APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1987, p. 1827–1832
0099-2240/87/081827-06$02.00/0

Vol. 53, No. 8

Correlation of In Vitro Challenge Testing with Consumer Use Testing for Cosmetic Products

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Received 19 February 1987/Accepted 11 May 1987

An in vitro microbial challenge test has been developed to predict the likelihood of consumer contamination of cosmetic products. The challenge test involved inoculating product at four concentrations (30, 50, 70, and 100%) with microorganisms known to contaminate cosmetics. Elimination of these microorganisms at each concentration was followed over a 28-day period. The test was used to classify products as poorly preserved, marginally preserved, or well preserved. Consumer use testing was then used to determine whether the test predicted the risk of actual consumer contamination. Products classified by the challenge test as poorly preserved returned 46 to 90% contaminated after use. Products classified by the challenge test as well preserved returned with no contamination. Marginally preserved products returned with 0 to 21% of the used units contaminated. As a result, the challenge test described can be accurately used to predict the risk of consumer contamination of cosmetic products.

The work described addresses the criticism that microbial challenge tests used in the cosmetics industry are unreliable as predictors of a product's ability to resist microbial contamination from consumer use. The criticism is based on the contention that challenge test data are not validated by consumer use data (17). The Food and Drug Administration expressed its concern about in-use preservative adequacy of cosmetics in a *Federal Register* notice in 1977. This notice stated that regulatory action would be taken "to remove from the market any cosmetic that poses an unreasonable risk of injury because of inadequate preservation to withstand contamination under customary conditions of use" (18). Industry has been responsive to this concern. As early as 1970, cosmetic trade associations and individual companies recommended that consideration be given to continued effectiveness of a cosmetic's preservative system under intended consumer use conditions (11, 20, 31). More recently, consumer test programs to assess in-use preservative adequacy have been described (21).

Preservative adequacy of cosmetics is typically evaluated by using microbial challenge tests (12, 13). There are, however, few documented reports showing that microbial challenge tests are predictive of consumer contamination potential. One study showed that, of three mascara formulas susceptible to the challenge test organisms, only one was actually contaminated due to consumer use (1). In a study on eye shadows, the microbial content of several consumer-used products was determined. Challenge testing of two of the products was conducted, but no attempt to correlate in-use contamination incidence with the challenge test results was made (16). In a study on shampoos, poor correlation was found between MIC results and a simulated "in use" test (15). Other studies have reported either contamination incidence of used cosmetic products (2, 3, 33) or preservative challenge test results (7, 26), but no attempts to correlate the data were made. To our knowledge this is the first published report to show that a microbial challenge test predicts consumer contamination potential for shampoo and skin lotion cosmetic products.

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MATERIALS AND METHODS

Products evaluated. Two product types (shampoos and skin lotions) at three preservative conditions were evaluated. Product containers were chosen to permit direct consumer contact with the product so protection due to package design would not be a significant factor.

The first shampoo was composed of the base product preserved with methylisothiazolinone and methylchloroisothiazolinone (Kathon) at 0.02 to 0.04%. For the second shampoo, methyl and propyl parabens at 0.28 to 0.32% were added to the base product instead of Kathon. Finally, the shampoo base was used without any preservative. The shampoo base was composed of water, ammonium lauryl sulfate, sodium lauryl sulfate, cocamide diethanolamide, polyquaterium 10, sodium phosphate, fragrance, SD alcohol 40, sodium chloride, disodium phosphate, EDTA, and color. Product was packaged in 16-oz (ca. 473-ml) bottles with screw-cap tops 24 mm in diameter.

Three skin lotions were prepared with different preservative systems. The first was preserved with imidazolindyl urea (0.08 to 0.12%) and methyl and propyl parabens (0.28 to 0.32%). The second lotion contained only the parabens, and the third lotion was prepared without preservative. The skin lotion base was composed of water, glycerol, petrolatum, cetyl alcohol, cyclomethicone and dimethicone copolyol, stearyl alcohol, isopropyl palmitate, dimethicone, sodium hydroxide, stearic acid, lanolin acid, polyethylene glycol 100 stearate, carbomer 934, EDTA, hydrogenated vegetable glycerides, phosphate, masking fragrance, and titanium dioxide. Product was packaged in 4-oz (ca. 118-ml) wide-mouth jars (62 mm in diameter).

Microbial challenge test. The challenge test used was a modification of the standard Cosmetics, Toiletries, and Fragrance Association procedure (13). The modification consisted of diluting products to four concentrations (100, 70, 50, and 30%) with double-reverse-osmosis water. The four product concentrations were then challenged with the

TABLE 1. Bacterial challenge testing data for shampoo made at three preservative conditions

Kind of shampoo	Product concn (%)	CFU/g of product at day postchallenge:						Interpretation
		0	1	7	14	21	28	
Unpreserved base	100	TNTC[a]	TNTC	TNTC	TNTC	TNTC	TNTC	Fail at all product concn
	70	TNTC	TNTC	TNTC	TNTC	TNTC	NA[b]	
	50	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
	30	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
Paraben preserved	100	TNTC	TNTC	TNTC	60	<20[c]	<20	Pass at 100% product concn
	70	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
	50	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
	30	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
Isothiazolinone preserved	100	TNTC	260	<20	<20	<20	<20	Pass at 30% product concn
	70	TNTC	1,200	<20	<20	<20	<20	
	50	TNTC	2,020	<20	<20	<20	<20	
	30	TNTC	11,000	<20	<20	<20	<20	

[a] TNTC, Too numerous to count, $>10^5$ CFU/g.
[b] NA, Not applicable
[c] Detection limit, 20 CFU/g.

following organisms: *Escherichia coli* ATCC 15597, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, and a variety of preservative-resistant environmental isolates including *Pseudomonas cepacia*, *P. aeruginosa*, *Pseudomonas maltophilia*, *Pseudomonas* spp., and *Serratia marcescens*. Products were also challenged with molds (*Aspergillus niger* ATCC 16404 and *Penicillium levitum* ATCC 10464) and yeasts (*Candida utilis* ATCC 8205 and *C. albicans* ATCC 10231).

Challenges were made with mixed cultures. Bacterial challenge levels were 10^5 to 10^6 CFU/g of product. Mold and yeast challenge levels were 10^3 CFU/g of product. All challenged product was incubated at ambient temperature during the test over 28 days.

Microbial content at each product concentration was assayed at 0, 1, 7, 14, 21, and 28 days. Trypticase soy agar (BBL Microbiology Systems) plus 1.5% Tween 80 was used for bacterial recovery. Mycophil agar (BBL) plus 1.5% Tween 80 was used for yeast and mold recovery. At each assay time, 1 g of product was diluted in 9 ml of sterile double-reverse-osmosis water, and then 0.5 ml of the diluted product was pour plated into 10 ml of the appropriate agar medium. The pour plates were incubated at 32 to 35°C for 3 days followed by 21 to 29°C for 2 days prior to counting.

In-use test. Approximately 30 subjects were randomly assigned to each product and asked to use the products as they normally would. All products provided to the subjects were free of detectable microorganisms (<20 CFU/g). Unexposed control products incubated during the test period remained below this limit throughout the test. Skin lotion products were returned after 2 weeks of consumer use. The shampoos were returned after 3 weeks of use.

Microbial-content testing was conducted on each returned product unit immediately upon receipt and again 4 to 7 days postreceipt. Standard techniques for microbial-content testing of cosmetic products were used (14). Ten grams of product was diluted in 90 ml of sterile double-reverse-osmosis water and thoroughly mixed, and 0.5 ml of the diluted product was pour plated with 10 ml of Trypticase soy agar plus 1.5% Tween 80. Plates were incubated at 32 to 35°C for 3 days followed by 21 to 29°C for 2 days.

A product was considered contaminated if >100 CFU/g was observed or if gram-negative bacteria at any level were detected at initial receipt and 4 to 7 days postreceipt. Bacteria were identified by using API systems (20E, NFT, Staph-trac; Analytab Products), Enterotube (Hoffmann-La Roche Inc.), or Oxi-ferm (Hoffmann-La Roche) rapid identification systems. Yeasts and molds were identified by morphological characteristics (6).

Statistical analysis. Chi-square and analysis of variance testing was performed on the data (34).

RESULTS

Microbial challenge testing. The bacterial challenge test results are shown in Tables 1 and 2. A product showing reduction and elimination of the inoculum over the 28-day challenge period at all four product concentrations was designated a pass at 30% (pass 30%) and was considered well preserved. A product showing reduction and elimination of the inoculum at only the 100% concentration (i.e., full strength or neat product) was considered marginally preserved (pass 100%). A product was considered poorly preserved if no reduction of the inoculum occurred (fail).

Bacterial challenge testing of the shampoo formulas (Table 1) showed that the isothiazolinone-preserved formula rapidly reduced and essentially eliminated the inoculum at all four product concentrations. The paraben-preserved shampoo showed reduction and elimination at only the 100% product concentration, while the upreserved base shampoo failed the test. Similarly, bacterial challenge testing of the skin lotion formulas (Table 2) showed that the imidazolidinyl urea/paraben-preserved formula reduced and eliminated the inoculum at all four product concentrations. The paraben-preserved lotion showed reduction and elimination at only the 100% product concentration; the unpreserved base lotion failed the test.

Table 3 summarizes the microbial challenge test results, including the end results of fungal challenge testing for the shampoos and skin lotions.

The unpreserved shampoo base had significant biocidal activity against yeast and molds due to the antifungal activity of the anionic surfactants in the formula. Based on the bacterial challenge testing results, the shampoos were classified as poorly preserved (base shampoo), marginally preserved (paraben-preserved shampoo), or well preserved (isothiazolinone-preserved shampoo).

The unpreserved skin lotion failed the fungal and bacterial challenges. It was classified as poorly preserved. The para-

TABLE 2. Bacterial challenge testing data of skin lotion made at three preservative conditions

Kind of skin lotion	Product concn (%)	CFU/g of product at day postchallenge:						Interpretation
		0	1	7	14	21	28	
Unpreserved base	100	TNTC[a]	TNTC	TNTC	TNTC	TNTC	TNTC	Fail at all product concn
	70	TNTC	TNTC	TNTC	TNTC	TNTC	NA[b]	
	50	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
	30	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
Paraben preserved	100	TNTC	180	<20[c]	<20	<20	<20	Pass at 100% product concn
	70	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
	50	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
	30	TNTC	TNTC	TNTC	TNTC	TNTC	NA	
Imidazolidinyl urea/paraben preserved	100	TNTC	80	<20	<20	<20	NA	Pass at 30% product concn
	70	TNTC	TNTC	<20	<20	<20	NA	
	50	TNTC	TNTC	<20	<20	<20	<20	
	30	TNTC	TNTC	40	20	<20	<20	

[a] TNTC, Too numerous to count, $>10^5$ CFU/g.
[b] NA, Not applicable.
[c] Detection limit, 20 CFU/g.

ben-preserved lotion passed the yeast and mold challenges at all four product concentrations, but passed the bacterial challenge only at the 100% product concentration. Therefore, it was classified as marginally preserved. The imidazolidinyl urea/paraben-preserved lotion passed the yeast, mold, and bacterial challenges; it was classified as well preserved.

In-use testing. In-use microbiological-contamination incidence for the shampoo and skin lotion formulas are shown in Table 4. Usage data are also shown.

Total shampoo uses as well as the amount of product used for each of the three shampoos were statistically equivalent. Contamination incidence (46%) was statistically higher for the poorly preserved base shampoo. A low level of contamination (21%) was seen in the marginally preserved (paraben) shampoo. The well-preserved shampoo showed no contamination.

Total skin lotion uses were not statistically different; however, total amount used was significantly higher for the well-preserved lotion. Despite this increased usage, neither the well-preserved nor the marginally preserved skin lotion products were contaminated upon return. The poorly preserved lotion showed a significantly higher (90%) contamination incidence.

The organisms isolated from the contaminated shampoo and skin lotion units are shown in Table 5. The most frequent isolates from the poorly preserved and marginally preserved shampoos were *Enterobacter* spp., *Serratia* spp., and *Klebsiella* spp. Additional isolates included *Citrobacter freundii.* and *Pseudomonas* spp. Organisms most frequently encountered from the unpreserved skin lotion included gram-positive cocci, *Enterobacter* spp., *Pseudomonas* spp., and *Bacillus* spp. Yeasts and molds isolated included *Rhodotorula* sp., *Scopulariopsis* sp., *Aureobasidium pullulans*, and *Penicillium* sp.

Table 6 correlates the challenge test results with the in-use test results for both shampoo and skin lotion. The base shampoo and skin lotion formulas that were classified as poorly preserved by the challenge test returned with a high incidence of contamination. The paraben-preserved shampoo, classified as marginally preserved by the challenge test, returned with a low incidence of contamination. The paraben-preserved skin lotion, also classified as marginally preserved by the test, returned uncontaminated. Both the isothiazolinone-preserved shampoo and the imidazolidinyl urea/paraben-preserved skin lotion, classified as well preserved by the challenge test, were returned uncontaminated after consumer use.

TABLE 3. Summary of microbial challenge testing results and preservation classifications of shampoo and skin lotion made at three preservative conditions

Cosmetic and preservative	Microbial challenge results[a]			Preservation classification
	Bacterial challenge	Yeast challenge	Mold challenge	
Shampoos				
Unpreserved base	Fail	Pass 30%	Pass 50%	Poor
Paraben preserved	Pass 100%	NT	NT	Marginal
Isothiazolinone preserved	Pass 30%	Pass 30%	Pass 30%	Well
Skin lotions				
Unpreserved base	Fail	Fail	Fail	Poor
Paraben preserved	Pass 100%	Pass 30%	Pass 30%	Marginal
Imidazolidinyl urea/paraben preserved	Pass 30%	Pass 30%	Pass 30%	Well

[a] Fail, No reduction of microbial challenge at any product concentration; pass 100%, reduction and elimination of microbial challenge at only the undiluted product concentration; pass 50%, reduction and elimination of microbial challenge at 100, 70, and 50% product concentrations; pass 30%, reduction and elimination of microbial challenge at all product concentrations. NT, Not tested.

TABLE 4. In-use testing results for shampoo and skin lotion made at three preservative conditions[a]

Cosmetic and preservative	No. of subjects	Avg amt used (g)	Avg no. of uses	% Returned contaminated
Shampoos				
Unpreserved base	26	116]	18]	46 (12/26)]
Paraben preserved	29	123]	18]	21 (6/29)]
Isothiazolinone preserved	29	119]	19]	0 (0/29)]
Skin lotions				
Unpreserved base	30	25]	17]	90 (27/30)]
Paraben preserved	30	25]	16]	0 (0/30)]
Imidazolidinyl urea/paraben preserved	30	42]	22]	0 (0/30)]

[a] Bracketed values are not statistically different ($\alpha = 0.05$).

DISCUSSION

Inadequately preserved cosmetic products may become contaminated with undesirable organisms during use (1–3, 16, 33), leading to product degradation or, if contaminated with pathogens, acting as a fomite to potentially spread infection to susceptible users (4, 22, 23, 25, 32). In addition, microbial insults to cosmetic products may occur during their manufacture (5, 8). Microbial insults from manufacture can be controlled by careful attention to sanitary processing and adequate preservation. Insults occurring during consumer use, however, are controlled primarily by product preservation and, to a lesser extent, container design (e.g., single-use vials). The cosmetics industry relies on microbial challenge tests to evaluate how well a product withstands microbial insults, particularly those occurring during use (9, 13, 20, 26). Therefore, it is important that this testing be a valid predictor of the product's ability to withstand these microbial insults.

The data presented indicate that the challenge test described was a valid predictor of the ability of a shampoo or a skin lotion to withstand microbial contamination during consumer use. The two types of products in this test that were classified as well preserved did not become contaminated from consumer use. Products that the test classified as poorly preserved became highly contaminated after use. The paraben-preserved shampoo, classified by the test as marginally preserved, had a low contamination incidence after use. The challenge test, however, classified the paraben-preserved skin lotion as marginally preserved when it was, in fact, able to withstand consumer use without becoming contaminated. Consequently, the challenge test may be overly conservative for skin lotions. It should also be noted that parabens, depending on the particular cosmetic formula, can be effective preservatives. That their use in the two formulas described here resulted in marginally preserved classifications does not preclude their use as effective preservatives in other formulas.

The difference observed between the in-use susceptibility of the marginally preserved skin lotion and marginally preserved shampoo may be explained by different consumer exposure conditions for the two paraben-preserved products. Shampoos are constantly exposed to both user and water contamination during use. Water typically contains organisms (e.g., some *Pseudomonas* spp.) resistant to

TABLE 5. Types and incidence of microorganisms isolated from contaminated shampoo and skin lotion samples after use

Organisms recovered	Recovery incidence from samples indicated[a]		
	Shampoos		Skin lotion (unpreserved base)
	Unpreserved base	Paraben preserved	
Acinetobacter calcoaceticus	0	0	2 (4.1%)
Citrobacter freundii	1 (5%)	0	1 (2.0%)
Enterobacter spp.[b]	9 (43%)	4 (40%)	8 (16.3%)
Klebsiella spp.[c]	4 (19%)	1 (10%)	2 (4.1%)
Pseudomonas spp.[d]	3 (14%)	5 (50%)	6 (12.2%)
Serratia spp.[e]	4 (19%)	0	0
Unidentified gram-negative rod[f]	0	0	1 (2.0%)
Bacillus spp.	0	0	8 (16.3%)
Micrococcus sp.	0	0	3 (6.1%)
Staphylococcus spp.[g]	0	0	9 (18.4%)
Rhodotorula sp.	0	0	1 (2.0%)
Aureobasidium pullulans	0	0	3 (6.1%)
Scopulariopsis sp.	0	0	1 (2.0%)
Penicillium sp.	0	0	2 (4.1%)
Unidentified molds	0	0	2 (4.1%)

[a] Values in parentheses are the percentages of each isolate per total number of isolates recovered from that particular product. Several isolates could be recovered from a single unit.
[b] *E. cloacae* and *E. agglomerans*.
[c] *K. pneumoniae* and *K. oxytoca*.
[d] *P. putida*, *P. aeruginosa*, *P. maltophilia*, *P. acidovorans*, and *P. fluorescens*.
[e] *S. liquefaciens* and *S. marcescens*.
[f] Oxidase-positive nonfermenter.
[g] *S. epidermidis*, *S. aureus*, and *S. saprophyticus*.

TABLE 6. Correlation of challenge test and in-use test results

Product tested	Challenge test preservation classification[a]	In-use test contamination incidence[b]
Shampoos		
Unpreserved base	Poor (fail)	High (46%)
Paraben preserved	Marginal (pass 100%)	Low (21%)
Isothiazolinone preserved	Well (pass 30%)	None (0%)
Skin lotions		
Unpreserved base	Poor (fail)	High (90%)
Paraben preserved	Marginal (pass 100%)	None (0%)
Imidazolidinyl urea/paraben preserved	Well (pass 30%)	None (0%)

[a] Data in parentheses show Table 3 results.
[b] Data in parentheses show Table 4 results.

parabens. Skin lotions are typically exposed only to the consumer's hands when used. The microbial population of the skin is typically susceptible to the antimicrobial activity of parabens. Thus, the marginally preserved skin lotion may have received less severe in-use microbial insult (i.e., no preservative-resistant organisms) and therefore was resistant to in-use contamination despite the challenge test's indication of marginal preservation.

The types of organisms recovered from both products after use reflects the microflora indigenous to humans and household environments. In studies of home environments, wet areas such as bathrooms and kitchens contain large numbers of *E. coli* and *Klebsiella*, *Citrobacter*, and *Enterobacter* spp. Gram-positive cocci and *Bacillus* spp. are also present. *P. aeruginosa* is rarely found, but other pseudomonads (e.g., *P. maltophilia*) are prevalent in the home (19, 28). *Staphylococcus* spp., *Bacillus* spp., molds, yeasts, and gram-negative bacilli have been isolated from human skin either as indigenous or as transient organisms (24). Therefore, the microbial contaminants found in the used shampoos and skin lotions were reflective of the environments to which they were exposed.

Microbial challenge testing of cosmetics typically includes organisms resistant to preservatives (13). Resistant organisms are common and well known in the trade (10, 27, 29, 30). Preservation against three types of organisms typically results in products well preserved against ordinary and customary use by the consumer. Consequently, preservative challenge testing, particularly when the test uses preservative-resistant microorganisms like those in the test described here, is a valid but perhaps strict means of assessing consumer contamination risk.

The challenge test described here was capable of accurately but conservatively predicting which of the cosmetic formulas tested (e.g., shampoos or skin lotions) were susceptible to consumer contamination. This test assessed preservative adequacy independent of container design and its potential for protecting consumer products. If container design provides adequate protection, even poorly preserved products could withstand consumer use. Additional studies are needed to assess these effects.

ACKNOWLEDGMENTS

A note of thanks is extended to P. A. Geis for help in identifying fungal isolates and to W. A. Apel for review of the manuscript.

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