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Influence of *Candida albicans* biofilms on the resistance of *Salmonella typhi* to antibiotics

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Abstract

In many countries around the world, *Candida albicans* - *Salmonella typhi* co-infection would represent a real health problem according to the potential interactions that exist between both pathogens. The aim of this work was to study the impact of *C. albicans* on the resistance of *S. typhi* to antibiotics and to determine the influence of fungal biofilms on its growth. Reactivated clinical isolates of *S. typhi* (n = 5) and *C. albicans* (n = 5) were co-cultured on specific media containing or free of $MgCl_2$. The colonies obtained were analysed by microscopy and an antibiogram was performed from it. The analysis indicated that in case of simultaneous growth of *S. typhi* and *C. albicans*, there is improvement in fungal biofilm formation. But when *S. typhi* infection is posterior to that of *C. albicans*, fungal biofilms stimulated in vitro are disorganized. Moreover, the sensibility of *S. typhi* to antibiotherapy *in-vitro* appeared to decrease in the presence of *C. albicans* , by approximately 100% with ofloxacin, amoxicillin, doxycycline, ciprofloxacin and ceftriaxone, 87.55% with cefoxitime and 80% with colistin. Altogether, the results of the present study suggest that the co-culture of both pathogens improves the infectious capacity and resistance of *S. typhi* to antibiotherapy through fungal biofilm.

Keywords: *S. typhi; C. albicans*; Biofilms; Antibiotics; Resistance

1 Introduction

C. albicans is the main responsible yeast in human mycosis, including cutaneous, genital and digestive candidiasis [1]. Commensal to the digestive tract, saprophytic and absorbotrophic, it is generally its passage from the blastopore to the filamentous form that leads to potentially serious fungal pathologies, this biological ability generally being associated with a failure in the functioning of the immune system [2]. As for most microorganisms, *C. albicans* is found in ecosystems under complex structures consisting of microorganisms and self-induced extracellular matrix called biofilm which facilitate their vital adaptation to all biotopes. The latter consist either of fungus only or of at least two groups of microorganisms [3]. Indeed beneficial or competitive interactions between *C. albicans* and bacteria have already been reported [4-5]. Some bacteria like *Pseudomonas aeruginosa* and *staphylococcus epidermidis* easily colonize tissues when *C. albicans* is found on the same tissue in its mycelium and/or unicellular form. On the other hand, they give fungi

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resistance against toxic agents [6]. Similarly, *Streptococcus mutans* amplifies the aggressiveness of thrush disease caused by *C. albicans*, in compensation to metabolites supply, necessary for bacteria growth [7]. Moreover, in previous research of our team, it has been demonstrated that infection with *Candida spp*. would increase the frequency of infection with *S. typhi* and *S. paratyphi* by 4 to 5 times [8]. Then, it seems that yeasts have an impact not only on the adaptation of bacteria to their living environment, but also, they constitute one of the factors motivating the escape of the latter to immune system action or exacerbating their resistance to conventional drugs [1]. This can be illustrated in the case of typhoid fever.

Indeed, typhoid fever affects approximately 20.000.000 people/year over the world and is responsible for more than 600.000 deaths per year with predominance of clinical indistinguishable paratyphoid fever [9-10-11]. The real frequency of the disease, which is underestimated in endemic areas, receives special attention from Governments' Ministries in charge of Public Health in concerned countries, with the recurrent development of treatment and diagnostic protocols. Despite these efforts, the resistance of *S. typh* to antibiotics is still in increase [9]. Cephalosporins and quinolones commonly used in the world in case of acute and/or severe typhoid fever are becoming increasingly inappropriate for patients [9-12-13]. Previous studies have shown that the emergence of certain infection caused by Gram negative bacteria such as *Escherichia coli, Salmonella typhi murium* and *Pseudomonas aeruginosa* are linked to their ability to camouflage under their latent form in certain cells, tissues and organs, such as macrophages, epithelial cells or in the gall bladder, or to cooperate with other microorganisms such as *Schistosoma* and yeasts [5-14-15]. However, the relationship between *C. albicans* and *S. typhi* and the impact on resistance to antibiotics have not been intensively elucidated. In the present study, we evaluated the effect of *C. albicans* biofilms on the resistance of *S. typhi* to antibiotics.

2 Material and methods

2.1 Renewal of clinical strains and quality control

S. typhi (n=5) and *C. albicans* (n=5) strains obtained from patients at Yaounde Pasteur Center (CPY), Lanquintinie Hospital (HL) and Bonassama District Hospital (HDB) in Douala were reactivated for 24 hours on Sabouraud + chloramphenicol and Muller Hinton Agar (MHA) media 24 hours along for respective cultures. A resistance profile control test was carried out using the diffusion method through discs impregnated with antibiotics and antifungals, respectively on MHA for *S. typhi* and on Sabouraud medium for *C. albicans*.

2.2 *In-vitro* study of the influence of *C. albicans* biofilm on the growth of *S. typhi*

Two batches of *S. typhi* + *C. albicans* co-cultures were considered, one in the presence of 10 mM $MgCl_2$ and the other without $MgCl_2$, on potato medium + D-glucose (PGB medium) for 48 hours, prepared with 200 g of potato filtrate and 5.5×10^3 mol/L of D-glucose. Magnesium chloride had the role of enduring the formation of fungal filaments [16].

The contents of the tubes placed in co-culture at 37 °C for 48 hours were observed by microscopy at 40X objective to assess the simultaneous growth on PGB medium to check the reliability of the growth medium and the purity of the strain respectively [17-18]. The Gram and Crystal Violet solution tests were used for that activity.

2.3 Study of the impact of *C. albicans* on the sensitivity of *S. typhi* to antibiotics analysis

After the co-culture of *S. typhi* and *C. albicans* on PGB (potato and glucose broth) medium, the strains were inoculated on MHA medium for the antibiogram test according to the Clinical and Laboratory Standard Institute (CLSI). For the purpose, the antibiotics used were colistin, cefoxitime, ofloxacin, doxycycline, amoxicillin, ciprofloxacin and ceftriaxone which are usually prescribed for severe and benign typhoid fever before treatment with kecotonazole. Isolates were pre-treated to ketoconazole and then pre-enriched on selenite-sodium biselenite medium for 24h. Fluconazole discs were used to check yeast growth in Petri dishes. An antibacterial susceptibility control test was performed on clinical bacterial isolates. Colonies formed were analyzed by microscopy respectively with 40X and 100X objectives, after Crystal Violet and Gram staining as described by CLSI M100 [28-29].

2.4 Statistics and reproducibility

The microscopic observations were recorded as photographs. Graphs were created with SPSS version 23.0. Correlation between antibiotics inhibition diameter means were analyzed by t-Student method. The results were considered statistically significant for p-value inferior to 0.05.

3 Results

3.1 Nature and resistance profile of the strains.

Tables (1A) and (1B) present the nature of the strains used for the study, respectively the yeast and the bacteria strains.

Table 1(A) Resistance profile of *C. albicans* strains (1A) and *S. typhi* strains used (1B)

Strains Ca	Fluconazole	Voriconazole	Amphotericin B	Itraconazole	Nystatin	Flucytosine
S1446	S	S	S	S	S	R
S13409	S	S	S	S	S	R
S15932	S	S	S	S	I	R
S8230	S	S	S	S	S	R
S36105	S	S	S	I	S	I

R : Resistant ; I : Intermediate ; S : Sensitive ; C.a *: C. albicans* . S1446, S13409, S15932, S8230, S36105: *C. albicans* strains reactivated and identified by Vitek ® 2 YST ID card method.

1(B)

S. typhi	OFX	AX	DO	CIP	CRO	FOX	CTL
ST01CP19	R	R	R	R	R	S	R
ST01CP21	S	I	S	S	R	R	R
ST01HB19	R	R	S	R	R	R	R
ST02CP19	R	R	S	S	R	R	R
ST02CP21	R	R	R	R	R	R	R

OFX : ofloxacin ; AX : amoxicillin ; DO : doxycycline ; CIP : ciprofloxacin ; CRO : ceftriaxone ; FOX : cefoxitime ; CTL : colistine ; R : Resistant ; I : Intermediate ; S : Sensitive. ST01CP19, ST01CP21, ST01HB19, ST02CP19, ST02CP21: *Salmonella* strains reactivated and identified by API 20E diagnostic with Kirby-Bauer Disk Diffusion method.

Table (1A) shows that the strains of *C. albicans* used in the context of this study are all sensitive to the antibiotics tested with some exceptions: S36105 was intermediate to itraconazole and flucytosine while S15932 was intermediate to nystatin. Except of S36105, all were resistant to flucytosine. *S. typhi* strains used in the experiment and presented in table (1B). Major sensitivities were expressed by ST01CP19 (cefoxitime), ST01CP21 (ofloxacin, doxycycline, ciprofloxacin), ST01HB19 (doxycycline) and ST02CP19 (doxycycline, ciprofloxacin).

3.2 Influence of *C. albicans* biofilm on *S. typhi* growth

$MgCl_2$ solution had an impact on the growth of *C. albicans* as shown by the microscopic analysis. The isolates obtained from co-culture of *S. typhi* with *C. albicans* in PGB medium makes it possible to observe the formation of the bacterial biofilm under the influence of the fungal strains previously cultured without $MgCl_2$ (figures A1 and B1). On the other hand, the bacterium seems to stimulate the formation of mycelial filaments. Photographs (A2 and B2) show destabilising mycelial filaments and fungal pseudospores. There is no visible biofilm of *S. typhi*.

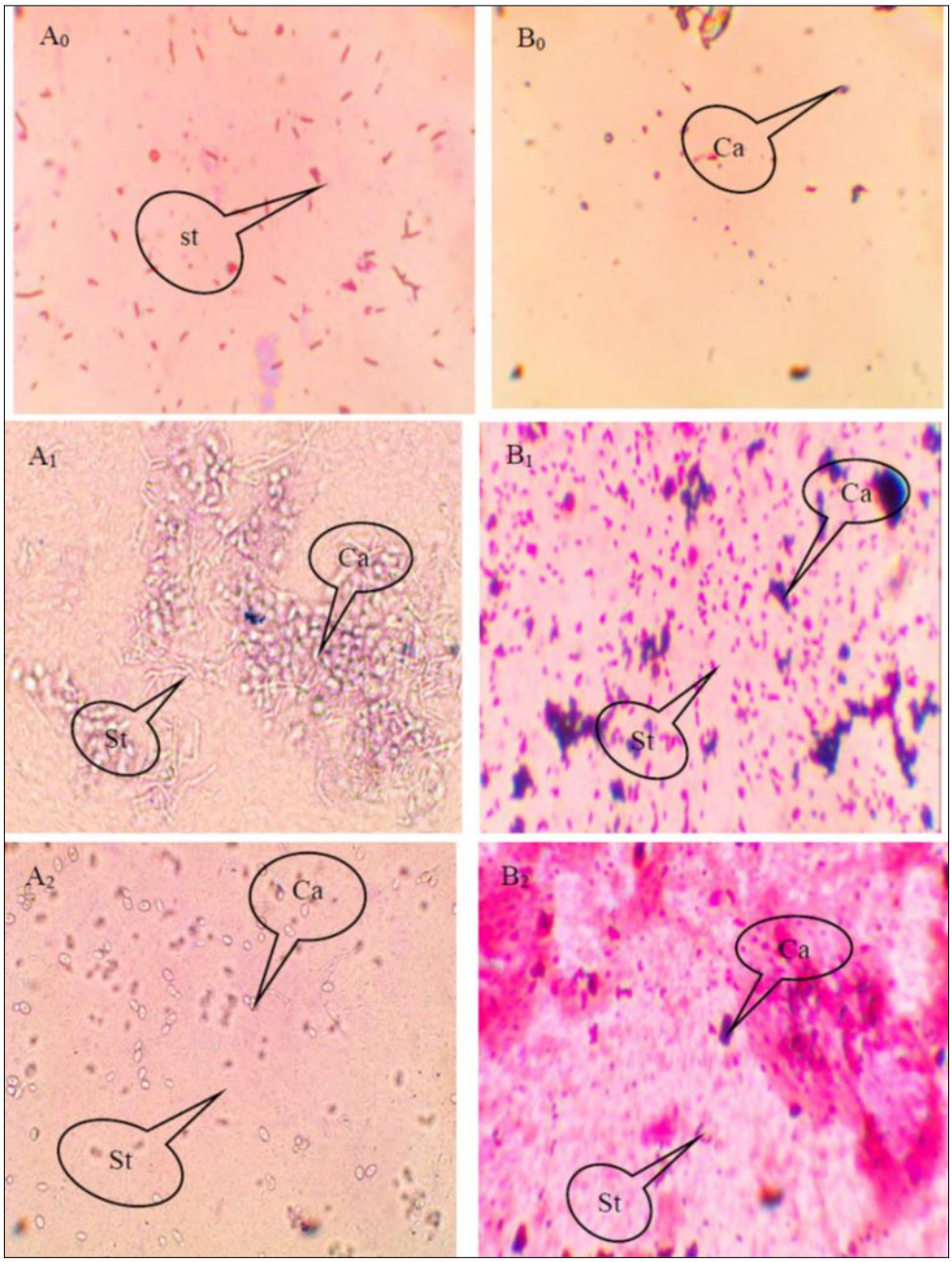

Figure 1 *C. albicans-S. typhi* in vitro co-cultured on PGB medium without $MgCl_2$ (A1, B1) and with $MgCl_2$ (A2, B2) seen under optical microscopy (40X objective) after staining with crystal violet solution (a) and (b) procedure. St: *S.typhi:* Ca: *C. albicans*. A0 and B0 are control strains respectively for *C. albicans* and *S typhi*

3.3 Impact of *C. albicans* on the sensitivity of *S. typhi* to antibiotics

The aim of this part was to check whether the sensitivity of *S. typhi* to antibiotics does not vary after co-culture with *C. albicans*. The result shows that the inhibition diameter of antibiotics decreases after *in vitro* co-culture of microorganisms (Table 2). There is significative difference in mean diameters for amoxicillin, ciprofloxacin and cefoxitime (in the absence of $MgCl_2$, $P = 0.042$) in the case of the co-culture of *S. typhi* and *C. albicans* without preliminary induction of fungal biofilm. This signifies the mean value of the inhibition diameters of antibiotics on *S. typhi* control strains is greater than those of co-cultured bacteria. Moreover, there is no statistical difference at $P < 0.05$ for ofloxacin, doxycycline, colistine and ceftriaxone, meaning that the mean inhibition diameters of antibiotics on control strains equal to those of co-cultured bacteria.

Table 2 Comparison of mean inhibition diameters values for antibiotics pairs in the presence or absence of $MgCl_2$

Pairs of antibiotics in the presence of $MgCl_2$	**Mean (mm)**	**Sig (bilateral)**	**Pairs of antibiotics in the absence of $MgCl_2$**	**Mean (mm)**	**Sig. (bilateral)**
Ofloxacin(st01-st1 with $MgCl_2$)	8.5±4.24	0.116	Ofloxacin(st01-st1 without $MgCl_2$)	8.5±4.24	0.116
Amoxicillin(st02-st2 with $MgCl_2$)	9.1±2.59	0,025	Amoxicillin(st02-st2 without $MgCl_2$)	9.1±2.59	0.025
Doxycycline(st03-st3 with $MgCl_2$)	8±3.16	0.065	Doxycycline(st03-st3 without $MgCl_2$)	14±5.31	0.058
Ciprofloxacin(st04-st4 with $MgCl_2$)	17.3±3.22	0,006	Ciprofloxacin(st04-st4 without $MgCl_2$)	14.6±4.26	0.027
Ceftriaxone(st05-st5 with $MgCl_2$)	4.8±2.31	0.107	Ceftriaxone(st05-st5 without $MgCl_2$)	8.7±3.60	0.073
Cefoxitime(st06-st6 with $MgCl_2$)	7.0±3.43	0.111	Cefoxitime(st06-st6 without $MgCl_2$)	7.2±2.43	0.042
Colistin(st07-st7 with $MgCl_2$)	5.5±2.28	0.074	Colistin(st07-st7 without $MgCl_2$)	6.9±3.03	0.085

st: *Salmonella typhi* ; st01 to st07: average of the diameters of growth inhibition by antibiotics for control strains; st1 to st7:

Average of the diameter of growth inhibition antibiotics for strains obtained after co-culture of *S. typhi* with *C. albicans.* By comparing antibiotics inhibition diameters obtained after applying the antibiotic to *S. typhi* in co-culture to the control cultures, it is found that regardless the experiment, the sensitivity of bacteria to tested antibiotics decreases (figure 2).

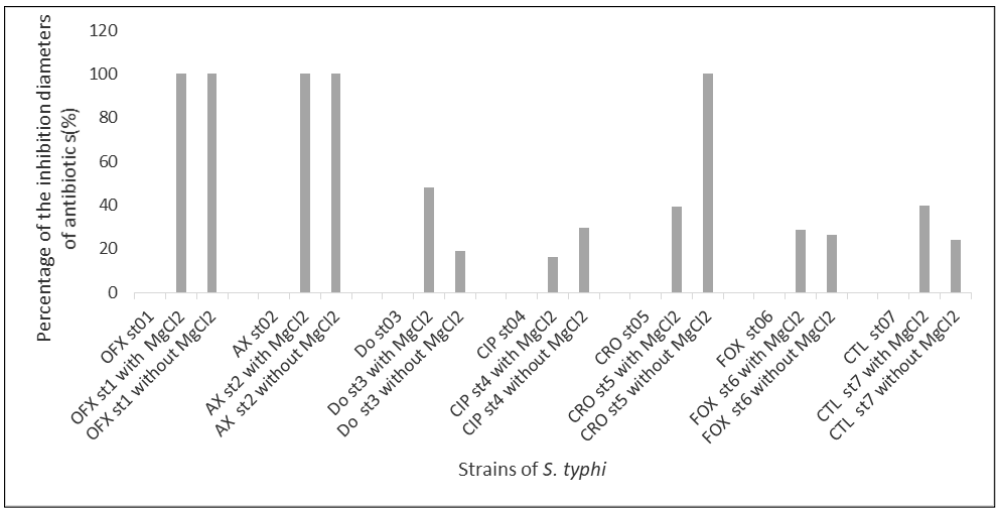

Figure 2 Comparison of *S. typhi* resistance after co-culture with *C. albicans* compared to witness strains by type of antibiotics. st: *Salmonella typhi* ; st01 to st07: average of the diameters of growth inhibition by antibiotics for control strains; st1 to st7: average of the diameter of growth inhibition antibiotics for strains obtained after co-culture of *S. typhi* with *C. albicans* . OFX : ofloxacin ; AX : amoxicillin ; DO : doxycycline ; CIP : ciprofloxacin ; CRO : ceftriaxone ; FOX : cefoxitime ; CTL : colistine

Comparing *S. typhi* strains inhibition diameters cocultured with *C. albicans* led to observation that the diameter of inhibition of bacterial growth by antibiotics when co-cultured with yeast in presence or absence of $MgCl_2$, is Less than those of witness strains.

4 Discussion

The aim of this study was to evaluate the influence of *Candida albicans* biofilm on the resistance of *Salmonella typhi* to antibiotics. This follows our first study which showed that the frequency of typhoid fever increases about 5 times in patients coinfected by *C.albicans* and *S. typhi* [8].

The co-infection of *S. typhi* with *C. albicans* on PGB medium shows that all fungal strains cultured without $MgCl_2$ influence the formation of the bacterial biofilm (figures A_1 and B_1). This reflects the resistance of *Salmonella* to yeast defense mechanisms. Indeed the modification of the shape of these bacilli *in vitro* corresponding to the formation of bacterial biofilm allows them to quickly adapt to their environment and save energy by exchanging molecules between cells, promoting their growth; this structure would ensure the distribution of nutrients and oxygen. As well, it allows wastes, enzymes and metabolites passage between cells [19]; the same applies to fungi [20]. However, if bacterial invasion of the culture medium is being subsequent to the colonization of filamentous and pathogenic *C. albicans* , the later would emit bactericidal or bacteriostatic molecules. The bacterium would therefore try to circumvent by minimising their impact on its physiology through the formation of a biofilm [17]. The molecules emitted by *S. typhi* can be antimicrobial peptides or toxins due, for example, to the activation of the hlyE gene which encodes for a porogenous homolysin toxin which is also well involved in its virulence or the systemic infection of the bacteria [21]. Indeed, the relationship between *C. albicans* and *S. typhi* would be conflicting. This assertion seems more obvious if we add to this hypothesis the competition of both pathogens for their carbon source i.e. glucose.

However, this competition would not be a brake on the invasion of *S. typhi* in an ecosystem initially colonised by fungi. The unchanged shape of bacteria after 48 hours of culture and mycelial filaments destroyed demonstrate in figures A_2 and B_2, the ability of bacteria to stimulate the formation of fungal biofilms (figures A_1 and B_1). These biofilms offer metabolic benefits and protection to bacteria for escaping the host defense system [22].

The co-infection of *S. typhi* with *C. albicans* decreases the sensitivity of *Salmonella* to antibiotics. Even the third-generation cephalosporins and quinolines used in this study and commonly administered to patients in healthcare centres for mild or severe typhoid fever are becoming obsolete (table 2) [23]. This would testify the appearance of new mutations on the *Salmonella pathogenicity island* (SPI)**,** responsible for the resistance of *S. typhi* to antibiotics. The resistance would be the result of two mechanisms: -competition, when the infection of the bacterium is posterior of the yeast; -symbiosis, when the infection of the bacteria is simultaneous with that of the yeast. The expression of molecules of the type III secretion system of *S. typhi*, in particular the *Salmonella* pathogenicity island 1 (SPI1-T3SS) would play an important role in this process as in *Pseudomonas aeruginosa* and *Salmonella typhimurium* [24]. The study conducted by Rakitin *et al.* (2021) showed that, the genome of *S. typhimurium*, very close to *S. typhi* resistant to antibiotics, contains the pathogenicity islands genes SPI1, SPI2, SPI4, SPI5, SPI9, SPI11, SPI13, SPI14 and CS54. Also, the decrease in the sensitivity of the pathogen to drugs is also due to the presence of integron plasmids [25]. So, decreased sensitivity of *S. typhi* to ciprofloxacin with *C. albicans* would be linked to a mutation in plasmids. Besides, studies have shown that point mutations in the Quinoline resistance determining region (QRDR) of the topoisomerase gyrA gene which codes for DNA gyrase could affect the genome of the bacterium and contribute to the emergence of resistance genes to this family of antibiotics. Genes also located on plasmids such as the HI1 and C incompatibility groups are vectors of drugs resistance in *S. typhi* which would complete the non-exhaustive list of other gene factors [26].

The antibiotics inhibition diameters obtained with the control strains decreased *in vitro*, after the co-culture of *S. typhi* with *C. albicans* regardless of the timing of the bacteria plant seeding on medium (figure 2). Summarising, the inhibition diameter values of the control strains are greater than those of the test strains. This would mean that in co-infection with *C. albicans* , bacteria develop drug resistance mechanisms to adapt to their ecosystems [27].

5 Conclusion

The evaluation of the influence of *Candida albicans* biofilms on the resistance of *Salmonella typhi* to antibiotics led to the conclusion that when the bacterial infection is posterior to the fungal invasion *in vitro*, *S. typhi* disorganizes the structure of the fungal biofilm in order to better recolonize the medium. On the other hand, when bacterial infection is simultaneous with that of the yeast, the bacteria stimulates the formation of the fungal biofilm which would then protect it against the action of antibiotics. Given the importance of these fungal biofilms in pathogenesis and antibiotic resistance of *S. typhi*, it is important to determine the molecular factors and mechanisms that would influence their establishment and consolidation in further studies.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors hereby certify that there is no conflicting interest of any kind in this study

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