Study on the protective effect of an innovative cow milk-based product against some human skin-bacterial pathogens

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Abstract

There is a growing interest related to the possible use of some components of cow milk that behave as immunomodulators, exert biological activity, and have anti-inflammatory factors. The aims of this study were: i) to investigate the chemical composition and antioxidant activity of Podolian cow milk; ii) to test the efficacy of some hand soaps supplemented with different percentage of cow milk compared to placebo; iii) to evaluate the antibacterial activity of milk added to soap. Antioxidant activity of Podolian milk resulted in mean 97.03% and 52.09% utilizing 2,2'-azinobis 3-ethylbenzthiazoline-6-acid and 2,2-diphenyl-1-picrylhydrazyl methods, respectively. The efficacy test showed that the most effective soap was obtained adding 5% of Podolian milk. For this soap, total counting bacterial test evidenced the highest reduction of bacterial hand contamination assessed by 98%.

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Contributions: CC suggested the research idea, analyzed and interpreted the out findings. HSE, CDA, GP, MM and RP carried out the sensorial test. CL carried out the HPLC and other chemical analysis. HSE performed the antibacterial test, wrote the manuscript and carried out the statistical analysis. IC and PF contributed in writing the manuscript.

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Introduction

A possible alternative employment of the Podolian milk could be an ingredient for the formulation of natural cosmetics, because milk of animals raised on mountain natural grazing does not contain residues of herbicides and chemical fertilizers. Cow's milk is a natural tensor, rich in vitamins, minerals, antioxidants, proteins, enzymes and lipids, that well promotes the epidermis protection and regeneration.[1]

In fact, cow milk components have a strong absorption capacity and water retention, promoting hydration of the skin, and preventing the degradation of the epidermal cells.[2,3] Moreover, it contains several antimicrobial factors which exert both specific and non-specific bacteriostatic and bactericidal activity which contribute to the protection against infectious diseases. Nowadays, many companies are interesting in isolating such specific substances from cow milk for applying in pharmaceutical, cosmetics and food industry.[4] Thanks to these properties, in the cosmetic market there are several products made from milk of different species whose preparations (face and body creams, cleansing milk, and tonic) are particularly appreciated by consumers called Lifestyle of Health and Sustainability.

Some studies have shown that lysozyme in milk can be successfully employed for controlling the growth of many bacterial species.[5-7] Lysozyme was first described in 1922 by Alexander Fleming as a bacteriolytic protein and a non-specific immunoprotective factor, whose powerful antibacterial activity is due to its capacity to catalyze the hydrolysis of the β (1-4) glycosidic links between *N*-acetylmuramic acid and *N*-acetylglucosamine in the bacterial cell wall polysaccharides.

Casein (or caseinate salt) is used as surface-active agent in soaps and in various cosmetics such as cold lotions, hair sprays and hand creams.[8] Casein hydrolysates could also be employed for skin hydration.[9] However, little is known about these applications and their markets.[1]

The abundance of xanthine oxidase (XO) in milk was assessed recently for its physiological and antimicrobial role. The purified XO was able to inhibit bacterial growth in the presence of a reducing substrate. In fact, Hancock *et al.*[10] reported the potential efficacy of cow milk on the metabolic activity of *Escherichia coli* under low conditions of oxygen. This oxygen concentration, known

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to be optimal for the generation of peroxynitrite by XO,[11,12] had no significant effect on bacterial viability.

The bibliographic research states no standard methodology to assess the effectiveness of liquid detergent soaps for hand hygiene. For these reasons, in this study we were first undertaken to prepare an effectiveness test for the evaluation of soaps made by standardizing the soiling with specific products of targets. Despite the increasing use of cow milk, few studies have been conducted on its antibacterial properties and perceived quality in a hand soap. The aim of the present work was to evaluate the perception of untrained consumers towards sensory aspects of liquid hand soap made with different percentage of milk obtained from Podolian cows. The soap was made for handwork targets with high exfoliating power.

Materials and Methods

Animals

The research was carried out on Podolian cows reared in a farm situated in Basilicata at an altitude of about 800 m a.s.l. Bulk milk derived from cows aged between 7 and 10 years, in the middle stage of lactation, and reared in free range management.

Chemical analysis of milk

After collection, 3 milk aliquots were immediately refrigerated at +4°C and transported to the laboratory for analysis. The content of protein, fat, lactose was determined by Milkoscan FT 6000 (ISO 9622:2013).[11] All measurements were carried out in triplicate.

High performance liquid chromatography fractionation of lysozyme

Lysozyme quantity was determined by High performance liquid chromatography using a reversed-phase column. Sample milk preparation, column equilibration and elution were performed by modified method of Pellegrino and Tirelli[12] and Cosentino *et al.*[13] The chromatographic separations were run on a Synergi MAX-RP 80 Å column (150×4.6 mm, 4 µm particle size) from Phenomenex (Torrance,CA, USA) with a MAX-RP guard column (4 mm×2 mm id). Injection volume was 20 µL and flow rate was 0.8 mL/min. The mobile phase consisted of a gradient of water (A) and acetonitrile (B) both containing 0.1% trifluoroacetic acid (v/v). Eluting conditions are: 0 min 80% A and 20% B; 9 min 60% A and 40% B; 15 min 60% A and 40% B; 20 min 80% A and 20% B. Detection was carried out by fluorescence detector (Jasco FP-2020 Plus-Intelligent-fluorescence detector) set at 280 nm excitation and 350 nm emission. Calibration curves were acquired with known amounts of HEW lysozyme in the concentration range of 5 to 100 mg/L. All measurements were done in triplicate.

Antioxidant activity

Antiradical activity was evaluated by using both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzthiazo line-6-acid) (ABTS) assayes.[14]

DPPH assay: The stock radical solution of DPPH was prepared by dissolving 20 mg of DPPH in 15 mL of ethanol. After 1 minute of agitation with Vortex, 1 ml of stock DPPH solution was diluted in ethanol (1:30). 50 µL of milk were added to 950 µL of DPPH solution and incubated into the darkness for 30 minutes at room temperature. After centrifuging (5 min, 8000 rpm), absorbance was measured at 515 nm against the reference solvent (ethanol) using spectrophotometer UV-Vis (LKB Biochrom 4050 Ultrospec II). Experiments were carried out in triplicate.

ABTS assay: The stock solution of the ABTS radical was prepared by dissolving 38 mg of ABTS in 10 ml of an aqueous sodium persulphate solution (2.45 mM). The mixture was dark stored for 12-16 hours. For the analysis, 1 ml of stock $ABTS^{\cdot+}$ solution was diluted in ethanol (1:30). 20 µL of milk sample was added to 980 µL of $ABTS^{\cdot+}$ solution. Milk samples were reacted with $ABTS^{\bullet+}$ working solution for 2 h in incubation into the darkness at room temperature. After centrifuging (5 min, 8000 rpm), absorbance was measured at 734 nm against the reference solvent (ethanol). The solutions were prepared fresh for the analysis and all determinations were carried out in triplicate.

Calculation: Antioxidant activity of cow milk was evaluated through the Radical Scavenging Activity (RSA%) utilizing the following formula: RSA%=(1−Ai/ A0)×100%, where Ai is the absorbance of sample and A0 is the absorbance of colorimetric radical substance.

Hand soap preparation

Hand soaps, supplemented with different percentage of milk, were prepared by specialized and certified laboratory (Ecocosmesicreativa, Italy). The liquid soap was prepared identifying handwork target audience (mechanical, butcher, carpenter, *etc.*). Abrasive micro-granules obtained by crushing the shell of hazelnuts and almonds have been added in equal amounts to all prepared formulations in order to increase their degreasing power. The surfactants used derived from corn starch and coconut oil. The fragrance was given by essential oils of orange and rosemary, able to eliminate bad odours while making the product a pleasant fragrance. Three liquid hand soaps were realized with same formulation and with different addition of cow milk: A) Podolian milk 25%; B) Placebo; C) Podolian milk 5%. All hand soaps were prepared and packaged in 100 mL white containers with dispenser.

The basic formula, according to International Nomenclature of Cosmetic Ingredients, is the following: water, Sodium lauroyl sarcosinate, Cocamidopropyl betaine, Acrylates copolymer, Lauryl glucoside, Lac, *Citrus autantium* dulcis oil, *Prunus amygdalus* dulcis shell and *Corylus avellana* shell, Glycerin, Benzyl alcohol, *Rosmarinus officinalis* oil, Sodium benzoate. All soaps of amber colour appear as homogeneous gel with microgranules. The pH value is 6.40.

Hand soap sensorial test

A semi-structured questionnaire consisting of 5 questions (Table 1) was administered to 8 women and 7 men, regular consumers of liquid soap recruited from our University. Their ages ranged between 22 to 54 years. The data collection phase was carried out simultaneously to contamination and individual washes, as described below, occurred at a distance of a minimum of 5 hours one from the other and with a maximum of 2 daily washes. Soiling and washing were performed to each tester in the same environment and conditions in order to guarantee equivalence of stimuli and to not stress the skin.

Evaluation of cleaning hands

For evaluating the effectiveness in removing dirt of tested soaps, the following soiling categories were utilized: mechanical (engine oil, grease workshop, used engine parts); carpenter (glue, plaster paste, water-based paint); housewife (garden soil, extra virgin olive oil, margarine); baker (bread dough, charcoal, butter) and butcher (animal fat, minced meat, suet).

Soiling procedure

Contamination and hand movements were made by all the testers providing precise instructions for 20 seconds for all the above mentioned soiling products.

Handwashing procedure

The individual washing took place with a soap standard amount (2.5 mL) provided with pressure dispenser. The washing was performed according to times and cleaning movements of hands recommended by Hygiene guidelines for hand drawn health care from the World Health Organization, 2006 - WHO.

Antibacterial efficacy of prefered hand soap

The preferred soap selected from the sensorial test was subjected to antibacterial efficacy test. This trial was carried out on the same testers. For the test, sterile water was utilized and conserved at room temperature 20±2°C and hand washing procedures respected methodology of WHO. The bacterial sampling was performed before and after hand washing using sterile cotton swab; dipping in sterilized tube containing 10 mL of sterile distilled water and subsequent decimal dilutions ranged from 10^{-1} until 10^{-3}. One hundred µL from each dilution was inoculated on Plate Count Agar media[15] following the scheme of preparation of ISO 2293: (1988).[16] The plates were cultured manually and instrumentally using Spiral Biotech Auto-plate (Advanced Instruments, Inc., Norwood, MA). The plates were incubated at 30±2°C and the bacterial enumeration was evaluated after 72-96 hours for both manual and instrumental method using stereoscopic microscope and DC-count, respectively. The antibacterial efficacy of the preferred hand soap was determined according to the following equation as a medium values among testers:

[T.BW – T.AW]/T.AW X 100

Where T.BW: the total bacterial colonies CFU/mL before hand-washing; T.AW: the total bacterial colonies CFU/mL after hand-washing.

Statistical analysis

Data were analyzed by a one way ANOVA. Differences were tested by Tukey *post-hoc* test with a probability of $P<0.05$ using *SPSS* statistical software package version 13.0 (2004).

Results and Discussion

Physico-chemical characteristics of podolian milk

Table 2 lists the physic-chemical characteristics of Podolian milk employed the liquid soap of this work. It is noted that it is rich in protein, fat and lactose giving the soap more nutrients properties for the skin. Moreover, the antioxidant activity is very high for both ABTS and DPPH tests. In fact, Butler *et al*.[17] showed that milk produced by grazing bovine has greater antioxidant capacity than that one produced by cows kept indoor. The greatest antioxidant activity of a cosmetic product provides to the skin greater benefits such as increased elasticity and protection against UVA.[18] The lysozyme is an important enzyme with antibacterial properties.[19] The cow milk has a trace content of lysozyme (generally <0.6 ppm) according to Claeys *et al*.[20] and its content varies depending on the lactation period; at the beginning of that period its quantity is higher.[21] Kuczyńska *et al*.[22] found that the cow milk kept on the pasture was characterized by a higher content of proteins, in comparison to the cows that have been reared indoor.

Hand soap sensorial test

Regarding the total preference (popularity scale of 1 to 10), the obtained results assigned the highest score (7.15) to soap C made adding 5% of milk, followed the soap A (6.37), made by 25%. The lowest scoring was observed in the Placebo soap B (5.95).

The following results (Figure 1) within the testers category showed that soap C, resulted medium in terms of exfoliating power for all categories except for baker profile where it resulted strong. The skin cleaning and skin hydration resulted elevated in all categories, reaching in both parameters analyzed, the maximum preference in the carpenter profile. The soap perfume, understood as the smell that the soap leaves on hands after washing, was considered pleasant except for butchery where it is indicated as significantly not so pleasant.

Total counting bacteria of hand pre and after washing

The total counting bacteria was carried out for examining the antibacterial efficacy of the more appreciated soap (D) selected by the testers. The obtained results of total counting bacteria (CFU/mL)

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Table 1. Questionnaire scheme.

Exfoliating power	Low		Medium			Strong		Too much	
Skin cleaning	Poor		Enough			Good		Very good	
Skin perception	Dried		Sufficiently hydrated			Hydrated		Very hydrated	
Perfume	Unpleasant		Not so pleasant			Pleasant		Very pleasant	
Overall evaluation	1	2	3	4	5	6	7	8	9	10

Table 2. Podolian cow milk: chemical composition and Radical Scavenging Activity, RSA (x±S.D.).

Protein g/100 g	Fatg/100g	Lactose g/100g	Lysozyme mg/L	RSA% (DPPH)	RSA% (ABTS)
3.60	3.96	5.11	0.25	52.09	97.03
±0.06	±0.05	±0.04	±0.04	±3.40	±1.06

DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azinobis (3-ethylbenzthiazoline-6-acid).

of hand pre and after washing evidenced the highly significant reduction (P<0.01) of bacterial colonies after washing (Figure 2). In particular, the percentage of bacterial growth inhibition was assessed by 96.62% and 98.76 % for manual and instrumental method, respectively. Cow milk may exert its antibacterial activity against a high number of bacteria by catalyzing the hydrolysis of b1-4 glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine in the bacterial cell walls polysaccharides. Furthermore, the milk may limit the migration of neutrophils into a damaged tissue and therefore might function as an anti-inflammatory agent and also may work synergistically with lactoferrin and immunoglobulins in antimicrobial functions.[23]

The antibacterial activity of bovine milk might be derived from some protein and enzymes found in milk such as, lysozyme, lactoferrin and lactoperoxidase.[24,25]

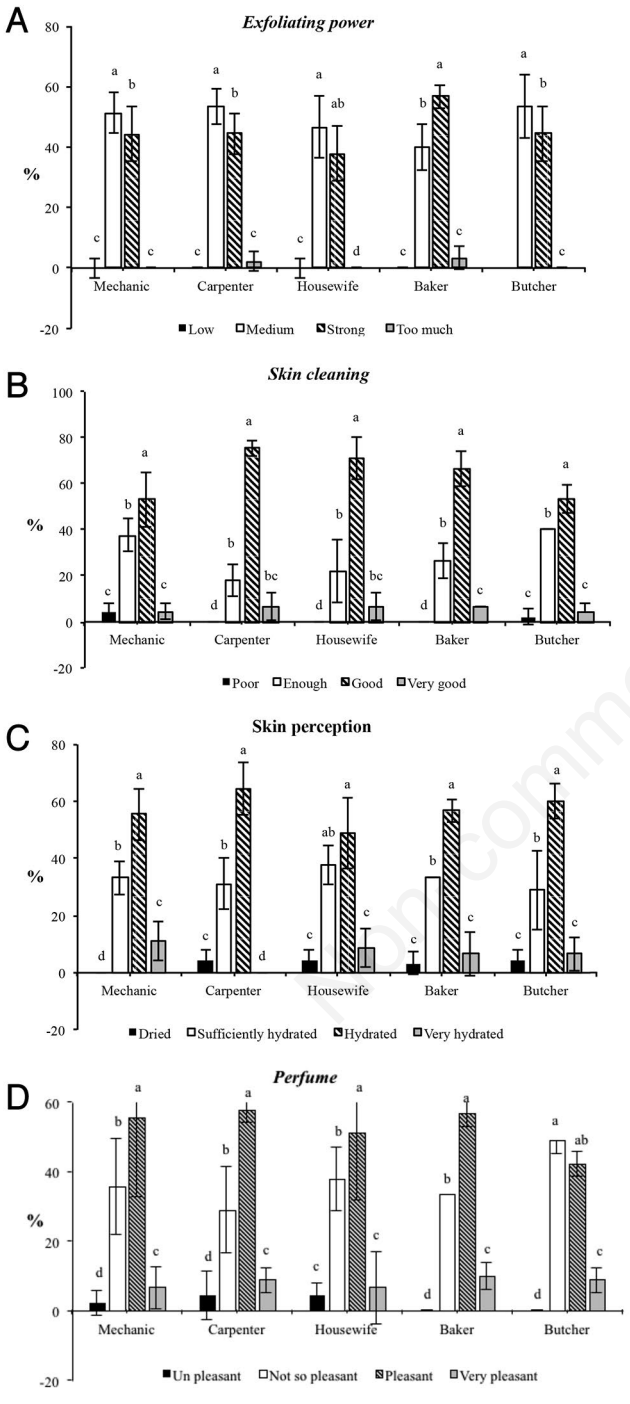

Figure 1. Sensorial test of soap D for A) Exfoliating power, B) Skin cleaning, C) Skin perception and D) Perfume. Bars with different letters for each category indicate means values significantly different at P<0.05 according to Waller Duncan test. Data are expressed as mean of three replicates ±SDs.

Conclusions

Cosmetics industry, in recent years, has not experienced moments of crisis as it instead happened in other economic and commercial sectors. In fact, the analysis of the industry literature shows a steady increase characterized by a positive trend, not only at national level but also European level. For this reason, we chose to use Podolian milk as a raw material for a skincare product. This research has highlighted a possible alternative use of the milk produced by grazing animals. The Podolian milk in particular has proved to be a good basis for the realization of a liquid soap. The placing of a new liquid soap hands in the cosmetic industry would allow to small breeders of Podolian cattle to improve their incomes. This could counter the current and widespread abandonment of livestock activities, which are involving a serious threat both economically and socially due to the depopulation of rural areas. The obtained data showed that the product realized with Podolian milk guarantee excellent results in terms of the respect of the skin in combination with a good cleansing and antibacterial efficacy in the professional categories identified as targets of consumers. In conclusion, the cosmetics industry, given the propensity for product innovation, may represent a new area of interest for quality productions of animal husbandry.

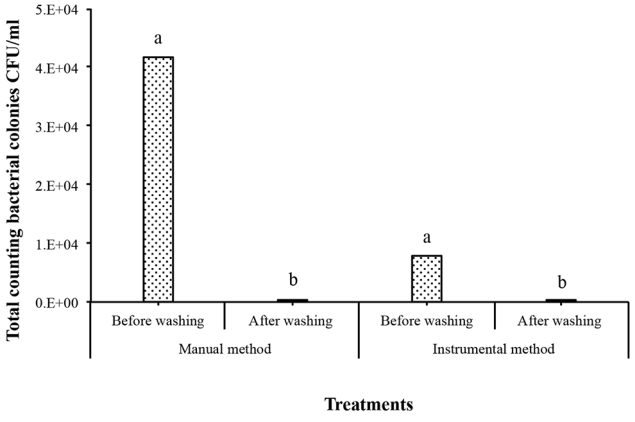

Figure 2. Total counting bacterial colonies before and after hand washing by soap. Bars with different letters for each method indicate means values significantly different at P<0.01 according to Tukey test. Data are expressed as mean of three replicates ±SDs.

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