Preserving cosmetic formulations amid multidrug resistance

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Abstract

The World Health Organization (WHO) has highlighted the growing concern of microbial drug resistance, which poses risks to global health. Recent incidents, such as infections linked to artificial eye drops, illustrate that preserved formulations can also harbor multidrug-resistant organisms. In response, safety agencies are recommending the evaluation of additional pathogens in cosmetic formulations to enhance public safety. However, the shift to milder preservatives may contribute to the emergence of new strains and resistance mechanisms in existing microorganisms. To address these challenges, a response has been developed aiming at the identification of effective solutions against multi-resistant microorganisms. This approach pinpoints potential causes of resistance and draws on our extensive experience in antimicrobials to offer viable alternatives. We focus on rapidly applying commercially available raw materials and blends that effectively target troublesome microorganisms while minimizing the risk of long-term resistance development. By collaborating with our customers, we aim to deliver solutions that protect their corporate reputation without necessitating extensive reformulations.

Introduction

The discovery of antibiotics is widely regarded as one of the most revolutionary inventions in human history [1]. Their introduction allowed modern medicine to take hold worldwide and led many to believe that mankind could "close the book on infectious diseases". However, this book had to be reopened with the emergence of multi-resistant microorganisms, which are now considered by the WHO to be one of the most serious global threats to public health and development [2].

Parallel observations are currently being made in the cosmetics industry with the emergence of an increasing number of multi-resistant bacteria. The focus of cosmetics has always been on providing low-risk products, beneficial to the wellbeing of the consumer [3;4]. With the introduction of the first preservatives, such as *p*-hydroxybenzoic acid esters in the 1920s, it became possible to reduce the microbiological risks of water-based cosmetics [5]. The continuing high number of microbiological contaminations in the 1960s and 1970s led to increased public attention, which resulted in the first regulations and standards in Europe and the USA [6]. The subsequent harmonization of the use of preservatives and the availability of additional preservatives closed the book on microbiological contamination in cosmetics. In the EU, the determination of the microbiological quality of products is based on the requirements of the EU Cosmetics Regulation EC No. 1223/2009 and is ensured through the mandatory performance of a preservative challenge test. Similar to the development of antibiotic resistance, an increasing tolerance to preservatives is observed; for example, microorganisms resistant to every classical preservative on the market have been identified [7;8]. It is therefore time to reopen the book on microbiological contamination in cosmetics. Growing toxicological concerns in combination with the clean beauty trend have led to a reduction in the use of listed preservatives and are increasingly reinforcing this trend. For example, the number of product recalls in the European Union is steadily increasing and the emergence of new human pathogenic species is being observed (Fig. 1). Links between infections in immunocompromised patients and contaminated cosmetics were drawn several times: i)

Pluralibacter gergoviae from a contaminated cleaning product caused nosocomial sepsis in a pediatric intensive care unit, ii) *Burkholderia cepacia* in a moisturizing body milk caused nosocomial infections in a multidisciplinary intensive care unit, and iii) a *B. cepacia* outbreak in the US was linked to a no-rinse cleaning foam [9;10;11]. Incidents with *P. gergoviae* led to a recommendation by the German Federal Institute for Risk Assessment that cosmetics should be free of *P. gergoviae* [12]. Since resistance of these microorganisms to preservatives can be linked to antibiotic resistance, infections with these germs are troublesome; as demonstrated by the outbreak of extensively drug resistant *Pseudomonas aeruginosa* associated with artificial tears [13].

These incidents demonstrate the need to add new pages to the book of microbiological contamination, including i) the identification of antimicrobial alternatives that account for microbial resistance, and ii) the development of new products and modes of action that address the emergence of resistance. This new approach will be demonstrated through one industrial case dealing with a recurrent contamination of *P. gergoviae*, isolated from a cream.

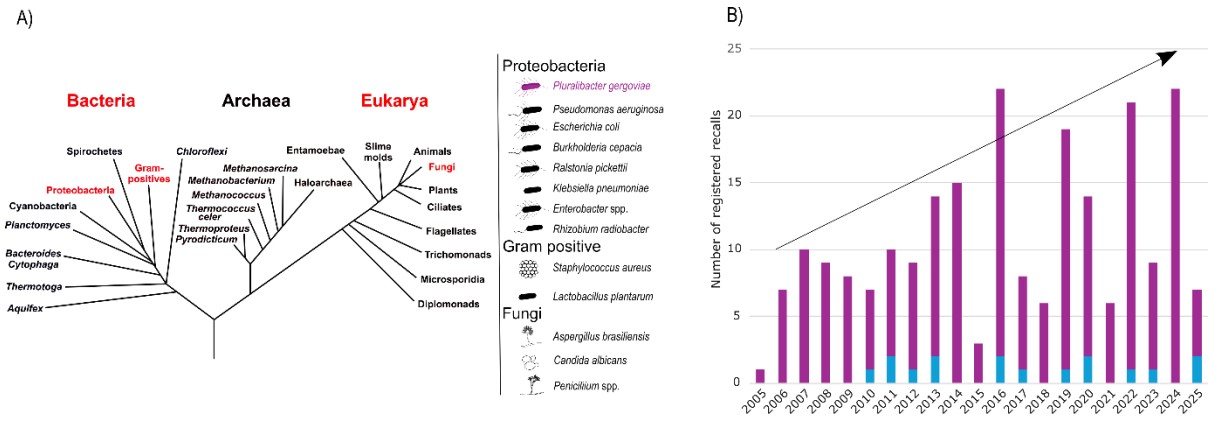

Figure 1: Microbiological risk of cosmetics.

A) Tree of life adapted from [14]. Microorganisms contaminating cosmetics origin from three taxa (red). Bacteria examined in this study are colored in deep purple. B) Number or product recalls due to microbial contamination registered in the Rapid Exchange of Information System of the European Union (RAPEX), with 2025 still ongoing. Incidents reporting *Pluralibacter gergoviae* are given in blue.

Material and Methods

Cream contaminated with *Pluralibacter gergoviae* – a case example

In this case example, a customer experienced recurring contaminations of *Pluralibacter gergoviae* in a product, already in the market, across multiple production batches, despite the formulation passing the challenge test as outlined in the European Pharmacopoeia. The cream was preserved with 1.5 % of a customer preservation system comprising 10 % caprylhydroxamic acid, 75 % glyceryl caprylate, and 15 % glycerin. The isolated strain was named *Pluralibacter gergoviae* K033.

Isolation of microorganisms and determination of germ load

One gram of formulation was added to 9 g sterile tryptone soya broth (TSB; casein peptone 17 g, soy peptone 3 g, Glucose 2.5 g, NaCl 5 g, K_2HPO_4 2.5 g, filled up to 1000 ml with distilled water, pH = 7). A tenfold dilution series was prepared in the described media and 100 µl of each dilution was plated on agar plates of the described media (containing 18 g agar) and incubated over night at 30 °C. Afterwards, colony forming units (CFU) were counted and the number of living bacteria per g of formulation was calculated using the weighted arithmetic mean. Bacteria were freshly prepared as described above and species were determined via 16S-rRNA gene amplification, followed by sequencing.

High throughput testing of antimicrobial molecules against a tolerant *Pluralibacter gergoviae*

To maintain resistance pressure and assess bacterial tolerance, an alternative MIC-based approach was developed. After cultivation, the bacterial load of 16 manufacturer-provided samples was determined as described above. To isolate *P. gergoviae*, tryptic soy agar (TSA) was prepared with 0.1–2 % of the customer preservation system (CPS; 10 % caprylhydroxamic acid, 75 % glyceryl caprylate, 15% glycerin). Bacteria were plated and incubated accordingly. Colonies were resuspended in 0.9 % NaCl to a McFarland standard of 0.5. A 100 µl aliquot was transferred into 9.9 ml Mueller-Hinton broth (Sigma-Aldrich) supplemented with 1 % CPS. This bacterial suspension was then mixed 1:1 with CPS-adapted MH broth, containing additional antimicrobials (Table I) and subjected to a serial dilution for MIC determination.

Table I: Antimicrobials tested in the adapted MIC determination.

Number	Antimicrobial system
1	Glyceryl caprate
2	Levulinic acid
3	1,2-Hexandiol
4	Benzoic acid
5	Phenylpropanol
6	*p*-anisic acid
7	Ethylhexylglycerin

Tolerance development to the recommended new preservation system

Tolerance to the CPS was overcome by the addition of levulinic acid and phenylpropanol. To test, if the isolated strain can become tolerant to the new preservation system, TSA was supplemented with the new preservation system (NPS) and *P. gergoviae* was cultivated on increasing concentrations, ranging from 0.1 – 1 % NPS. The bacterium was re-cultivated on the same concentration three times, before transfer. A tolerance to the used concentration within the formulation would be defined as resistance.

Challenge test

With low amounts of sterile formulation, a NPS was identified via a modified MIC determination. Challenge tests were carried out as described in the European Pharmacopoeia (Ph.Eur.). The newly developed NPS was challenged with i) the isolated *P. gergoviae K033*, cultivated on TSA containing CPS and ii) the commercial strain usually used in challenge testing *P. gergoviae* DSM9245.

Identification of anti-resistance multifunctionals

The observed degradation of antibacterial glyceryl caprylate, a key component of the preservation system (CPS), suggests that lipases are crucial for tolerance. To assess the impact of lipase inhibitors, TSA with 1.5 % CPS was placed adjacent to agar containing 5 % of the antimicrobial lipase inhibitor triethyl citrate. A control contained only TSA and CPS. The interface between the two agar was inoculated with 2 CFU of *P. gergoviae* K033, resuspended in 0.9 % NaCl. For further analysis, one CFU of *P. gergoviae* K033 was grown in TSB overnight at 37 °C. Lipases were extracted from the supernatant using ammonium sulfate precipitation [15] and desalted via Amicon centrifugation (10 kDa cut-off). A 230 µl aliquot was tested for

lipolytic activity by adding 20 µl of substrate (0.0663 g 4-nitrophenyloctanonate in 25 ml isopropanol and 0.1 M Tris buffer 1:1), measuring absorbance at 410 nm. A final concentration of 2 % glyceryl esters was added to the fraction with the highest lipolytic activity and incubated overnight at 37 °C. Controls included a denatured fraction, glyceryl esters without the desalted fraction, 2 mM purified lipase from *Burkholderia cepacia* (Sigma-Aldrich), and a fraction with 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The MIC of each fraction was determined. Synergy between glyceryl esters and antimicrobial lipase inhibitors (glucogalactone, epigallocatechin-3-gallate, rosmarinic acid, triethyl citrate, and tormentil rhizome extract) against *P. gergoviae* DSM924 was evaluated using the fractional inhibition concentration (FIC), with synergy defined as FIC ≤ 0.5 [16].

Results

Identification of a functional new preservation system

The manufacturing of a skin cream was halted due to contamination with *P. gergoviae*. Sixteen market samples were analyzed, revealing contamination levels from 0 to 4.9 × 10^5 CFU/g. To address *P. gergoviae* K033, which exhibits resistance to the CPS, the MIC of additional molecules was determined in a modified system that maintained the selection pressure of the original preservation system. *P. gergoviae* K033 did not grow on TSA containing 1.5 % CPS, the concentration in the formulation, but did grow on agar plates with 0.2 % CPS. Tolerance was reintroduced through stepwise cultivation with increasing CPS concentrations (Fig 2). Levulinic acid displayed the lowest MIC (Table II) among the molecules usable in this formulation. This product was available in a blend with the antifungal *p*-anisic acid; both acids were mixed and 1 % of this blend was used (20 % levulinic acid, 7 % *p*-anisic acid, 35 % water, and 38 % glycerin). Also, glyceryl caprylate was replaced with 1 % phenylpropanol to create the NPS. In contrast to the CPS, no tolerance up to in-use concentrations could be achieved through recultivation, and the NPS eliminated populations of the isolated *P. gergoviae* K033 as well as the typically tested *P. gergoviae* DSM924, achieving A-criteria according to Pharm. EU.

Table II: Adapted minimal inhibition concentration of antimicrobials.

Number	Preservation system	MIC [%]
1	Glyceryl caprate	>2
2	Levulinic acid	0.3
3	1,2-Hexandiol	0.7
4	Benzoic acid	0.6
5	Phenylpropanol	0.5
6	p-Anisic acid	0.5
7	Ethylhexylglycerin	0.4

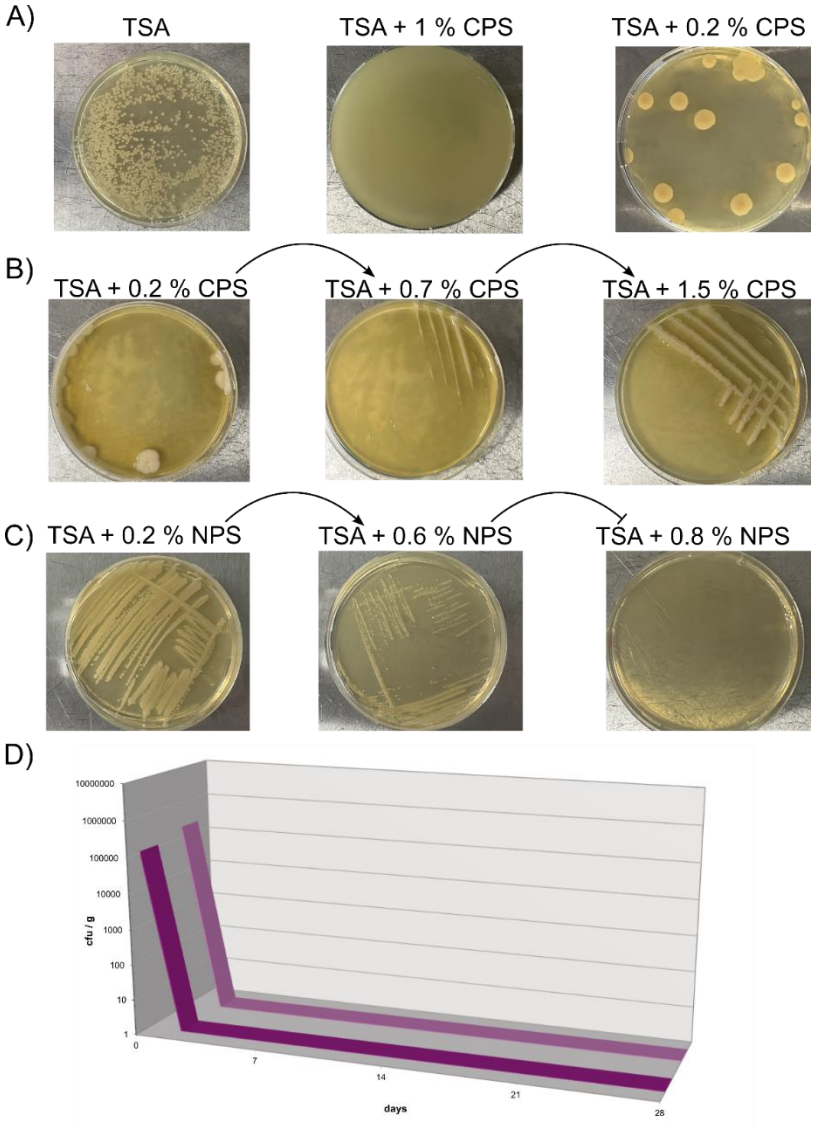

Figure 2: Identification of a new preservation system.

Isolation of *Pluralibacter gergoviae* K033 from formulation. The bacterium did not grow, when transferred to TSA containing same CPS concentrations as given within the formulation. Growth of a different phenotype was observed, when cultivated on 0.2 % of the CPS. B) Resistance towards the original amounts of CPS were reintroduced by stepwise increase in the concentration of antimicrobials. C) Stepwise increase did not lead to resistance, when the NPS was used. D) Challenging the original formulation with added NPS using *P. gergoviae* K033 (deep purple) and *P. gergoviae* DSM9245 (pink).

Identification of anti-resistance multifunctionals

With the NPS, a solution was provided with lower chance of developing resistance. Since no pre-made blend was available, dealing with such contaminations, research on possible systems was conducted. As clearance of the CPS by *P. gergoviae* K033 was observed on agar plates, we hypothesized the involvement of lipases (Fig. 3). Using *P. gergoviae* DSM9245, cultivated without the presence of CPS, the MIC of glyceryl caprylate was 0.125 % and the MIC of glyceryl caprate was 0.25 %. When combined with purified lipase from the culture supernatant of *P. gergoviae* K033, or commercially available lipase, the MIC increased to 0.5 % in the case of glyceryl caprylate and 1 % in case of glyceryl caprate. As a control, the addition of purified lipases had no effect on the MIC, either when mixed with the lipase inhibitor PMSF or when denatured enzymes were used. Furthermore, the antimicrobial lipase inhibitor triethyl citrate [17] visually stopped the clearance of the CPS, when added into the agar (Fig 3). Combined with glyceryl esters, triethyl citrate generated a FIC of 0.375, indicating synergy. The same is true for glucogalactone (0.375), epigallocatechin-3-gallate (0.5), rosmarinic acid (0.25), and tormentil rhizome extract (0.5).

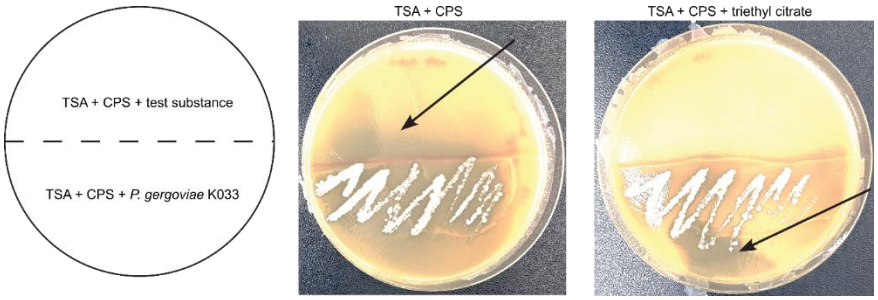

Figure 3: Effect of triethyl citrate on the clearance of the CPS.

P. gergoviae K033 was plated on TSA containing the CPS. Next to this agar, i) the same, or ii) agar also containing 5 % triethyl citrate was poured. Clearence of the CPS was observed visually and did not occur next to ii. Plates were incubated for 1.5 days, until the clearance was observable.

Discussion

Antimicrobial resistance is a significant global issue affecting healthcare, agriculture, and cosmetics. In the cosmetics sector, microbial resistance was acknowledged in 1997, prompting discussions on the necessity of combining different preservatives [18]. Increasing reports of resistant strains, including *P. gergoviae*, which shows resistance to many preservative systems, have underscored the urgency for manufacturers to address this challenge [7;19]. This study presents solutions for a bacterial contamination in a cream formulation, originally preserved with combined antimicrobials, and emphasizes the implementation of innovative post-contamination procedures.

Antimicrobial resistance mechanisms include: i) removal of the antimicrobial substance from the cell, ii) destruction or modification of the molecule, and iii) mutation of target structures. These adaptations are driven by selection pressure, particularly from antimicrobials such as traditional preservatives or multifunctional agents with antimicrobial properties [20]. Our study demonstrated that selection pressure based resistance explains, why *P. gergoviae* K033, isolated from a cream containing the CPS, was non culturable on TSA containing similar amounts of CPS. As demonstrated before [21], the resistance towards glyceryl esters was mediated by the increased secretion of lipases. We hypothesized that *P. gergovaie* K033 was intrinsically resistant towards weak acids present in the CPS [22]. Therefore, antibacterial efficacy relied on glyceryl esters present in the formulation. After chemical hydrolysis, the disappearing selection pressure decreased tolerance to the CPS. Still, resistance could be reintroduced using isolated and slightly tolerant individuals (Fig 2). In this study, a modified high throughput MIC determination were proposed as a suitable tool to identify potent antimicrobials against resistant germs.

In contrast to healthcare and agriculture, the use of antimicrobials in cosmetics fundamentally depends on i) the type of formulation and ii) acceptance by the end customer; who is most often unaware about microbiological risks [23]. This is, why the replacement of glyceryl esters with the ether ethylhexylglycerin, resistant to chemical degradation by lipases, was discarded. The implementation of novel test methods allowed the development of applications designed to combat resistance in cosmetics by protecting multifunctional from chemical degradation.

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