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Postbiotics Cosmetic Formulation: *In Vitro* Efficacy Studies on a Microbiome Friendly Antiperspirant

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ABSTRACT: Antiperspirants are used many times a day to prevent bad odor in the armpit. Bad odor under the armpit is called organic volatile molecules. It is formed by the metabolism or breakdown of sweat secreted from sweat glands by microorganisms in the axillary region. Antiperspirants prevent sweat secretion by blocking the sweat glands thanks to the active ingredients in the antiperspirants and prevent the formation of bad odor by stopping the bacteria from metabolizing the secretions. These active substances are still used today as aluminum and its salts. Although the damage of aluminum salts on the skin is not fully known, their accumulation in the body causes toxicity. The search for new active substances to eliminate the negative health effects of the active substances used is still continuing today. The purpose of this research is to produce formulations containing active cosmetic ingredients with natural components that can be used in new formulations. Moreover, it includes the evaluation of the efficacy of active substances that will provide inhibition of bacteria that cause bad odor in the axillary microbiota by adding them to formulations. In studies conducted by adding *Lactobacillius* lysates *(*Acilac ATA-LTW12- *Lactobacillus* ferment lysate extract*)* obtained from probiotics combined with *Humulus lupulus* extract, *Salvia_officinalis* leaf extract to the formulation, a decrease in microorganisms causing bad odor formation was observed. Microbiota differences between the formulation with and without postbiotics applied in an artificial axillary microbiota environment were observed. It was observed that the *Lactobacıllus ferment lysate extract* component used in the reduction of bad odor-causing microorganisms supported specific inhibition in the natural armpit microbiota and normalized the microbiota. There was no statistically significant difference between the negative control (PBS) and the antiperspirant without postbiotics. Significant statistical differences were observed between the antiperspirant containing postbiotic (Acilac ATA-LTW12- *Lactobacillus* ferment lysate extract) and the antiperspirant without postbiotic, and between the antiperspirant containing postbiotic and negative control. As a result, a new one has been added to the roll-on type antiperspirant formulation studies with natural ingredients free of aluminum salts, sustainable to nature, and presented to the literature for development.

KEYWORDS: Cosmetic; formulation; postbiotic; antiperspirant; axillary microbiota

1. INTRODUCTION

In many cultures and societies, unpleasant body odors that may occur as a result of excessive sweating may cause social embarrassment or negative perception of the individual [1]. Man is a homeotherm (warm-blooded) organism and tends to keep his internal temperature constant. When the body temperature increases or decreases, the body regulates thermoregulation by regulating some physiological events to reach the ideal temperature. Sweating is one of the physiological reactions of the body to lower its temperature. People are faced with sweating many times during the day. Sweating can occur after intense exercise, during excitement or stress, in the presence of a hot and humid environment [2]. These events can be uncomfortable for the person by causing hyperhidrosis, which leads to the formation of bad odor or wetting of clothes as a result of excessive sweating. Eccrine, apocrine and apoeccrine sweat glands are involved in sweat formation [3]. Eccrine glands provide thermoregulation. Sweat secreted by the eccrine glands is an aqueous solution of organic and inorganic ions and is usually odorless. The apocrine gland provides individual odor formation rather than thermoregulation. Sweat of apocrine origin contains components such as isovaleric acid, ammonia, lipids [4, 5]. Moisture generated by eccrine and apocrine sweat glands contributes to the proliferation of axillary

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microbiota[4]. Many bacterial species are present in the axillary flora and the bacterial species that cause bad odor are mainly *Corynebacterium spp*. and *Staphylococus spp*. [6, 7]. Many formulations have been developed and continue to be developed to prevent bad odor. Antiperspirants and deodorants are now used continuously to prevent underarm odor. In the USA, the commercial volume of antiperspirants and deodorants accounts for a significant proportion of all cosmetics and 90% of consumers use either antiperspirant or deodorant products every day [1]. Although antiperspirants and deodorants differ in their active ingredients and mechanisms, they are often confused by users and used interchangeably. Deodorants are formulations containing active ingredients with antiseptic, bacteriostatic or bacteriocidal activity that mask the bad odor caused by sweating or prevent the formation of bad odor [8]. Antiperspirants, on the other hand, reduce sweating by blocking the eccrine sweat glands thanks to the active substance and prevent the axillary microbiome from creating bad odor [3]. There are concerns about the safety of the active ingredients used in antiperspirants and deodorants. Aluminum salts are one of the leading active ingredients used in antiperspirants and are known to be the most common metal used in formulations of the 21st century [1]. Aluminum is a metal that can accumulate in the body and has a risk of toxicity. In many studies, aluminum exposure has been associated with cancer, environmental pollution, hormonal disorders and bone pain [8-11]. As stated in many studies, natural formulations need to be developed. It is necessary to obtain natural products by adding active ingredients prepared from natural products instead of chemical components used as active ingredients in antiperspirant and deodorant formulations. For this purpose, *humulus lupulus, salvia officinalis* add to the formulation to inhibit odor-causing bacteria in the armpit and to obtain antiperspirant efficacy naturally.

Sweating is one of the most powerful autonomic thermoregulatory events for the human body in which water loss through the skin occurs. In more general terms, eccrine or apoeccrine refers to the active secretion of secretions produced in sweat glands to the body surface [12]. The evaporation of sweat prevents the body from overheating and balances body fluids. A person loses fluid by sweating an average of 0.5-1.0 L per day. Some studies have shown that the sweat produced by the human body varies between 1.5 and 2.5 L/hour [12]. When playing sports or walking on a hot day, the amount of sweating can quadruple [13]. Most of the sweat produced is converted into heat by some physiological events and removed from the body. In addition to temperature regulation, sweating also removes waste substances and maintains skin pH [14]. Although the general composition of sweat is water, it contains many substances such as salts and urea. Human sweat glands have the ability to reabsorb the substances they secrete. Thus, sweat retains most of the salts necessary for secretion before evaporation. Without this protection mechanism, humans would lose a lot of sodium chloride salts. This salt conservation ability is enhanced in people living in hot environments. Sweat glands found on the entire surface of the body are called eccrine sweat glands. The larger sweat glands are called apocrine sweat glands and are found only in certain areas. There is a third type of sweat gland, called apoeccrine sweat glands, located in the armpits and lymph nodes [15].

Apocrine sweat glands are sweat glands that are present from birth but become active with hormonal transitions during puberty. Apocrine glands do not open directly to the skin but open to the hair ducts and the skin together with the sebaceous glands. Therefore, apocrine glands are limited only to the areas of hair growth on the body. In a study, the density of apocrine glands was examined and it was shown that there are approximately 40-50 apocrine glands within 1 cm^2 . The density of apocrine glands varies according to race and gender. Apocrine glands are limited to the genital and perineal region, breast and axillae. The apocrine gland produces the individual odor rather than thermoregulation [16].

There are three types of sweating in our body: emotional, thermal and sweet sweating. Thermal sweating involves a system in which eccrine glands, which are distributed throughout the body in hot conditions depending on the heat load, are used to lower the temperature. The activity of eccrine sweat glands is controlled by the hypothalamus, the center of thermoregulation, and the nervous system. Thermal sweating is influenced by many factors other than body temperature, such as gender, age, menstrual cycle [17].

Emotional sweating is a physical response that can occur throughout the body under anxiety, fear, pain and stress, but occurs in the palms, soles and armpits where the response to stimuli is greater. Emotional sweating disappears when the factor is removed or during sleep. It is activated by stimulation of cholinergic and adrenergic fibers of the sympathetic system. Emotional sweating is a type of sweating of apocrine origin and is not seen in the armpit during childhood [18].

Sweet sweating is a type of sweating that is explained as a result of swallowing or consuming foods with intense spice content together with the temperature factor. Sweating occurs in the upper part of the body, including the face, scalp and neck. This sweating also occurs when the capsaicin from spicy foods binds to the temperature receptors in the oral cavity and stimulates the sweat glands to release heat over the entire body surface [19].

Sweat odor is mainly apocrine in origin because it contains many organic compounds that allow bacteria to grow. The volatile molecules formed by the breakdown of these organic compounds in the armpit allow us to perceive the bad odor. The rapid growth of bacterial colonizations in alkaline or neutral environments further facilitates this phenomenon.

A culture-based approach to isolate odor-producing bacteria has helped to identify *Corynebacterium* and *Staphylococcus* species, particularly *Corynebacterium striatum, C. jeikeium,* and *Staphylococcus haemolyticus*, which are involved in the formation of odorous volatiles [20-22].

Excessive sweat and body odor can be very uncomfortable for social life and therefore many different products such as antiperspirants, deodorants, and wet wipes are designed to combat these undesirable features [23].

Aluminum salts, especially aluminum chlorohydrate (ACH), are generally used in antiperspirant formulations. In these formulations, the concentration is between 10-20% depending on the type of salt used. Table 2 shows the active ingredients used in antiperspirants [24]. Antiperspirants include the following: zirconium, iron, chromium, lead, mercury and zinc salts, which are mostly astringent and cause the precipitation of proteins, with the consequent blockage of the holes of the secretory ducts. Tannins have been used in formulations with satisfactory results in preventing excessive sweating. Anticholinergic compounds have also been used as antiperspirants in formulations [25].

Table 1. *Aluminium sourced active ingredients and CAS numbers used in antiperspirants.*

	Active ingredient	CAS Number		Active ingredient	CAS Number
1.	Aluminum Bromohydrate	39431-98-6	13.	Aluminum Sesquichlorohydrex PG	245090-60-2
2.	Aluminum Chloride	7446-70-0	14.	Aluminum Sulfate	10043-01-3
3.	Aluminum Chlorohydrate	12042-91-0	15.	Aluminum Zirconium Octachlorohydrate	98106-55-9
4.	Aluminum Chlorohydrex	53026-85-0	16.	Aluminum Zirconium Octachlorohydrexgly	174514-58-0
5.	Aluminum Chlorohydrex PEG	242812-76-6	17.	Aluminum Zirconium Pentachlorohydrate	98106-54-8
6.	Aluminum Chlorohydrex PG	245090-52-2	18.	Aluminum Zirconium Pentachlorohydrex Gly	125913-22-6
7.	Aluminum Citrate	813-92-3	19.	Aluminum Zirconium Tetrachlorohydrate	98106-52-6
8.	Aluminum Dichlorohydrate	10284-64-7	20.	Aluminum Zirconium Tetrachlorohydrex Gly	134910-86-4
9.	Aluminum Dichlorohydrex PEG	242812-79-9	21.	Aluminum Zirconium Trichlorohydrate	98106-53-7
10.	Aluminum Dichlorohydrex PG	245090-53-3	22.	Aluminum Zirconium Trichlorohydrex Gly	134375-99-8
11.	Aluminum Sesquichlorohydrate	11089-92-2	23.	Ammonium Alum	7784-25-0
12.	Aluminum Sesquichlorohydrex PEG	242812-86-8	24.	Ammonium Thiocyanate	1762-95-4

Antiperspirants are designed to control wetness and odor in the underarm area of the body. The chemicals used in antiperspirants can act as antiperspirants and reduce underarm moisture. Metal salts (ACH, zirconium cholorohydrate), anticholinergics, aldehydes (formaldehyde, glutaraldehyde), anti-adrenergics, metabolic inhibitors and many alcohols and other organic acids and other similar substances are used in antiperspirants [26].

Although there is limited scientific data on the application of hops ingredients in skin care products, the properties described for hops plant extracts or beer by-products as well as for their active compounds make hops a promising ingredient for skin care cosmetics. *Humulus lupulus* L. (hops) is a herbaceous perennial plant species, one of the three species of *Humulus* in the family *Cannabeaceae* [27].

Its active ingredients include lipophilic components such as essential oils and bitter acids. Myrcene is the main component of hop essential oil with a share of up to 70% by volume [28]. It protects UVB-treated human skin fibroblasts against photoaging by decreasing the expression of reactive oxygen species (ROS), matrix metalloproteinase (MMP)-1, MMP-3, interleukin (IL)-6 and increasing transforming growth factor (TGF)-ß and Type I procollagen secretion. Myrcene is therefore used in sunscreens or anti-aging products [29]. Bitter bitter acids make up 30% of the secondary metabolites in lupulin glands [30]. Bitter acids are composed of a-acids (humulone and its derivatives) and B-acids (lupulone and its derivatives). They act as radical scavengers and inhibit lipid peroxidation. Humulone and lupulone show antibiotic effects against many Gram-positive bacteria (*Bacillus, Lactobacillus, Micrococcus, Streptococcus* and *Staphylococcus*) [31].
The leaves and essential oils of sage (*Salvia officinalis* L.), a member of the Lamiaceae family, are widely used in the food, pharmaceutical and perfumery industries [32]. Sage leaf extract shows antimicrobial, antioxidant, antisecretory, antiperspirant, anti-inflammatory and astringent activity. The main constituents of sage essential oils are 1,8-cineole, camphor, a-thujone, B-thujone. The antimicrobial activity is mainly due to the tujones in the essential oil. Externally applied tujones appear to have antihidrotic activity as they block the nerve endings in the sweat glands. Sage leaves also have a well-known antioxidant activity due to their content of phenolic compounds. Sage leaves can be used in antiperspirant formulations as dry extracts up to 1% and glycolic and liquid extracts up to 5% [33].

The skin acts as a physical and chemical barrier that protects our body against potential attacks by pathogens and toxic substances. Human skin is an ecosystem of different habitats supporting various microorganisms in many folds, recesses and special niches on a surface area of 1.8 m^2. The skin provides a point of connection with the outside world, in which a vast ecosystem of bacteria, viruses, fungi, mites and archaea maintain a dynamic and symbiotic relationship with the skin microbiome. Microbiota is a description given to all microbial cells living within a specific region. Axillary microbiota includes all microbial cells that live in a specific region and on the surface of the armpit. The moist, warm environment provided by the structural anatomy of the armpit and the dense coating of hair is an event that increases bacterial proliferation. The axillary region is home to more than 100 microorganisms per cm^2, including colonized bacteria [34, 35].
Bad odor occurs when apocrine sweat secretions are metabolized in the axillary microbiota. Although the main task of the eccrine glands is to provide thermoregulation, in the presence of sweat glands, it causes a hydration that supports the humidification of the environment and the proliferation of surface microbiota [14].
Corinobacteria are also involved in the formation of volatile short fatty chain acids. Through the enzyme Na-acylglutamine aminosylase, they convert apocrine released volatile fatty acids into short, medium and branched volatile fatty acids and provide odor formation [36].
Another product that causes axillary malodor is androstenol and androstenone molecules, which are steroid derivatives. The resulting odor is known to be musk-like and urine- like [37].
Propionibacteria and *Staphylococci* metabolize glycerol and lactic acid, which are abundant on the skin surface, to form acetic and propionic acids, which encourage bacteria to grow in the axillary microbiota and cause underarm odor [38].

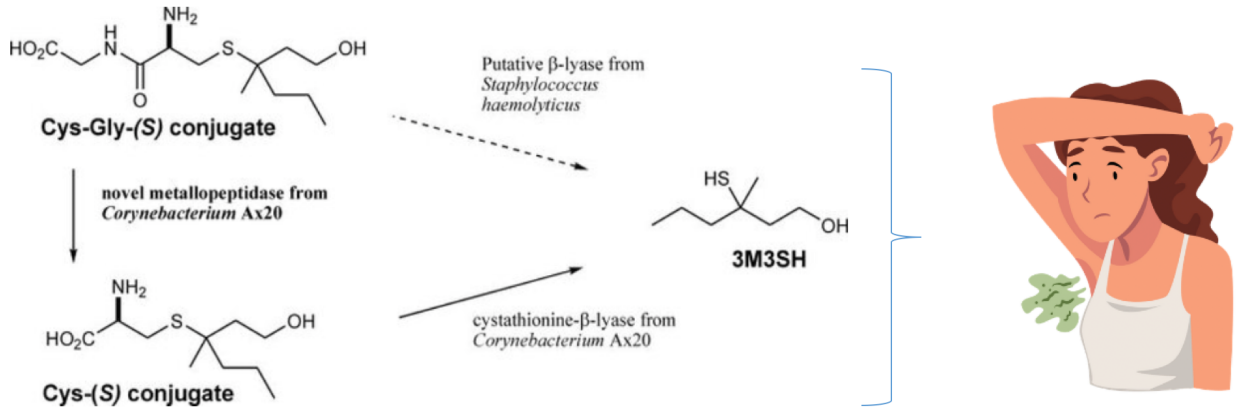

Figure 1. Shematic representation of bad odor formation

More than 100 trillion microorganisms live in the human body and microorganisms play a role in human health and many diseases by affecting the microbiota. Microorganisms differ according to their location in the human body, age, gender, race and diet. Prebiotics, probiotics and synbiotics found in

functional foods help regulate the composition and activity of the gut microbiota. They can also directly influence the immune response 'Biotics' refers to nutrients used to improve human health by affecting the gut microbiota. The term biotic is derived from the Greek word 'biōtikós' (related to vitality) and is defined as substances produced by living organisms. Probiotics are live non-pathogenic microorganisms present in the gut microbiota that provide health benefits to the host. They act through molecular and cellular mechanisms that antagonize pathogenic bacterial adhesion, enhance innate immunity, reduce pathogen-induced inflammation and promote intestinal epithelial cell survival, barrier function and protective responses. Postbiotics are defined as a mixture of cellular components and metabolic by-products released from probiotic or fermenting bacterial lysates, or bioactive substances produced by live bacteria during fermentation [39, 40].

Specific postbiotics benefit human health by modulating the skin microbiota and thus promoting the growth of beneficial microbial species as well as inhibiting the growth and activity of potential pathogens. Among the biotechnological solutions presented, the addition of postbiotics as active ingredients has proven to be one of the most promising, due to the lack of risks of bacteremia and fungemia, as well as natural stability during industrial processes and shelf life. The formulation of cosmetic products containing postbiotics has also proven to be less costly, as there is no need to maintain cell viability in the final product during transportation and storage [41, 42].

2. RESULTS

Minimal changes in skin microbiota were observed after the application of the postbiotic-free formulation. A significant change in the number of bacteria present in the skin microbiota was observed after the use of an antiperspirant emulsion containing postbiotics. The change in favor of the postbiotic indicates that this formulation provides inhibition of bad odor-causing bacteria and has positive results on the growth of *Lactobacillus acidophilus* bacteria necessary for skin integrity (Table 2).

Table 2. *Effect of postbiotic-free and postbiotic content antiperspirant formulation on skin microbiota simulation*

Microorganisms (cfu/mL)	Negative Control (PBS) (cfu/mL)	Postbiotic-free antiperspirant (cfu/mL)	Postbiotic containing antiperspirant (cfu/mL)	Negative Control (PBS) log10	Postbiotic-free antiperspirant log10	Postbiotic containing antiperspirant log10
Malassezia (pityrosporum) folliculitis	3,60E+03	3,55E+03	3,00E+03	3,6	3,6	3,5
Staphylococcus hominis	3,60E+03	3,60E+03	2,80E+03	3,6	3,6	3,4
Staphylococcus epidermidis	3,60E+03	3,55E+03	2,60E+03	3,6	3,6	3,4
Staphylococcus aureus	3,60E+03	3,61E+03	2,60E+03	3,6	3,6	3,4
Staphylococcus capitis	3,60E+03	3,60E+03	2,80E+03	3,6	3,6	3,4
Cutibacterium (Propionibacterium) acnes	3,60E+03	3,60E+03	2,80E+03	3,6	3,6	3,4
Corynebacterium striatum	3,60E+03	3,60E+03	2,70E+03	3,6	3,6	3,4
Candida albicans	3,60E+03	3,60E+03	2,60E+03	3,6	3,6	3,4

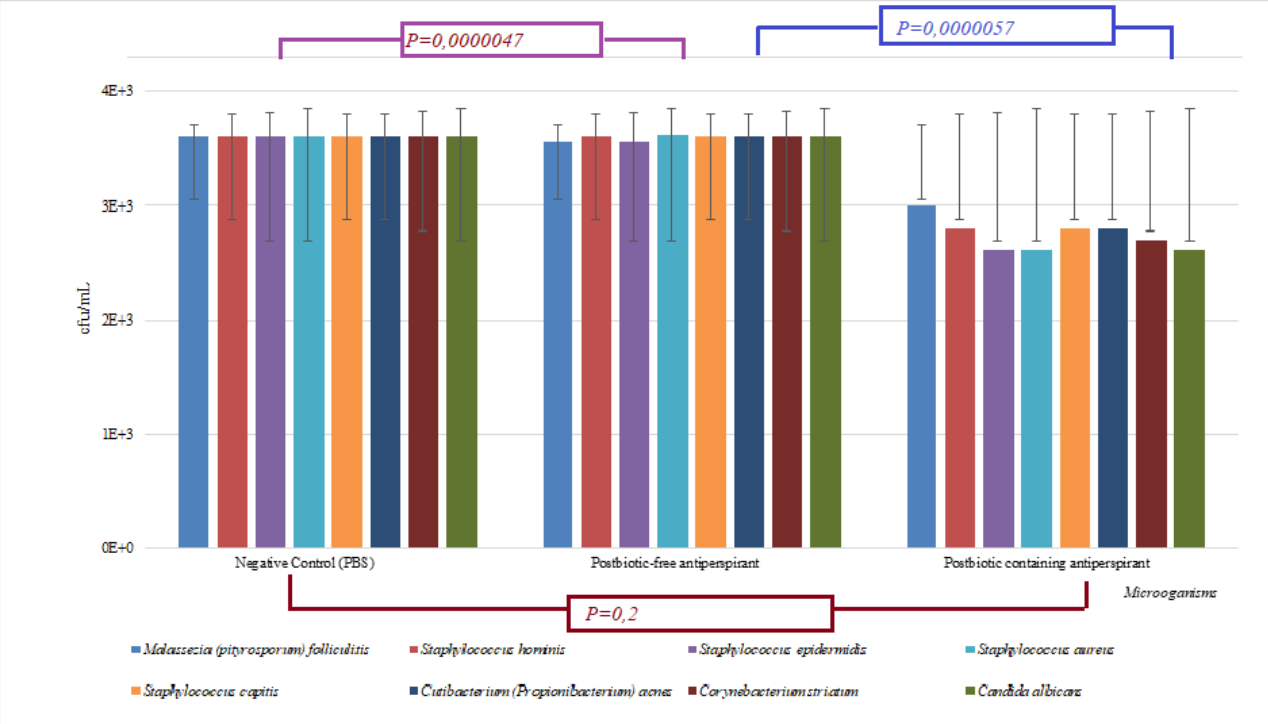

Figure 2. Significant change in microbiota diversity. Different variances, two-way t-test graph.

3. DISCUSSION

The formation of bad odor in the armpit is caused by organic volatile odor molecules formed as a result of the metabolism of the fluids secreted by the perspiratory glands by bacteria in the axillary region. Metal salts, which are currently used as active ingredients in antiperspirant formulations, block prepisratory glands and prevent the outflow of sweat. Aluminum salts are the most commonly used metal salts in antiperspirant formulations. Alternative formulations to these metal salts have been studied due to their known toxic effects. In this way, by using postbiotics obtained from probiotics [43] that will provide a natural balance in the axillary microbiota, it is aimed to inhibit the bacteria that cause the formation of bad odor due to antiperspirant use in the region.

Artificial axillary microbiota was established under *in vitro* conditions. In these *in vitro* conditions, two formulations with and without postbiotics were studied. In the formulation containing postbiotics, it was observed that the bacteria causing bad odor formation as a result of sweating decreased. It has been reported that *Humulus lupulus* extract and *Salvia officinalis* leaf extract described in the cosmetic ingridient (CosIng) data base, which includes the cosmetic directives of the European Union, can be used in antiperspirant formulations thanks to their antiperspirant properties. Considering the CosIng data, *Humulus lupulus* extract and *Salvia officinalis* leaf extract were used as active ingredients in roll-on type antiperspirant, and slightly successful results were obtained.

When the Table 2 was evaluated, there was no statistically significant difference between the negative control (PBS) and the natural antiperspirant without postbiotics (p=0.2). Significant statistical differences were observed between the antiperspirant containing postbiotic (Acilac ATA-LTW12- *Lactobacillus* ferment lysate extract) and the antiperspirant without postbiotic, and between the antiperspirant containing postbiotic and negative control (p=0.0000047, p=0.0000057, respectively).

4. CONCLUSION

As an alternative to aluminum salts, it was tried to design a natural, microbiome friendly antiperspirant formulation that is sustainable in nature, new generation and aluminum-free, does not participate in systemic circulation and does not contain harmful metals.

Overall, our results suggested there is no significant effect herbal and natural antiperspirant formula without postbiotic. However, our results clearly indicated that microbiome friendly postbiotic (Acilac ATA-LTW12- *Lactobacillus* ferment lysate extract) containing antiperspirant fomulation were induced harmful

bacteria species. Also, this formula regulate the axillary microbiota. This research study was carried out in order to leave more sustainable, effective and cleaner cosmetic products for future generations.

5. MATERIALS AND METHODS

Two postbiotic and non-postbiotic formulations containing *Humulus lupulus* and *Salvia officinalis* were created. These formulations were planted on artificial skin microbiota created under *in vitro* conditions. These two groups of formulations were sampled by swab technique and bacterial counts were performed.

Table 3. *Microbiome friendly antiperspirant formulation containing postbiotics*

INCI/Product Name	CAS No	% Concentration	Function
Aqua	7732-18-5	72,95	Solvent
Glycerin	5343-92-0	1	Moisturizer
Butylene Glycol	107-88-0	5	Moisturizer
Sodium Chloride	7648-14-5	0,5	Thickener
Xanthan Gum	11138-66-22	0,8	Thickener
***Lactobacillus* Lysate Postbiotic (*Acilac ATA-LTW12*)**		2	Antimicrobial
***Humulus lupulus* Leaf Extract**	8060-28-4	0,2-1	Active substance
***Salvia officinalis* Leaf Extract**	8022-56-8	1-5	Active substance
Ecocert Zinc Oxide	1314-132	20	Antimicrobial
Non-Allergenic Fragrance		0,2	Perfume

Table 4. *Postbiotic-free formulation*

INCI/Product Name	CAS No	% Concentration	Function
Aqua	7732-18-5	72,95	Solvent
Glycerin	5343-92-0	1	Moisturizer
Butylene Glycol	107-88-0	5	Moisturizer
Sodium Chloride	7648-14-5	0,5	Thickener
Xanthan Gum	11138-66-22	0,8	Thickener
***Humulus lupulus* Leaf Extract**	8060-28-4	0,2-1	Active substance
***Salvia officinalis* Leaf Extract**	8022-56-8	1-5	Active substance
Ecocert Zinc Oxide	1314-132	20	Antimicrobial
Non-Allergenic Fragrance		0,2	Perfume

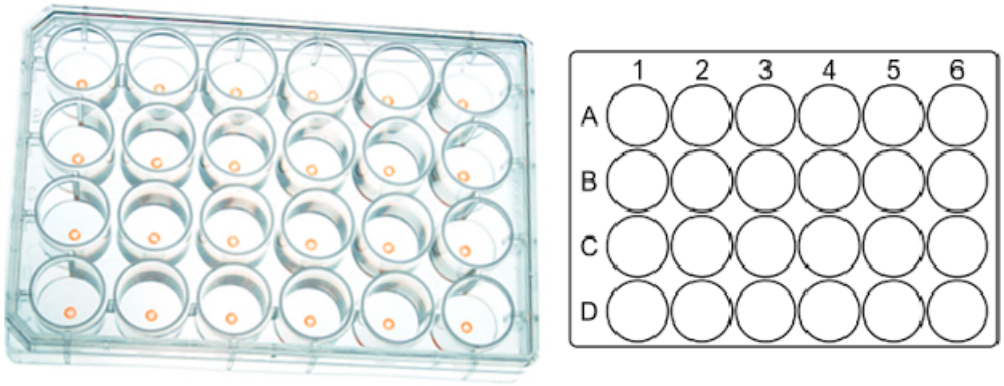

Figure 3. Field sample prepared for control or study groups.

5.1. Strains

Table 4. *Strain used in artificial axillary microbiome simulation*

Malassezia (pityrosporum) folliculitis ATA-LTM 08147	GV, facultative anaerobic, non-spore yeast
Staphylococcus hominis ATA-LSM 0198051	GP, immobile, facultatively anaerobic, non-spore cocci
Staphylococcus epidermidis ATA-LSE 0198052	GP, immobile, aerobic non-spore, cocci
Staphylococcus aureusATA-LSA 011204	GP, non-motile, aerobic non-spore, cluster cocci
Staphylococcus capitisATA -LSC 0201201	GP, non-motile, aerobic non-spore, cluster cocci
Cutibacterium (Propionibacterium) acnesATA-LPC 0204221	GP, immobile, anaerobic/aerotolerant, non-spore bacillus
Corynebacterium striatumATA-LCST 0101224	GP, immobile, aerobic bacillus
Candida albicans ATA-LTCA 0504212	GV, facultative anaerobic, non-spore yeast

5.2. Microorganisms' preparation

If the culture to be used has been opened and the subculture prepared, the subculture is removed from the freezer. A bead is removed from the subculture tube under aseptic conditions and bacteria are streaked on TSA, MRS and SDA medium. Incubate for 18-24 hours at 37 C ±1° C for bacteria and 22±1° C for mold and yeast. At the end of incubation, the most distant colony can be selected and cultured again and a new subculture can be formed. This process can be repeated three times. Place 5 g of glass beads in a sterile tube and add 10 mL of diluent (saline, MRD). With the help of a pipette, remove microorganisms from the subculture and add them to the bead tube. Mix with a soft vortex for a maximum of 3 minutes. Stirring can be maximum 3 minutes. After homogenization and bacterial bead contamination, the microorganism suspension is withdrawn and transferred to another sterile tube. The number of microorganisms in (TSA) mL is determined by inoculation from the homogeneous suspension. For use, 1E+7 cfu/mL for bacteria and 10-10^5% cfu/mL for mold and yeast are prepared in Mc Farland device.

5.3. Analyzing and evaluating the sample

There are 24 single-type microorganisms in the working plate. Each well contains 3.6E+3 CFU lyophilized microorganisms. In this study, 24 plates containing 3.6E+3 CFU lyophilized microorganisms were used (Figure 2). Antiperspirants with and without postbiotics and PBS as negative control were applied to the plates in 100 µL to be spread on the bottom. It was kept for 15 min contact time. At the end of the period, 0.9 mL of neutralizer (TSB+Tween80) was added to the plates with the help of a micropipette. After mixing was completed, the mixtures were added to Falcon tubes containing 9 mL of neutralizer (TSB +Tween 80) (1/10 dilution) with the help of a micropipette. Each falcon tube was diluted a second time (1/100 dilution). The

contents of each tube were inoculated into MRS agar general medium for anaerobics, TSA medium for aerobics, yeasts as 1 mL in SDA medium by the pour plate method.

Aerobic bacteria were incubated in aerobic conditions at 37 °C for 48 h, anaerobic bacteria in anaerobic conditions at 37 °C for 48 h, yeasts in aerobic conditions at 22 °C for 72 h. At the end of the period, the colonies were counted. The first row (4n) was left blank for each sample. 20n studies were performed for each microorganism and the average was taken. The calculation was made according to the formula below:

N= Total C/ Vx(n1+0,1n2) x d

N = number of microorganisms in 1 gram or 1 mL

C = Total number of colonies in all counted petri dishes

V = Volume transferred to the counted petri dish (1 mL)

n1 = Number of petri dishes counted from the first dilution (1/10)

n2 = Number of petri dishes counted from the second dilution (1/100)

d= Dilution ratio (1/10) of the most concentrated of the 2 consecutive dilutions.

5.4. Statistical analysis

The results of difference test are expressed as mean ± standard deviation (SD). Data were analyzed by analysis of variance (ANOVA) followed by least significant difference (LSD) test for multiple comparisons using SPSS version 25.

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Conflict of interest statement: The authors declared no conflict of interest.

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