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Interactions entre parabènes et levures du mycobiote cutané

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Abstract

Parabens are substances with antifungal and antibacterial properties, suspected to be endocrine disruptors and widely used as preservatives in cosmetics. In this case, exposure to these compounds is mainly dermal and interactions may occur with skin components including cutaneous mycobiota.

This work explored the *in vitro* interactions between three parabens (methylparaben, ethylparaben and propylparaben) and two yeasts of the human cutaneous mycobiota (*Candida parapsilosis* and *Cryptococcus uniguttulatus*) by studying the effect of these parabens on fungal growth and the fungal capacity to metabolize the tested compounds.

The growth of three strains of *C. parapsilosis* was not influenced by the presence of parabens; this could be explained by the low concentrations tested. On the other hand, growth of *C. uniguttulatus* was completely inhibited by ethylparaben from the first day of contact, whereas the same fungus was not sensitive to the two other parabens, even after seven days of incubation. The presence of a capsule in this fungus as well as the physico-chemical properties of ethylparaben could explain this selective inhibition.

C. parapsilosis degraded 90 to 100% of propylparaben after seven days of incubation, but had no effect on the other tested parabens. The enzymes of *C. parapsilosis* thus seem to only degrade the long chain parabens. In the same conditions, *C. uniguttulatus* did not degrade any paraben. This inability may be due to the absence of fungal enzymes able to degrade parabens or to the possible inaccessibility of an intracellular enzyme due to the polysaccharide capsule.

Our work has shown that parabens can act differently from one fungus to another within the cutaneous mycobiota, which could lead to a state of dysbiosis in people using cosmetic

products containing parabens. This cutaneous mycobiota can also be involved in the degradation of parabens and thus reduce, according to the produced metabolites and their activities, the risk of endocrine disruption they can induce.

Keywords: parabens, endocrine disruptors, fungi, cutaneous mycobiota, *Candida parapsilosis*, *Cryptococcus uniguttulatus,* degradation.

Introduction

Parabens (PBs) are a group of substances widely employed as preservatives, mainly in pharmaceuticals or food and industrials products (1–5) and are regarded as the most common ingredient of cosmetics (5,6). According to Dulio and Andres, PBs are present in more than 80% of hygiene products and toiletries such as shampoos, moisturizing creams, shaving creams and cleansing gels (7).

In European Union (EU), the maximum authorized concentration of PBs in cosmetic products is 0.4% for single ester and 0.8% for mixtures of esters (8). Initially used at the same concentrations, butyl and propylparaben were reduced to a percentage of 0.14% for each individual amount (9).

In terms of chemical structure, PBs are esters of *p*-hydroxybenzoic acid, with alkyl substituents ranging from methyl to butyl or benzyl groups (10,11). We can mention in particular methylparaben (MePB), ethylparaben (EtPB), propylparaben (PrPB), butylparaben (BuPB), and benzylparaben (BePB) (11). Among them, MePB and PrPB are the most commonly used and are often present in the products together (11,12).

On the other hand, the length of the alkyl chain is of considerable importance for the physicochemical properties of these products. In general, with the increase in the alkyl chain length, the solubility in water decreases (1,11,13). MePB and EtPB (short chain parabens) are more water soluble than PrPB and BuPB (long chain parabens). However, the antimicrobial properties of PBs are directly proportional to the length of alkyl chain (11,14). For example, BuPB has 4-fold greater ability to inhibit microbial growth than EtPB, but on the other hand solubility of BuPB is 12 times lower than EtPB (11). For this reason, in many products,

mixtures of MePB and PrPB or BuPB are used to ensure optimal antimicrobial activities and acceptable solubility (14).

PBs are also known for endocrine disrupting effects at low exposure levels. Experimental studies in animals have evidenced adverse effects on sperm production and testosterone levels following exposure to parabens with longer side chains, this same kind of PBs being also more estrogenic *in vitro* and *in vivo* (15). Moreover, it has been shown that MePB accelerates skin aging and causes damage to DNA during sun exposure (16).

The human skin is densely colonized with a microbiota influenced by many host and environmental factors. Changes in this microbiota clearly play a role in the pathobiology of many types of skin disease and cosmetic disorders (17). This cutaneous microbiota refers to all the microbial communities that predominate and / or are permanently adapted to the skin. Even if one million bacteria inhabit every square centimeter of skin, the microbiota also contains other less well-explored kingdoms such as that of fungi called "mycobiota" (18–20). A study of Findley et al. has improved the knowledge of cutaneous mycobiota by sequencing and analysis of fungal communities at different cutaneous sites in healthy adults (21). The results of their study showed a great fungal diversity of this mycobiota including *Candida*, *Malassezia*, *Epicoccum*, *Aspergillus*, *Cryptococcus* and *Rhodotorula* species, variable depending on the anatomical site.

Several host-related factors, such as regular application of cosmetics on skin could affect the composition and variations of mycobiota. It is known that dermally applied PBs are taken up by skin and metabolized by esterases. In general, skin permeation decreases with increased chain length (15). After uptake, PBs and their metabolites are conjugated and excreted in urine and bile (15). On the other hand, the cutaneous mycobiota could participate in the metabolism of PBs via its enzymatic arsenal. It is therefore interesting to study the role that this mycobiota

could play in the degradation of PBs and consequently in the potential reduction of the risk of their systemic toxicity.

The aim of this work was to explore the interactions between the most used PBs in cosmetic products and species from human cutaneous mycobiota by studying *in vitro* the fungal ability to metabolize the tested compounds and the effect of these PBs on the fungal growth.

Materials and methods

Fungal growth and parabens

The three strains of *Candida parapsilosis* and the strain of *Cryptococcus uniguttulatus* used in this study have been isolated from skin samples of healthy subjects. They have been identified by MALDI-TOF mass spectrometry (Vitek MS, BioMerieux). Before experimentation, *Candida* strains were grown for 48h on Sabouraud agar medium at 30°C and *C. uniguttulatus* was grown for 96h at 27°C on the same medium. The yeasts were then harvested in LB Broth (Sigma-Aldrich Inc. St. Louis, USA) and incubated at 30°C or 27°C during 24h before experimentation. The yeasts were resuspended at a concentration of 10^3/mL in LB medium diluted ½ in sterile distilled water containing PBs (Sigma-Aldrich) first dissolved in DMSO, in order to obtain in the experimentation medium a final concentration of 1mM for MePB and EtPB and of 100µM for PrPB. Growth controls were first carried out by incubating yeasts in DMSO at concentration used for PBs dilution. The assays were then carried out at pH 5.2 in glass tubes. At day 0, 1, 3 and 7, monitoring of the fungal growth was carried out by seeding a part of fungal suspensions on Sabouraud agar medium. The agar plates were then incubated during 48h at 30°C for *C. parapsilosis* and during 72h at 27°C for *C. uniguttulatus* before numeration of the CFU (colony forming unit). Controls of fungal growth were also carried out in the same medium without PBs.

Each experimentation has been carried out twice, each time in triplicate.

Determination of parabens

At day 0, 1, 3 and 7, 300 µL of fungal suspension were in parallel centrifuged at 3000g for 15 min to pellet the cells, and 100µL of the supernatant were collected for the determination

of PBs. Medium supplemented with each paraben, but without microorganisms, was used as negative control of parabens degradation.

PBs were assayed in LB broth using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. PBs analytical standards (MePB, EtPB and PrPB) were obtained from Sigma-Aldrich Inc. Calibration standards were prepared by spiking known amounts of each PB in LB broth to construct a 5-point calibration curve. Calibration standard final concentrations ranged from 10 ng/mL (limit of quantification) to 200 ng/mL. Similarly, quality control samples were also prepared in LB broth to obtain final concentrations in the low (10 ng/mL), medium (25 ng/mL) and high (100 ng/mL) ranges.

As a first step, study samples were diluted 1/1500 and 1/15000 using a mixture of water and methanol for PrPB and MePB/EtPB, respectively. Deuterated internal standards (MePB-d4, EtPB-d5 and PrPB-d7) obtained from LGC Standards (Molsheim, France) were added to each sample (standards, QCs and study samples). Then, these samples were diluted 1/50 with a mixture of water and methanol (50/50, v/v) and injected directly into the LC-MS/MS system.

A UHPLC system from Shimadzu (Nexera X2 (Kyoto, Japan)) coupled to a 6500+ QTrap mass spectrometer from Sciex (Concord, Canada) was used for analysis. Target analytes were separated using a 3.5 µm CSH column (Waters® Acquity CSH C18, 2.1 x 100 mm, Milford, USA) heated at 40° C and a mobile phase composed of pure water (phase A) and acetonitrile (phase B) delivered at 0.35 mL/min in the gradient mode. Gradient elution started at 0% of B (0-1 minute); then linearly increased to 20% of B (1-4 min); then to 98.6% of B (4-7 min); maintained at 98.6% of B for 1 minute (7-8 min); then decreased to 0% of B (8-8.1 min) and finally maintained at 0% of B for 2.4 minutes (8.1-10.5 min) for re-equilibration of the column.

Target analytes were then transferred to the mass spectrometer using an electrospray ionization interface, operating in negative ionization mode. For each compound, multiple reaction monitoring mode (MRM) with two specific daughter fragment transitions for one parent precursor ion was used. The first was used for quantification while the second was used for confirmation. Specific transitions used for each compound are reported in table 1.

Data were acquired with Analyst® software and quantification was obtained with MultiQuant® software. Ratios of peak areas of target analyte over its corresponding deuterated internal standard were used for calculations. Results were expressed as percentage of residual PBs, calculated using tubes containing PBs without microorganisms as reference.

Results and discussion

Effect of parabens on fungal growth

PBs are preservatives widely used in cosmetics thanks to their antifungal and antibacterial activity. They are generally more active against yeasts than against bacteria (22). Once in contact with the skin, these molecules could interact with its constituents, including cutaneous mycobiota.

To explore the effect of PBs on *C. parapsilosis* and *C. uniguttulatus,* the yeasts have been incubated during 7 days in LB culture media without and with 1 mM of MePb and EtPb and 100 µM of PrPb, then the fungal CFU have been numbered at Day 0, 1, 3 and 7. The results showed that the growth of the three *C. parapsilosis* strains has not been influenced by the presence of PBs (Figure 1). The count of CFUs of the three strains in the presence of each PB is similar to that of the same strains without PB after 7 days of incubation. The absence of a fungicidal effect of these compounds could be explained by the concentrations used in our study: 1 mM for MePb and EtPb and 100 µM for PrPb corresponding to 0.152 g/L, 0.166 g/L and 0.018 g/L respectively. These concentrations are low compared to those usually used in cosmetics that are close to the maximum concentrations authorized by European regulations: 0.4% (≃4 g/L) for EtPb and MePb and 0.14% (≃1.4 g/L) for PrPb (9). Moreover, Miceli et al. have reported that 0.08% (0.8 g/L) EtPB or 0.01% (0.1 g/L) PrPB was required to inhibit growth of *C. albicans* by 50% (23). On the other hand, the growth of *C. uniguttulatus* has been completely inhibited by 1 mM of EtPb from the first day of contact, whereas this same fungus was not sensitive to the two other PBs even after 7 days of incubation (Figure 1). The inhibition of *C. uniguttulatus* growth by EtPb could be explained by the mechanism of action of PBs which depends on their physicochemical properties and their cellular target.

As emphasized by many authors, despite the fact that these compounds have been used for more than 50 years, the exact mechanism of antimicrobial activity demonstrated by PBs has not yet been elucidated (24–27). Most studies have focused on bacteria and it was postulated that these compounds are capable of protein synthesis inhibition (including key enzymes, such as ATPases and phosphotransferases) (27–29) or of the inhibition of the synthesis of DNA and RNA (28–30). Other studies pointed out that PBs may interfere with the microorganisms respiration (22,26,27,31) by the inhibition of oxygen consumption of mitochondria in fungi, especially in mitochondrial complex II (25,28); however, it was also postulated that the mode of action of aliphatic PBs was based on their ability to disrupt a cell membrane by the modification of its integrity and permeability (32,33), the induction of a potassium efflux (34), or the alteration of the transmembranal potential (24,26,27). These substances may have multiple biological effects, but their inhibitory effects on membrane transport and mitochondrial processes are considered essential for their antifungal activity (1,22,25,35).

The importance of antimicrobial activity of PBs is related to their chemical structure. A linear relationship exists between this antimicrobial activity and the length of the alkyl chain from methyl to octyl paraben (11,36). PrPB is considered more active against most bacteria than MePB (29).

In addition, it has been shown that the same intracellular concentrations of MePB, EtPB or PrPB were required to cause a 50% decrease in the rate of *Escherichia coli* growth, suggesting that their different antimicrobial activities could be explained by differences in their extent of uptake by the cell (37).

Indeed, simultaneously with the increase of the length of the alkyl chain, the value of octanol–water partition coefficient rises, which results in decrease of water solubility and increase lipophilicity (1,11,13). Studies have shown a correlation between the increase in anti-

microbial activity of PBs and an increase in their lipophilicity, supporting the hypothesis of a mechanism associated with solubilization in the cell membrane (37). Consequently, the lowest minimal inhibitory concentration for *Aspergillus*, *Fusarium* or *Penicillium* is recovered for BuPB and PrPB compared to MePB and EtPB (22).

Surprisingly, concerning our results, we observed an inhibition of the growth of *C. uniguttulatus* only in the presence of EtPB whereas PrPB is more liposoluble than EtPB. The yeasts from *Cryptococcus* genus present the particularity of having a capsule of polysaccharide nature. So, PBs have to interact with the fungal capsule before reaching the cell membrane. Considering the stability of the concentration of EtPB from day 0 to day 7 of incubation and the total inhibition of the growth of *C. uniguttulatus* under EtPB, two hypotheses could explain our result: i) EtPB could act specifically on a target located at the capsule level, thus inhibiting the yeast growth, or ii) the capsule could behave differently with EtPB only, allowing this compound to cross the capsule to act on the cell membrane or an intra cytoplasmic target. During this action, EtPb would not be degraded, explaining the absence of decrease in its concentration between day 0 and day 7 of incubation. The specific passage of the capsule by EtPB could be explained in this case by the chemical structure of this PB with a water solubility/liposolubility ratio allowing it to cross the capsule while MePB is more water-soluble and PrPb more liposoluble.

Therefore, according to these results, the effect of EtPB differs from one fungus to another within the human cutaneous mycobiota.

Effect of tested fungal strains on parabens

The determination of PBs has been carried out after Day 0, 1, 3 and 7 of incubation in the supernatant of culture of *C. parapsilosis* and *C. uniguttulatus* incubated with each PB. Our results showed no decrease of MePB and EtPB concentrations in presence of *C. parapsilosis* strains. On the contrary, these three same strains have been able to degrade PrPB up to 100% after 7 days of incubation: 6 to 8% at D1, 16 to 36% at D3 and 90 to 100% at the end of the incubation time (Figures 2). Moreover, no decrease of the PBs has been observed in the experimental medium without yeasts. *C. parapsilosis* appears to possess enzyme(s) capable of acting selectively on PrPB and sparing MePB and EtPB. It can therefore be suggested that this (ese) probable enzyme(s) act(s) only on long-chain alkyl PBs.

Our results are consistent with the few studies published in the literature. Koseki et al. isolated and characterized an *Aspergillus oryzae* protein capable of hydrolyzing the ester bond of EtPB, PrPB and BuPB (38). This esterase named AoPrbA decreased the antimicrobial activity of these PBs. The highest hydrolytic activity of this esterase was against BuPB (0.42 U/mg protein) and decreased with respect to shorter alkyl chain PBs (0.28 U/mg protein for PrPB and 0.094 U/mg protein for EtPB). A recent study by Zhu and Wei showed that the fungus *Fusarium solani pisi* has a cutinase enzyme (FsC) also capable of hydrolyzing the ester bond of PBs (39). Authors expressed this enzyme on the surface of *Saccharomyces cerevisiae* and showed that it degraded only 3% of MePB and 20% of EtPB. On the other hand, it degraded PrPB and BuPB with an efficiency of 89% and 97%, respectively, after 24 hours of incubation.

Another class of fungal enzymes capable of degrading PBs is laccases (40–47). This family of oxidases has attracted increasing interest in wastewater treatment processes in recent years due to its ability to degrade PBs into non-toxic or easily removable products (47). Macellaro et al. studied the ability of four fungal laccases to degrade PBs including MePB and

BuPB (40). They showed that these enzymes were able to degrade these two PBs in the presence of a natural mediator, and that BuPB was more susceptible to degradation than MePb. Mizuno et al. demonstrated that both iso-BuPB and n-BuPB were almost completely removed (95%) after 2 h of treatment and disappeared after 4 h of treatment with 0.5 U/mL of laccase activity in the presence of 1-hydroxybenzotriazole (HBT) as mediator (40,41).

Our results as well as those of the literature showed that PBs with a relatively long alkyl side chain would be preferentially degraded by fungal enzymes, compared to those with a short alkyl side chain. In order to explain this finding, Zhu et al. calculated the binding energy between the active site of the FsC cutinase enzyme and various PBs (39). They found that this energy increased, and thus that the binding of PBs with the FsC became stronger, with increased length of their alkyl side chain (39). It can therefore be concluded that the preferential degradation of long-chain alkyl PBs is not related to their liposolubility but rather to the enzyme-paraben binding energy.

Our results obtained with *C. parapsilosis* as well as those of the fungal enzymes published in the literature are at the opposite of the results found in bacteria. Valkova et al. showed that the specific activity of PrbA esterase produced by the bacterium *Enterobacter cloacae* was high with EtPB (100%) and MePB (91%). The activity declines with longer chain-length parabens, with 70% activity with PrPB and 35% activity with BuPB (48). *Enterobacter gergoviae* also showed a significant degradation capacity, removing 97.9% of the MePB (49). Amin et *al*. compared the degradation of MePB and PrPB by *Pseudomonas beteli*. The latter degraded 100% MePB and 5% PrPB in 10 days (50). Bacterial esterases degrade more efficiently PBs with short alkyl side chains.

The main product of parabens hydrolysis is *p*-hydroxybenzoic acid (*p*-HBA) (1,15,51). Some microorganisms like *Pseudomonas sp* can use the *p*-HBA as a carbon source via the β-

ketoadipate pathway (50). This pathway is one of the routes by which a microorganism can attack the six-carbon nucleus of an aromatic compound and convert it into an aliphatic acid. This acid is further broken down to acetyl-CoA and succinate, which then enter the tricarboxylic acid cycle (Krebs cycle) (50).

Concerning metabolites, in a study comparing the cellular toxicity of PrPB in the presence of an inhibitor of esterase and the toxicity of its metabolites, the metabolites -*p*-HBA or the alcohol derived from PrPB- did not exhibit any significant toxicity (1). The increased toxic action of PrPB in the presence of esterase inhibitor suggests that PrPB itself, rather than its hydrolysis product, was responsible for the toxicity observed (1). No such data exist regarding the endocrine disrupting properties of these metabolites.

Unlike *C. parapsilosis*, *C. uniguttulatus* is unable to degrade PrPb, MePb and EtPb after 7 days of incubation (Figure 2). This inability may be due to the absence of fungal enzymes capable of degrading PBs or the inaccessibility of a possible intracellular enzyme to PBs because of the polysaccharide capsule.

Conclusions and perspectives

Our work showed that yeasts of the cutaneous mycobiota could be involved in the degradation of PBs used in cosmetic preparations and consequently in the potential decrease of endocrine disruption risk. Further studies would be necessary to explore if their enzymatic arsenal has a role in this degradation that need to be explore through the identification and assay of the metabolites of PrPB. We also demonstrated in this *in vitro* study that the relationships between fungi and PBs could represent one of the factors contributing to the dysbiosis of cutaneous mycobiota. Nevertheless, studies could be performed with other cutaneous fungal species, such as *Malassezia*.

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Table and figures

Table 1. Retention times and MRM transitions for target analytes.

Analyte	Retention time (min)	MRM transition (m/z)
MePB	5,73	151,0 -91,9
		151,0-136,0
MePB-d4	5,70	155,0-96,0
		155,0-140,0
EtPB	6,53	165,0-91,9
		165,0-136,8
EtPB-d5	6,51	170,0-91,9
		170,0-135,6
PrPB	6,98	179,1-91,9
		179,1-136,8
PrPB-d7	6,96	186,1-91,9
		186,1-136,0

Figure 1. Colony Forming Units (CFU) numeration of the three *C. parapsilosis* strains (CP1, CP2, CP3) and of the *C. uniguttulatus* (CU) strain in culture medium without (Control) or with parabens (Methylparaben-MePB, Etylparaben-EtPB, Propylparaben-PrPB) at Day 0,1,3 and 7.

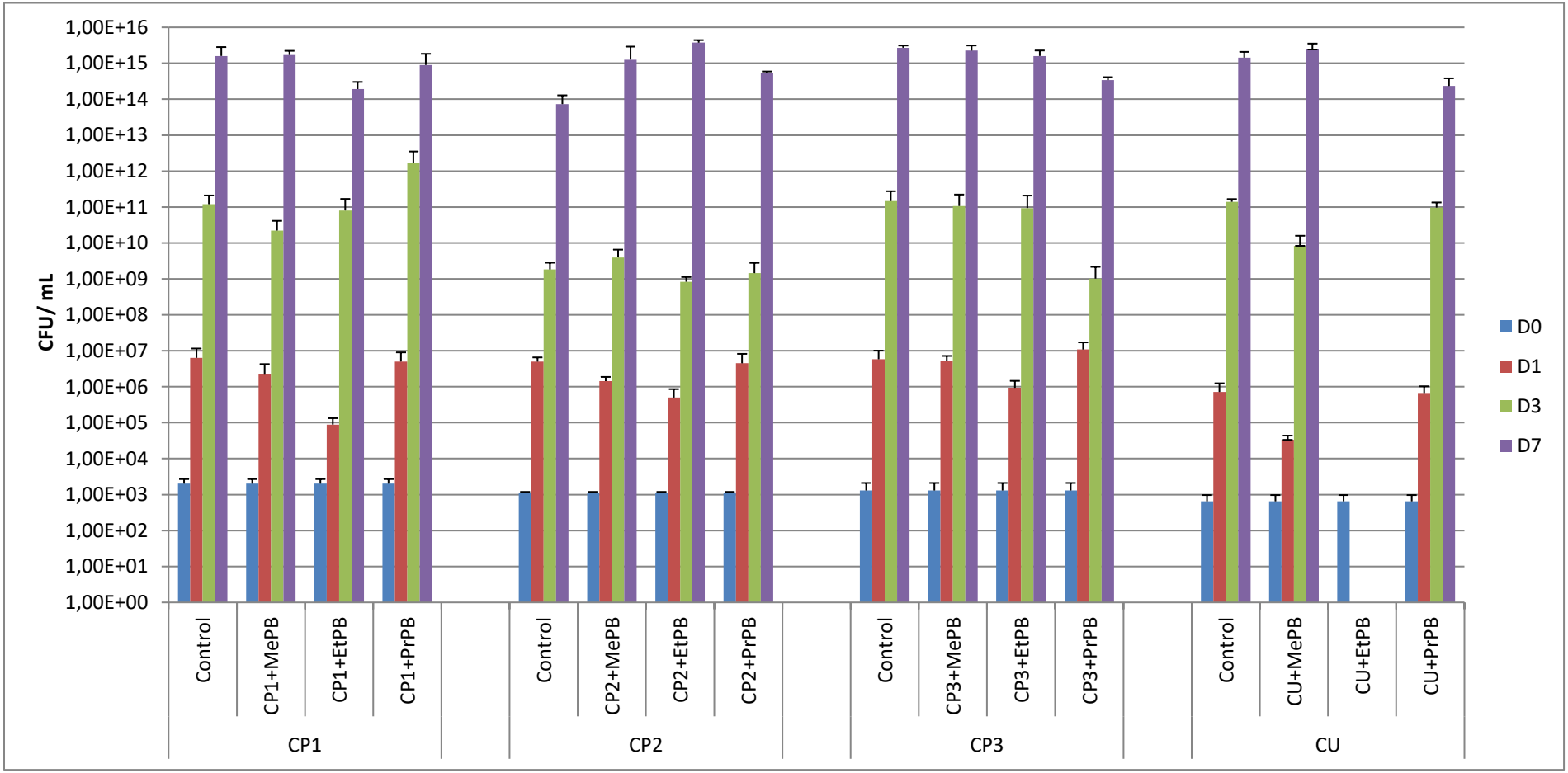
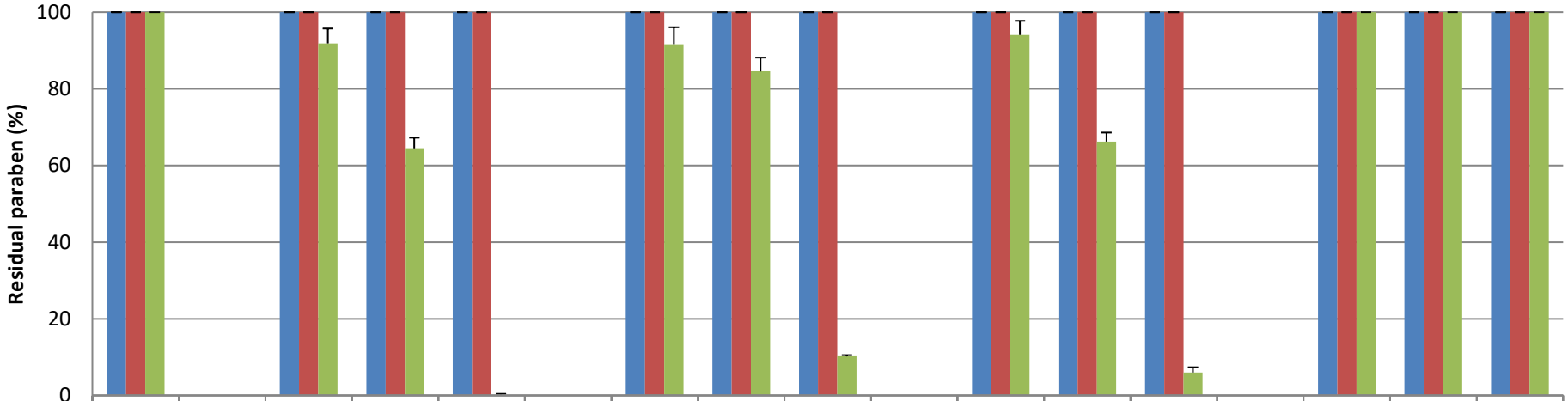


Figure 2. Residual parabens (%) (Methylparaben-MePB, Etylparaben-EtPB, Propylparaben-PrPB) during incubation times with *C. parapsilosis* (CP1, CP2, CP3) and *C. uniguttulatus* (CU) strains. A determination of parabens (CTR) has been carried out after 7 days of incubation in the experimental medium without yeasts.



	D7 CTR		D1	D3	D7		D1	D3	D7		D1	D3	D7		D1	D3	D7
			CP1				CP2				CP3				CU		
MePb	100		100	100	100		100	100	100		100	100	100		100	100	100
EtPb	100		100	100	100		100	100	100		100	100	100		100	100	100
PrPb	100		91,84	64,47	0		91,63	84,54	10,19		94,04	66,23	5,98		100	100	100

Résumé

Les parabènes sont des substances dotées de propriétés antifongiques et antibactériennes, suspectées d'être des perturbateurs endocriniens et très largement utilisées comme conservateurs dans les produits cosmétiques. L'exposition aux parabènes s'effectue alors principalement par voie cutanée et ces produits peuvent alors interagir avec les constituants de la peau dont le mycobiote cutané.

Ce travail a permis d'explorer les interactions entre trois parabènes (méthylparabène, éthylparabène et propylparabène) et deux levures du mycobiote cutané humain (*Candida parapsilosis et Cryptococcus uniguttulatus*) en étudiant *in vitro* l'effet des parabènes sur la croissance fongique et la capacité de ces microorganismes à métaboliser les parabènes testés.

La croissance des trois souches de *C. parapsilosis* n'a pas été influencée par la présence de parabènes, ce qui pourrait être expliqué par les faibles concentrations testées. En revanche, la croissance de *C. uniguttulatus* a été complètement inhibée par l'éthylparabène dès le premier jour de contact, alors que ce même champignon n'était pas sensible aux deux autres parabènes, même après sept jours d'incubation. La présence d'une capsule chez ce champignon ainsi que les propriétés physico-chimiques de l'éthylparabène pourraient expliquer cette inhibition sélective.

Les trois souches de *C. parapsilosis* ont dégradé 90 à 100% de propylparabène après sept jours d'incubation, mais n'ont eu aucun effet sur les autres parabènes. Les enzymes de *C. parapsilosis* ne semblent ainsi dégrader que les parabènes à chaine longue. *C. uniguttulatus* n'a dégradé aucun parabène, même après sept jours d'incubation. Cette incapacité peut être due à l'absence d'enzymes fongiques capables de dégrader les parabènes ou à l'inaccessibilité possible d'une enzyme intracellulaire aux parabènes en raison de la capsule polysaccharidique.

Nos travaux ont ainsi montré que les parabènes peuvent agir de façon différente sur un champignon ou un autre au sein du mycobiote cutané ce qui pourrait entrainer un état de dysbiose chez les personnes utilisant des cosmétiques contenant ces composés. Ce mycobiote cutané peut également être impliqué dans la dégradation des parabènes et ainsi diminuer potentiellement le risque de perturbation endocrinienne dont ils sont responsables.

Mots clés : parabènes, perturbateurs endocriniens, champignons, mycobiote cutané, *Candida parapsilosis*, *Cryptococcus uniguttulatus*, dégradation.

SERMENT

En présence des Maîtres de cette école, de mes chers condisciples et devant l'effigie d'Hippocrate, je promets et je jure d'être fidèle aux lois de l'honneur et de la probité dans l'exercice de la médecine. Je donnerai mes soins gratuits à l'indigent et n'exigerai jamais un salaire au-dessus de mon travail. Admis dans l'intérieur des maisons mes yeux ne verront pas ce qui s'y passe ; ma langue taira les secrets qui me seront confiés, et mon état ne servira pas à corrompre les mœurs ni à favoriser le crime. Respectueux et reconnaissant envers mes Maîtres, je rendrai à leurs enfants l'instruction que j'ai reçue de leurs pères.

Que les hommes m'accordent leur estime si je suis fidèle à mes promesses ! Que je sois couvert d'opprobre et méprisé de mes confrères si j'y manque !