Note

Effects of Parabens and Isothiazolinone on the Microbiological Quality of Baby Shampoo: The Challenge Test

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An *in vitro* microbial challenge test has been developed to predict the likelihood of consumer contamination of baby shampoo. Four preservatives were tested in our study: the parabens Medcide D, Medcide PB, Sepicide HB. and isothiazolinone Methylisothiazolinone/Chloromethylisothiazolinone [MI/MCI]. These preservatives were tested separately and in combination. The challenge test involved inoculating the product with *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Aspergillus brasiliensis* and *Candida albicans*. Inhibition growth of these microorganisms at each preservative concentration was followed over a 28 d period. The test was used to classify products as poorly preserved, marginally preserved, or well-preserved. Interestingly, it was the combination (0.1% Isothiazolinone [MI/MCI] and 0.1% Sepicide HB) which inhibited most the microbial growth of microorganisms while preserving the physicochemical properties of the product. As a result, the challenge test described can be accurately used to predict the risk of consumer contamination of cosmetic products.

Key words : Baby shampoo / Parabens / Isothiazolinone [MI/MCI] / Challenge test / Microbial growth.

Cosmetics are occasionally contaminated with spoilage microorganisms, and the biggest concern regarding contamination is the possible presence of pathogens that may pose health threats (Cowen and Steiger, 1976). In cosmetics, contaminating microorganisms may cause spoilage of the product and present a serious health risk for consumers worldwide (Becks and Lorenzoni, 1995; Behravan et al., 2005). Even non-pathogenic spoilage microbes in a cosmetic may cause disease under certain conditions. For example, a microbe contaminating a cosmetic product may be invasive if the cosmetic is applied to cover a blemish or break in the skin (FDA, 2001). The microbiologist uses a variety of chemical preservatives to prevent contamination by pathogens or

spoilage microorganisms, and their use can extend the shelf life of the products. The preservatives include benzyl alcohol, boric acid, sorbic acid, chlorhexidine, formaldehyde, parabens, quaternary ammonium compounds, phenol, imidazolidinyl compounds, and others. Several excellent reviews cover this topic (Durant and Higdon, 1991; Chapman, 1987).

Parabens are the most widely used preservatives in commercially available shampoos (Fox, 1988). The most common parabens used in cosmetic products are methylparaben, propylparaben, and butylparaben. Fox (1988) reported that propylparaben is most commonly used as an antimicrobial in water-based products such as cosmetics, shampoos, moisturizers, conditioners and sunscreens. Typically, more than one paraben is used in a product, and they are often used in combination with other types of preservatives to work against a broad range of microorganisms. The combined use of parabens and other

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preservatives could reduce microbial levels while increasing the shelf life of products. Among these preservatives, the most frequently used is isothiazolinone: Methylisothiazolinone / Chloromethylisothiazolinone [MI/MCI]. MI is used as a co-biocide with MCI and has been recently introduced as a stand-alone preservative. Activities of organic acids are profoundly compromised in neutral and alkaline formulations as is the stability of isothiazolinone preservatives in the presence of amines (Collier et al., 1990).

Assessment of the preservative efficacy in cosmetics is usually performed using the challenge test (ISO/WD 11930, 2008). This test provides assurance regarding the microbiological levels of the product at the time the test is performed. Russell (2003) suggested that challenge test should be undertaken at the beginning, during and at the end of the shelf life of the product.

Test-challenging a product with appropriate organisms is a major concern in determining how effective a preservative must be, and is based on the inoculation of the cosmetic product with bacteria, followed by incubation and sampling for survivors during the storage period (Russell, 2003).

Considering the promising properties of additives and the growing interest in their efficient use, this study postulates that parabens (Medcide D, Medicide PB and Sepicide HB) and isothiazolinone [MI/MCI] which are naturally abundant and available may represent efficient additives. These agents could replace the commonly used expensive preservatives and improve the cost-effectiveness of the overall production process, and hence, the final end product. Accordingly, this study used the challenge test to focus on the value of adding parabens and isothiazolinone [MI/MCI] to baby shampoo separately and in combination at various concentrations.

The baby shampoo used in this study was prepared by the manufacturer (Parfumerie Africa) in a well-defined form. The shampoo consisted of the following: (1) water, (2) anionic surfactants Zetesol MGS (magnesium lauryl-2 sulfate) and Texapon N70, the non-ionic surfactant Puritan CFD (cocamide diethanolamide) and the amphoteric surfactant sabosol PB (cocamidopropyl Betain) [all surfactants were provided by Zschimmer & Schwarz Italiana S.p.A], (3) Polyquaternium 10 (Chematek S.p.A), a detangling agent, (4) Crovol Maracuja, PEG-60 Maracuja Glycerides (Croda Chemical Europe, England), an anti-irritating agent, (5) EDTA (Sigma-Aldrich, USA), a chelating agent, (6) Triethanolamine (Virginia Dare Extract Co., Inc., USA), a pH regulator, and (7) Linalool (Simagchem Corporation, China)

In our study, four types of preservatives were tested. First was Medcide D (MD), a mixture of (diazolidinyl urea) and methyl and propyl parabens in propylene glycol (Medolla Italia, Italy). It is a clear liquid and can be easily incorporated in cosmetic formulations at any stage of the formulating process. This preservative is effective in a wide range of pH and suitable for cold formulations of shampoos. It is used at concentrations from 0.5 to 1.0% in preparations. The second preservative was Medicide PB (MPB) which consists of a selected combination of methyl, ethyl, butyl, propyl and isobutyl parabens in phenoxyethanol (Medolla Italia, Italy). It is a clear liquid and added into end products at temperatures between 0°C and 40°C. pH tolerance is given for an acidic and neutral environment. It is used at concentrations from 0.3 to 1.0% in preparations. Another preservative used in this study was Sepicide HB (SHB), a mixture of methyl, ethyl and propylparaben in phenoxyethanol (Seppic, France). This is a slightly colored viscous liquid, slightly soluble in water. It is used at concentrations from 0.5 to 1.5% in preparations. The final preservative used was Isothiazolinone (Chloromethylisothiazolinone/Methylisothiazolinone) (MI/MCI), an electrophilic preservative mixture. It is a slightly yellow liquid, soluble in water and used when the product pH is between 3 and 8. It was used in concentrations at 0.01-0.1% (Simagchem Corporation, China).

Firstly, these preservatives were added separately to the baby shampoo at 1.0% MD; 1% MPB; 1.5% SHB and 0.1% [MI/MCI]. Thereafter, preservatives were used in combination according to the following concentrations: 0.5% MD + 0.5% MPB, 0.5% MD + 0.5% SHB, 0.1% MD + 0.1% [MI/MCI], 0.5% MPB + 0.5% SHB, 0.1% MPB + 0.1% [MI/MCI] and 0.1% SHB and 0.1% [MI/MCI]. A baby shampoo sample without any additives was used as the control.

For the microbial challenge test, each of the test formulations was inoculated with each of the following microorganisms: *Micrococcus luteus* LB 14110, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella enterica* ATCC 43972, *Pseudomonas aeruginosa* ATCC 9027, *Aspergillus brasiliensis* ATCC 16404 and *Candida albicans* ATCC 10231, using the test procedure modified from United States Pharmacopeia (USP, 1994). Microorganisms were grown in aerobic conditions. *S. enterica* ATCC 43972, *P. aeruginosa* ATCC 9027 and *M. luteus* LB 14110 were grown 24 h at 30°C in Luria Bertani medium LB (10 g/l peptone; 5 g/l NaCl; 5 g/l yeast extract; pH 7.2) while *E. coli* ATCC 8739 and *S. aureus* ATCC 6538 were grown at 37°C on the same medium (LB). Yeast and fungal strains were cultivated on

sabouraud dextrose agar (Difco Laboratories) at 25 °C for 48 h and 1 wk, respectively. All microorganisms were harvested by washing the cells from the slants with sterile saline. The growth in terms of colony forming units per milliliter (CFU/ml) of each organism was determined. Twenty ml samples were placed into sterile glass bottles, one for each organism and inoculated with the test microorganism (10^5 - 10^6 colonies or spores/ml sample). The containers were incubated at room temperature (35°C) for a total of 28 d and examined periodically. Examination was done initially and after 3, 7, 14, 21 and 28 d of inoculation. Plate counts were performed using media corresponding to those in primary cultivation.

For the stability test at ambient conditions, the formulations prepared were evaluated in terms of their clarity, foam producing ability and fluidity (Aghel et al., 2007) and the pH of 10% shampoo solution in distilled water was determined at room temperature 25°C (Mainkar and Jolly, 2000). The cylinder shake method was used for determining foaming ability. Fifty ml of the 1% shampoo solution was put into a 250 ml graduated cylinder, covered and shaken 10 times. The total volumes of the foam contents after 1 min shaking were recorded. Immediately after shaking, the volume of the foam was calculated each min for 4 min (Klein, 2004). For stability studies, clear liquid shampoos were formulated. The samples in glass containers were stored at room temperature (35°C) for three months. After the storage, the preservative efficacy was tested. The thermal stability of formulations was studied by placing the samples in glass tubes in a humidity chamber at 45°C and 75% relative humidity. Their appearance and physical stability were inspected each month for a period of three months (Barel et al., 2001).

All measurements were carried out in triplicate, and all microbial counts were converted into base-10 logarithms of colony forming units per ml of the samples ($\log_{10}$ CFU/g). Data were subjected to analysis of variance (ANOVA) using the General Linear Models procedure of the Statistical Analysis System software of the SAS Institute (SAS, 1990). Differences among the mean values of the various treatments and storage periods were determined by

the least significant difference (LSD) test, and the significance was defined at $P < 0.05$. The differences equal to or greater than the identified LSD values were considered statistically significant.

For microbiological evaluation, sterility is not necessary but the shampoos must not be contaminated with pathogenic microorganisms and they should not harbour microbial contaminants in high numbers (Ravita et al., 2009). With the aim of evaluating the microbiological effect of additives on baby shampoo, combinations of various parabens (MPB, MD and SHB) and Isothiazolinone (MI/MCI) were prepared at different concentrations (Table 1). Thus, we studied their capacities to inhibit Gram positive bacteria (*M. luteus* LB 14110 and *S. aureus*), Gram negative bacteria (*E. coli* ATCC 8739, *S. enterica* ATCC 43972, *P. aeruginosa* ATCC 9027 and moulds (*C. albicans* ATCC 10231 and *A. brasiliensis* ATCC 16404).

The initial population of *M. luteus* LB 14110 in unpreserved samples was $6 \log_{10}$ CFU/ml (Table 2). This population slightly increased until the seventh d reaching $6.9 \log_{10}$ CFU/ml, then it was decreased to $4.18 \log_{10}$ CFU/ml at the end of storage time in non-treated samples (sample 1). Nevertheless, for all treated samples a significant reduction in *M. luteus* population was observed in comparison with sample 1. For example on the 21st d of storage, MPB at 1.0% reduced the number of *M. luteus* to 99.99%. Used alone, MPB was the best preservative among the parabens studied. 0.1% [MI/MCI] reduced the microbial load of *M. luteus* in treated samples after 14 d of storage. On the other hand, samples treated with MPB / [MI/MCI] in combination showed delayed growth for *M. luteus* in comparison with controls. A 99.99% reduction in bacterial cell number ($P < 0.01$) was observed after 3 d in the tested cosmetic product according to the challenge test (Table 2); similarly, a logarithmic reduction of 5 ($P < 0.01$) by the 7th d was noted in the sample treated with SHB / [MI/MCI] combination (Table 2).

Staphylococcus aureus ATCC 6538 is a common skin organism (Geis, 2006). Most preservative challenge test methods use it to examine cosmetic products because it is the most common contaminant that

TABLE 1. Different percentages of preservatives used in formulations

Preservatives	Concentration used in formula No. (%)										
	1	2	3	4	5	6	7	8	9	10	11
MD	—	1.0	—	—	—	0.5	0.5	0.1	—	—	—
MPB	—	—	1.0	—	—	0.5	—	—	0.5	0.1	—
SHB	—	—	—	1.5	—	—	0.5	—	0.5	—	0.1
[MI/MCI]	—	—	—	—	0.1	—	—	0.1	—	0.1	0.1

TABLE 2. Effect of preservatives on the microbial load of Gram positive Bacteria: *Micrococcus luteus* LB 14110 and *Staphylococcus aureus* ATCC 6538

Sample N°	Microorganisms (Gram+ Bacteria)	Microbial load (log ₁₀ CFU/ml)					
		Initial	3 d	7 d	14 d	21 d	28 d
1	<i>M. luteus</i> LB 14110	6.0 ^a	6.33±0.14 ^a	6.9±0.06 ^b	6.23±0.10 ^a	5.96±0.05 ^a	4.18±0.08 ^c
	<i>S. aureus</i> ATCC 6538	5.7 ^a	5.61±0.08 ^a	5.14±0.07 ^a	4.83±0.12 ^b	3.12±0.09 ^c	2.31±0.06 ^c
2	<i>M. luteus</i> LB 14110	6.0 ^a	5.66±0.05 ^a	4.01±0.07 ^b	3.18±0.09 ^b	1.81±0.14 ^c	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	5.50±0.04 ^a	3.89±0.03 ^b	2.68±0.1 ^c	<1	<1
3	<i>M. luteus</i> LB 14110	6.0 ^a	5.32±0.06 ^a	3.67±0.03 ^b	1.22±0.09 ^c	<1	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	4.65±0.04 ^a	2.22±0.09 ^b	<1	<1	<1
4	<i>M. luteus</i> LB 14110	6.0 ^a	5.44±0.08 ^a	3.91±0.03 ^b	2.69±0.07 ^b	1.11	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	4.92±0.06 ^a	3.77±0.03 ^b	2.45±0.04 ^b	<1	<1
5	<i>M. luteus</i> LB 14110	6.0 ^a	4.82±0.07 ^b	2.28±0.08 ^c	<1	<1	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	3.56±0.1 ^b	1.82±0.07 ^c	<1	<1	<1
6	<i>M. luteus</i> LB 14110	6.0 ^a	3.12±0.05 ^b	1.67±0.06 ^c	<1	<1	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	2.22±0.08 ^b	<1	<1	<1	<1
7	<i>M. luteus</i> LB 14110	6.0 ^a	4.44±0.07 ^b	2.61±0.05 ^c	1.43±0.11 ^c	<1	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	3.92±0.04 ^b	2.33±0.08 ^c	<1	<1	<1
8	<i>M. luteus</i> LB 14110	6.0 ^a	3.55±0.1 ^b	1.18±0.06 ^c	<1	<1	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	1.26±0.04 ^b	<1	<1	<1	<1
9	<i>M. luteus</i> LB 14110	6.0 ^a	4.77±0.07 ^b	2.33±0.09 ^c	1.21±0.11 ^c	<1	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	2.75±0.13 ^b	1.89±0.1 ^c	<1	<1	<1
10	<i>M. luteus</i> LB 14110	6.0 ^a	<1	<1	<1	<1	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	<1	<1	<1	<1	<1
11	<i>M. luteus</i> LB 14110	6.0 ^a	1.42±0.11 ^b	<1	<1	<1	<1
	<i>S. aureus</i> ATCC 6538	5.7 ^a	<1	<1	<1	<1	<1

± : Standard deviation of three replicates

a-c: Averages with different letters in the same column are different ($P<0.05$).

may pose threats to consumers (Cowen and Steiger, 1976). It is used as a representative Gram-positive cocci in many tests. At the same time, since its nutrient needs are comparatively demanding, it does not always seem to be a logical choice as a challenge inoculum.

The initial population of *S. aureus* in samples without preservatives was 5.7 log₁₀ CFU/ml (Table 2). This value decreased with the storage time reaching 2.31 log₁₀ CFU/ml on the 28th d of storage. However, for all the treated samples, the initial load of *S. aureus* decreased rapidly until it was completely eliminated. In fact, after three d of storage, the absence of *S. aureus* was observed in samples treated with MPB / [MI/MCI] and SHB / [MI/MCI] combinations. Paraben blends with MPB / MD, MPB / SHB and MD / SHB showed a progressive decrease in the CFU values after 7, 14 and 28 d of storage respectively. (Table 2)

Jacobs and Sade have reported in 1984 that methyl paraben at 0.5 mM sensitizes anoxic buffered suspensions of *S. aureus* to gamma radiation by over 150%. During storage, *M. luteus* and *S. aureus* counts increased in non-treated samples but it was

clear that all treatments resulted in a significant population reduction ($P<0.05$) in baby shampoo.

E. coli ATCC 8739 is a fermentative Gram-negative rod. It is a member of one of the largest bacterial families, the Enterobacteriaceae, and is considered an indicator of fecal contamination (Geis, 2006). During storage, *E. coli* counts slightly increased in sample 1 in the 3 first d of storage and then decreased to a final load of 3.33 log₁₀ CFU/ml but it was clear that all treatments resulted in a significant population reduction even since the first d of storage ($P<0.05$). Interestingly, samples treated with the MPB / [MI/MCI] combination delayed significantly the *E. coli* growth in comparison with controls (Table 3). In fact, *E. coli* loads were less than 1 log₁₀ CFU/ml from the 3rd d of storage. In addition, a reduction of 99.99% in the number of the microorganisms was observed by the 7th d of challenge for combinations MD / [MI/MCI] and SHB / [MI/MCI]. A 99.99% reduction in bacterial cell number ($P<0.01$) was observed after 14 d in the tested cosmetic product treated with [MI/MCI], MD/MPB and MD/SHB.

Maximal reduction of *S. Enterica* ATCC 43972

count was shown by the MPB / [MI/MCI] combination, (Table 3) followed by samples treated with MD / [MI/MCI]. Same inhibition levels were observed for samples treated by the MPB / MD and SHB / [MI/MCI] combinations. By the end of the storage period (day 28), all *S. enterica* counts of samples treated with different combinations were reduced to 99.99%.

P. aeruginosa is a non-fermentative Gram-negative rod and a well-known and highly ubiquitous pathogen. It also shows high resistance to many preserva-

tives (Geis, 2006). The *P. aeruginosa* challenge test results are shown in Table 3. The results showed a reduction and an elimination of the pathogen over the 28 d of the challenge period for the ten preservative concentrations used. On the other hand, *P. aeruginosa* challenge testing showed that the isothiazolinone-preserved formula [MI/MCI] used in combination with parabens rapidly reduced and eliminated the microorganism whereas paraben-preserved shampoo showed elimination of the *Pseudomonas* load only after 21 and 28 d of storage (Table 3).

TABLE 3. Effect of preservatives on the microbial load of Gram negative Bacteria: *Escherichia coli* ATCC 8739, *Salmonella* Enterica ATCC 43972 and *Pseudomonas aeruginosa* ATCC 9027

Sample No.	Microorganisms (Gram—Bacteria)	Microbial load (log ₁₀ CFU/ml)					
		Initial	3 d	7 d	14 d	21 d	28 d
1	<i>E. coli</i> ATCC 8739	5.4 ^a	5.76±0.06 ^a	5.54±0.07 ^a	5.53±0.09 ^a	4.9±0.05 ^a	3.33±0.04 ^b
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	6.29±0.04 ^a	5.64±0.07 ^a	4.98±0.09 ^a	3.3±0.03 ^b	2.97±0.1 ^b
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	5.84±0.07 ^a	4.88±0.04 ^a	4.29±0.08 ^b	3.0±0.02 ^b	2.28±0.04 ^c
2	<i>E. coli</i> ATCC 8739	5.4 ^a	5.21±0.1 ^a	3.77±0.12 ^b	2.65±0.09 ^b	1.43±0.06 ^c	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	5.70±0.06 ^a	4.19±0.04 ^b	2.92±0.03 ^c	1.55±0.17 ^c	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	5.71±0.14 ^a	4.78±0.09 ^a	3.68±0.05 ^b	2.11±0.04 ^c	<1
3	<i>E. coli</i> ATCC 8739	5.4 ^a	4.41±0.05 ^a	2.17±0.08 ^b	1.18±0.05 ^c	<1	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	4.89±0.03 ^a	2.41±0.1 ^b	1.31±0.11 ^c	<1	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	5.59±0.14 ^a	3.98±0.12 ^b	3.02±0.08 ^b	1.76±0.07 ^c	<1
4	<i>E. coli</i> ATCC 8739	5.4 ^a	4.78±0.07 ^a	3.71±0.04 ^b	2.41±0.14 ^b	1.4±0.06 ^c	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	5.13±0.08 ^a	4.07±0.03 ^b	2.65±0.08 ^c	1.55±0.05 ^c	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	5.64±0.06 ^a	4.24±0.08 ^b	3.33±0.03 ^b	2.0±0.04 ^c	<1
5	<i>E. coli</i> ATCC 8739	5.4 ^a	3.35±0.05 ^b	1.82±0.04 ^c	<1	<1	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	3.79±0.08 ^b	2.03±0.03 ^c	1.23±0.06 ^c	<1	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	5.32±0.04 ^a	2.98±0.14 ^b	1.77±0.05 ^c	<1	<1
6	<i>E. coli</i> ATCC 8739	5.4 ^a	2.29±0.06 ^b	1.27±0.04 ^c	<1	<1	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	2.54±0.11 ^b	1.43±0.09 ^c	<1	<1	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	4.55±0.04 ^a	3.12±0.14 ^b	1.67±0.13 ^c	<1	<1
7	<i>E. coli</i> ATCC 8739	5.4 ^a	3.77±0.06 ^b	1.92±0.08 ^c	<1	<1	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	4.15±0.04 ^b	2.44±0.06 ^c	1.84±0.03 ^c	<1	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	5.20±0.05 ^a	4.56±0.09 ^b	2.66±0.1 ^c	1.69±0.09 ^c	<1
8	<i>E. coli</i> ATCC 8739	5.4 ^a	1.44±0.04 ^b	<1	<1	<1	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	1.58±0.1 ^b	<1	<1	<1	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	4.67±0.05 ^a	3.66±0.09 ^b	1.33±0.02 ^c	<1	<1
9	<i>E. coli</i> ATCC 8739	5.4 ^a	2.71±0.08 ^b	1.92±0.05 ^c	<1	<1	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	3.05±0.09 ^b	2.12±0.11 ^c	1.18±0.1 ^c	<1	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	5.0±0.04 ^a	4.27±0.08 ^b	3.64±0.05 ^b	1.61±0.09 ^c	<1
10	<i>E. coli</i> ATCC 8739	5.4 ^a	<1	<1	<1	<1	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	<1	<1	<1	<1	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	<1	<1	<1	<1	<1
11	<i>E. coli</i> ATCC 8739	5.4 ^a	2.21±0.07 ^b	<1	<1	<1	<1
	<i>S. Enterica</i> ATCC 43972	5.9 ^a	2.45±0.09 ^b	1.13±0.1 ^b	<1	<1	<1
	<i>P. aeruginosa</i> ATCC 9027	6.0 ^a	3.00±0.07 ^b	1.54±0.04 ^c	<1	<1	<1

± : Standard deviation of three replicates

a-c: Averages with different letters in the same column are significantly different ($P<0.05$).

Interestingly, the MPB / [MI/MCI] blend was the best combination that inhibited the growth of this microorganism. In fact, it led to the elimination of the microbial load from the third d of storage.

O'Neill and Mead (1982) studied the effect of different parabens on *E. coli* and *P. aeruginosa*. They reported that the initial kill rates of *E. coli* by methyl, ethyl and propyl parabens were similar at equal saturation fractions. Zedan and Serry (1984) demonstrated that *P. aeruginosa* was unable to grow on methyl paraben alone except after prolonged incubations (15 d or more) where selection of resistant cells occurred.

The initial population of *C. albicans* and *A. brasiliensis* was 6 log₁₀ CFU/ml. This value decreased during the storage period in non-treated samples, and treated samples with different preservatives showed a significant decrease and elimination ($P < 0.05$) of the two microbial strains from the baby shampoo samples. Table 4 shows that parabens MPB, MAD and SHB and even the combination MPB / [MI/MCI] could eliminate *C. albicans* ATCC 10231 and *A. brasiliensis* ATCC 16404 only after 21 d of

storage. However, parabens blend MPB / MD and SHB / MD could inhibit mould growth after 14 d of storage. Interestingly, *C. albicans* and *A. brasiliensis* were completely inhibited by the MPB / SHB combination at a final concentration of 1%. On the other hand, the isothiazolinone-preservative formula [MI/MCI] was not effective in eliminating *C. albicans* ATCC 10231 and *A. brasiliensis* ATCC 16404 from the inoculated samples. Finally, the best combination that eliminated the microbial strains was a parabens combination (SHB / MPB) followed by the combinations MD / MPB, MD / SHB and SHB / [MI/MCI] that respectively showed an inhibition after 3 and 14 d of storage. (Table 4)

In accordance with our findings, Acott and Labuza (1975) reported that methyl- and propyl parabens inhibited growth of *A. brasiliensis* at 0.05 and 0.1% concentration during the period of storage for 9 months.

The study of the effect of different combinations of preservatives on the microbiological quality of baby shampoo showed that alone or in combination with isothiazolinone, parabens were able to reduce and in-

TABLE 4. Effect of preservatives on the microbial load of yeast and fungi: *Candida albicans* ATCC 10231, and *Aspergillus brasiliensis* ATCC 16404

Sample No.	Microorganisms (Yeast and fungal)	Microbial load (log ₁₀ CFU/ml)					
		Initial	3 d	7 d	14 d	21 d	28 d
1	<i>C. albicans</i> ATCC 10231	6.0 ^a	5.66±0.07 ^a	4.38±0.09 ^b	3.89±0.05 ^b	2.34±0.09 ^c	1.69±0.04 ^c
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	5.59±0.06 ^a	4.47±0.09 ^b	3.91±0.08 ^b	2.58±0.02 ^c	1.53±0.07 ^c
2	<i>C. albicans</i> ATCC 10231	6.0 ^a	4.92±0.09 ^b	2.19±0.09 ^c	1.22±0.03 ^c	<1	<1
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	5.0±0.07 ^a	2.48±0.09 ^b	1.3±0.02 ^c	<1	<1
3	<i>C. albicans</i> ATCC 10231	6.0 ^a	4.6±0.04 ^b	3.42±0.1 ^b	2.0±0.11 ^c	<1	<1
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	4.69±0.09 ^b	3.63±0.03 ^b	2.13±0.07 ^c	<1	<1
4	<i>C. albicans</i> ATCC 10231	6.0 ^a	5.20±0.04 ^a	4.63±0.08 ^b	2.08±0.05 ^c	<1	<1
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	4.92±0.09 ^a	2.19±0.07 ^b	1.22±0.04 ^c	<1	<1
5	<i>C. albicans</i> ATCC 10231	6.0 ^a	5.68±0.09 ^a	4.56±0.1 ^a	4.0±0.7 ^a	2.75±0.06 ^b	1.52±0.09 ^c
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	5.71±0.04 ^a	4.78±0.06 ^a	3.28±0.09 ^b	2.13±0.06 ^c	1.17±0.1 ^c
6	<i>C. albicans</i> ATCC 10231	6.0 ^a	3.4±0.07 ^b	1.94±0.08 ^c	<1	<1	<1
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	3.6±0.06 ^b	1.98±0.09 ^c	<1	<1	<1
7	<i>C. albicans</i> ATCC 10231	6.0 ^a	4.92±0.05 ^a	2.71±0.09 ^b	<1	<1	<1
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	4.12±0.04 ^a	2.99±0.09 ^b	<1	<1	<1
8	<i>C. albicans</i> ATCC 10231	6.0 ^a	4.23±0.03 ^a	3.11±0.1 ^b	2.37±0.11 ^b	1.07±0.04 ^c	<1
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	4.55±0.14 ^a	3.03±0.08 ^b	2.0±0.09 ^c	1.76±0.05 ^c	<1
9	<i>C. albicans</i> ATCC 10231	6.0 ^a	<1	<1	<1	<1	<1
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	<1	<1	<1	<1	<1
10	<i>C. albicans</i> ATCC 10231	6.0 ^a	5.0±0.07 ^a	2.66±0.09 ^b	1.67±0.07 ^c	<1	<1
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	4.5±0.04 ^a	3.92±0.08 ^b	2.2±0.03 ^c	<1	<1
11	<i>C. albicans</i> ATCC 10231	6.0 ^a	3.89±0.09 ^b	2.48±0.07 ^b	<1	<1	<1
	<i>A. brasiliensis</i> ATCC 16404	6.0 ^a	3.8±0.06 ^b	2.22±0.04 ^b	<1	<1	<1

± : Standard deviation of three replicates

a- c: Averages with different letters in the same column are significantly different ($P < 0.05$).

hibit the bacterial growth, and these results are in accordance with those showed by Maddox (1982). In addition, combinations of parabens were shown to have a synergistic effect on bacteria as earlier described by Doron et al. (2001) and Ma and Marquis (1996). Furthermore, Soni et al. (2001) concluded that parabens were likely to have multiple targets and biological effects and could be used as antimicrobial preservatives, particularly in cosmetic products. Our findings are in agreement with those results.

[MI/MCI] has been used extensively for more than twenty years as an antimicrobial in cosmetics (e.g. skin creams and shampoos). [MI/MCI] has broad-spectrum antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, yeasts and fungi, at very low concentrations and over a broad pH range (Law and al., 1984). Our study showed the effect of [MI/MCI] against *M. luteus* and *S. aureus* (Gram positive bacteria), *E. coli*, *P. aeruginosa* and *S. enterica* (Gram negative bacteria) and fungi (*A. brasiliensis* and *C. albicans*).

For the stability test at ambient conditions, all treated samples had the same characteristics as the non-treated one regarding foaming properties (data not shown). Consequently, the preservatives used do not affect the physical appearance of the baby shampoo.

The pH of shampoos has been shown to be important for improving the hair quality and minimizing eye irritation. The current trend to promote shampoos of lower pH is one of the ways to minimize damage to the hair. Mild acidity prevents swelling and promotes tightening of the scales, thereby inducing shine. All the shampoos were acid balanced and in the range from 5.5 to 5.9, which is near to the skin pH (data not shown). All the eleven formulations of shampoo showed similar foaming characteristics in distilled wa-

ter. All samples had significantly comparable foaming properties (Table 5). Therefore, the preservatives used did not affect the foam stability of the baby shampoo. Stability and acceptability of organoleptic properties (odor and color) of formulations during the storage period indicated that they were chemically and physically stable (data not shown).

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REFERENCES

- Acott, K.M., and Labuza, T.P. (1975) Inhibition of *Aspergillus niger* in an intermediate moisture food system. *J. Food Sci.*, **40**, 137-139.
- Aghel, N., Moghimipour, B., and Dana, R.A. (2007) Formulation of a herbal shampoo using total aponins of *acanthophyllum squarrosum*. *Iranian J. Pharm. Res.*, **6**, 167-172.
- Barel, A.O., Paye, M., and Maibach, H.I. (2001) *Handbook of Cosmetic Science and Technology*, pp. 423, 583-588, 773-775, Informa Healthcare, New York.
- Becks, V., and Lorenzoni, N. (1995) *Pseudomonas aeruginosa* outbreak in a neonatal intensive care unit: a possible link to contaminated hand lotion. *Am. J. Infect. Control.*, **23**, 396-398.
- Behravan, J., Bazzaz, F., and Malaekheh, P. (2005) Survey of bacteriological contamination of cosmetic creams in Iran. *Int. J. Dermatol.*, **44**, 482-485.
- Chapman, D.G. (1987) Preservatives available for use. In *Society for Applied Bacteriology Technical Series 22: Preservatives in the Food, Pharmaceutical and Environmental Industries* (Board, R.G., Allwood, M.C., and Banks, J.G., Eds.), Blackwell Scientific, Bedford, U.K.
- Collier, P.J., Ramsey, A., Waigh, R.D., Douglas, K.T., Austin, P., and Gilbert, P. (1990) Chemical reactivity of some isothiazolinone biocides. *J. Appl. Microbiol.*, **69**, 578.
- Cowen, R.A., and Steiger, B. (1976) Antimicrobial activity: a critical review of test methods of preservative efficacy. *J. Soc. Cosmet. Chem.*, **27**, 467.
- Doron, S., Friedman, M., Falach, M., Sadovnic, E., and Zvia, H. (2001) Antibacterial effect of parabens against planktonic and biofilm *Streptococcus sobrinus*. *Int. J. Antimicrob. Ag.*, **18**, 575-578.
- Durant, C., and Higdon, P. (1991) Methods for assessing antimicrobial activity. In *Society for Applied Bacteriology Technical Series 27: Mechanisms of Action of Chemical Biocides* (Denyer, S.P. and Hugo, W.B., Eds.), Blackwell Scientific, Bedford, U.K.
- FDA (Food and Drug Administration). (2001) Bacteriological analytical manual. (8th ed), Washington, D.C.
- Fox, C. (1988) Introduction to the Formulation of Shampoos. *Cosmet Toilet.*, **103**, 25-58.
- Geis, P.A. (2006) *Cosmetic microbiology: Apractical approach* (Second Edition), pp. 111-146, Taylor and

TABLE 5. Foam retention of baby shampoo formulations with preservatives

Sample No	Time (min)				
	1	2	3	4	5
1	165.3	163.3	162.0	161.4	159.3
2	155.2	154.2	152.3	150.8	148.2
3	140.1	136.8	134.4	132.5	130.2
4	143.9	142.5	141.3	140.2	139.7
5	156.8	155.0	150.5	146.4	143.0
6	160.9	156.9	153.7	154.2	155.9
7	179.4	177.7	176.3	175.5	173.5
8	170.0	169.3	168.7	166.4	164.4
9	180.9	179.1	178.0	176.5	175.5
10	185.6	184.3	182.3	180.0	179.6
11	188.7	186.6	184.0	182.2	180.0

- Francis Group, New York.
- ISO/WD 11930. (2008) Evaluation of the antimicrobial protection of a cosmetic product.
- Jacobs, G.P., and Sade, N. (1984) Hypoxic radiosensitization by the antimicrobial methyl paraben. *Int. J. Radiat. Oncol. Biol. Physiol.*, **10**, 1217-1220.
- Klein, K. (2004) Evaluating Foam. *Cosmetics and Toiletries Magazine.*, **119**, 32-35.
- Law, A.B., Moss, J.N., and Lashen, E.S. (1984) Kathon CG: a new single component, broad spectrum preservative for cosmetics and toiletries. In *Cosmetic and Drug Preservation: Principles and Practice* (Karaba, J.J., ed.), pp. 129-141, Marxel Decker, New York.
- Ma, Y., and Marquis, R.E. (1996) Irreversible paraben inhibition of glycolysis by *Streptococcus mutans* GS-5. *Lett. Appl. Microbiol.*, **23**, 329-333.
- Maddox, D.N. (1982) The role of *p*-hydroxybenzoates in modern cosmetics. *Cosmet. Toiletries.*, **97**, 85-88.
- Mainkar, A.R., and Jolly C.I. (2000) Evaluation of commercial herbal shampoos. *Int. J Cosmet. Sci.*, **22**, 385-391.
- O'Neill, J.J., and Mead, C.A. (1982) The parabens: bacterial adaptation and preservative capacity. *J. Soc. Cosmet. Chem.*, **33**, 75-84.
- Ravita, T.D., Tanner, R.S., Ahearn, D.G., Arms, E.L., and Crockett, P.W. (2009) Post-consumer use efficacies of preservatives in personal care and topical drug products: relationships to preservative category. *J. Ind. Microbiol. Biot.*, **36**, 35-38.
- Russell, A.D. (2003) Challenge testing: principles and practice. *Int. J. Cosmet. Sci.*, **25**, 147-153.
- SAS. (1990) SAS/STAT user's guide. Cary, NC, USA: Statistical Analysis System Institute, Inc.
- Soni, M.G., Burdock, G.A., Taylor, S.L., and Greenberg, N.A. (2001) Safety assessment of propylparaben. *Food Chem. Toxicol.*, **39**, 513-532.
- USP: United States Pharmacopeia. (1994) Antimicrobial preservatives efficacy, (Rockwell, MD), p. 1681.
- Zedan, H.H., and Serry, F.M. (1984) Metabolism of esters of *p*-hydroxybenzoic acid by a strain of *Pseudomonas aeruginosa*. *Egypt J. Microbiol.*, **19**, 41-54.