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Evaluation of Manuka Honey as an adjuvant antimicrobial preservative in a O/W emulsion

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Summary

Cosmetics and personal care products need to be protected against microorganism contamination to prevent microbial spoilage and consumers' health hazards. The use of parabens, the most common preservatives added to cosmetic products, is associated with allergies, and some studies suggested that they could cause hormone disruption; therefore, the need of efficient and safer alternatives to parabens is increasing. The present study investigates the preservative efficacy of Manuka honey, a New-Zealand honey well known for its pronounced antimicrobial activity, in a O/W emulsion, in comparison with the traditional preservative methylparaben. An emulsion containing 10% Manuka honey was tested against bacterial (Escherichia coli ATCC 8739, Staphylococcus aureus ATCC 6538, Pseudomonas aeruginosa ATCC 9027) and fungal strains (Candida albicans ATCC 10231, Aspergillus brasiliensis ATCC 16404) by a challenge test, a procedure in which the efficacy of a preservative system is challenged by contamination with specified bacterial and fungal strains, according to the European Pharmacopeia procedures. The unpreserved emulsion and the emulsion with 0.2% methylparaben were reference samples. Manuka honey showed a good ability to protect the emulsion from contamination with standard bacterial inocula; in particular, it has been proven more effective than methylparaben against Pseudomonas aeruginosa, and showed the same efficacy as methylparaben against E.coli. Conversely, there was no evidence of a protective effect activity of Manuka honey against fungal contamination in our experimental conditions. Manuka honey, if added to a formulation for its humectant and moisturizing activity, also exerts an antibacterial activity, and can therefore be considered as an effective candidate for alternative preservation systems intended for use in cosmetic and personal care products.

Riassunto

I conservanti devono essere presenti nei cosmetici per ridurre il rischio di contaminazioni microbiche, che potrebbero portare ad un deterioramento del prodotto e ad effetti negativi sulla salute dei consumatori. I parabeni, i conservanti antimicrobici per lungo tempo più frequentemente impiegati nei cosmetici, sono associati con manifestazioni allergiche ed alcuni studi, peraltro non confermati, li indicano come potenziali interferenti endocrini; benchè la loro sicurezza sia stata riconosciuta, i

consumatori si stanno sempre più orientando verso prodotti per la cura personale che non li contengano, e per questo motivo c'è una costante ricerca di conservanti alternativi ai parabeni che siano sicuri ed altrettanto efficaci.

Lo scopo del presente lavoro è stato quello di valutare l'efficacia conservante del miele di Manuka, un miele proveniente dalla Nuova Zelanda di cui è nota la spiccata attività antimicrobica, in una emulsione O/A, paragonandola con quella del tradizionale conservante metilparaben. A questo scopo un'emulsione contenente il 10% di miele di Manuka è stata sottoposta ad un challenge test, una procedura che consiste nel verificare l'efficacia di un conservante in una formulazione contaminata artificialmente con specifici ceppi di batteri (Escherichia coli ATCC 8739, Staphylococcus aureus ATCC 6538, Pseudomonas aeruginosa ATCC 9027) e miceti (Candida albicans ATCC 10231, Aspergillus brasiliensis ATCC 16404). Il miele di Manuka ha mostrato una buona efficacia nel proteggere l'emulsione dalla contaminazione batterica, risultando attivo quanto il metilparaben nei confronti di E.coli e addirittura più attivo nei confronti di P.aeruginosa. Nelle condizioni sperimentali adottate, invece, il miele di Manuka non si è rivelato utile nel proteggere la formulazione dalla contaminazione con i ceppi fungini. In base ai risultati ottenuti in questa indagine, si può concludere che il miele di Manuka, se aggiunto ad una formulazione topica per la sua azione umettante ed idratante, può anche esplicare un'apprezzabile azione antibatterica, e può quindi essere considerato come il possibile componente di sistemi preservanti alternativi destinati a cosmetici e prodotti per la cura personale.

INTRODUCTION

Cosmetic products do not need to be sterile, but they must be adequately protected from microbial contamination and spoilage. Microbial contamination of cosmetics can occur during manufacture processes or can be inadvertently caused by the consumer during use; microorganisms can be very versatile in their metabolism and can grow and reproduce inside the product. Microbial multiplication can cause product degradation, with visible growth, colour and odour changes and gas production; moreover, the presence of pathogenic and even nonpathogenic microorganisms in personal care products constitutes a threat to consumer safety, especially when cosmetic formulations are intended for use in areas of particular concern (e.g., the eye area) or when they are used by children or immunocompromised individuals (1). For these reasons, antimicrobial preservatives are needed in personal care product formulations to prevent both primary (during production) and secondary (after container opening) microbial contamination. The European Regulation (EC) N°1223/2009 on cosmetic products states that manufacturers can only use preservatives listed in the Annex V of the same Regulation (2). However, in recent years the safety of some of these preservatives has been called into question; several studies were published claiming a link between parabens and breast cancer (3-5), and, although these studies were subsequently rejected by a number of scientists and international health authorities (6-8), Regulation (EU) No 358/2014 recently amended Annex V of Regulation (EU) No 1223/2009 to prohibit the use of isopropylparaben, isobutylparaben, phenylparaben, benzylparaben and pentylparaben in cosmetics. Moreover, Regulations (EU) No 1003/2014 and 1004/2014 lowered the maximum concentration allowed for propylparaben and butylparaben, and prohibited their use in products for children. Because of the issues of traditional preservatives, the consumer perception of these ingredients is not very positive, and, as a consequence, cosmetic manufacturers are increasingly looking for alternative preservation systems.

There is a large number of compounds, included in skin care products for their beneficial effect, that may coincidentally contribute to the antimicrobial protection of cosmetic formulations; these compounds are not, strictly speaking, preservatives, since they are not listed in Annex V of the Regulation (EU) N° 1223/2009. The presence of these ingredients allows manufacturers to promote cosmetic and toiletry products with marketing claims such as “preservative free” or “contains no preservatives”, although these statements are sometimes considered misleading for consumers, and the term “self-preserving” would be more appropriate (9). Alternatives to traditional preservatives are, for example, multifunctional ingredients, approved for cosmetics without restrictions, which combine an antimicrobial action with other favourable functions (e.g., caprylyl glycol, phenethyl alcohol, ethyl hexylglycerin, pentylene glycol) (9). There are also a number of botanical extracts (grapefruit seeds, usnic acid, Japanese honeysuckle, rosemary) and essential oils (thyme, tea tree, neem seeds) with remarkable antimicrobial activity that can be used as a part of a preservative strategy of cosmetic formulations (10-11). A natural-based ingredient that can be brought to the attention of formulators is honey, used for centuries in ethnomedicine as a topical treatment of a wide range of burns and wounds, and currently revalued for its antiseptic and healing properties (12-13). These biological effects are essentially attributed to two factors: the presence in honey of hydrogen peroxide, which is produced by the bee-derived enzyme glucose oxidase, and hyperosmolarity, due to its very high sugar content (14). Processing with heat and filtration can

reduce peroxide-based antimicrobial activity of honey (15). Certain honey types present additional antimicrobial factors; in particular, Manuka honey, derived from Manuka tree (Leptospermum scoparium), a bush found throughout New Zealand, has been demonstrated to possess significant non-peroxide antimicrobial properties, attributed to its methylglyoxal (MGO) levels (16-18). On the other hand, honey is a cosmetic ingredient often present in personal care products, in which it is frequently present as a humectant and as a skin moisturizer (19-21). The purpose of this study was therefore to evaluate the preservative efficiency of Manuka honey in a simple O/W cream formulation and to compare it with methylparaben, chosen as a reference preservative.

MATERIALS & METHODS

Materials

Manuka honey with MGO® 400+ (certified to contain at least 400 mg/kg of methylglyoxal) was purchased via the Internet by Manuka Health Europe Ltd, Warrington, UK. Tryptone Soy Agar (TSA), Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), Sabouraud Dextrose Agar (SDA), Sabouraud Liquid Medium (SLM), Peptone Water and phosphate-buffered saline tablets (PBS, Dulbecco A, pH 7.3) were purchased from Oxoid-Thermofisher Scientific (Rodano, Italy). Methyl parahydroxybenzoate (methylparaben) and all other chemicals were obtained by Sigma-Aldrich, Gallarate, Italy. Culture media, PBS and other solutions were prepared with MilliQ water. The test organisms used in this study were as follows: Escherichia coli (ATCC 8739) (Gram-negative bacillus), Staphylococcus aureus (ATCC 6538) (Gram-positive coccus), Pseudomonas aeruginosa (ATCC 9027) (Gram-negative bacillus), Candida albicans (ATCC 10231) (yeast), Aspergillus brasiliensis (niger) (ATCC 16404) (mold) (all purchased from Oxoid-Thermofisher Scientific, Rodano, Italy).

Cream formulation

The cream used in our experiments was "Macrogol cetostearileteres crema base (FUI XII)" (22), a white, odourless O/W hydrophilic base cream; it is for external application as an emollient and moisturizer, and for use as a diluent in medicinal external preparations. Cream emulsion formula was composed of two phases: a lipophilic phase, containing petroleum jelly (petrolatum) g 15, paraffinum liquidum (mineral oil) g 6, cetostearyl alcohol g 7.2, and a hydrophilic phase, containing Cetomacrogol 1000 (polyethylene glycol hexadecyl ether) g 1.8, purified water g 70.

To obtain the cream the components of the lipophilic phase were molten together at 70°C; separately, cetostearyl alcohol was dissolved in distilled, freshly boiled water cooled at 80°C. The aqueous phase was added to the oil phase and the mixture emulsified by using a mixer homogenizer (DS3 MultiGel, Sambuca-Tavarnelle, Firenze, Italy), stirring until it congealed. To prepare the cream containing methylparaben, this preservative was added at 0.2% to the boiling water; to prepare the cream containing Manuka honey (10%), honey was simply added to the aqueous phase (water amount was reduced to 60 g). The addition of 10% honey did not significantly affect the viscosity of the emulsion (data not shown). The concentrations of Manuka honey and methylparaben to be added to the creams were decided on the basis of the results of MIC evaluation (see next paragraph).

Determination of antimicrobial activity of Manuka honey and methylparaben

Preliminarily, antimicrobial activity of Manuka honey and methylparaben against our microbial

strains was determined as Minimum Inhibitory Concentration (MIC) by using an agar dilution test (23). Mother solution of Manuka honey was prepared by dissolving honey in sterile water to obtain a 50% wt/vol solution, which was sterilised by filtration; mother solution of methylparaben was obtained by dissolving it in DMSO at 5% wt/vol. Petri plates (5 mm diameter) containing increasing concentrations of Manuka honey (2.5%, 5%, 7.5%, 10%, 12.5% wt/vol) or methylparaben (two-fold serial concentrations from 0.025% to 0.2% wt/vol) were prepared by mixing appropriate volumes of mother solutions and molten (45°C) agar media (Mueller Hinton Agar for bacteria and Sabouraud Dextrose Agar for fungi; total volumes 10 mL). After solidification of the medium, the agar surface was inoculated with 2 μL of microbial suspensions containing about 10^4 c.f.u.; plates were inverted and incubated at 30-35°C for 18-24 hours (for bacteria), at 20-25°C for 48 hours (for Candida) and at 20-25°C for a week (for Aspergillus). After incubation, plates were visually checked for bacterial growth, and MICs were defined as the lowest concentrations at which no growth was observed. All test were conducted at least in triplicate; at the concentrations tested, DMSO had no inhibitory effect on microorganisms' growth. Results are reported in Table I.

TABLE I

Minimum inhibitory concentration (MIC) of Manuka honey and methylparaben against selected microbial strains.

	Manuka honey (wt/vol)	**Methyl paraben (wt/vol)**
Escherichia coli ATCC 8739	10%	0.2%
Pseudomonas aeruginosa ATCC 9027	>12.5%	0.2%
Staphylococcus aureus ATCC 6538	10%	0.2%
Candida albicans ATCC 10231	>12.5%	0.05%
Aspergillus niger ATCC	>12.5%	0.1%

Challenge test

In agreement with European Regulation, the microbiological stability of a cosmetic must be evaluated by a challenge test and its results must be included in the Cosmetic Product Safety Report; however, Regulation does not specify the protocol for this test. Among several protocols established to verify the antimicrobial protection of cosmetics (24), we decided to apply the challenge test described in European Pharmacopoeia 7 (25).

Briefly, the test consisted of artificially contaminating the preparations, in the final container, with a standard inoculum of suitable microorganisms. Inoculated formulations are stored at a prescribed temperature, and samples are withdrawn from the containers at specified inter-

vals of time, and surviving microorganisms counted on agar plates. Test was carried out on creams without any preservative, creams containing appropriate Manuka honey concentrations and, for comparison, creams containing methylparaben, a conventional pharmaceutical preservative widely used in cosmetics (26).

To prepare microorganisms inocula, bacterial strains were grown at 30-35°C for 18-24 hours on TSA plates, C. albicans at 20-25°C for 48 hours on SDA plates, and A. brasiliensis at 20-25°C for a week on SDA plates. To harvest the bacterial cultures, we used sterile saline solution, obtaining suspensions whose turbidity was adjusted to McFarland standard N° 4; these suspensions were further diluted 1:10 with saline to reduce the microbial counts to about $1x10^8$ microorganisms per milliliter. 400 μL of these final suspensions were added and thoroughly mixed to the formulation samples (50 g), contained in sterile plastic containers with screw cap, obtaining an inoculum of 10^5 to 10^6 microorganisms per gram of preparation. C. albicans inocula were prepared in the same way, but Candida number was determined performing a manual counting with a Nageotte chamber. Finally, to harvest A. brasiliensis spores, we used sterile saline containing 0.5 g/L of polysorbate 80 (Tween 80), and the spore enumeration was performed with a Nageotte chamber. Also in the case of Candida and Aspergillus the final inoculum was of 10^5 to 10^6 microorganisms per gram of the preparation.

Immediately after inoculation, 1 g of each cream was removed in sterile conditions and transferred into a sterile becher with a magnetic stirring bar, brought to a volume of 10 mL with Peptone Water and mixed on a magnetic stirrer at room temperature until a homogeneous suspension was obtained, and tenfold dilution were done in sterile saline. Any residual antimicrobial activity of the product was eliminated by dilution + the use of 0.5% Tween 80 (27). Triplicate plating of each dilution was performed by using appropriate media (Mueller Hinton Agar for bacteria, Sabouraud Dextrose Agar for Candida and Aspergillus) (zero hour count) and plates incubated at 37°C for 24 hours for bacteria and Candida and at 25°C for a week for Aspergillus. After incubation, the count of colony forming units (C.F.U.) per plate was counted and the number of surviving microorganisms per gram of tested cream was determined. After the first count, the inoculated formulations were maintained at 20-25°C protected from light, and, at appropriate time intervals, subjected to the enumeration of viable microorganisms as described above. Results are reported in Figures 1-5.

RESULTS

Antimicrobial activity of Manuka honey and methylparaben

Table I shows that, in our experimental conditions, Manuka honey is able to inhibit the growth of Gram-negative bacteria E.coli and S.aureus at a concentration of 10% wt/vol (concentration corresponding to 0.04% MGO, based on manufacturer's declarations); the growth of P. aeruginosa, C. albicans e A. brasiliensis was not inhibited at the highest honey concentration tested (12.5% wt/vol). Concentrations above 12.5% were not tested because the addition to the media of such amounts of honey made the agar excessively soft. MIC values obtained in our experimental conditions for Manuka honey tested are consistent with those reported by other Authors (28).

Methylparaben showed inhibitory activity against bacterial strains tested at 0.2% wt/vol and against A. brasiliensis at 0.1% wt/vol; it resulted more active against C. albicans (MIC 0.05% wt/vol). These values these values are in agreement with those reported in the literature (29).

Results of Challenge Test

A concentration of Manuka honey of 10%, although not inhibitory against all the strain tested, was added to creams, because higher quantities modify rheological properties of the formulation, making it too runny.

The challenge test is considered to pass the European Pharmacopoeia method if the following criteria (preparations for cutaneous application) are fulfilled: bacterial reduction = 99% (2 Log) reduction 2 days after inoculation and >99.9% (3 Log) reduction 7 days after inoculation; and fungal reduction = 90.0% (1 Log) reduction 7 days after inoculation and 99% (2 Log) reduction 14 days after inoculation.

The population of P. aeruginosa was effectively controlled by 10% Manuka honey, since bacteria number was reduced more than 99,9% (3 log) 7 days after inoculation and no longer increased; however, the test would have not fulfil the criteria fixed by the European Pharmacopoeia method, because bacteria count 2 days after inoculation was not reduced by 99% (2 log) (Fig. 1).

Anyhow it is noteworthy that 0.2% methylparaben in the same experimental conditions was not able to inhibit Pseudomonas, whose growth was comparable to the control for the whole duration of the experiment. Interestingly, honey concentration able to protect the emulsion against P. aeruginosa was not able to inhibit the growth of the same bacterium in the test for M.I.C evaluation. Manuka honey was also able to inactivate the inocula of E. coli at 7 days, with a reduction < 2 log after 2 days; similar inhibition was obtained with methylparaben (Fig. 2). Unpreserved formulations supported the growth of both Gram-negative bacteria during the test period (Fig. 1 and 2). Manuka honey and methylparaben exhibited similar inhibitory activity against S.aureus, as in both cases bacteria inocula were totally inactivated in 7 days; it is interesting that in the unpreserved formulation viable count of S.aureus decreased spontaneously, even though more slowly than in the preserved creams (Fig. 3). Finally, no appreciable protective activity against Candida and Aspergillus was found in our experimental conditions (Fig. 4 and 5).

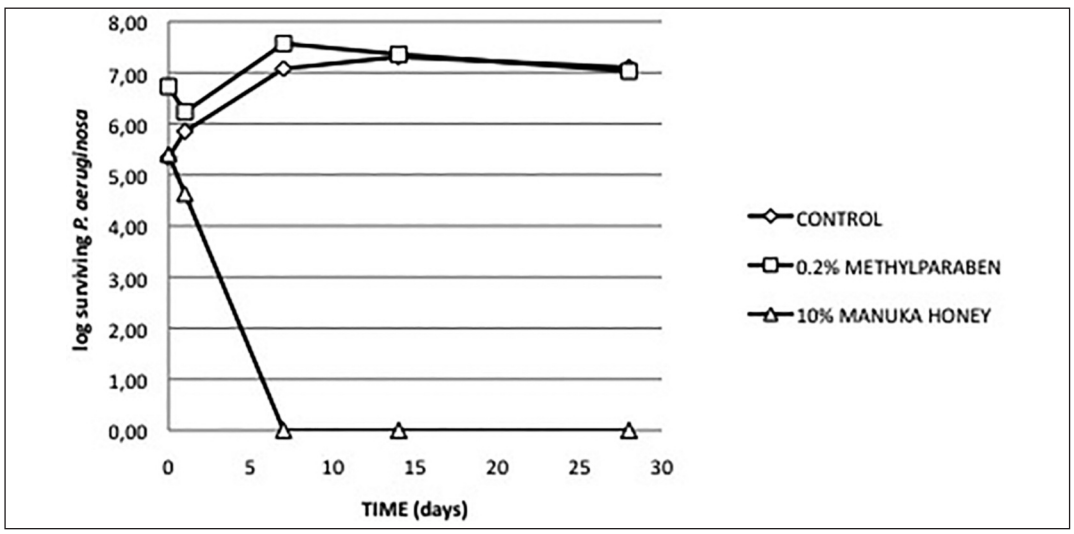

Fig. 1 *Survival of Pseudomonas aeruginosa ATCC 9027 in unpreserved emulsion and in emulsions containing 10% Manuka honey or 0.2% methylparaben. The results are mean of three experiments.*

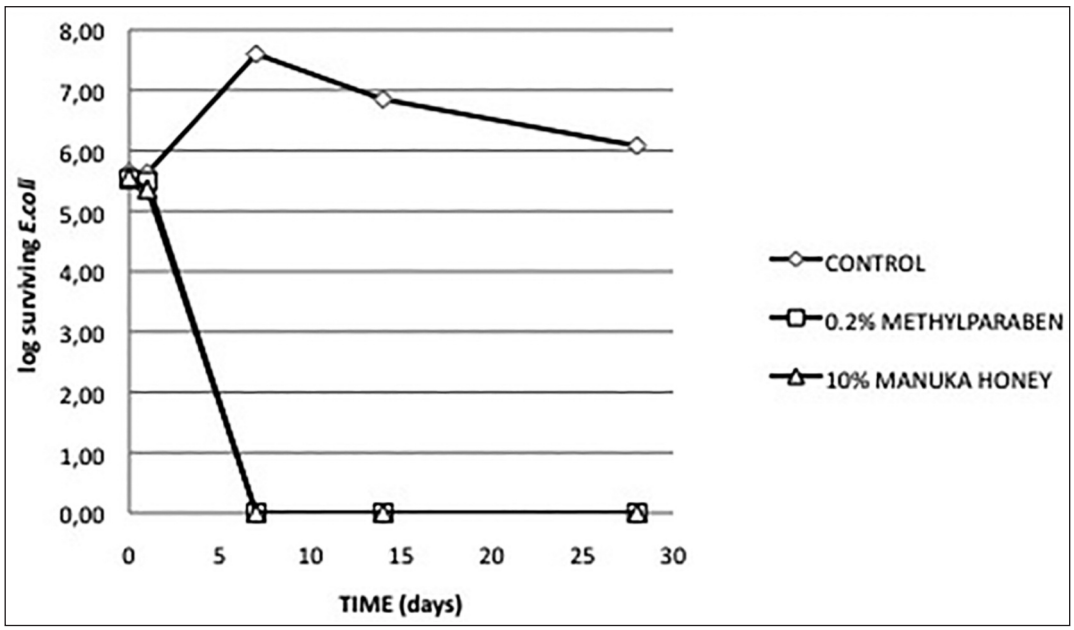

Fig. 2 *Survival of Escherichia coli ATCC 8739 in unpreserved emulsion and in emulsions containing 10% Manuka honey or 0.2% methylparaben. The results are mean of three experiments.*

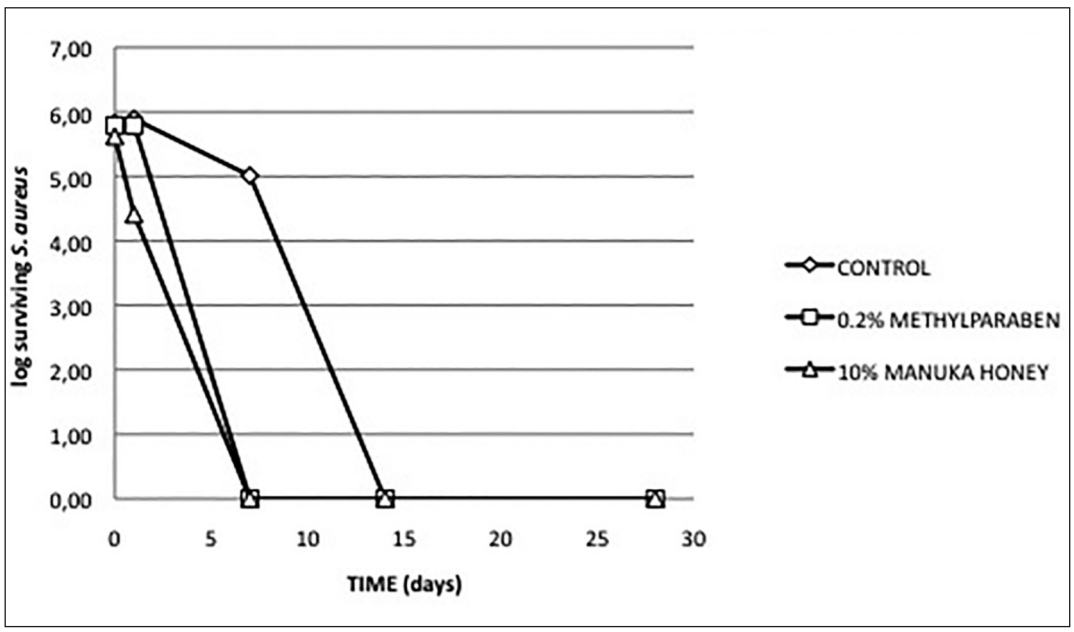

Fig. 3 *Survival of Staphylococcus aureus ATCC 6538 in unpreserved emulsion and in emulsions containing 10% Manuka honey or 0.2% methylparaben. The results are mean of three experiments.*

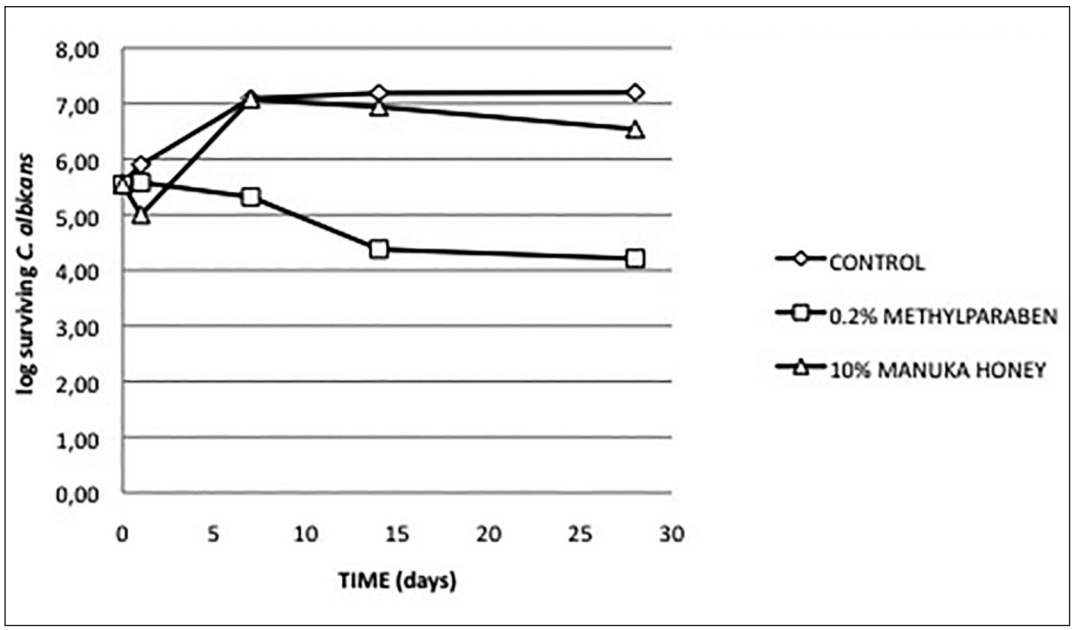

Fig. 4 *Survival of Candida albicans ATCC 10231 in unpreserved emulsion and in emulsions containing 10% Manuka honey or 0.2% methylparaben. The results are mean of three experiments.*

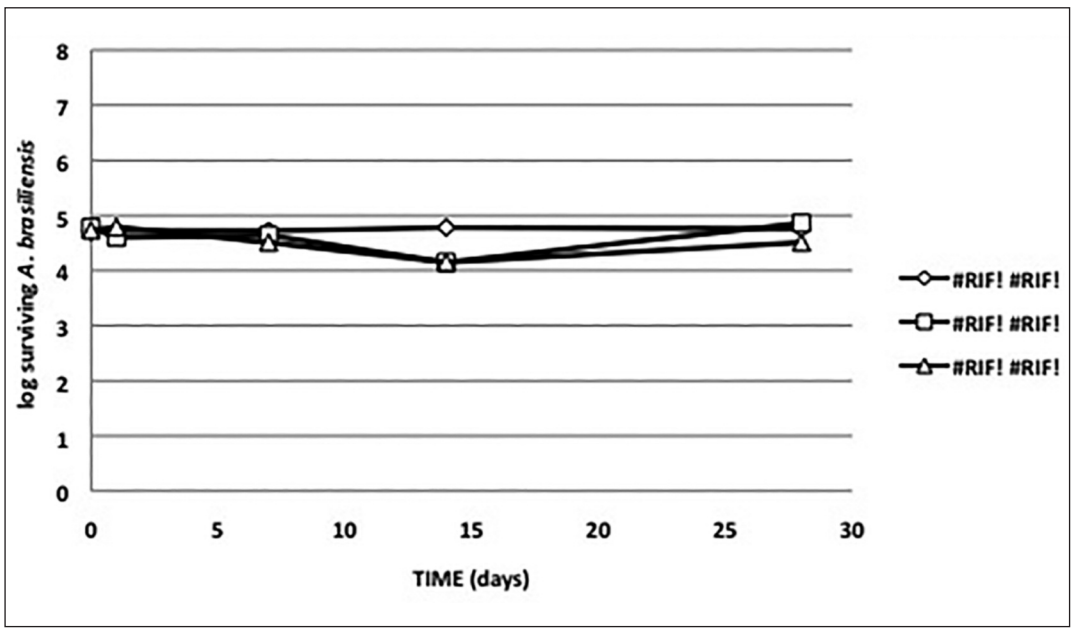

Fig. 5 *Survival of Aspergillus brasiliensis ATCC 16404 in unpreserved emulsion and in emulsions containing 10% Manuka honey or 0.2% methylparaben. The results are mean of three experiments.*

DISCUSSION

Particularly in leave-on cosmetic products, a blend of parabens is a standard preservative option; however, due to ongoing discussions regarding the safety of these compounds, cosmetic formulators are increasingly facing with the need to find reliable alternatives, possibly of natural origin, to protect microbiological quality of products during their use and storage.

The challenge tests performed on the cream preserved with 10% Manuka honey demonstrated that this product is able to protect the formulation against standard inocula of Gram-negative bacteria P. aeruginosa and E. coli equally or even better than methylparaben, although it does not fully meet the requirements of European Pharmacopoeia; a similar inhibition was obtained against S. aureus, while there was no evidence of an antifungal activity in our experimental conditions.

The antimicrobial activity of Manuka honey and methylparaben observed in the challenge test was substantially different from that found in *in vitro* conditions; in particular, although MIC of Manuka honey was >12,5%, a concentration of 10% in the emulsion has proven to be effective in reducing microbial load of 3 log after 7 days. These findings corroborate the results reported in previous papers (30-31) and confirm that physicochemical interaction with the ingredients of cosmetic formulations can enhance or reduce the efficacy of an antimicrobial preservative. Indeed parabens are effective against bacteria and fungi in standard microbiological tests (29), but when they are introduced in complex multi-phase products, such as creams or lotions, an appropriate antimicrobial efficacy may not be achievable, because they partition into the oil phase of the emulsions, requiring higher concentrations to remain active (32).

For this reason, the addition to cosmetic formulations of appropriate concentrations of Manuka honey (primarily intended as a moisturizer), featuring a documented antimicrobial activity and a remarkable water solubility, could help preserve cosmetics and consequently could allow to reduce the levels of traditional preservatives needed to avoid microbial spoilage, especially of cosmetics with high water content.

The use of a natural product as an ingredient of cosmetic formulations could pose some concerns related to microbial contamination and the reproducibility of its chemical composition. However, honey sample used in our experiments did not reveal any detectable microbial contamination when subjected to total viable counts (data not shown); in any case, even if a sterilization should be requested, some Authors have demonstrated that there was no significant changes in Manuka honey antibacterial activity when it was subjected to a commercial sterilization procedure using gamma-irradiation (33). As far as eventual variations in composition are concerned, it is possible to standardise accurately Manuka honeys as of their physicochemical and antimicrobial properties (34).

In conclusion, our results suggest that Manuka honey can be considered as a cosmetic ingredient, primarily usable for its humectant and skin conditioning effects, that presents the additional benefit of an antibacterial activity; it could be added to selected cosmetic formulations to reduce the concentration of conventional preservatives required, or as a component of alternative preservative combinations.

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