Microbiological activity of an innovative Intimate Hygiene cleanser against vulvovaginal Candidiasis

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Candida is usually present in the vaginal microbiota in which lactobacilli (LB) are predominant. An imbalance in LB production leads to yeast overgrowth and to the occurrence of vulvovaginal candidiasis. This common vaginal infection is caused by Candida species, is associated with intense itching, swelling, and irritation, and frequent relapses. A new cleanser for feminine intimate hygiene (SA2.1) was designed to increase the microbiological activity of the traditional cleanser, adding zinc, surfactant with antibacterial properties and a mucous-adhesive system. This study evaluated the in-vitro effect of SA2.1 on Candida spp. compared with the reference SA in a double-blind design. Two cleansers for intimate hygiene were provided: the reference one containing thymol from Thymus vulgaris extract at pH 3.5 (SA = Saugella Attiva, Mylan), and the test one (SA2.1 = Saugella Attiva new, Mylan) in which Zinc Cocethsulfate with antimicrobial action, and Xanthan gum and Chondrus crispus extract as mucous-adhesive agents were added to the reference formula. The anti-fungal activity of the products above described was evaluated in 12 strains of Candida spp. isolated from vaginal swabs from women with vaginitis, and in two strains from the American Type Culture Collection (ATCC-MYA 276 C. glabrata, ATCC-24433 C. albicans) belonging to the same species as the wild samples. The minimum inhibitory concentration (MIC) was determined. MIC values showed a statistically significant difference considering all Candida species: 0.18% vs 0.29% (p=0.001 at Student’s t test two-tail) in favour of SA2.1. SA2.1 evidenced a significant improvement in the microbiological potency, showing comparable MICs between C. Albicans and C. glabrata, even if the last one is more virulent. Comparing the MIC within each species, SA was 20.7% and 44.4% less potent than SA2.1, respectively for C. albicans and C. glabrata. Detergents for feminine intimate hygiene should have documented effective formulations for maintaining genital health and preventing bacterial and fungal contamination. The combination of thymol, zinc and antibacterial surfactant improved the inhibitory activity of the traditional cleanser against Candida albicans and Candida glabrata.

Keywords: Candida; intimate hygiene; plant extracts; cleanser pH

INTRODUCTION

The female genital tract is particularly exposed to possible contamination due to the close contact with the external environment and the anatomical configuration. The vaginal epithelium, mucus, pH, and ecosystem represent the natural barrier opposing to the proliferation of pathogens. The vaginal microbiota is populated by...
lactobacilli (LB), the predominant commensal microorganisms (MO), in balance with other saprophyte, opportunist and pathogen MO. Some MOs often become pathogens within the vagina only as consequence of alterations of the ecosystem, particularly Candida and Gardnerella vaginalis, major responsible for bacterial vaginosis.

Candida is a fungus usually present in the vaginal microbiota as opportunist MO, i.e. convertible in pathogens in peculiar conditions. Its growth is controlled by LB function, through the formation of lactic acid. An imbalance in LB presence leads to an overgrowth of candida spp, and the occurrence of vulvovaginal candidiasis. [Guaschino S, 2008]. This is a common vaginal yeast infection, caused by Candida species, associated with intense itching, swelling, and irritation. Candida infection frequently relapses.

The first steps aimed to protect the integrity of vaginal ecosystem in a natural way are the correct lifestyle and the daily intimate hygiene using adequate products. Different formulations of detergents for feminine intimate hygiene are available, and their antimicrobial and pharmacological activity need scientific evidence supporting the claim.[European Directive,2012]

A detergent based on Thymus vulgaris extract (SA = Saugella Attiva) showed a significant selective antimicrobial, anti-inflammatory and antioxidant action, due to thymol and carvacrol, the main active ingredients of the extract.[Braga PC, 2005; Braga PC, 2006a; Tacconi E, 2003] Thymus vulgaris is titrated in thymol, and is the most potent antimicrobial agent among plant extracts. [Vardar-Unlu G, 2003]

In a comparative microbiological study in candida infections, the formulation that proved to be more effective contained thymol and was formulated at acid pH. [Guglielminetti ML, 2015]

To improve the clinical performance of this detergent, the research and development paid specific attention to increasing the antimicrobial potency and extending the persistence of the active ingredients in situ, aimed to prolong their clinical action. In view of achieving that goal, Zinc cocethsulfate, surfactant with antibacterial properties, and a muco-adhesive system based on the association of xanthan gum and Chondrus crispus (carrageenan) were taken into consideration.

Zinc inhibits the growth of Gram+ and Gram-bacteria normally found on human skin and oral cavity, being anaerobic Gram-negative and aerobic Gram-positive bacteria are the most susceptible to its action.[Johannsson A, 1995; Söderberg TA, 1990] Additionally, zinc protects intact cells against bacterial cytotoxicity preventing the leakage of ions (Na+, K+) through membrane lesions in host cells.[Bashford CL, 1988] It also favours the re-epithelisation and the decrease of inflammation [Agren MS, 1990]. These properties support the rationale for the use of zinc in the local healing of wounds and infections. Moreover, Zinc cocethsulfate, disodium capryloyl glutamate, and coco-glucoside represent mildly aggressive surfactants.

Xanthan gum and lambda carrageenan association can establish a strong interaction with mucin, due to the presence of muco-adhesive polymers.[Parente ME, 2015, Rossi S, 2014]

The combination of xanthan gum, carragénans and glyceryl oleate has emollient and moisturizing properties. A new and modern cleanser indicated for feminine hygiene, containing thymol at pH 3.5, zinc, xanthan gum, Chondrus crispus and glyceryl oleate and an innovative surfactant system (SA2.1) has been developed with the pivotal characteristics of an antimicrobial and anti-inflammatory activity to counteract candida strains and bacterial vaginosis.

Aim of this study was to evaluate the in vitro effect of SA2.1, test cleanser, on Candida spp. compared with the reference cleanser (SA) based on traditional surfactants in a double-blind design.

**METHOD**

The study was planned and carried out following a controlled, randomised, double blind design. The tested products were SA2.1 (Mylan Company) versus SA as reference traditional cleanser.

The main ingredients of SA2.1 are: Thymus vulgaris extract, Zinc cocethsulfate, disodium capryloyl glutamate, and coco-glucoside, propylene glycol, glycerine as humectant, lactic acid, xanthan gum and Chondrus crispus as muco-adhesive agents, glyceryl oleate as Emollient, and α-ketoglutaric Acid as deodorant. SA2.1 formulation is characterised by the addition of zinc, muco-adhesive polymers, and moisturising agents to the traditional cleanser, as well as the adoption of innovative non-aggressive surfactants.

To maintain double blind condition of the investigator up to the end of the study, the two solutions were supplied in identical bottles, labelled only with a progressive number randomly assigned to each product. The key code was delivered in a sealed envelope to the investigator that disclosed it only after the completion of the determinations and the statistical analysis.

The anti-mycological activity of the products above described was evaluated in 12 strains of Candida spp. (C. glabrata and C. albicans), identified by Vitek (bioMérieux AS, Marcy-l'Etoile, France) and isolated from vaginal swabs from women with vaginitis provided by the Laboratorio di Analisi Mediche San Giorgio Srl in Pavia, Italy (LSG), and in two strains from the American Type Culture Collection, (ATCC C. glabrata MYA 276, ATCC 24433 C. albicans) belonging to the same species as the wild samples, and provided by LGC Standards from the CBS strain database.
The minimum inhibitory concentration (MIC) of the products was determined.

Quantitative testing was based on the evaluation of the minimum inhibitory concentration (MIC), defined as the lowest concentration at which an absence of visible growth is observed and expressed in % of product using the broth microdilution test in RPMI-1640 medium (Sigma Aldrich). Fungal inoculants, according to CLSI criteria (Clinical and Laboratory Standards Institute), were prepared to obtain a final concentration of 0.5x10^3 to 2.5x10^3 CFU/mL in RPMI-1640 broth. Multiwell microdilution plates were kept at thermostatic conditions at 35°C. The MIC readings were taken at 24 and 48 hours.

The statistical analysis was performed applying the two-tailed Student’s t test for independent data. The level of significance was taken to be α=0.01 (type 1 error) and power β=0.90 (type 2 error).

### RESULTS

MIC values showed a statistically significant difference considering all candida species: 0.18% vs 0.29% (p<0.001 at two-tailed Student’s t test) in favour of SA2.1. (Table 1) The MIC of the reference product resulted 35.5% higher than the one of the test cleanser. The new formulation evidenced a significant improvement in the microbiological potency that was more important on *C. glabrata*. In fact, SA2.1 shows comparable MICs between *C. Albicans* and *C. glabrata*, even if the last one is more virulent. On the contrary, SA is more potent on *C. albicans* than on *C. glabrata*.

Comparing the MIC within each species, SA mean values were 20.7% and 44.4% than the ones of SA2.1, respectively for *C. albicans* and *C. glabrata*. The power of the statistical significance between the test and the reference product greatly exceeded the 90% for all the comparisons carried out.

### DISCUSSION

The local or systemic infections of Candida species is increasing and may lead to serious consequences. *Candida glabrata*, the most widespread pathogenic fungi in non-*Candida albicans* Candida species in humans, is extremely aggressive due to many virulence factors contributing to a low therapeutic response and frequent recurrent candidiasis. In particular, its ability to form very resistant biofilms plays an important role in antifungals failures. [Rodrigues CF, 2017]

*C. glabrata* is the emerging species as the main Vulvovaginal candidiasis (VVC) agents, the most frequent female genital disorders. A study on *C. Glabrata* vaginal isolates screened for sensitivity or resistance to the antifungal fluconazole, showed that isolates resistant to fluconazole adhered more efficiently to the vaginal ring and were statistically more able to form biofilm. Probably fluconazole selects this species, that through adhesion, causes colonization or VVC symptomaticity. [Nakamura-Vasconcelos SS, 2017]

Considering the worse virulence of *C. glabrata*, the severity of the disease outcomes, and the emerging presence in vulvovaginal candidiasis it is of huge importance that cleansers for intimate hygiene as SA2.1 active against the growth of Candida as whole species.

In fact, the present study evidences that the in-vitro effect of SA2.1 is superior to the traditional cleanser SA in inhibiting Candida spp. growth, as shown by the statistically significant lower MIC. Moreover, SA2.1 is equipotent on *C. albicans* and *C. glabrata*, while SA is less powerful on *C. glabrata* than on *C. albicans*.

The main active ingredients of SA2.1 are thymol, present also in the original formulation, and zinc, besides the acid pH.

In a previous study, SA showed significantly higher inhibition halo of Candida spp. isolated from women with vaginitis versus other commercially available cleansers indicated for feminine hygiene. The mycological study demonstrated the importance of ingredients derived from plant extracts with antimicrobial and anti-inflammatory

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**Table 1:** The Minimum Inhibiting Concentration of Candida, expressed in % of product, for the test and the reference cleanser. Mean ± SE, Two-tailed Student’s t test for independent data, **p<0.01.**

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<tr>
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<th>SA2.1 candidaspp.</th>
<th>SA candidaspp.</th>
<th>SA2.1 <em>C. albicans</em></th>
<th>SA <em>C. albicans</em></th>
<th>SA2.1 <em>C. glabrata</em></th>
<th>SA <em>C. glabrata</em></th>
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<tr>
<td>Mean</td>
<td>0.184</td>
<td>0.286</td>
<td>0.170</td>
<td>0.214</td>
<td>0.199</td>
<td>0.357</td>
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<tr>
<td>SE</td>
<td>0.008</td>
<td>0.029</td>
<td>0.011</td>
<td>0.008</td>
<td>0.013</td>
<td>0.053</td>
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<tr>
<td>max</td>
<td>0.3</td>
<td>0.9</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
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<td>min</td>
<td>0.09</td>
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<td>0.1</td>
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<tr>
<td>n</td>
<td>42</td>
<td>42</td>
<td>21</td>
<td>21</td>
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p       **   **   **
Power (α=0.01) 99.76% 98.33% 98.39%
action, such as thymol, and of the detergent acid pH that proved to be more active than alkaline pH in Candida infections. The data also confirmed the higher resistance of C. glabrata. [Guglielminetti ML, 2015]

Thymol and carvacrol are the active components of Thymus vulgaris extract, that evidenced a synergistic and selective antibacterial and antifungal activity, leaving Lactobacilli unchanged. [Didry N, 1994; Friedman M, 2002; Kalemba D, 2003; Tacconi E, 2003]

Thymol affects the fungal virulence inducing morphostructural damages of the Candida envelope antagonising Candida filamentation [Braga PC, 2007; Culici M, 2006]. Moreover, Thymol reduces the bacterial and fungal adhesion to human vaginal epithelial cells at concentrations below the minimum inhibitory concentration, allowing the inactivation of pathogens even at low doses. [Braga PC, 2010a; Culici M, 2005; Dal Sasso M, 2006]

Carvacrol and eugenol cause morphological changes in food spoiling mould, probably due to their action on cell wall enzymes, such as citinases and glucanases. The highest activity against microorganisms and their broadest spectrum are documented for thymol, carvacrol and eugenol, being explained by the acidic nature of the hydroxyl group, forming a hydrogen bond with an enzyme active centre. [Kalemba D, 2003].

Terpenes as carvacrol and thymol enter the fatty acyl chains of the membrane lipid bilayers and disrupt the lipopolysaccharide outer layer, with a consequent partial disintegration of the outer membrane, altering the membrane permeability.

Biofilms are known to be important factors underlying microbial infections, including both C. Albicans and G. vaginalis, the predominant species in bacterial vaginosis, and allow microbes to tolerate higher concentrations of antibiotics, thus increasing the possibility of recurrence. Thymol antagonises native and mature biofilms of Candida and Gardnerella, hindering virulence and pathogenicity of these pathogens having a high tendency to develop biofilms. [Braga PC, 2008; Braga PC, 2010b]. The action of thymol is reinforced by the acid pH of the formulation, useful in preventing or treating bacterial and fungal infections. [Guerra B, 2006].

The pharmacological action of thymol is completed by antioxidant [Braga PC, 2006b; Lee KG, 2002] and anti-inflammatory actions. [Braga PC, 2006a]

In conclusion, extract of Thymus vulgaris has properties that make it suitable for the use in preparations intended for daily intimate feminine hygiene.

The presence of Zn can explain the increased potency of SA2.1, evidenced by the improved MIC, now similar for C. albicans and C. glabrata.

Zinc salts showed in vitro the ability to inactivate herpes simplex virus (HSV) [Arenas M, 2000], to inhibit human immunodeficiency virus type 1 (HIV-1) infection, [Haraguchi Y, 1999] and the growth of Chlamydia trachomatis [Greenberg SB, 1985], Trichomonas vaginalis. [Gardner WA Jr, 1981]

Zinc inhibits virus production in HeLa cells infected with human rhinovirus type 1A, probably because zinc complexes with rhinovirus coat proteins, hindering their function as substrates for proteases or as reactants in the assembly of the virus particles. [Korant BD, 1976]

The SOS response is a global response to bacterial DNA damage in which the cell cycle is arrested and DNA repair and mutagenesis are induced. The SOS response may play a role in the development of antibiotic resistance and the effect of zinc suggests ways to prevent it. In fact, Zinc inhibits the virulence of diarrheagenic E. coli by inducing the envelope stress response and inhibiting the SOS response and the hypermutator phenomenon in E. coli as well as in Klebsiella pneumoniae, and was more effective in inhibiting the SOS response than other metals. [Bunnell BE, 2017]

Moreover, zinc provides a surfactant action, combining skin cleansing with skin care, as it intervenes in epidermis maturation and skin integrity, through collagen synthesis, fibroblast proliferation and repair processes. Zinc deposition onto the skin after long term use of Zinc cocethsulphate was determined by tape-stripping method in volunteers. A significant deposition of zinc is observed in the outer layers of epidermis. The deodorant and dandruff-reducing actions of Zinc cocethsulphate when used as skin cleanser are reported for sensitive skin and restitutio cleansing at low pH. Zinc showed to accelerate skin cells renewal and to have anti-irritant properties. [Rigano L, 2005]

The muco-adhesive system given by the association of Xanthan Gum and Carrageenan assures a prolonged contact of the formulation on vaginal mucosa, allowing to increase drug efficacy and to improve patient compliance thanks to a posology reduction. [Gasparrì F, 2017] The capability of muco-adhesive formulations to adhere to mucosal substrates is due to the presence of mucous-adhesive polymers.[Andrews GP, 2009; Smart JD, 2005] Experimental results indicate that the formulation based on xanthan gum/lambda carraghenan association is able to establish a greater interaction with mucin. The capability of xanthan gum/lambda carrageenan-based formulation to interact with mucosal substrate is responsible for a stronger resistance towards removal by wash-away.

The natural substances examined here demonstrated strong pharmacological and clinical activity, as shown by many independent and international sources. The maintenance of a healthy vaginal ecosystem and its restoration from mild/moderate inflammatory/infectious conditions, concomitantly, if needed, with pharmacological therapy, can prevent the complications of bacterial vaginosis, which, although not considered life-threatening, is recognised as a contributing factor in premature membrane rupture, premature birth and low birth weight.
CONCLUSIONS

The use of active ingredients in the woman's daily intimate hygiene is the essential issue for adequate protection of genital area. These cleanser formulations should scientifically prove their antimicrobial effectiveness necessary premise for maintaining genital health and preventing bacterial and fungal contamination. The data here presented suggest that formulation tested have an improved inhibitory activity against Candida albicans and Candida glabrata.

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