

Preservative and antimicrobial susceptibility of non-fermenting bacilli recovered from solid waste of beauty salons in Brazil

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ABSTRACT

This work aimed to isolate Non-fermenting Gram-Negative Bacilli (NFGNB) from packages of hair care products, discarded as common solid waste by beauty salons and to assess their susceptibility to antibiotics and preservatives. Samples from hair care products were collected during eight weeks from seven salons in the metropolitan area of the city of João Pessoa, Brazil. Twelve NFGNB were recovered and subjected to biochemical identification and susceptibility testing. Species of the genus *Burkholderia* were more frequent, followed by *Pseudomonas* and *Aeromonas*. The highest antibiotic resistance rates were observed among quinolones, meropenem, and sulfonamide. Six isolates showed multidrug-resistance profile and survived different concentrations of parabens, imidazolidinyl urea, and triclosan. However, no correlation between resistance to antibiotics and the preservatives was found. Multidrug-resistant opportunistic pathogenic species found in the material remaining inside the used packages may represent a risk to human health and to the environment when discarded improperly with normal household waste.

INTRODUCTION

Due to the expanding influence of the beauty industry over the last decade, Brazil has experienced an increase in the number of beauty salons. These establishments dealing with cosmetics treatment as well as provide extended services related to the use of several chemicals (Nkansah *et al.*, 2016). Cosmetics and personal care products (CPCPs) are commodities formulated in order to cleanse, mask and correct imperfections, real or imaginary, and are therefore related to positive human emotions and rather than being considered as relating to environmental issues by most users (ANVISA, 2005). However, CPCPs may affect the environment in two ways: (1) by the nature of the

substances themselves, as yet an underexplored theme and; (2) by means of the generation of packaging waste (Zulaikha *et al.*, 2015; Fendall and Sewell, 2009).

As they are products for external use, CPCPs are designed to last since they must maintain their organoleptic and/or cosmeceutical characteristics for consumption up to 36 months (Bu *et al.*, 2013). However, many substances included in the formulations can be used as nutritional sources for microorganisms, requiring the use of different classes of preservatives in order to prevent spoilage of a product during its lifetime (Flores *et al.*, 1997). In addition, certain compounds may easily be decomposed by microorganisms, thus releasing toxic substances into the environment (Xu *et al.*, 2009).

Although CPCPs are not required to be sterile preparations, when opened, all cosmetic products are subjected to secondary contamination from the handler microbiota or from the storage environment (Zapka *et al.*, 2011). Biological contaminants in cosmetics may include pathogenic organisms, opportunistic

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pathogens and saprophytes, particularly Non-fermenting Gram-Negative Bacilli – NFGNB (Shaqra *et al.*, 2014). Microbial contamination from cosmetics is a global concern with possible negative economic consequences as well as risks to public health, when considering the potentials for product loss integrity, consumer infection, and the onset of microbial multidrug-resistance phenomenon, due to the resilience of preservatives present in the formulations (Lin *et al.*, 2010; Richardson, 2007).

In Brazil, the cosmetics industry generates nearly US\$ 110 billion, growing on average by 10% each year, and taking the 7th position as producer, 24th as exporter and 3rd as a global consumer, particularly of hair care products, makeup and for oral hygiene and personal care (ABIHPEC, 2018). The most consumed CPCPs are intended for the treatment of hair, representing an increase of 38.2% in sales of these inputs in one year alone. Most CPCPs costumers in Brazil are women visiting beauty salons at least once a week, representing in the last 10 years an increase in consumption in all social classes: 84% (A and B), 78% (C) and 63% (D and E) (IBOPE, 2017).

In many beauty salons, the different CPCPs are handled incorrectly. Solid waste and wastewater may contain any number of chemical and biological contaminants, making these establishments an important source of pollution (Ajuzie and Osaghae, 2012). In this context, the waste produced may adduce many pathogens to the environment, posing a serious risk in terms of human and environmental health. This work aimed to recover NFGNB from packages of hair care products, discarded as common solid waste by beauty salons and to assess their susceptibility to antibiotics and preservatives.

MATERIAL AND METHODS

Beauty salons and collection of solid waste

Samples of solid waste from opened cosmetic packages were collected from disinfected collectors distributed in seven salons in the cities of João Pessoa and Cabedelo, Brazil. Salon employees, who were responsible for the use and collection of the material had been previously instructed on how to dispose of the packages in the collectors properly to avoid further contamination of the material or to mix their contents with organic material. The accumulated waste was manually separated, using the recommendations of the Brazilian legislation (ANVISA, 2004).

Samples were taken by swabbing the inner wall of the package containing the remaining material. In containers with larger openings, the scraping was performed at the bottom, close to the grooves or slots, while with the threaded opening or flip top type of container, the scraping was conducted near the region of the caps. NFGNB were isolated (APHA *et al.*, 2012) and staining pattern and identification were conducted as described by Vieira *et al.* (2011), using the Bactray III® (Laborclin, Pinhais, Brazil).

Antibiogram

The susceptibility of the isolates to seven antibiotics used in the empirical antipseudomonal therapy scheme was tested by the Kirby-Bauer disk diffusion method (NCCLS, 2003). The tests were performed in duplicate.

Susceptibility testing of preservatives

All NFGNB resistant to at least one antibiotic were subjected to contact test with different concentrations of four substances used as preservatives in hair care product formulations to determine the minimum inhibitory concentration (MIC). The method and media used were the same as in the antibiogram. The preservative concentrations were based on biocidal concentrations already identified as to effect, given the current Brazilian legislation on the use of these substances in cosmetics (ANVISA, 2012). These tests were performed in duplicate.

Data processing and statistical analysis

To obtain the resistance ratio of an organism to the antibiotic, the number of times that this response occurred was divided by the total number of antibiotics tested. The index of resistance to preservatives was calculated in the same way. The correlation between these two indices was determined by the Pearson test and considered significant if $p < 0.05$ using R version 3.3.3.

RESULTS AND DISCUSSION

Solid waste generated by beauty salons

A total of approximately 14 kg of used hair care product containers was produced by the selected beauty salons over eight weeks. Most of these packages (96.6%) were made from plastic material, followed by metal and glass, totaling 3.4%. The collection included 146 packages of 73 types of national and foreign products, especially toners or highlighters (17.1%), shampoos (15.8%), neutralizers (15.1%), hair dyes (10.3%) and bleaches (8.9%). Other cosmetic materials (32%) included hairsprays, hair masks, conditioners, and moisturizers. Using the Cosmetic Ingredient Review specifications (2018), eight preservatives with known toxicity or irritability were listed: DMDM hydantoin, ethylparaben, imidazolidinyl urea, methylparaben, methylisothiazolinone, propylparaben, sodium benzoate and triclosan. Among them, parabens were found in 77.7% of the products.

Isolation of microorganisms

Twelve NFGNB were recovered from the packaging and are summarized in Table 1. Three genera were identified: *Burkholderia*, *Pseudomonas*, and *Aeromonas*. All detected species are rods associated with opportunistic pathogens and known for their metabolic versatility by which they can easily adapt to selective pressures in their environment (Hernández-Montes *et al.*, 2012).

Secondary contamination of CPCPs generally occurs due to improper manipulations by the handlers and improper storage of the products, which can result in premature deterioration of the CPCPs (Shaqra *et al.*, 2012). Because most of the ingredients used in the formulation of CPCPs are degradable, preservatives are usually included in CPCPs to extend the stability of the end product to up to 36 months (Eck, 2006). Given this, cosmetic raw materials used for hair care provide a rich nutrient source for microorganisms, for example, fatty acids, vegetable extracts, hydrocarbons and natural polymers, synthetic or semisynthetic (Geis, 2006).

Table 1: NFGNB recovered from the beauty salon.

Hair care products	Isolates
Hair moisturizer	RX01 (<i>Pseudomonas aeruginosa</i>)
	RX02 (<i>Burkholderia cepacia</i>)
	RX04 (<i>Aeromonas hydrophila</i>)
	RX11 (<i>Burkholderia cepacia</i>)
Hair straightening cream	RX03 (<i>Burkholderia cepacia</i>)
	RX12 (<i>Burkholderia cepacia</i>)
Toner	RX05 (<i>Burkholderia cepacia</i>)
Conditioner	RX06 (<i>Burkholderia cepacia</i>)
Shampoo	RX07 (<i>Burkholderia cepacia</i>)
	RX08 (<i>Pseudomonas aeruginosa</i>)
Hair mask	RX09 (<i>Burkholderia pseudomallei</i>)
Highlighter	RX10 (<i>Pseudomonas aeruginosa</i>)

Antibiotic resistance

Table 2 shows the results of the antibiotic susceptibility test. Six isolates showed resistance to between one and four antibiotics. Although the six other isolates were sensitive to all antibiotics tested, well-defined colonies with clear sharp edges were observed in the zone of inhibition. Invasion in the halo region was observed in RX03, RX05, and RX08 which were sensitive to gentamicin, imipenem, polymyxin B and sulfonamide.

Table 2: Number and frequency of antimicrobial resistant NFGNB and mutant subpopulation recovered from beauty salons.

Antibiotic	Gram-negative bacilli		Resistant mutant subpopulation	
	n	%	n	%
CIP	1	8.3	2	16.7
NOR	3	25.0	7	58.3
IMP	0	0.0	3	25.0
MER	2	16.7	4	33.3
GEN	0	0.0	4	33.3
SUL	3	25.0	6	50.0
POL	5	41.7	7	58.3

CIP–ciprofloxacin 5 mg.mL⁻¹, NOR–norfloxacin 10 mg.mL⁻¹, IMP–imipenem 10 mg.mL⁻¹, MER–meropenem 10 mg.mL⁻¹, GEN–gentamicin 10 mg.mL⁻¹, SUL–sulfonamide 300 mg.mL⁻¹, POL–polymyxina B 300 mg.mL⁻¹.

Exposure of bacteria to different substances, among them, preserving agents, peroxides, and surfactants, however, may create an environment whereby the microbiota exhibits a high frequency of mutation, characterizing the emergence of hypermutable strains, which represent a risk (Orús *et al.*, 2015). This is related to the occurrence of some microorganisms being resistant to different classes of substances, including antibiotics (Green *et al.*, 2018; Xu *et al.*, 2009). With the purity of cultures confirmed, this result may be related to the motility of the bacteria tested or the slow diffusion of the antibiotic over the agar, but the selective pressure exerted by the substances present in cosmetics should also be considered in terms of the promotion of multi-resistant genotypes.

This phenomenon has already been discussed for all species detected in this study: *B. cepacia* (Pope *et al.*, 2010), *P. aeruginosa* (Moradali *et al.*, 2017) and *A. hydrophila* (Martino *et al.*, 2013). Establishment of hypermutable strains results from the process of microbial adaptation when bacteria is inserted in a particularly hostile environment, represented in this case by inoculating the cosmetic after the breaking of the seal, in the context of secondary contamination. Additionally, according to Maciá *et al.* (2004), when an antibiogram reveals the presence of mutant subpopulations within the inhibition zones of three or more antibiotics, this clearly identifies hypermutable strains, as seen in isolated RX05 and RX08.

Generally, *P. aeruginosa* and *B. cepacia* are similar in their multidrug-resistance modulation systems as a result of overexpression of efflux genes promoted by mutational events, for example, the selective pressures caused by components of cosmetic formulations. As a result, a widely-specific efflux system is responsible for expelling antibiotics and, may involve different classes of molecules such as dyes, detergents, antimetabolites, organic solvents and molecules involved in quorum-sensing (Schweizer, 2003). In addition, other mechanisms may also be involved in this complex phenomenon, exemplified by expression of enzymes (Mlynarczyk *et al.*, 2009) or change in the antibiotic target (Lambert, 2005).

RX04 and surprisingly RX01 showed resistance to polymyxin B. The polymyxins are polypeptides that interact with lipopolysaccharide (LPS), disrupting the outer membrane in Gram-negative bacteria, resulting in an increase in membrane permeability and rapid loss of cell content (Trimble *et al.*, 2016). Some bacteria, such as *B. cepacia*, have intrinsic resistance to polymyxin B, however, the susceptibility observed in some naturally sensitive microorganisms may occur due to the modification of the structure of the polysaccharide core of LPS, particularly by the addition of aminoarabinose at the 4' position of the lipid phosphate (Gunn *et al.*, 1998).

Resistance to preservatives

Six isolates showed resistance to at least two of the tested preservatives. RX04, RX05, and RX08 proved to be the most resistant bacilli (Table 3), however, there was no correlation between resistance to preservatives and antibiotics (correlation = -15.0%, $p = 0.776$). This result repeated what has been observed in previous studies (Shaqra *et al.*, 2014; Osungunna *et al.*, 2010; Flores *et al.*, 1997).

Table 3: Minimal inhibitory concentration of preservatives against multidrug-resistant bacilli recovered from beauty salons in at least two replicates.

Isolates	Minimal inhibitory concentration (%)				
	I	M	P	M+P	T
RX01 (<i>Pseudomonas aeruginosa</i>)	S	S	0.38	3.00	S
RX02 (<i>Burkholderia cepacia</i>)	1.50	S	S	3.00	1.50
RX03 (<i>Burkholderia cepacia</i>)	1.50	1.50	0.75	0.38	1.50
RX04 (<i>Aeromonas hydrophila</i>)	6.00	R	R	R	6.00
RX05 (<i>Burkholderia cepacia</i>)	6.00	S	0.38	R	6.00
RX08 (<i>Pseudomonas aeruginosa</i>)	6.00	S	1.50	R	6.00

Range of concentration: imidazolidinyl urea (I): 6.0-0.38%; methylparaben (M), propylparaben (P) and triclosan (T): 3.0-0.19%. Letters R and S indicate that isolates were resistant or susceptible to all concentrations tested.

In the case of imidazolidinyl urea, only RX01 was sensitive. Given this, 5 isolates exhibited a disturbing resistance pattern as their MIC ranged from 2.5 to 10 times the reference value, 0.6% (ANVISA, 2012), inferring that the microorganisms had already adapted to the presence of the new generations of preservatives.

Imidazolidinyl urea is a formaldehyde-releaser found in 15% of CPCPs (Geis *et al.*, 2006). Generally, it is used in association with other preservatives in the cosmetic industry, particularly in formulations intended for skin care and the eye area or those areas that remain in contact with mucous for hours (Noureddine *et al.*, 2013). Its mechanism of action involves alkalization of the amino or sulfhydryl groups of proteins or of the nitrogen ring in the purine bases, being an effective biocide against Gram-negative bacilli in concentrations greater than 0.5%. The maximum concentration allowed in CPCPs, however, is only 0.6% in Brazil (ANVISA, 2012).

Most of the isolates were susceptible to triclosan in all concentrations tested, with the exception of RX04, whose MIC was detected at 1.5%. With respect to parabens, methylparaben was the most efficient as a biocidal agent compared to propylparaben or an association of the two. RX04, RX03, RX08, and RX05 exhibited the most significant resistance profiles. For example, RX04 did not form halos in all parabens concentrations tested. Traditionally, triclosan and parabens are considered of low toxicity and are found in more than 85% of the CPCPs (Cosméticos and Perfumes, 2008). However, these substances are highly absorbed when swallowed and there is a known risk of multiple adverse effects when in contact with skin, mucosa, and conjunctiva (Ocaña-González *et al.*, 2015).

In concentrations ranging from 0.5 to 3%, triclosan acts by inhibiting the synthesis of fatty acids and therefore presents a broad spectrum of activity against bacteria and fungi (Heath *et al.*, 1999). However, due to the mutagenic and genotoxic nature of this compound (Wang *et al.*, 2017), the maximum concentration allowed for use in cosmetics is 0.3% (ANVISA, 2012), i.e. less than five times the MIC determined, which may justify the isolation of RX04 in the capillary moisturizer.

On the other hand, parabens in the range between 0.015 and 0.3% are believed to block the electron and membrane transport systems (Denyer, 1995). Considering their toxicity, these compounds separately are allowed to be at the most 0.4% in formulations, reaching 0.8% when combined with other parabens (ANVISA, 2012). This group is more effective against filamentous fungi, yeasts, and Gram-positive bacteria, but less active against Gram-negative caused by intrinsic resistance mechanisms, which may explain the test results (Crovetto *et al.*, 2017).

Besides the discussion on toxicity, the possibility that parabens can cause endocrine disruption must be considered. In this case, preservatives block the receptors of naturally occurring hormones, which alters the metabolism of the cells and synthesis and distribution of other hormones. Medical conditions have been reported in the literature as being related to parabens, such as increased incidence of breast, testis and prostate cancer; malignant melanoma; endometriosis and reduced volume of semen (Noureddine *et al.*, 2013). Additionally, in animals, alterations in egg hatching, male feminization and changes in the immune system have already been observed due to preservatives (Bila and

Dezotti, 2007). Given the toxic and endocrine disruption effects, many countries, including Brazil, have limited the maximum concentration of these compounds to well below 1% (ANVISA, 2012).

An adult uses on average nine CPCPs per day, with formulations that may result in 126 different chemical compounds (Bocca *et al.*, 2014). Given the risks of pollution from discharge or biological contamination related to cosmetic waste, beauty salons should be recognized as a potential environmental contamination source. It is noteworthy that these types of establishments are still an unexplored field of research. There is a current gap in the literature regarding the quality and risk of waste produced by beauty salons when compared to common issues such as air quality of the exposed public in confined spaces (Raymer *et al.*, 2009; Mounier-Geyssant *et al.*, 2006), labor conditions (Vogel, 2011), disease transmission by fomites (Atei *et al.*, 2010) and quality of service and customer satisfaction (Yim *et al.*, 2007).

This work was the first study in the literature that has explored the microbiological quality of the waste produced in these establishments in Northeastern Brazil. The project for which this work was conceived received the Elo-Cidadão Award 2015, acknowledged by the Dean of Extension and Community Affairs of the Federal University of Paraíba.

CONCLUSIONS

This study highlights the risk that opportunistic pathogens found in cosmetic preparations and their packaging discarded as common domestic waste pose for human and environmental health. Besides evoking the need to change the handling practices of cosmetics in beauty salons, this study demonstrates that some bacteria recovered from cosmetic packaging are resistant to antibiotics and certain classes of preservatives, demanding the need of new preservatives, as well as the development of policies for the appropriate management of these residues.

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CONFLICT OF INTERESTS

The authors declare that no conflict of interest is associated with this work.

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