Correlation of Biomarker and Biophysical Parameters with Microflora at Different Skin Sites in Young Women (20-21yrs.) of Meerut

Sonia Sharma[1*], Alka Sagar[1], Saurabh Singh Yadav[2], Pramod W. Ramteke[3]

[1]*Meerut Institute of Engineering and Technology, Meerut, India*
[2]*Swami Vivekanand Subharti University, Meerut, India*
[3]*Sam Higginbottom University of Agriculture Technology and Sciences, Allahabad, India*

*Corresponding author

A B S T R A C T

Dermal health is the outcome of dynamic interactions between the microflora and immune networks to influence the health and overall wellbeing. Biophysical parameters reflect the functional integrity of skin. Biomarkers LL-37 and HBD 2, 3 have broad spectrum amino acid activity that plays role in epithelial defense system. The aim of the present work was to find out exiting correlation between the isolated genera, biophysical parameters and biomarkers of skin. Methods of isolated genera from seven cutaneous site from 45 young girls (21-22 yrs.) were ccorrelated with Biophysical parameters such as trans-epidermal water loss, epidermal hydration, skin pH, dermal health, level of biomarkers LL-37 and HBD, Meerut Institute of Engineering and Technology (MIET), Meerut. One hundred six (136) isolates presumptively identified as *Staphylococcus epidermidis, Pseudomonas* sp., *Lactobacillus* sp. *Propionobacterium* sp. on the basis of selective media and biochemical characterization were analyzed. Total viable count was moderately correlated with LL-37, HBD-2,3, epidermal hydration and skin gloss, bears weak positive correlation with TEWL and skin lesions and weak negative correlation with pH, redness, scales and skin integrity. The study findings suggested that areas having better dermal health are positively correlated with microbial load and biomarker LL-37 and HBD 2,3. Dermal health is a consequence of normal flora and antimicrobial peptides can be used in human skin ointments for treatment of skin disorders.

Introduction

Skin is the semipermeable filtration system having many dynamic interactions with microbes that reside on skin surface. Many experiments had revealed the mutual beneficial relationship of these bacteria with the host (Cogen *et al.,* 2008).

The most direct benefit is the production of lysozyme, acidity (pH 4.5-5.9) which impart antifungal and antimicrobial properties, and defensins (HBD-2,HBD-3), antimicrobial peptide marker LL-37, HBD-2,HBD-3,lantibiotics, and other molecules that act to resist co-colonization by pathogens(Cogen *et al.,* 2010; Nakatsuji *et al.,* 2013; Lai *et al.,*

2009,2010,2013; Naik *et al.,* 2012) stimulate host immune system by enhancing antibody production, stimulate phagocytosis and clearance mechanisms, and augment interferon and cytokine production (Moon, 2012; Nipa, 2015).

In contrast, some of the bacteria present on skin surface such as *Staphylococcus aureus* when applied to the surface of mouse skin can initiate harmful events that exacerbate the allergic responses Nakamura *et al.,* 2013.

LL-37 derives its name due to two leucine leading residues with overall length of 37 amino acids. HBD are the human beta defensins which are cysteine rich and have broad spectrum amino acid activity play role in epithelial defense system (Zheng *et al*., 2016, Hans *et al*., 2014; Crack *et al*., 2012).

Certain factors such as the amount of perspiration, anatomic location, the amount of sebum and sweat production, age and hormonal level of the host determines the type and density of bacteria (Chiller *et al*., 2001).

Previously individual role of LL-37, HBD and microbes had been studied in cutaneous defense in some diseases (Zheng *et al*., 2016; Hans *et al.,* 2014; Crack *et al.,* 2012).

It is hypothesized that there exist some relation between the concentration of these antimicrobial peptides with the density and type of microbes present at different anatomical sites on skin.

For this purpose the present work was planned to find out the correlation between microbial load, biophysical parameters such as Trans epidermal water loss, epidermal hydration, skin pH, dermal health, level of biomarkers LL-37 and HBD2,3 using healthy young females.

Materials and Methods

Study design

This study was carried out for one month from January to February 2019 in the department of Biotechnology & Microbiology at Meerut Institute of Engineering & Technology affiliated to CCS University (U.P.), Meerut, India. The sample size was calculated using website.

http://www.raosoft.com/samplesize.html. 45 (Forty Five) healthy woman (21-22yrs) were included in this experimental study so that correlation can be found out between healthy skin and biophysical parameters. Participants had not received antibiotics 15 days before the sampling avoid any other medicine during the test period and provided informed consent to participate in the study.

The average high and low temperature during study was 10-17^{0}C with maximum of 24 ^{0}C and 56% R.H. The body sites chosen for study were washed 12 hrs before the study. The participants were allowed to bathe only with water and restrict the use of any shampoo soap and beauty product during study period.

Isolation of bacteria

In this study, different areas: (alar crease) corner of nose, hand back, (reticuloauricular crease) backside of ear, volar forearm, toe webs, interdigital space, antecubital fossa from each individual were sampled after swabbing the site in three replicates (thrice a week) (Grice *et al.,* 2008) and inoculated on different media like mannitol salt agar aerobically incubated, lactobacillus selection agar, thioglychollate broth, blood agar (anaerobic jar). After incubation at 37- 40^{0}C for 24-48hrs, bacterial colonies were isolated.

Bacterial cultures were maintained on slants at -20 ^{0}C. (Ian *et al.,* 2016; Julie *et al.,*2019).

Biochemical identification of isolated bacteria

The identification of bacteria was based on morphological characteristics and biochemical tests carried out on the isolates.

Morphological characteristics observed for each bacteria colony after 24 h of growth included colony appearance; shape, elevation, edge, optical characteristics, consistency, colony surface and pigmentation.

Biochemical characterizations using Gram staining techniques, Catalase, Methyl red, Voges-proskaeur, Indole, Sugar fermentation, Starch hydrolysis, Citrate, Urease, Gelatin hydrolysis for tentative identification of bacteria (Olutiola *et al.,* 2000).

Study of biomarkers

Collection of stratum corneum was standardized, using the same tape, pressed against the skin for 10 seconds stored at -20 °C.

Samples were aliquoted into Eppendorf tubes and stored at −80 °C. Tape-strippings were analysed for the presence of biomarkers of AMPs (LL-37, β- defensins (HBD-2, HBD-3)).

For quantification, LL-37 and β- defensins were extracted from last 4/5 tapes using 15 mL Tris bufered saline and the extract was kept at 4°C overnight. Each extract was filtered through polytetrafluoroethene (PTFE) membrane and the trapped corneocytes in the membrane filter were analysed using chemiluminescence immuno-detection method (Maja-Lisa Clausen *et al.,* 2018·)

Biophysical parameters

The measurement of biophysical parameters of the skin was performed using 4 different instruments: Skin pH-Meter® (PH905), Skin-Glossymeter® GL 200, Tewameter TM 210®, Corneometer CM 820. The skin of study participants was cleaned with mild soap 2hrs before taking the measurement. (Harvey *et al.,* 2017).

Statistical analysis

All experiments were carried out in triplicates. Data obtained were analyzed by T test and one-way analysis of variance (ANOVA). Means were compared by Duncan's New Multiple Range test (SPSS Statistics for Windows, version 10.0) (SPSS Inc., Chicago, Ill., USA). Differences were considered significant at $p < 0.05$.

Results and Discussion

The body sites were chosen in this study on the basis of the varied bacterial compositions of different skin areas. Superficial swabs were employed because this method was less invasive and was reported to yield similar microbial profile to that associated with skin scraping of epidermis or punch biopsy of full thickness of epidermis and dermis (Grice *et al.,*. 2008). Samples were spread-plated onto the surface of BA (Blood Agar) and MSA (Mannitol Salt Agar), LSA (Lactobacillus Selective Agar), CLED (Cystine Lactose Electrolyte-Deficient Agar) plates and the plates that contained visible growth were counted. The minimum TVC was at antecubital fossa with maximum count at reticulo-auricular crease shown in table no.1. *S.epidermidis* form white colored colonies, *S. aureus* was observed as golden coloured colonies on MSA and deep yellow colonies on CLED, white colonies observed at LSA were of *Lactobacillus*.

Table.1 Total viable count of microorganisms at different skin sites (Mean±S.E.)

S.No.	Samples	No of TVC
1	Volar forearm	102±25
2	Retroauricular crease	136±20
3	Alar crease	106±15
4	Toes web space	109±20
5	Hand back	104±30
6	Inter digital space	107±15
7	Antecubital fossa	94±20

Table.2 Comparative growth of microorganisms from skin samples on different media

S.No.	Samples	Mannitol salt agar		LBS agar	CLED agar	Blood agar
1	Volar forearm	$4^{*\blacktriangle}$	42^{ns}	$8^{\square\bullet\blacksquare}$	24^{ns}	$24^{\blacktriangle\blacksquare}$
2	Retroauricular crease	$10^{*\blacktriangle\blacksquare\bullet\square\,\sun}$	46^{ns}	12^{ns}	28^{ns}	$36^{*\blacktriangle\blacksquare\bullet\square\,\sun}$
3	Alar crease	$12^{*\blacktriangle\blacksquare\bullet\square}$	41^{ns}	10^{ns}	29^{ns}	$14^{\bullet\spadesuit}$
4	Toes web space	$1^{\blacktriangle\spadesuit}$	39^{ns}	$20^{\blacktriangle\bullet\,\sun}$	34^{ns}	$15^{\spadesuit\,\bullet}$
5	Hand back	$1^{\blacktriangle\spadesuit}$	37^{ns}	$16^{\bullet\,\sun}$	30^{ns}	$20^{\spadesuit}$
6	Inter digital space	$1^{\blacktriangle\spadesuit}$	40^{ns}	16^{ns}	32^{ns}	$18^{\spadesuit}$
7	Antecubital fossa	$1^{\blacktriangle\spadesuit}$	38^{ns}	$6^{\blacksquare\,\square}$	28^{ns}	$20^{\spadesuit}$
	Tentative identification	*S.aureus*	*S.epidermidis*	*Lactobacillus*	*Pseudomonas*	*Propioni bacterium*

* $P<0.05$ compared to Interdigital space, ■ $P<0.05$ compared to toe web space, ♠ $P<0.05$ compared to reticuloauricular crease, ▲ $P<0.05$ compared to alar crease, ● $P<0.05$ compared to volar forearm, ◘ $P<0.05$ compared to hand back, ☼$P<0.05$ compared to antecubital fossa.

Green colonies with typical matted surface and rough periphery on CLED were of *Pseudomonas*. *Propionibacterium* were observed as pinprick-sized white glossy opaque neat-edged colonies on the blood agar. The details of colonial growth on different media are shown in table no.2.

The fig.1 shows the effect of abiotic stress on the growth of bacteria isolated from different sites. The microflora from reticuloauricular crease and alar crease are able to tolerate the stress whereas the flora from volar forearm is somewhat sensitive to stress compared with other sites. The biochemical characterization (fig.2) in addition to growth on specific media (table no. 2) leading to their tentative identification revealed that *S.epidermidis* is the predominant organism at all the sites taken into account in the study and *S. aureus* is the least found organism.

Pseudomonas is the second highest organism in toe web and interdigital space with *Propionibacterium* occupying the third place in its abundance followed by *Lactobacillus* in the study.
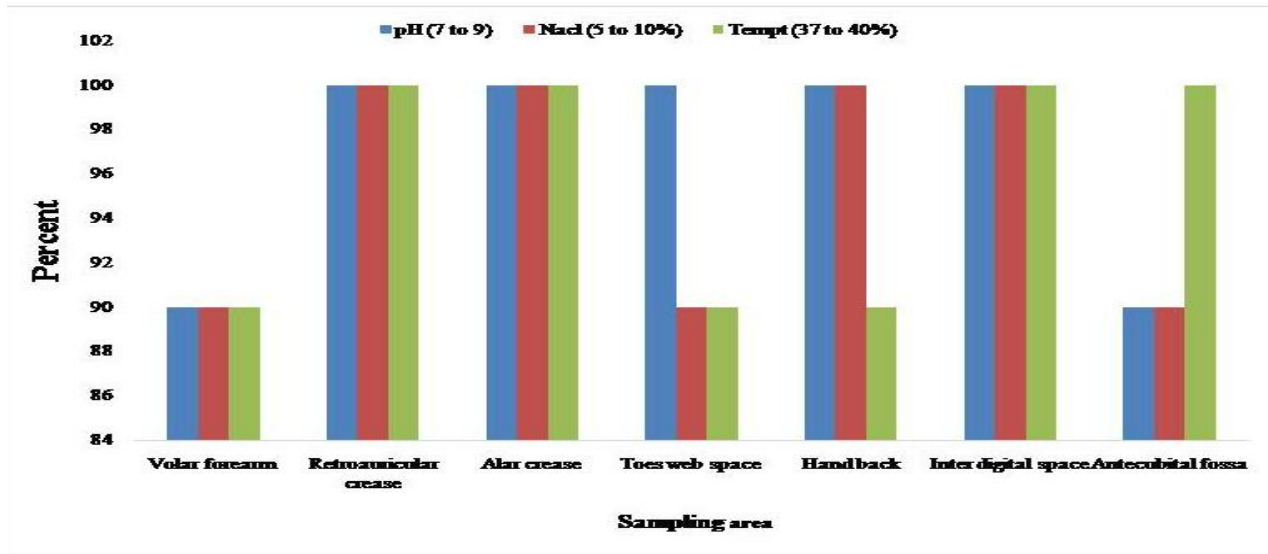
Fig.1 Characterization of isolated bacteria for Abiotic stress
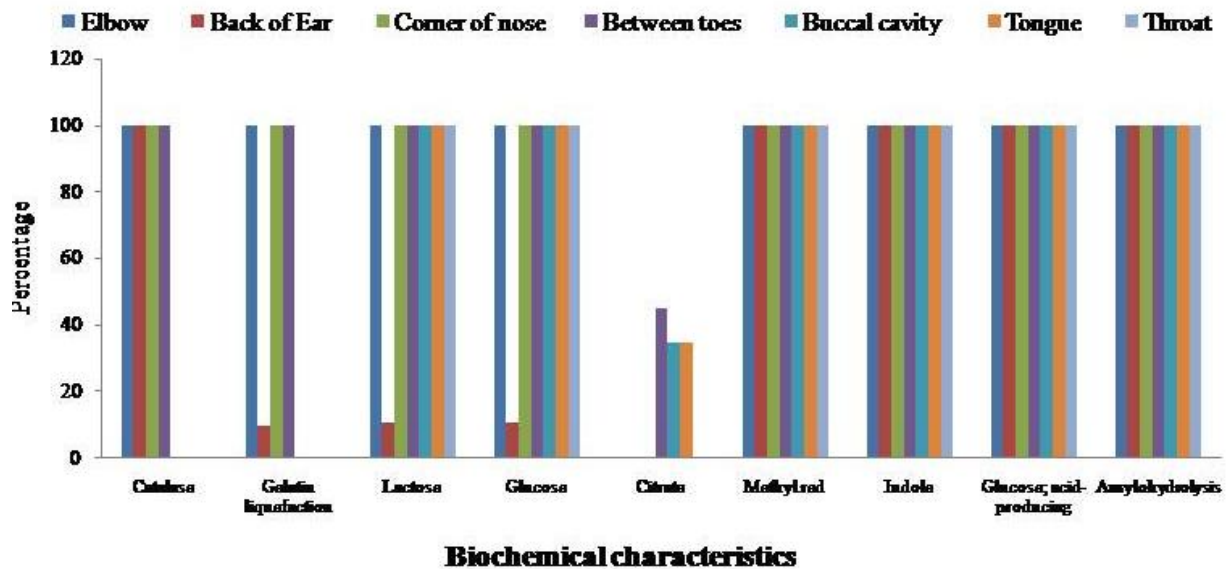

Fig.2 Biochemical characteristics of isolated bacteria

The highest concentration of biomarker are present at alar crease which has shown in table.3. Lowest concentration of defensins HBD-3 and canthalidins LL-37 at interdigital space and HBD-2 at toe web space.

The evaluation of biophysical parameters as revealed from table no. 4 the epidermal hydration was highest at reticuloauricular crease and lowest at interdigital space.

The transepidermal water loss was lowest at antecubital fossa and highest at toe web space. The skin gloss was highest at reticuloauricular crease and lowest at antecubital fossa.

Table.3 Level of different biomarkers (antimiocrobial peptides) at different skin sites.

S.no.	Samples	Biomarkers		
		HBD-2(pg/ml)	HBD-3(pg/ml)	LL-37 (pg/ml)
1	Volar forearm	0.41 ±0.02□	0.07 ±0.008□	0.18 ±0.04 ns
2	Retroauricular crease	0.43 ± 0.02□	0.50 ±0.03*□	0.91 ±0.04*□
3	Alar crease	0.39 ±0.04*□	0.54 ±0.06*□	0.98 ±0.09*□
4	Toes web space	0.12 ± 0.09☼	0.10 ±0.04☼	0.16 ±0.01☼
5	Hand back	0.21±0.04☼	0.12 ±0.03☼	0.20 ±0.03 ☼
6	Inter digital space	0.11 ± 0.08 ☼	0.07 ±0.01☼	0.12 ±0.04☼
7	Antecubital fossa	0.18 ± 0.09 ☼	0.10 ±0.06 ☼	0.21 ±0.088 ☼

* $P<0.05$ compared to, volar forearm,□ $P<0.05$ compared toe web space Interdigital space, antecubital fossa,handback,☼ $P<0.05$ compared to alar crease and reticuloauricular crease.

Table.4 Biophysical parameters of various skin sites

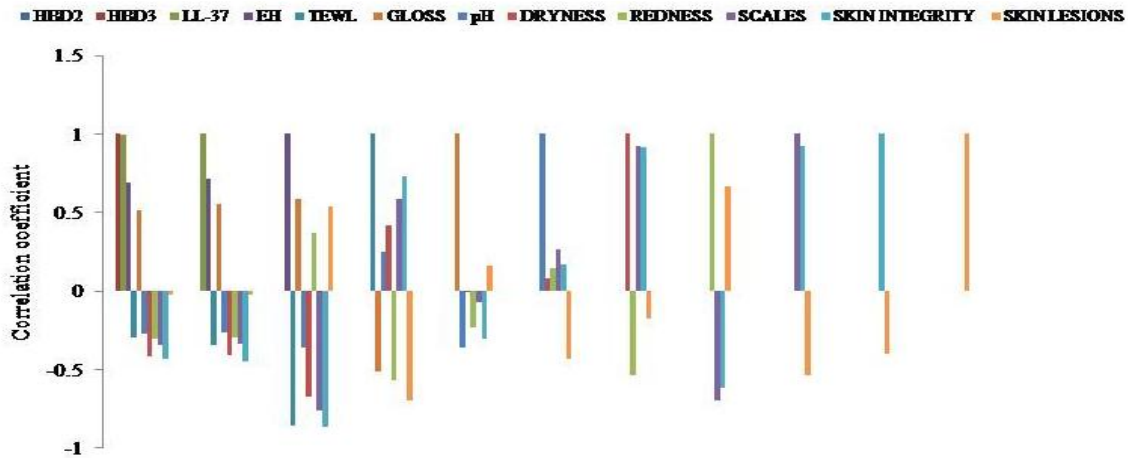| Biophysical Parameter	Sampling area						
	Volar forearm	Retroauricular crease	Alar crease	Toes web space	Hand back	Inter digital space	Antecubital fossa
Epidermal Hydration	42.12 ±1.21*♠■	72.12±4.21●*■▲☼	59.12 ±2.94■*♠	17.24±1.54●♠☼*▲■	51.42±4.32■*♠	14.24±2.21●♠*▲■☼	54.12±2.84■*♠
TEWL (g/m2h)	5.12 ±1.20■*	9.24 ±2.34■*	10.86 ±0.85■*☼	38.51±2.12●♠☼*▲■	9.28±1.24■*	38.51±5.64●♠☼*▲■	2.88±0.84■*▲
Gloss, Glossimeter units	9.14 ±0.86ns	9.48 ±0.28 ns	7.16 ±1.23 ns	5.14± 0.64 ns	6.12±0.89 ns	5.14± 0.89 ns	4.91±0.94 ns
pH	5.5 ±0.34 ns	5.2 ±0.71■	5.4± 0.21 ns	6.1± 0.25□♠	5.0± 0.6☼■	5.2± 0.6 ns	5.8±0.43□

* $P<0.05$ compared to Interdigital space, ■ $P<0.05$ compared to toe web space, ♠ $P<0.05$ compared to reticuloauricular crease, ▲ $P<0.05$ compared to alar crease, ● $P<0.05$ compared to volar forearm, □ $P<0.05$ compared to hand back, ☼ $P<0.05$ compared to antecubital fossa.

Table.5 Grading of skin sampled for various dermal parameters

S.no.	Samples	Dryness	Redness	Scales	Skin integrity	Skin lesions
1	Volar forearm	2.34±0.12♠☼■	0.54±0.08 ns	1.84±0.21♠*■☼	1.62±0.31♠☼	0.86±0.12■
2	Retroauricular crease	0.27±0.07•*▲■■	0.54±0.12 ns	0.34±0.05• *▲■■	0.64±0.09• *▲■■	0.71±0.21■
3	Alar crease	1.48±0.32♠☼	0.49±0.14 ns	1.28±0.24♠■☼	1.42±0.13♠☼	0.78±0.03■
4	Toes web space	1.76±0.14♠☼	0.54±0.15 ns	1.62±0.32♠■☼	1.85±0.43♠☼	0.52±0.08■
5	Hand back	1.25±0.13♠☼•	0.64±0.13 ns	0.52±0.09•*▲■■	1.25± 0.11♠☼	1.32±0.09••♠ *▲☼■
6	Inter digital space	1.84±0.23♠☼	0.46±0.09 ns	1.78±0.08♠■☼	1.92±0.31♠☼	0.43±0.28■
7	Antecubital fossa	0.34±0.09• *▲■	0.68±0.07 ns	0.41±0.05•*▲■■	0.72±0.04•*▲■■	0.84±0.12■

* $P<0.05$ compared to Interdigital space, ■ $P<0.05$ compared to toe web space, ♠ $P<0.05$ compared to reticuloauricular crease, ▲ $P<0.05$ compared to alar crease, ● $P<0.05$ compared to volar forearm, ▫ $P<0.05$ compared to hand back, ☼$P<0.05$ compared to antecubital fossa.

Fig.3 Correlation coefficient of microbial load with biophysical and biomarkers

The Skin tolerance test showed skin tolerance grading was lowest at reticuloauricular crease depicting better dermal health and highest at volar forearm as shown in table no. 5.

The statistical analysis depicted that samples collected from different sites vary significantly from each other in above parameters undertaken in the present study.

The correlation analysis as shown in fig no. 3 reveals total viable count was moderately correlated with LL-37, HBD-2,3, epidermal hydration and skin gloss, bears weak positive correlation with TEWL and skin lesions and weak negative correlation with pH, redness, scales and skin integrity. HBD-2 bears a strong positive relation with LL-37, skin gloss and epidermal hydration. HBD-3 was found

to be strongly correlated with LL-37 and moderately correlated with skin gloss.LL-37 was positively correlated with epidermal hydration.

Epidermal hydration was found to be positively correlated with TVC and biomarkers LL-37, HBD-2,3 and skin gloss. Skin pH was positively correlated with skin lesions. TEWL has positive correlation with skin lesions. Dryness was positively correlated with skin lesions and negative correlation with rest all traits.

Scales were positively correlated with dryness, TEWL and skin integrity in the present study. Skin lesions had positive correlation with redness. Skin lesions had positive correlation with redness and epidermal hydration negative or weak positive correlation with all other traits.

Skin is a complex ecosystem as it harbor different microbial communities depending on the local microenvironment prevailing at specific site (Domingos *et al.,* 2001) individual health status, eating behavior and environmental contacts, age related differences (relative dominance of lactobacilli in neonatal skin as compared to *Propionibacteria* in the mother) (Tacconelli *et al.,* 2001).

The development and maturation of systemic immune system depends on exposure of young child to microbes (Teruki *et al.,* 2018) influencing the growth and function of all organ systems, including the brain. In the present study *Staphylococcus epidermidis* was the most common isolate of the cutaneous microbiota.

This inference is supported by the investigation done by (Overturf *et al.,* 1990) which explained that more than 90% of all aerobic resident microbiota is *Staphylococcus*

epidermidis, it amplifies the keratinocyte response to pathogens by educating the skin's immune system through the production of antibacterial peptides (bacteriocins: epidermin, epilancin K7, epilancin 15X, Pep5 and staphylococcin 1580), lantibiotics: lanthionine-containing antibacterial peptides(Bierbaum G *et al.,* 1996), immunomodulatory properties (inhibition of inflammatory cytokine production), tumor necrosis factor receptor-associated factor (TRAF 6),TRAF1(Lai *et al.,* 2009) pheromone cross-inhibition(δ-haemolysin, δ-toxin or δ-lysin) (Nakamura *et al.,* 2013) and enhanced expression of tight junction protein which promote barrier function, promotes the expression of host AMPs such as cathelicidins and β-defensins causes microbial lipid membrane leakage (Nakatsuji *et al.,* 2017), blocks NF-kB inhibition (Wanke *et al.,* 2011) and inhibit colonisation with potential pathogens. *S. aureus* is found in low numbers as compared to other commensal organisms because host skin cells constantly sample the microorganisms inhabiting the epidermis and dermis via pattern recognition receptors (PRRs).

The activation of agr loci leads to a downregulation of virulence factors as signal is sent to the bacterium that an appropriate density is reached (Otto *et al.,* 2001) and ultimately lead to colonization inhibition (Otto 2001).

The commensal species thus prevent pathogen growth and maintain the stability of the resident cutaneous community (Cogen *et al.,* 2007).(Hoyle *et al.,* in 1991)) reported the presence of toll-like receptors (TLRs) TLR2 mediated by LTA (lipoteichoic acid) which specify pathogen-associated molecular patterns called bacterial priming and mast cell-mediated antiviral immunity (Wanke *et al.,* 2011) thereby activating the innate immune receptors providing efficient and

effective response of keratinocytes (Dekio *et al.,* 2005). Some strains of *Staphylococcus epidermidis* produce 6-N-hydroxyaminopurine (6-HAP), a DNA polymerase inhibitor which selectively inhibits proliferation of tumor lines (Teruki *et al.,* 2018) suggesting that the microbiome can act across the epidermis and interact with cells in the dermis and may confer protection against skin cancer.

AMPs are the defense molecules exhibit immunomodulatory activity by inducing proliferation differentiation and migration of cells along with regulating apoptosis of neutrophils and epithelial cells. Cathelicidin LL-37 is one of the few human bactericidal peptides with potent anti-*Staphylococcal* activity against *S. epidermidis* biofilms in vitro (Saporito *et al.,* 2018) forms an effective barrier against bacteria (Nelson *et al.,* 2009).

P.acnes and *Pseudomonas* bacteria possess next highest numbers in the samples collected in our study followed by *Lactobacillus*. Specific genes encoding for triacylglycerol lipase and lysophospholipase were identified in the *Propionibacterium* species, these enzymes specifically promotes triglyceride hydrolysis and propionic acid secretion resulting in an acidic pH which limits the growth of *Pseudomonas* (Megyeri, 2018), reduce Methicillin- resistant *S. aureus* (MRSA), *S. pyogenes* (Moon, 2012) and promote the growth of lipophilic yeasts including *Malassezia* species (Platsidaki, *et al.,* 2018)

Pseudomonas produces *pseudomonic* acid and *pyrrolnitrin* which works against *Staphylococcal and Streptococcal, Helicobacter pylori, Candida krusei, Candida albicans, Torulopsis glabrata*, *Saccharomyces cerevisiae* and *Aspergillus fumigatus*. A dense glycocalyx biofilm is formed on CA

filaments, Type IV pili, phospholipase C, phenazine compounds, including pyocyanin, phenazine-1-carboxylic acid (PCA), 1-hydroxyphenazine and phenazine-1-carboxamide (Xu *et al.,* 2017) kills the fungus (Xu *et al.,* 2014)and dermatophytes (Treat *et al.,* 2007).

Lactobacillus part of natural moisturizing factor (NMF) exerts beneficial effect on skin by producing lactic acid, hyaluronic acid, diacetyl improves the stratum corneum barrier function and enhance the production of ceramides by keratinocytes (Lew *et al.,* 2012) reduce the appearance of wrinkles, tightens pores and contains exfoliating properties, remodelling of epidermal and dermal cells and improves dry and dull skin, reduces skin microflora, activation of TLR, modulate angiotensin vascular endothelial growth factor and cytokines secretion reducing erythema (Muizzuddin *et al.,* 2012) pLTA, which has anti-photoaging effects on human skin by regulating matrix meralloprotionase-1 (MMP-1) expression. (Jeong *et al.,* 2016) reported that *Lactobacillus* prevented skin inflammation and reduced biomarkers of inflammation in mice suffering from psoriasis(Schauber *et al.,* 2008).

These results suggest a symbiotic relationship that leaves the host more prepared to combat pathogenic infection. Furthermore, the release of the AMPs LL-37 & HBD2,3 is amplified by these microbes conferring dual attack on pathogens as is depicted by the positive correlation between concentration of AMPs and microbial load.

The clinical assessment of skin tolerance by the dermatologist revealed that exposed areas such as hand back has better dermal health compared to antecubital fossa, may be due to higher level of LL-37 as the sunlight induces the synthesis of vitamin D this increased level of LL-37 activates pro and anti-inflammatory

mediators.

Moreover the greater microbial diversity of exposed skin sites is due to its higher interaction with external environment promoting better skin tolerance. Furthermore, exposure could also modulate resident microflora by encouraging evaporation of water, reducing the accumulation of secretions and maintaining the skin pH.

These factors may contribute to better dermal health in exposed compared with unexposed sites. When the bacterial ecosystem is balanced and differentiated, the skin remains healthy.

Clinical research has shown that blemish-prone skin has a less-diverse skin microbiome, over-populated with pathogens and damaging stressors—compared to those with healthy skin (Prescot *et al.,* 2017).

Little is known about many of the other bacterial species on skin due to their low abundance and apparent harmlessness.

In the present era skin diseases are on the verge of increasing day by day, the last decade has seen drastic increase in antibiotic resistance paving a challenge to combat skin infections. In addition to causing diseases some of the microbes potentially also play an opposite role by protecting the host.

The reticuloauricular crease has maximum TVC, biomarkers, epidermal hydration and better dermal health depicting a positive correlation between all these parameters. The present findings suggests that microenvironment i.e. biophysical characters influence type of microflora which in turn affects the level of biomarkers and health status of skin by preventing the growth of pathogenic organisms.

The complex host–microbe and microbe–microbe interactions on the surface of human skin pave a way to use these symbiotic microbes by selective modulation of microflora (pre- and/or skin probiotics) as novel therapeutic approach to dermatological diseases.

Future prospects

Further work should be done by studying large samples to standardize the CFU of commensal bacteria to be used in skin ointments for therapeutic purpose.

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