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Ability of Laboratory Methods To Predict In-Use Efficacy of Antimicrobial Preservatives in an Experimental Cosmetic

J. K. FARRINGTON,[1] E. L. MARTZ,[1] S. J. WELLS,[1] C. C. ENNIS,[1] J. HOLDER,[1] J. W. LEVCHUK,[2] K. E. AVIS,[2] P. S. HOFFMAN,[3]† A. D. HITCHINS,[4]* AND J. M. MADDEN[4]

Schering-Plough Health Care Products, Memphis, Tennessee 38151[1]; Department of Pharmaceutics, University of Tennessee, Memphis, Tennessee 38163[2]; Department of Microbiology and Immunology, University of Tennessee, Memphis, Tennessee 38155[3]; and Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, D.C. 20204[4]

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The abilities of nine antimicrobial systems to preserve an experimental water-based cosmetic formulation were evaluated by six microbiological challenge tests: the U. S. Pharmacopeia test; the British Pharmacopeia test; the Cosmetic, Toiletry, and Fragrance Association test; the rapid screen test; the sequential challenge test; and the post-use test. The antimicrobial systems contained various combinations and amounts of two parabens and a quaternary compound in order to provide a broad range of preservation. The results obtained were compared with the abilities of the formulations to support maintenance and growth of microorganisms in microfloras obtained from human axilla areas and finger skin during an 8-week simulated in-use test. Without statistical analysis all of the tests predicted the results obtained with well-preserved or poorly preserved formulations. The rapid screen test was the best test for predicting differences at intermediate levels of preservation. Statistically, all of the tests were equivalent predictors of preservation efficacy in the in-use test ($P = 0.05$). At the $P = 0.10$ level, only the U.S. Pharmaceopeia, British Pharmacopeia, rapid screen, Cosmetic, Toiletry, and Fragrance Association tests were significantly predictive. The results of prediction by a test, based on the preservative levels used, agreed well with the in-use test results ($P = 0.01$). A total of 20% of the formulations that contained excessive microbial levels contained human axilla microorganisms. The levels of preservation in failed products were similar to the levels of preservation in unused controls.

Most cosmetics are not manufactured or marketed as sterile products; rather, they are manufactured and marketed as products that do not contain microorganisms that cause harm if the products are handled and used as intended by the manufacturer. However, cosmetic products that are handled improperly can become contaminated during manufacture, storage, or consumer use. Microbial contamination that causes obvious degradation of products does not pose a serious health risk, because such products are usually discarded before initial or further use. Antimicrobial preservation systems are often included in cosmetic products to prevent microbial growth. The Food and Drug Administration (FDA) requires cosmetic manufacturers to provide safe products. A product must not contain unacceptable levels of microorganisms at the time of purchase and, in some instances, during use. Although the FDA does not have a recognized cosmetic preservative efficacy test (6), various tests for determining the efficacy of drug preservation are available (5, 12).

In this study we evaluated several preservative efficacy tests (2) which have been described or proposed for controlled in-use testing of experimental test cosmetics, in order to identify the tests that most reliably predicted the performance of cosmetic preservation systems during 8 weeks of simulated human use. The results of this study may also be relevant to the preservation of pharmaceuticals.

* Corresponding author. Mailing address: Division of Microbiological Studies (HFS-516), Food and Drug Administration, 200 C Street, S.W., Washington, DC 20204. Phone: (202) 260-0874. Fax: (202) 205-4270.

† Present address: Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7.

MATERIALS AND METHODS

Microbial cultures. Microorganisms were obtained from the American Type Culture Collection, Rockville, Md., or from laboratory stock cultures. Strains were maintained either as described in the protocols of the various preservative efficacy tests or on nonselective growth media. Before use, each microbial strain was identified by appropriate biochemical and cultural tests; these tests included gram-positive and gram-negative Micro ID panel tests (Micromedia Systems Division, Medical Specialities, Inc., Cleveland, Ohio) for bacteria and API 20C kit tests (Analytical Products, Plainview, N.Y.) for yeasts. Lyophilization or low temperatures were used for long-term storage (1).

Human subjects. Human subjects were selected randomly from a large pool of screened applicants who were found to be qualified on the basis of the following characteristics: no history of asthma, atopic diseases, or reactions to topically applied substances; no dermatological disorders; no antibiotic use within the past 30 days; and willingness and availability to follow the assigned protocol for 12 consecutive weeks. The study protocol included the following requirements: topical application in both axillae had to be limited to the nonantibacterial soap and deodorant furnished by us; personal hygiene activities and drug and cosmetic use had to be entered daily in a logbook; the test formulation had to be inoculated daily for 8 weeks; underarm and formulation sampling had to be done weekly.

Before the study, each subject was given complete verbal and printed instructions. Compliance was checked throughout the study by technicians. Privacy, confidentiality, and full regard for the subjects' welfare remained uncompromised throughout the study.

A total of 40 subjects were selected for each of two 12-week

TABLE 1. Nominal and observed preservative and pH levels of formulations during the test period

Product	Composition of antimicrobial system (%, wt/vol)			pH
	Propyl-paraben	Methyl-paraben	Quaternium 15	
A	0.10 (0.092–0.102)[a]	0.20 (0.146–0.190)	0.00 (—)[b]	7.73–7.84
B	0.00 (—)	0.30 (0.237–0.306)	0.00 (—)	7.66–7.89
C	0.05 (0.044–0.050)	0.10 (0.074–0.094)	0.00 (—)	7.66–7.93
D	0.025 (0.019–0.025)	0.05 (0.036–0.049)	0.00 (—)	7.74–7.96
E	0.05 (0.046–0.056)	0.10 (0.076–0.095)	0.05 (0.030–0.041)	7.73–7.88
F	0.10 (0.095–0.101)	0.20 (0.146–0.191)	0.30 (0.222–0.258)	7.59–7.76
G	0.05 (0.042–0.051)	0.15 (0.108–0.134)	0.10 (0.066–0.081)	7.68–7.79
K	0.30 (0.257–0.313)	0.00 (—)	0.00 (—)	7.71–7.87
L	0.00 (—)	0.00 (—)	0.30 (0.138–0.283)	7.61–7.87
Control	0.00 (—)	0.00 (—)	0.00 (—)	7.60–7.89

[a] The values in parentheses are the observed levels (the ranges of values found over a period of 3 to 4 months at the ambient temperature during the human study).
[b] —, no antimicrobial agent found.

study periods; in both study periods we used the same investigative protocol. The two groups of subjects were referred to as human study group I (HSG I) and HSG II, and during each study period the subjects' axillae were sampled weekly. Test formulations were distributed during the fourth week for daily inoculation by the subjects for the remaining 8 weeks of the study. The subjects were required to submit to 12 weekly samplings; the daily inoculation period was extended for those who missed weekly samplings. The subjects were not to cease daily inoculations in the event of missed appointments.

Approximately 8% of the 80 subjects were involuntarily withdrawn from the study during the first 4 weeks of each period because of noncompliance or other difficulties. Replacement subjects were selected randomly from the applicant pool early enough to complete at least 7 weeks of sampling. There were no voluntary withdrawals. The subjects received previously agreed upon honoraria when the tests were completed.

Preparation of test formulations. The three groups of test formulations consisted of a base formulation that contained various levels of preservative mixtures similar to mixtures found in many commercially available formulations. One formulation was used for the initial experiment; two other formulations were manufactured for use with HSG I and HSG II. Each formulation was manufactured separately (a stock base formulation was not used). The manufacturing equipment, personnel, and methods used were in full compliance with current good manufacturing practice regulations (4, 11). Before use, all materials were examined for uniformity of the raw materials used to manufacture each lot.

The cosmetic formulation ingredients used were as follows: 5.00% (wt/wt) anhydrous lanolin (Croda, Inc., New York, N.Y.); 5.00% (wt/wt) cetyl alcohol (Proctor and Gamble Co., Cincinnati, Ohio); 2.00% (wt/wt) stearic acid (Humko/Witco Chemical Co., Memphis, Tenn.); 1.00% (wt/wt) triethanolamine (99% stock; McKesson Chemical Co., San Francisco, Calif.); 5.00% (wt/wt) sorbitol (70% stock; ICI Americas, Inc., Wilmington, Del.); 1.00% (wt/wt) sodium caseinate (Sigma Chemical Co., St. Louis, Mo.); and 0 to 0.30% (wt/wt) antimicrobial systems. The following antimicrobial agents were used in various combinations in the antimicrobial systems: propylparaben (Napp Chemicals, Inc., Lodi, N.J.), methyl-paraben (Dow Chemical Co., Midland, Mich.), and Quaternium 15 (Mallinckrodt, St. Louis, Mo.) (Table 1). Purified USP quality water was added to obtain the desired volume. Caseinate was added so that the unpreserved base formulation would simulate a preparation that supported microbial growth.

The test formulations were added (by weight) through the tops of wide-mouth, 8-oz (ca. 237-ml) opalescent glass cosmetic jars (Smith Container Corp., Memphis, Tenn.), and the jars were capped with phenolic screw caps with polyliners. The test formulations were subjected to stability testing at room temperature and at 37°C at a relative humidity of 75%. The preservative levels and pH values were determined at monthly intervals during storage while in-use tests were being performed (Table 1).

Distribution of formulations. All nine test formulations were assigned randomly to the 40 subjects in the first test period. For the second period, seven formulations were assigned randomly to the remaining 40 subjects (formulations C and D were omitted as a result of data obtained during the first period). Thus, data were obtained for at least 8 weeks from at least nine subjects for the seven formulations used in the second period. These data were augmented by data obtained from subjects who were reassigned to different formulations.

If a product failed (i.e., if plated microbial CFU were too numerous to count), a fresh container of the same formulation was given to the subject. Another randomly assigned formulation was given to a subject if the subject experienced two consecutive failures with his or her original formulation or if the formulation was withdrawn from the study (e.g., when the number of failures for a formulation represented most of the subjects assigned to it). After withdrawal of a formulation, and for subject safety, all originally assigned subjects were randomly assigned to other formulations regardless of their product failure histories.

Use of formulations. For daily inoculation of test formulations, the subjects rubbed two fingers of a hand in the opposite axilla. The subjects were instructed to wash their hands before rubbing to remove most microorganisms from the fingers. The subjects chose the most convenient hand to use; the axilla to be sampled was not specified because initial evaluations revealed only marginal differences between right and left axillary microfloras. Immediately after rubbing, the fingers were immersed deeply in the cream, and the cream was mixed by the fingers for several seconds. All excess cream on the fingers was removed on the lip of the jar. The subjects were instructed about the need to minimize procedurally introduced contamination during inoculation. Product weight was checked weekly to assess compliance with correct inoculation procedure.

Sampling protocol. A trained technician sampled each subject's axilla weekly by using a sterile dacron swab (Baxter, Scientific Products Division, McGaw Park, Ill.) moistened with isotonic phosphate vehicle (12) supplemented with 0.1% Tween 80 and rendered sterile. The swab was placed in a test tube containing 2 ml of phosphate-buffered saline (PBS)-

Tween 80 and transported to the laboratory. The tube was vortexed for 10 s, and 0.1 ml portions were removed and inoculated onto plates containing blood agar (Edge Biologicals, Memphis, Tenn.), cetrimide-nalidixic acid blood agar, and MacConkey agar (Gibco Laboratories, Madison, Wis.). The inoculum was spread over the agar surface of each plate with a glass rod, and the plates were incubated at 37°C for 24 h.

After incubation, the plates were examined, and the number of CFU per plate was determined for each medium. Values were estimated if a plate contained >300 CFU. Representative colonies were inoculated into brain heart infusion broth; the plates were then incubated for 24 h and examined for additional growth. After 4 h of incubation at 37°C, the broth cultures were used to inoculate Micro ID panels. Isolates from colonies grown on cetrimide-nalidixic acid medium were identified with gram-positive panels, and isolates grown on MacConkey agar were identified with gram-negative panels. We identified five organisms per subject. If more than five organisms were present, the organisms present at the greatest concentrations were identified.

Product was removed from each formulation with the tip of a sterile 5-ml syringe, which was capped and sent to the laboratory. A 1-g amount of product was placed in 9 ml of PBS, and the preparation was vortexed; 1-ml aliquots were spread onto tryptic soy agar and blood agar plates. Isolates were incubated and identified by using the methods described above for isolates obtained from human axillae.

Test formulations that contained >1,000 CFU/g or significant levels of gram-negative microorganisms were removed from the study. These and all other test formulations were later cultured to determine their microbial contents and were assayed by chromatography (13) to determine their preservative levels. Perkin-Elmer series 2 instruments (Perkin-Elmer Corp., Norwalk, Conn.) were used for chromatography. Parabens were extracted with hot isopropanol and were analyzed chromatographically at room temperature by using a Supelcosil LC-18 column (150 by 4.6 mm; pore size, 5 μm); Supelco, Inc., Bellefonte, Pa.). The mobile phase (flow rate, at 2 ml/min) consisted of acetonitrile and water, acidified with acetic acid (35:65:0.5). The detection wavelength was 254 nm. Quaternium 15 was acid hydrolyzed to release formaldehyde, which was derivatized with 2,4-dinitrophenylhydrazine. The derivative was chromatographed at room temperature on a Supelcosil LC-18 column (250 by 4.6 nm; pore size, 5 μm). The mobile phase (flow rate, 1.5 ml/min) consisted of acetronitrile, water, and trimethylammonium hydroxide (50:50:0.1), and the detection wavelength was 365 nm. A pH meter (Corning Glassworks, Corning, N.Y.) was used to measure sample pH values (accuracy, ±0.01 pH unit).

Laboratory efficacy test methods. Six laboratory efficacy tests, along with the weekly in-use test, were performed in duplicate with each of three separately manufactured formulations. All tests were performed in sterile glass jars with lids. The six tests used were the U. S. Pharmacopeia (USP) test (12), the British Pharmacopeia (BP) test (5), the Cosmetic, Toiletry, and Fragrance Association (CTFA) test (8), the rapid screen (RS) test, the sequential challenge (SC) test, and the FDA post-use test.

BP test. The challenge microorganisms used in the BP test were *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231, *Pseudomonas aeruginosa* ATCC 9027, and *Staphylococcus aureus* ATCC 6538. Each microorganism was inoculated separately at a level of 10^6 CFU/g or 10^6 CFU/ml, and the preparations were incubated at 20 to 25°C. Samples were removed for microorganism enumeration after 6, 24, and 48 h and 7, 14, and 28 days. A properly preserved topical product was defined as a product in which the bacterial counts were reduced by ≥99.9% within 48 h of the initial challenge, with no survivors after 7 days, and in which the fungal counts were reduced by ≥99% within 7 days of challenge, with no increase thereafter.

USP test. The challenge microorganisms used in the USP test were *C. albicans* ATCC 10231, *A. niger* ATCC 16404, *Escherichia coli* ATCC 8739, *P. aeruginosa* ATCC 9027, and *S. aureus* ATCC 6538. Each product was inoculated with 10^5 to 10^6 CFU/g or 10^5 to 10^6 CFU/ml, and the preparations were incubated at 20 to 25°C for 7, 14, 21, and 28 days. Preservation was judged to be effective if the bacterial counts were reduced by ≥99.9% by day 14, the fungal counts were reduced by 100% by day 14, and the counts for all microorganisms remained at or below designated levels for the last 14 days of the 28-day period.

CTFA test. The CTFA provides the CTFA test as a guideline for its members. The microorganisms used in this test were *Staphylococcus epidermidis* ATCC 12228, *P. aeruginosa* ATCC 9027, and *Candida parapsilosis* ATCC 7330. The cultures were maintained on tryptic soy agar and were transferred twice in tryptic soy broth (inoculum, 10%). The last transfer was the inoculum suspension. At zero time, 50 g of each test formulation was inoculated with 0.1 ml of one of the three inoculum suspensions. Samples were cultured on Letheen agar (9) on days 3, 7, 14, 21, and 28 after challenge. This cycle was repeated twice, with the same organisms reinoculated on days 28 and 56. A reduction in the number of each organism, as in the USP test, was required after each challenge in order for the formulation to pass the test.

RS test. A 50-g portion of each test formulation was challenged by inoculating it with 0.1 ml of a broth suspension containing one of three tripartite microbe panels. The microorganisms which we used were laboratory strains unless indicated otherwise. The panel members and inoculum level used for each microorganism were as follows: for the standard bacterial panel we used 1.4×10^6 CFU of *E. coli* ATCC 8739 per g, 0.8×10^6 CFU of *P. aeruginosa* ATCC 9027 per g, and 1.45×10^6 CFU of *S. aureus* ATCC 6538 per g; for the in-house panel we used 1.75×10^5 CFU of *Pseudomonas cepacia* per g, 3.3×10^6 CFU of *Pseudomonas* sp. strain G (*P. aeruginosa*) per g, and 2.95×10^5 CFU of *Enterobacter cloacae* per g; and for the yeast and mold panel we used 6.2×10^3 CFU of *C. albicans* ATCC 10231 per g, 5.35×10^3 CFU of *C. parapsilosis* per g, and 5.24×10^3 CFU of *A. niger* ATCC 9642 per g. Samples were removed 3 and 7 days after the formulation was challenged and were plated onto microbial content test agar (Difco Laboratories, Detroit, Mich.). The bacterial plates were incubated at 37°C, and the fungal plates were incubated at 30°C.

Formulations that exhibited a 99.9% reduction in bacterial numbers or a 90% reduction in yeast and mold numbers at 7 days after challenge were inoculated with 0.1-ml portions of a broth suspension containing *P. aeruginosa* ATCC 9027 to give a concentration of 100,000 CFU/g of product. Each formulation was cultured at 3 days after the *Pseudomonas* challenge, and the preparations exhibiting no recovery in 1-g test samples were considered preserved. Formulations that exhibited either recovery after the second challenge or an insufficient decrease in microbial numbers after the primary challenge were considered improperly preserved.

SC test. As proposed by the FDA, 20 g of each test formulation was inoculated with a broth suspension of *S. epidermidis* (10^6 CFU/g of product). Inoculation was repeated for 4 days. On day 5, *P. aeruginosa* (10^5 CFU/g) was added. The formulation was cultured to determine its microbial content on

day 8. This *Staphylococcus-Pseudomonas* inoculation procedure was repeated six times or until a reproducing population of *P. aeruginosa* was established (i.e., until the number of organisms was greater than the number of organisms in the inoculum).

FDA post-use challenge test. Each used test product container was challenged by adding *P. aeruginosa* (10^6 CFU/g). The test products were examined to determine their microbial contents at 24, 48, and 72 h and 7 days after challenge. The results were recorded as recovery or no recovery at each time of examination. A formulation was considered to have failed if any *P. aeruginosa* cells were recovered on day 7.

Statistical analysis. The Kendall coefficient of concordance and the Spearmann rank correlation coefficient tests were used to compare the product rankings that resulted from the different preservation efficacy methods used (10).

RESULTS AND DISCUSSION

The cosmetic preservative efficacy results obtained with various laboratory procedures were compared with the results obtained in the human in-use test. A major source of contamination during skin cosmetic application was assumed to be the microflora of the skin itself. Our test protocol required subjects to inoculate test products with their axillary skin microfloras obtained with freshly washed fingers. The resident microflora of skin is relatively invariable (7), and the axillary skin, since it is somewhat more protected from transitory microorganisms, is a relatively constant source of microbes that is easy to monitor. During this study, the in-use challenge inocula consisted mainly of microorganisms from axillae and, to a lesser extent, microorganisms from washed finger skin.

We expected a significant number of failures with the test formulations which we used, and casein was added to provide a source of organic matter to further tax the preservative systems. All formulations exhibited the expected levels of stability during the tests, and the maximum level of preservative loss was about 25% (Table 1). The values obtained in chemical preservative assays with formulations that failed the in-use tests were within the ranges of values observed with the unused products. This finding suggests that the microbes to which the formulations were exposed during use did not degrade the preservatives. Unchallenged preserved formulations were microbiologically stable throughout the study.

Despite ranking variability and tied ranks, all tests performed as well as the in-use tests. This finding was confirmed by calculating the Kendall coefficient of concordance from the observed product rankings, which was significant at the $P = 0.001$ level. Similarly, in comparisons of the rankings obtained with each of the six tests with the rankings obtained with the in-use test, the calculated Spearman rank correlation coefficient values showed that all tests performed as well as the in-use test ($P = 0.05$); however, at $P = 0.01$, only the BP, CTFA, USP, and RS tests ranked the products as well as the in-use test did. The less significant predictability of the post-use and SC tests at the $P = 0.01$ level was related to the tendency to rank marginally preserved products with well-preserved products. In contrast, the BP and RS tests tended to rank marginally preserved products with poorly preserved products, a conservative tendency which erred on the side of safety. The CFTA test exhibited neither tendency to as great a degree. However, differences in the results obtained with these four tests were not statistically significant.

Formulations A through D and K (Table 2) contained undesirable levels of microorganisms as determined by the in use test. Formulations E and F were considered marginal since

TABLE 2. Laboratory and in-use challenge test results

Formulation	No. of challenge test failures/no. of trials[a]						
	USP test	BP test	SC test	CTFA test	RS test	Post-use test	Human in-use test[b]
Base	2/2	2/2	2/2	2/2	2/2	NA	NA
A	2/6	6/6	0/6	5/6	6/6	0/15	12/15 (75–86)
B	6/6	6/6	0/6	5/6	6/6	0/15	12/15 (63–100)
C	4/6	6/6	6/6	4/4	6/6	4/8	7/8 (88)[c]
D	4/4	4/4	4/4	4/4	4/4	5/6	6/6 (100)[c]
E	0/6	0/6	0/6	0/6	2/6	0/17	1/17 (0–9)
F	0/6	0/6	0/6	0/6	0/6	0/17	0/17 (0)
G	0/6	0/6	0/6	0/6	1/6[d]	0/18	0/18 (0)
K	6/6	6/6	2/6	6/6	6/6	2/15	10/15 (63–71)
L	2/6[e]	0/6	0/6	0/6	0/6	0/16	1/16 (0–10)

[a] See Materials and Methods for the pass-fail criterion used for each test. NA, not applicable or not tested. A total of 6 to 11 subjects were used for each formulation in each study group (see Materials and Methods).

[b] The values indicate the number of failures that occurred at any time during the 8 week in-use test. The values in parentheses are the ranges of failure data, expressed as percentages based on the results obtained with each study group (see Materials and Methods).

[c] Data for only one study group because the formulation was withdrawn from the study.

[d] One yeast-mold panel contained a *Pseudomonas* strain.

[e] *C. albicans* was persistent.

1 failed in-use marginal performance out of 17 was defined as an in-use failure; poor performance was defined as two or more in-use failures. Except for formulation L, these results were predicted correctly only by the RS test (Table 2). Formulation L contained only one preservative, and the single failure was probably the result of an adaptive strain of *P. aeruginosa*. Such failures should be anticipated when a single antimicrobial agent is used in formulations that have no inherent antimicrobial activity (3). When we retested single test portions of formulation E and L which exhibited in-use failures 3 weeks after removal from the study, no microorganisms were found. We assumed that the organisms observed originally were transient organisms that were not able to survive in the test formulations. The BP test correctly predicted all of the results except the results obtained with marginal formulations E and L. The CTFA procedure correctly predicted the results obtained with formulations C, D, and K, but gave inconsistent results for formulations A and B. The USP procedure predicted the in-use results obtained with formulations B through D and K but failed to reveal a problem with formulations A and E. The SC test revealed potential problems with formulations C, D, and K but not with formulations A, B, E, and L.

The post-use test was designed to identify test formulations that may have encountered organisms which were able to destroy their preservative systems during use. In the absence of such an encounter, this test is merely a single-challenge test in which a single organism is used. Formulations A and C produced typical results. Formulation C contained 0.15% (wt/wt) total paraben, and formulation A contained 0.3% (wt/wt) total paraben. We observed no failures with formulation A, but the level of failure with formulation C was 50%.

The majority of the 48 individual samples that failed during the in-use test contained either a single microorganism or a predominant microorganism when more than one spoilage microorganism was present. The relative numbers and types of microorganisms isolated from the human subjects and from the product samples that failed are shown in Table 3. The isolates obtained from failed products were mainly gram-positive bacteria which resembled bacteria found in human skin microflo-

TABLE 3. Frequencies and types of microorganisms isolated from axillary areas of human subjects and used test formulations

Taxon	Frequency (% of samples)[a]							
	Human subjects				Used formulations			
	Rare	Few	Moderate	Numerous	Rare	Few	Moderate	Numerous
Gram-positive bacteria								
Bacillus spp.	<2	<1	0	0	7	<1	0	0
Diptheroids, nonhemolytic bacteria	20	15	9	9	<3	0	<1	<1
Micrococcus spp.	13	8	<1	<1	<2	<1	0	0
Staphylococcus epidermidis	6	21	7	36	<2	<1	0	<2
Staphylococcus aureus	8[b]	10	<2	10	6	<2	<1	<1
Streptococcus group D	<1	<1	<1	5	<2	<2	<1	7
Streptococcus viridans	4	<2	1	<1	<2	0	0	0
Streptococcus salivarius	<1	<1	<1	<1	0	0	0	0
Gram-negative bacteria								
Acinetobacter anatratus	<2	<1	0	<1	0	0	0	0
Acinetobacter lwoffi	9[b]	<1	0	<1	0	0	0	0
Aeromonas hydrophila	<1	0	0	0	<1	0	0	0
Enterobacteria aerogenes	<1	<1	0	0	0	0	0	0
Enterobacter cloacae	<1	<1	<1	<1	0	0	0	0
Escherichia coli	<1	<1	<1	<1	0	0	0	0
Klebsiella oxytoca	<7[b]	0	<1	0	0	0	0	0
Klebsiella pneumoniae	<1	1	<1	<1	0	0	0	0
Proteus mirabilis	<1	<1	<2	2	0	0	0	0
Pseudomonas aeruginosa	<1	<1	0	<1	0	0	0	<1
Pseudomonas cepacia	2	<1	<1	0	0	0	0	0
Pseudomonas stutzeri	<1	0	0	0	0	0	0	0
Serratia marcescens	<1	<1	<1	<1	0	0	0	0
Serratia rubidia	<1	0	0	0	0	0	0	0
Fungi								
Fungus species	4	<1	0	0	<2	<1	0	0
Yeasts	<1	<1	<1	<1	0	<1	0	<1

[a] Average results for HSG I and HSG II. Rare, <10 CFU per swab; few, <100 CFU per swab; moderate, <1,000 CFU per swab; numerous, >1,000 CFU per swab. Except as noted below, the frequencies for each taxon were well within the ranges represented by the averages ±3%.

[b] The frequencies were within the ranges represented by the averages ±6%.

ras. However, product failure isolates which were identical to strains in the users' microfloras were detected in only 14 of the 48 failures. Strains of a species were distinguished by differences in single characteristics that were not critical for species identification.

The strains obtained from the 14 failed products which were indistinguishable from strains in the subjects' microfloras included five strains that were either sole or predominant spoilage microflora strains and nine strains that were minority members of the spoilage microflora. The fact that some methicillin-resistant staphylococci were isolated during this study suggested that a few subjects, who were health facility personnel, may have had atypical skin microfloras. However, these strains did not explain the discrepancy between the user and product strains in the 34 other instances of spoilage. This discrepancy does not necessarily mean that the spoilage strains did not originate from the users' skin microfloras. They could have been suppressed among the users' skin microfloras to levels that were too low to be detected in the presence of the predominant microorganisms in the skin microfloras. If this is so, subsequent selection against the predominant microorganisms by the conditions of the in-use challenge test could explain the subsequent detection of the suppressed strains. The relatively low temperature of the in-use challenge test compared with the recovery temperature used to characterize the subjects' microfloras and/or different relative levels of susceptibility to preservatives could have resulted in selection of strains.

If selective pressures were not operating, the strain identity discrepancy may be explained by spoilage caused by nonaxillary microorganisms, such as members of the digital skin microflora. However, the contribution of such microorganisms was probably minimal since subjects apparently complied with our emphatic instructions to wash their hands properly before digitally sampling their axillary skin.

Overall, our results at the genus-species level are consistent with the common belief that normal resident microfloras of users are associated with preservation failures in multiuse products. Strain differences were most likely due to selection among resident and incident axillary skin microorganisms.

In this study we found that laboratory microbiological tests can be used to predict the in-use efficacy of antimicrobial preservatives in products such as cosmetics and pharmaceuticals. We identified totally inadequate preservation by using laboratory procedures. Using the RS test, we differentiated adequately preserved and well-preserved formulations. This testing approach should allow formulators to avoid the common practice of overpreserving products and to identify the minimum effective level of a preservative. Of all of the procedures used in this study, the RS test required the least time and the fewest materials. The differences between results of microbiological methods which we observed were obtained only by using a fairly high number of replicates, and serious consideration should be given to devising a method for predicting preservative efficacy on the basis of preservative concentrations and types.

The predicted preservative efficacies of cosmetics were

reflected by the results of the in-use test ($P \leq 0.01$). Ours predictions were based on the total preservative concentration in a product, assuming that of two products with equal total preservative levels, inclusion of the quaternary compound would result in greater efficacy. The observed ranking of formulations (in increasing order of preservation efficacy as determined by the in-use test) was as follows: D, C, A or B, K, E or L, and F or G. Similarly, the theoretical, predicted ranking formulations was: D, C, A or B or K, E, L or G, and F. Thus, although theoretical predictions of preservative efficacy are possible, such predictions must be validated on a formula-by-formula basis until more data on the inherent preservative actions and synergistic-antagonistic activities of cosmetic components are available.

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