
12.5	0.396	0.33	**2.78**	0.77	**93.46**

Effect of sodium bicarbonate against clinical isolates of chronic vulvovaginal candidosis

The effect of $NaHCO_3$ on the expression of virulence traits of 12 vulvovaginal clinical isolates was also assessed. We found that *Candida* spp. biofilms were dispersed in the presence of $NaHCO_3$, in a similar fashion as observed for *C. albicans* ATCC10231 (Figure 3). Different *Candida* species were equally affected by $NaHCO_3$. In particular, the highest concentration in test, 50 mg/mL of $NaHCO_3$, dispersed the biofilm biomass, resulting in 9-75% of biomass in comparison to the untreated control. The same result was obtained

regarding the metabolic activity of the biofilms. In addition, *C. albicans* clinical isolates also showed an impairment of germ tube formation in the presence of $NaHCO_3$, (Table 4); specifically, broth supplemented with 12.5 mg/mL provoked an average reduction of more than 90% on the number of hyphae in solution, in comparison with untreated control.

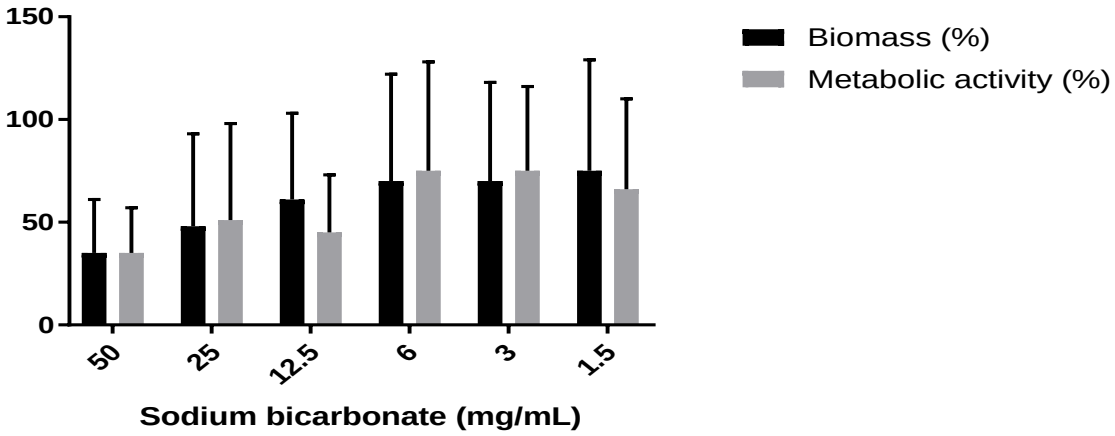

Figure 3. Average biofilm biomass (%) and metabolic activity (%) of biofilms of vulvovaginal clinical isolates after treatment with 1.5-50 mg/mL of sodium bicarbonate. The standard deviation considering results for all clinical isolates is also shown.

Table 4. Effect of $NaHCO_3$ on germ tube formation among vulvovaginal clinical isolates. The average proportion of hyphae is showed (%), as well as the respective standard deviation (SD).

Sodium bicarbonate (mg/ml)	**Hyphae cells / total (%, SD)**	**Reduction(%)**
0	21.06 (9.25)	-
1.56	14.75 (13.95)	29.97
3.13	12.55 (8.67)	**40.39**
6.25	10.57 (10.85)	**49.81**
12.5	1.49 (2.25)	**92.91**

Discussion

In this study we focused on the antifungal effect of sodium bicarbonate ($NaHCO_3$). Previous studies have showed that $NaHCO_3$ has antimicrobial abilities [253][254]. $NaHCO_3$ has been used in clinical practice to treat empirically vulvovaginal candidosis [170]. Therefore, its anti-*Candida* effect has already been explored by our research group, to treat this condition [256]. However, there are not enough studies that focus on its in-vitro effect against *Candida* spp.

We found that $NaHCO_3$ had a direct inhibitory effect against *Candida*. The effect was more pronounced in YPD broth, which is an unbuffered culture medium. Nevertheless, the effect was clearly pH-independent, since the same pH adjustment in culture medium, obtained with NaOH, did not inhibit *C. albicans* ATCC10231 growth. Most relevant, $NaHCO_3$ was equally effective against fluconazole resistant/susceptible isolates. Recent reports have highlighted an increased proportion of fluconazole resistant vulvovaginal *Candida spp*. isolates [256][185]. Therefore, our result points towards a promising alternative to treat resistant cases.

Sodium bicarbonate was also equally effective against different species of *Candida*. These results suggest for a general mechanism of action in the inhibition of *Candida* growth. The effect was probably fungistatic; the observed impairment of growth rate in the presence of $NaHCO_3$ also supports this hypothesis. The mechanism of inhibition of the growth of *Candida* spp. by $NaHCO_3$ has not been extensively studied. Previous studies suggest a role of the Na+ ion through an imbalance of the intracellular K+/Na+ ratio, whose homeostasis is crucial for the physiology of the fungal cell. In the presence of $NaHCO_3$, the excess of extracellular sodium would cause a massive influx of Na+ by a non-specific transport mechanism, and the intracellular accumulation of Na+ would be responsible for a growth arrest of the cell [257]. Other studies suggest a role of the HCO3- ion [258], since other salts such as $KHCO_3$ and NH_4HCO_3 also have an antifungal activity, possibly by alkalinizing the medium and favoring the stability of KH and NH_4, which would in its turn damage the cell wall and compromise the membrane.

In order to rule out structural effects caused by exposure to $NaHCO_3$, such as cell wall damage, we observed treated cells with SEM. We conclude that cells showed no significant structural damage. Previous studies have showed that $NaHCO_3$ has an impact in filamentation; in smaller amounts it induces filamentation, while in higher concentrations it is inhibited [259]. In this study, we found that proportion of hyphae was reduced in the presence of $NaHCO_3$; and that cells collected from culture medium

supplemented with 12.5 mg/ml (MIC), showed that the yeast cells were not damaged, but disturbed hypha were observed.

We also investigated the role of sodium bicarbonate in the dispersal of biofilms, which is one of the most important virulence factors for the onset of VVC [100][17]. We found that biofilm biomass was severely reduced after treatment with $NaHCO_3$, evidencing that its local use on the vaginal mucosa might help the dispersal of *Candida* spp. biofilms. In fact, formulations with $NaHCO_3$ have already been proved to be efficient for this purpose [256]. The metabolic activity of biofilms was also impaired, which is an indication that $NaHCO_3$ could be a valuable adjuvant in therapy, combined for instance with azole compounds.

We conclude that $NaHCO_3$ is an active agent against vulvovaginal *Candida spp*. isolates, acts in a pH-independent manner, and could potentially integrate a therapeutic strategy, particularly in the context of sVVC. In addition, *C. albicans*'s virulence is attenuated in the presence of sodium bicarbonate, and this compound might be an excellent adjuvant for therapy targeting control of chronicity of VVC.

Chapter 8. SUMMARIZING THE DISCUSSION

Discussion

Vulvovaginal candidosis is a mucocutaneous infection that affects the vulva and vagina and is caused by fungi of the genus *Candida* spp. This fungus is considered to be emergent, since the relationship between this agent and disease is recent, moving from colonizer to disease-causing agent [260], as it can be found in the vagina as an asymptomatic colonizer [261][10]. This transition occurs as a consequence of hormonal changes, as well as untreated chronic diseases, as is the case of diabetes, especially Type II, and diseases that affect immunity in the host, creating conditions for the development of the infection. This infection can manifest itself in a chronic or sporadic form, with its chronicity being the least incident form, but also the one that causes the most inconvenience in the woman's life, since its symptoms greatly affect the quality of life of the host [262].

In this work, we performed a comparison between *Candida* spp isolates from a group of women with sporadic vulvovaginal candidosis manifestation (a clinical episode and a *Candida* spp. isolate only involved) and a second group in which the strains were all obtained from women who presented chronic candidosis with several episodes and isolates of *Candida* spp. obtained at different stages of infection.

We verified such as others authors, that prevalence within general *Candida*, was ever for species *Candida albicans* [261] [260]. *Candida glabrata* was the second species more isolated in our collection, being the most prevalent non-albicans species, a result also obtained by others authors [263][140]. The high prevalence of *Candida albicans* can be explained by its ability to adapt to the human body, especially in stressful environments (pH and temperature variations, for example)[62].

The chronicity of vulvovaginal candidosis is in this context the most worrying condition of this infection, which derives from several factors such as the case of azole resistance, commonly used as first-line therapy in the treatment of this infection [264], but very little clarified [181]. Fluconazole is, for example, identified as one of the causes of resistance, when used for a long period [181][127][265]. In this context, there was a need to assess susceptibility to antifungals, due to the importance they can play in the medical prescription of these drugs, avoiding, for example, using a certain antifungal for a species previously evaluated as being resistant to that antifungal. Bearing in mind the importance of both fluconazole and clotrimazole in the treatment of this infection, we tested these two antifungals against all strains in our collection. Conducting an assessment by species, it was found that there was a high resistance to the two antifungals

of the species *C. albicans*, although this is considered by most authors as a species with very high levels of susceptibility [180][266]. The group of non-*C. albicans* species, on the other hand, considered the group with the highest resistance to most azoles in several studies, were those that showed greater susceptibility compared to *C. albicans* strains [88]. With regard to azole resistance, the relationship to the frequency of azole resistance, course of treatment and distribution by each of the clinical groups considered in our study, in which we did not find significant differences that justify the chronicity of vulvovaginal candidosis, because most likely there are others factors that lead to chronic VVC, factors inherent to the host or linked to the expression of virulence factors by the fungus. According to Annabel Lanes 2020, several factors inherent to the organism, may predispose women to develop chronic VVC (use of antibiotics, estrogen level in the organism, comorbidities, use of contraceptives and other behavioral factors that this author related to the development of chronicity) [267]. Jack Sobel, 2016 in addition to the predisposing factors mentioned above, mentioned others, which are linked to genetic variations of the host [28].

Virulence factors play an important role in the development of an infection. In the case of fungal infections, they are related to the adhesion of the microorganism to the host cell, while inhibiting the proper functioning of the immune system, allowing resistance by fungi to many antifungals and also allowing the adaptability of *Candida* spp., to variations of the host's physiological conditions [215]. In this work, we initially evaluated the phenotypic expression of three virulence factors (which are characteristics produced by the microorganism, in response to the conditions found in the host's vaginal mucosa, in order to maintain its subsistence). The three factors evaluated were: the ability to form biofilms, the ability to produce germ tube (for *C. albicans*) and the ability to produce phospholipases. We initially chose these virulence factors because of their importance, which are closely linked with the ability to persist in the vaginal mucosa, as they are described as important factors in the development of the disease, promoting invasion, adhesion, and resistance to antifungals (germ tube and biofilms), as well as the destruction of the host's cells (production of phospholipases)[140][265], These were evaluated comparatively to the group of strains obtained from women with sporadic candidosis, and we verified that there was a reduction in the expression of virulence factors, in strains obtained from women with chronic candidosis, since the virulence factors studied here were associated with adherence, invasion and immunological modulation [268]. In this context, we can hypothesize that the persistence of *Candida* spp. in the vaginal mucosa can be achieved by the negative regulation of these processes (although not totally). This negative regulation could probably cause numbness in the

innate immune system, since inflammatory processes - as a response mechanism to infection would be practically non-existent. Medeiros and collaborators [112] Comparing the two groups regarding virulence factors and did not find significant differences between cVVC and sVVC. similar results were obtained by Kallanci and collaborators studying as virulence factors phospholipase and proteinase production , adhesion to epithelial cells and MIC [269]. And Consolaro and collaborators, comparing groups and evaluating different virulence factors (hyphae formation, biofilm production, hydrophobicity of cellular surface and adhesion to epithelial cells), also not obtained significate difference between groups [94].

We evaluated other virulence factors, cell surface hydrophobicity, protease production capacity and evaluated the ability to resist high pHs, as well as the ability of *Candida* to resist phagocytosis in macrophages in a perspective of comparing groups (sVVC and cVVC). *Candida* may limit immune system response [269]. Results indicated that yeast isolates involved in sporadic infection adhered in a higher proportion to HeLa cells, which was related with their ability to produce biofilm ($p<0.05$). Gerwien *et al* found variation in *Candida albicans* response the group had no influence on macrophage resistance [231]. The ability to resist macrophage activity is strongly related to the ability to form germ tube [20], In our study, we did not find relevant differences between *C. albicans* and *C. glabrata*, although previous studies have highlighted differences related with the expression of germ tubes (hyphae), the ability of candida to escape phagocytosis by macrophages is strongly related to this virulence factor, piercing of the macrophage cell membrane [270].

We also obtained a relationship between the ability to evade phagocytosis was related with elevated production of proteases ($p<0.05$), virulence factor highly related to vulvovaginal candidosis by Shirkani and collaborators [271] and others authors [268][268], contrary, Ozcan and collaborators find results that reduce the importance of this factor for *Candida* pathogenicity in VVC [272]. In conclusion, the ability of cVVC isolates to evade macrophages may be related to the production of proteases, although more studies are needed to understand the role of this virulence factor in the chronic vulvovaginal candidosis and there are different opinions regarding the influence of the production of proteases on *Candida* spp virulence in VVC in the literature.

The hydrophobicity of cell walls in *Candida* is associated with the capacity to produce biofilms and adhesion to *Candida* spp, which are essential factors for the virulence of this fungus. [273][274][275] Hydrophobic cells of *Candida* are more resistant to phagocytosis than hydrophilic cells [276], the constituents of the cell wall of this fungus

(glucan, chitin, and mannoproteins) have the ability to modulate the the immune response of host[277]. Regarding chronic isolates, while episodic isolates' resistance to phagocytosis was related with a higher hydrophobicity of cell walls ($p<0.05$). We conclude that overall yeast strains involved in vulvovaginal candidosis showed an equal ability to evade macrophage mediated phagocytosis and to adhere to epithelial cells.

Vaginal infections by *Candida* spp., are often associated with their chronicity by the same strain. However, in some cases there may be genotypic variation of the same strain or its replacement by another [61]. In this study we tried to evaluate the genotypic variation of *Candida* isolates from a sample of 17 women with chronic cases of vulvovaginal candidosis. We verified whether there was a change in the strains or not (by RT-PCR) and if the same strain remained in the vagina[273]l mucosa, we evaluated the behavior over time of susceptibility to fluconazole and the ability to form biofilm. It is important to mention that in most cases we obtained replacement of the strain causing chronicity (9/17 cases), this data contradicts results found by other authors who evaluating the permanence or not of the strain causing cVVC obtained the same strain over time or in some cases, the same strain with small genotypic variations [220][221].

We restricted ourselves to a small sample of 24 strains (the 8 cases where we verified sequential strains, 6 clinical cVVC and 2 statistically cVVC) in which we verified that there was no significant relationship in susceptibility to fluconazole and the ability to form biofilms over time between these two correlated factors, which led us to think that this may have been influenced by the sample size. It is worth mentioning that we obtained a correlation between the ability to form biofilms and resistance to fluconazole only for the species *Candida glabrata*. This species is described as resistant to azoles especially to fluconazole [278] and with a strong capacity to form biofilms, leading to great adaptation and persistence in the vaginal mucosa [279].

Sodium bicarbonate has been used empirically to combat vaginal infections [104], but few in vitro studies have been developed to know its real effect on fungi of the genus *Candida* spp. In this study, the effect of sodium bicarbonate on *Candida* was evaluated, taking into account its ability to inhibit virulence factors (germ tube and biofilms). pH changes under the influence of sodium bicarbonate were also evaluated. We found that sodium bicarbonate inhibited *Candida*'s ability to form biofilms and germ tube, we also found that pH variation had no significant influence on the inhibitory effect of virulence factors by sodium bicarbonate and also on *Candida*'s susceptibility to this. product. Tomas M *et al* 2020 performed tests in vitro using formulations-based sodium bicarbonate, obtaining growth inhibition of several species of *Candida* spp tested with

these formulations [256]. In this context, we can conclude that for the prevention of resistance to antifungals by *Candidas*, sodium bicarbonate can be used to reduce possible persistence in the vaginal mucosa, we see its effect on the reduction of *Candida* biofilms and germ tube, factors that have been proven to be related to host cell adhesion and invasion (virulence factors) [268].

Final considerations

The evaluation of this collection carried out on the various parameters from the behavior of fungi in terms of virulence factors, in vitro behavior in co-culture with Hela cells and with macrophages, as well as the evaluation of possible alternative therapy based on sodium bicarbonate, led us to several important discoveries. The group of strains that caused sporadic vulvovaginal candidosis were able to form biofilm and germ tubes, which we were able to relate to adhesion to Hela cells (and therefore to the vaginal epithelium), and this must be related in some way to the hydrophobicity of the wall, this virulence factor being important for adhesion. On the other hand, Candida strains isolated from chronic vulvovaginal candidosis, on the other hand, showed a poor ability to form biofilm and a poor adhesion to Hela cells (therefore they do not adhere well to the epithelium), but showed a strong ability to better resist phagocytosis which is closely linked to the production of proteases and this group are also more resistant to pH variations. The chronic vulvovaginal candidosis isolates also showed the ability to model virulence factors over time, which is a capacity that allows them to persist according to the characteristics of the host.

In short, we think that our work reinforces the existence of a strong link between the behavior of virulence factors and the adhesion, penetration and persistence of Candida fungi in the vaginal epithelium and we also see the differences (in most cases not significant) between the two target groups of the work, which may underlie the chronicity of vulvovaginal candidosis.

Chapter 8. General Conclusion

Conclusion

Given the clinical importance of chronic vulvovaginal candidosis, due to the increased incidence, concern is growing and the cause must be analyzed both in the pathogen *Candida* spp. and in the host, seeking prevention strategies. Chronic vulvovaginal candidosis may be associated with a set of virulence factors expressed by *Candida* spp. that result in antifungal resistance. On the other hand, the expression of microorganism virulence factors can inhibit the performance of the host's immune system. Many epidemiological data and experimental studies justify the assumption that virulence factors are implicated in antifungal resistance and the consequent occurrence of cVVC. An example is the ability to form biofilms, considered one of the most important virulence factors due to the ability of *Candida* spp. resist antifungals. In addition, the microorganism's virulence factors alone would not be sufficient for the occurrence of cVVC; in fact, the host's pre-availability is also important to ensure the success of the infection. With the results obtained from this PhD project, we were able to emphasize the higher incidence of *Candida albicans*, in relation to other non-a*lbicans* species. It was also shown that *Candida glabrata* may be the most incident non-*albicans* species. We can verify that the susceptibility of *Candida albicans* to antifungals is not literally certain, because our results suggest an increased resistance to albicans strains. cVVC strains have a tendency to decrease the development of virulence factors, to decrease the action of the immune system and consequently remain in this mucosa, promoting chronicity. This leads us to conclude that although most strains have a tendency to reduce the development of vitulence factors, chronicity is not fully explained by this aspect, leaving room for future studies.

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