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A STUDY ON THE MICROBIOME OF FOOTWEAR OF DIFFERENT KINDS AND MAKE

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ABSTRACT

Microbial interaction between human associated objects and the environment we inhabit is of great relevance to human health and disease transmission. Saprophytic bacteria and fungi may be transferred from floor surface to footwear. There is also the likelihood of transfer of pathogens from footwear to floor surface. In the current study the microbiome, particularly bacteria and fungi, present on 5 cm^2 area of the inner surface of ladies footwear of various kinds and make was estimated by serial dilution and spread plate method. The microbial load obtained was higher for leather shoes, both for bacteria and fungi. A bacterial load of 318 x 10^5 cfu and fungal load of 16 x 10^7 cfu was obtained on the surface of leather shoes. The bacterial load was considerably higher for canvas and athlete shoes also (240 x 10^5 cfu and 167 x 10^5 cfu, respectively). The lowest levels of bacterial and fungal load were observed on the surface of plastic and rubber shoes. The bacterial count obtained for these shoes were 38 x 10^5 and 7 x 10^5 cfu, respectively. A higher load of fungi was observed on athlete shoes (67 x 10^5 cfu) followed by rubber shoes (57 x 10^5cfu). The mold levels on the surface of plastic and canvas shoes were of the order of 19 x 10^5 and 33 x 10^5cfu. A preliminary attempt was also made to identify the bacteria and fungi present on the surface of the footwear types using Gram staining and Lactophenol Cotton Blue staining, respectively. Gram positive cocci in clusters characteristic of *Staphylococcus* were identified from the surface of footwear made of leather, plastic, rubber and athlete shoes. Gram positive spore forming rods characteristic of *Bacillus* were identified from the surface of shoes made of leather. Gram negative rods were observed in all the footwear types except for that made of plastic. The fungus *Aspergillus* was found to be present on the surface of all the footwear types included in this study. *A. niger* was identified from rubber sandal and canvas shoes and *A. flavus* from canvas and athlete shoes. *Penicillium* was isolated from the surface of plastic, canvas and athlete shoes. Species of *Helminthosporium* was observed on the surface of athlete shoes.

KEYWORDS: Shoe microflora, microbial signature, human microbiome, forensic science, identification.

INTRODUCTION

Microbial interaction between humans or human associated objects and the environment has been the major focus of microbial ecologists in the past few decades. Recently studies have been focussing on the analysis of microbiome on personal belongings such as shoes and mobiles phones.[1] The human microbe interaction has been very dynamic and has been making revolutionary changes in the microbial ecology of our homes, offices, hospitals and cities.[2,3,4] The microbial ecology and the built environment play a significant role in human health and also in the transmission of human diseases.

'Human microbiome' is the term coined to denote the collection of microorganisms that we humans are associated with. They comprise of the normal bacterial flora which is the population of microorganisms routinely found growing on the body surface of healthy individuals.[5,6] The normal flora includes both non pathogenic commensals (not harmful to the host) and those with mutualistic existence. They are often associated with different regions of the body such as skin, mouth and gastrointestinal tracts.[7] These regions house diverse communities of bacteria and other microorganisms which may also vary from individual to individual. Within the same individual the flora can change depending on the health status and age of the host. This unique nature of microflora of each individual is increasingly gaining the attention of researchers and forensic scientists as a personal microbial signature.

Lax et al have observed a variety of microbial communities in phone and shoes and they were also able

to correlate between the floor environment and the microflora of shoe sole.[1] They have noticed that shoes that have travelled on different types of surfaces show distinct microbial signatures. They opine that the microbiome of mobile phones could also be used in this way.[1] Meadow et al has also suggested that smart phones carry the skin microbiome of their owners on its surface.[4] Lax et al has studied the microbiome of multiple home surfaces in which they had observed that the microbiome of a family was contributed by the individual microbial signature of the different family members. They are of the opinion that this individual microbial signatures could be effective in differentiating the individuals within a family.[8]

Fierer et al has proposed the forensic importance of skin bacterial communities in the identification of criminals or culprits.[9] Similarly, Lax et al has identified uniqueness in the structure and communities of microbes based on the surface type, identity of the person interacting with the surface, and his or her geographic location.[1] This would possibly help to infer individual identities, especially those associated with their personal belongings. These microbial communities are now gaining importance in forensic applications since it not only reveals the microbial signature of the individual, but also demonstrate where they have been before the sampling. The different floor microbial community play a major role in the shaping of the microbiome of shoes used by an individual. When the suspects walk at a crime scene their shoes show distinct microbial signatures based on the different surfaces on which they have travelled and also demonstrate where they have been before sampling. This may be of immense help in the identification of culprits or criminals, especially to trace their track when the crime was happening.[1]

Footwear has been identified as potential source of microorganisms in food processing industries also. Maintenance of hygienic environment in manufacturing area is a great concern for in-process as well as for finished products in food industries. Food safety, in many industries, is ensured by footwear sanitation that prevents the ingress and spread of pathogens into and among the production area. Many of the industries use special plant-only foot wears for preventing ingress of microorganisms from outside sources.[10] Procedures to sanitize footwear are widely used in the food industry for preventing cross contamination of finished products from potentially contaminated areas or sources such as raw materials, raw in-process food materials, debris from floor sweepings and dust and this is most often considered as a Good Manufacturing Practice (GMP).

The microbiome of foot wears are also contributed by the material from which it is made. Foot wears made of plastic and rubber contain fewer microbes contributed by the material since they are complex polymers that are less attacked by microorganisms. Footwear made of leather are deteriorated easily by bacteria and fungi, often resulting in discolorations, pigmentations or even foul smell.[11] Canvas shoe and athlete shoes are made of cloth, hides and other synthetic materials which also may be subjected to degradation, partly or fully, by a variety of microorganisms. The degrading microorganisms in this footwear types may also contribute to the load of microbiome on their surface.[11]

The present study aimed at investigating the microbiome of footwear of different kinds and make. In this study the microbial load of footwear were quantitatively estimated. Attempts were also made to identify the bacterial and fungal types prevailing in various kinds of footwear.

MATERIALS AND METHODS

Collection of Shoes

The present study evaluated the microbiome on different kinds of footwear. Ladies footwear made of different materials like rubber, plastic, leather, canvas and athlete shoes (5 number each) were collected from female respondents of age group 18-20.

Collection of Microflora from Shoes

The microflora of shoes of various kinds and make were cultured and quantitatively assayed. An area of 5 cm^2 each was marked in the inside of the shoes. Sterile cotton swabs soaked in saline were rubbed back and forth on the marked area to collect microorganisms from the shoe surface. The microorganisms collected were immediately suspended in 9 ml sterile bacteriological saline and used for preparing serial dilutions.

Culturing of Shoe Microflora

The skin microflora of the respondents was enumerated using the standard plate count method by means of spread plate type of bacterial culture. For this the shoe microflora collected from the inside of shoes of the respondents was serially diluted using the procedure of Aneja.[12] The dilutions were prepared by suspending the microorganisms collected from shoes on sterile cotton swabs in 9 ml bacteriological saline. This was mixed well to obtain the 10^{-1} dilution. One ml of the suspension was transferred to 9 ml saline and mixed well to obtain 10^{-2} dilution. From 10^{-2} dilution, 1 ml of the suspension was transferred to 9 ml saline and mixed well and was labeled 10^{-3} dilution. The procedure was continued and upto 10^{-7} dilution was prepared for each sample.

The microorganisms were cultured on Nutrient Agar (HiMedia, Mumbai) plates for the quantitative assay. From the serially diluted suspensions, 0.1 ml each of the sample from 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions were added on to Nutrient Agar plates and spread evenly on the agar surface using an L-shaped sterile glass rod. The plates were incubated at 37°C overnight. The colonies developed on the plate for each dilution were counted and recorded.

For enumeration of fungi, 0.1 ml each of serially diluted samples from 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions were

plated on to the surface of Potato Dextrose Agar (HiMedia, Mumbai). The plates were incubated at 30 °C for 3-5 days and the colonies developed were enumerated and recorded.

Gram Staining

The bacterial cultures on Nutrient Agar plates were studied for their Gram reaction by using the standard procedure of Gram staining.[12] The smear was observed under 100X objective of a bright field microscope (Olympus).

Lactophenol Cotton Blue Staining for Identification of Fungi

The fungal strains were stained using Lactophenol Cotton Blue staining following the procedure of Aneja.[12] A drop of LCB stain was placed on a clean glass slide. A tuft of fungal mycelia was collected from the PDA plate using a sterile needle and placed in the stain. The mycelia was teased into separate hyphal filaments and allowed to stand in the stain for 1 minute for the hyphae to take up the stain. A cover slip was placed over the preparation carefully, without the formation of air bubbles inside. The preparation was observed under 10X and 40X objectives of a bright field microscope.

RESULTS

The present study focused on the enumeration of the microbial load of ladies shoes of various make collected from respondents of age 18-20. For this the shoe microflora including bacteria and fungi was quantitatively assayed from an area of 5 cm^2 inside the shoes made of materials like leather, rubber, plastic, canvas and athlete shoes. The maximum level of bacterial load was obtained for leather shoes (318 x 10^5 cfu /5 cm^2 shoe area). The least number of bacteria was obtained for rubber shoes followed by plastic shoes (7 x 10^5 cfu /5 cm^2 area and 38 x 10^5 cfu /5 cm^2 area, respectively). An average bacterial count of 240 x 10^5 cfu /5 cm^2 shoe area was obtained for canvas shoes and for athlete shoes an average bacterial count of 167 x 10^5 cfu /5 cm^2 area was obtained (Table 1).

Table 1: Enumeration of Bacteria from Shoes of Various Kinds and Make.

Shoe type	Bacterial Load in Shoe Microbiome expressed as cfu/5 cm^2 shoe area
Leather	318 x 10^5
Rubber	7 x 10^5
Plastic	38 x 10^5
Canvas	240 x 10^5
Athlete	167 x 10^5

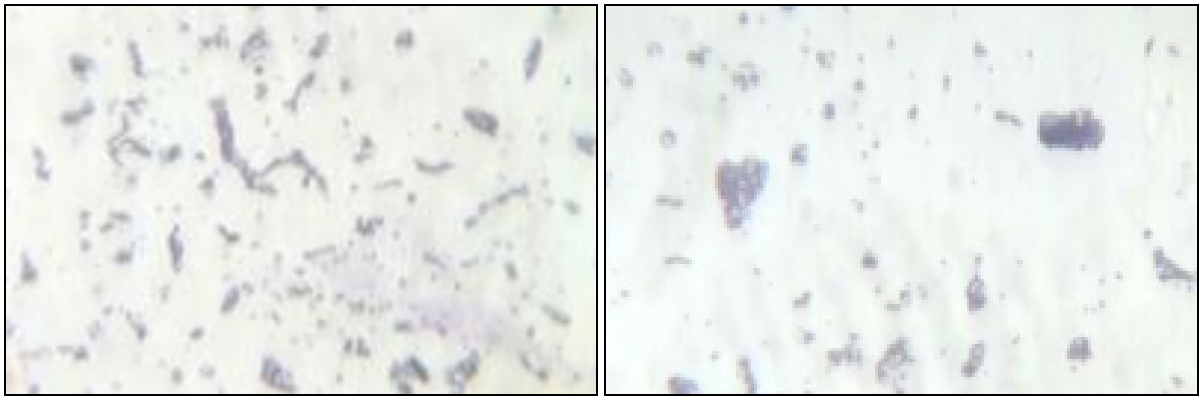
The fungal load also varied for the different varieties of shoes studied. The average mold count calculated using the fungal colonies obtained on PDA was highest for shoes made of leather where a confluent growth of fungi was obtained for all the three dilutions of $10^{-4,}$ 10^{-5} and 10^{-6}. An average mold count of 16 x 10^7 cfu /5 cm^2 shoe area was obtained for leather shoes while using 10^{-7} dilution for the enumeration. This was followed by athlete and rubber shoes with an average microbial count of 67 x 10^5 and 57 x 10^5 cfu /5 cm^2 area of shoes, respectively. An average mold count of 33 x 10^5 cfu /5 cm^2 shoe area was obtained for canvas shoes. The least fungal growth was obtained for plastic shoes with a fungal load of 19 x 10^5 cfu /5 cm^2 shoe area.

Table 2: Enumeration of Fungi from Shoes of Various Kinds and Make.

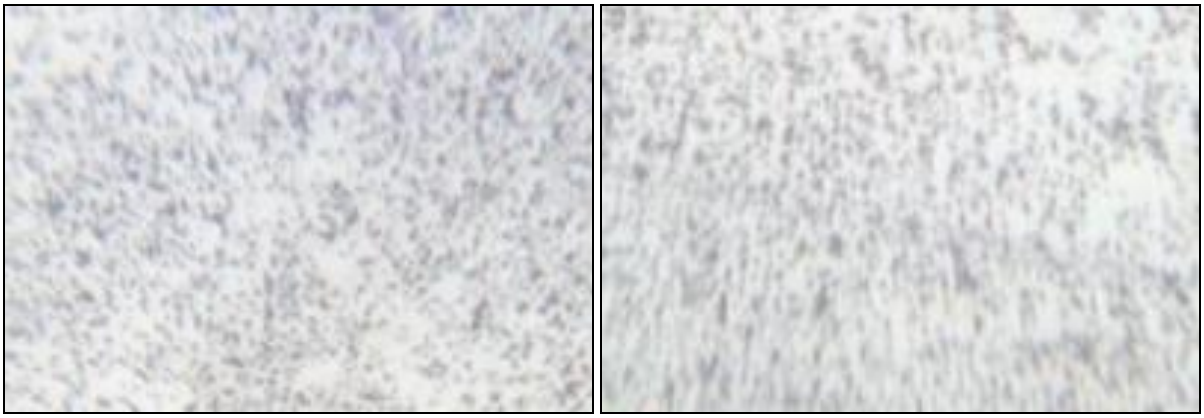
Shoe type	Fungal Load in Shoe Microbiome expressed as cfu/5 cm^2 shoe area
Leather	16 x 10^7
Rubber	57 x 10^5
Plastic	19 x 10^5
Canvas	33 x 10^5
Athlete	67 x 10^5

This study also tried to make a primary level identification of the bacterial and fungal strains prevailing in the various shoe types by observing the colony morphology as well as the staining reactions (Figure 1). The bacterial load in plastic shoes was found to include both Gram positive cocci and rods. Both Gram positive cocci in clusters characteristic of *Staphylococcus* and Gram negative rods were identified from bacteria isolated from rubber shoes. In leather shoes where the highest load was observed for bacteria, the major bacterial types included Gram positive cocci in clusters characteristic of *Staphylococcus*, Gram positive spore formers characteristic of *Bacillus* and Gram negative rods. Both Gram positive and Gram negative rod shaped bacteria were observed among the microflora of canvas shoes. The athlete shoes also bore Gram positive and Gram negative rods as well as Gram positive cocci.

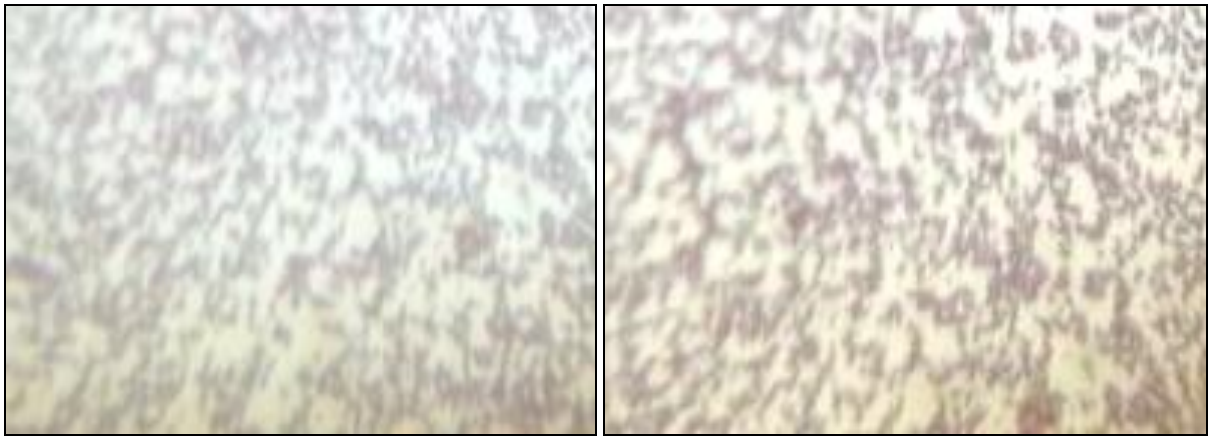
The fungal strains obtained from the various shoes types were identified after observing their colony morphology on PDA and spore morphology after LCB staining. In this process *Aspergillus, Penicillium and Helminthosporium* were undoubtedly identified from the various shoes types (Figure 1). *Aspergillus* and *Penicillium* were identified from plastic shoes while *A. niger* was identified from rubber shoes. Profuse growth of a fungal strain showing orange pigmentation was obtained from leather shoes which were not identified. However the leather shoes also contained *A. flavus* and *A. niger.* Fungal strains of *A. niger, A. flavus and Penicillium* were identified from the microflora of canvas shoes. The fungi *A. flavus, Penicillium* and *Helminthosporium* were identified to be present on athlete shoes.



Gram-positive cocci in clusters

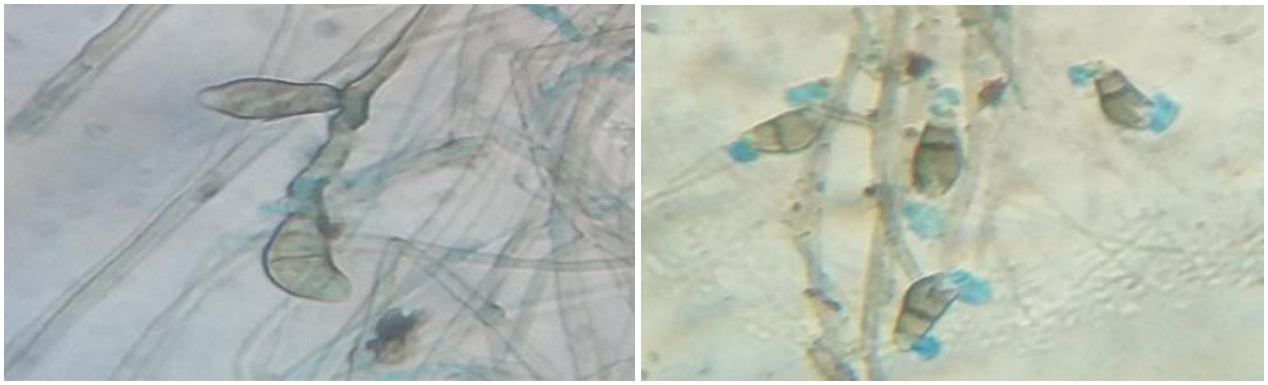


Gram-positive rods

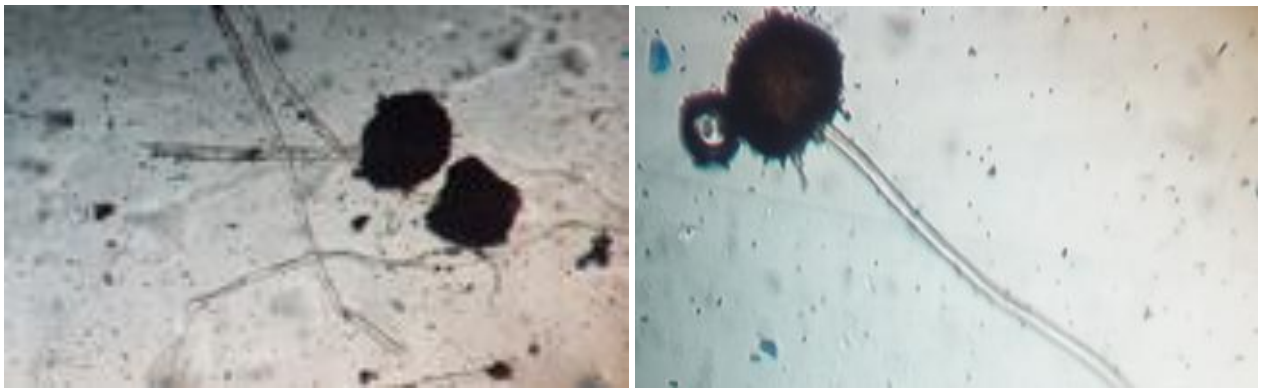


Gram-negative rods

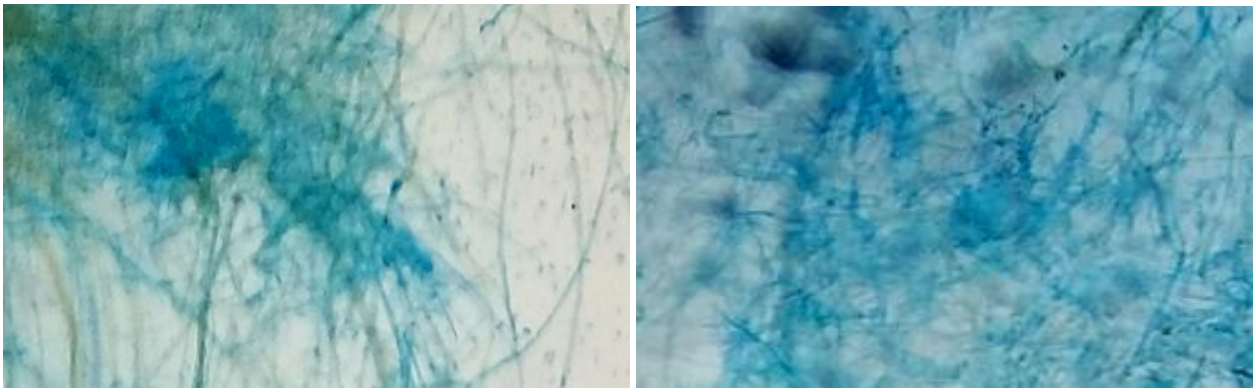
Figure 1: Gram reaction of bacteria obtained from footwear



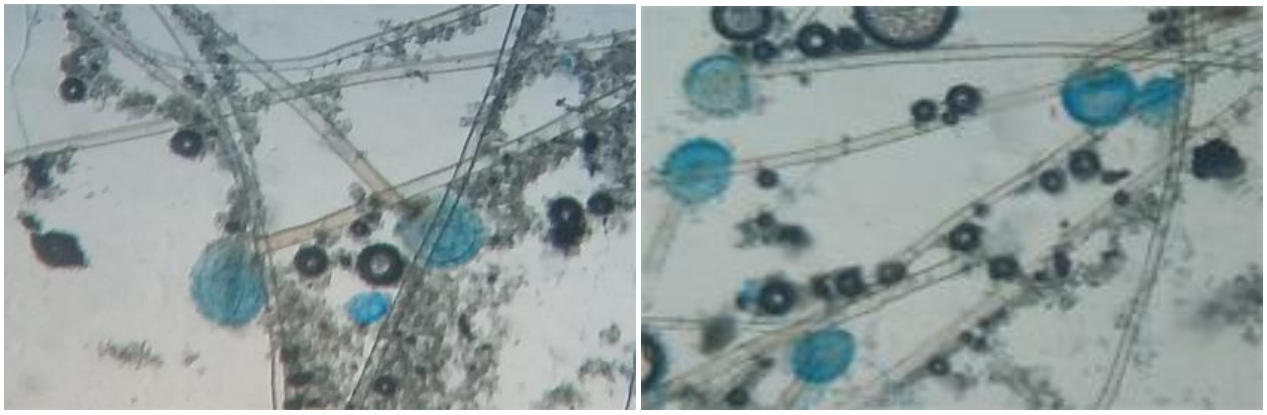
Helminthosporium



Aspergillus niger



***Penicillium* SP.**



Aspergillus flavus

Figure 2: Spore morphology after Lactophenol Cotton Blue staining of fungi isolated from footwear

DISCUSSION

Microorganisms are small, diverse and often specific to certain environments, organisms or individuals. Microbial communities show unique structure and composition. However, it varies with surface type, identify of the person interacting with the surface, and geographic location. The footwear we use can take in microbiota from the ground surface, that result in more diverse microbiome, characterized by a community of microorganisms that may be healthy or defensive. Studies have shown that microbial assemblages developed on the surface of human associated objects such as phones and shoes may also come from individuals who leave their skin microbiome on the surface of these materials. Hence the microbial communities on the surface of these objects are potentially valuable for forensic applications.[4] Microbial ecology of footwear fluctuates from person to person; this can be used as microbial signatures for the identification of individuals.

Studies have shown that skin health and human microbiome are affected by the type of sandals we wear.[10] Wearing shoes and socks create a humid environment that encourages the growth of fungus. The present study enumerated, and also comparatively analyzed, the variations in microbial flora of ladies footwear made of rubber, plastic, leather, canvas and athlete shoes of respondents at an age group of 18-20. The microbiome of an area of 5 cm^2 each on the inside surface of various footwear types was collected using sterile moist swabs, serially diluted and plated on Nutrient Agar and PDA, respectively, for bacteria and fungi.

The rubber footwear had a microbial load of 7 x 10^5 cfu of bacteria and 57 x 10^5 cfu of fungi on 5 cm^2 area of its surface inside (Tables 1 and 2). The microbial load of plastic shoes were also of the same order on 5 cm^2 area with 38 x 10^5 cfu of bacteria and 19 x 10^5 cfu of fungi, respectively (Tables 1 and 2). Both these materials are comparatively less attacked by bacteria and fungi due to their complexity in structure.[11] The microbial communities present on these footwear types are most likely contributed by the transient flora getting on to their surface from the floor and ground soil and also from the normal flora of human skin, particularly those on the foot.

The microbiome levels were extremely higher, even in the higher dilutions, for leather shoes. The bacterial load of leather shoes was obtained as 318 x 10^5 cfu (Table 1). Mold count was also higher for leather shoes. All the three dilutions from 10^4 to 10^6 produced confluent or mat like growth on the surface of PDA. However, a fungal load of 16 x 10^7 cfu was observed for the leather shoes (Table 2). The fungal species *A. flavus* and *A. niger* were identified from leather shoes in this study. Leather is an animal product made from the hide or skin of animals that contain 33 % protein, 1.5 % lipid and 65 % water.[11] The higher levels of bacterial and fungal load on leather shoes could be attributed to the rich levels of organic matter present in the material that support the growth of microorganisms. Also, the storage of leather materials such as bags, shoes, belts under high humidity favours the growth of fungi.[11] Microbes may also enter on to leather from the ground surface. Microbes decompose leather and produce foul smell. Formation of coloured spots and dull spots has been observed on leather products due to the microbial degradation of colouring substances. Halotolerant bacteria such as *Micrococcus luteus*, *M. roseus* and *Halococcus* sp. has been reported to produce yellow to red spots on salted raw hide. Finished leather or their products is deteriorated by *Aspergillus* and *Penicillium*.[11]

The microbiome of canvas shoes may come from the deteriorating bacteria and fungi or those entering from soil, floor surface and also from the skin flora of the person who wears it. In the present study a mold count of 33 x 10^5 cfu was obtained for canvas shoes. (Table 2). The bacterial flora was also observed in large numbers on the inner surface of canvas shoes (240 x 10^5 cfu/5 cm^2 area inside shoe surface, Table 1). The canvas shoes used in this study was of cloth make which could absorb moisture and the associated microorganisms from the floor surface. The microcapillaries in clothes serve as reservoir niche where rich supply of moisture and nutrients accumulate and it is extremely favourable for the growth of microorganisms. Textile fibres may be attacked by microorganisms under moist and warm conditions or where they come in contact with the soil. Bacteria and fungi capable of producing cellulase break down the cellulose molecules in textiles into cellobiose and glucose.[11] Cloth also contains other organic nutrients that could be utilized by microorganisms for their growth.

The athlete shoes are of covered type rather than open sandals. This will provide a humid or moist micro-environment inside the shoes which is beneficial for the growth and multiplication of microorganisms. Also, the microbes entering in to it from the floor surfaces or human skin may get retained inside it. Wearing socks along with such closed type shoes enhances sweating and increases the chance for microbial multiplication on the shoe surface inside. In the present study the bacterial and mold numbers was considerably higher in athlete shoes. The bacterial and mold counts obtained for athlete shoes in the current study were of the order 167 x 10^5 cfu and 67 x 10^5 cfu respectively per 5 cm^2 surface area (Tables 1 and 2).

Footwear can be potential source of pathogens that may pose serious safety risks if not properly maintained. The deadly pathogenic *Listeria monocytogenes* has been detected in a footbath located in a processed meat plant.[10] Besides enumerating the microbial load, the present study also tried to identify the kinds of bacteria and fungi present on the surface of various foot wear

types. For bacterial identification Gram staining was carried out to differentiate bacteria into Gram positive and negative groups. For identification of fungi the colony morphology on PDA and also the spore morphology after Lactophenol Cotton Blue staining were employed. Both Gram positive and Gram negative bacteria were observed in almost all the foot wear types used in this study. The plastic and canvas shoes contained Gram positive cocci and rods. Gram positive cocci in clusters characteristic of *Staphylococcus* were identified from the surface of footwear made of leather, plastic, rubber and athlete shoes. Gram positive spore forming rods characteristic of *Bacillus* were identified from the surface of shoes made of leather. In spite of their similarity in Gram reaction, the colonies of bacterial strains obtained in the present study varied considerably for the various shoe types. Studies have to be conducted further for identifying the biochemical properties of the bacteria for them to be identified to the genus and species level.

The fungal microbiome contained *Aspergillus* sp. in all the footwear types of the present study. *A. niger* was identified from rubber sandal and canvas shoes. *A. flavus* was isolated from canvas and athlete shoes. *Penicillium* was also isolated from the various footwear types, particularly from plastic, canvas and athlete shoes. Athlete shoes also contained species of *Helminthosporium* on their surface inside. A confluent growth of orange pigmented fungi was obtained in case of leather shoes which often overgrew the other types.

The revelations on personalized nature of the human microbiome and the distinct community types associated with the varied environments and the personal belongings will probably find great application in forensic investigations in the near future. The current study has laid a foundation in this area by identifying and enumerating the microbiome present on the surface of various footwear types. The study has shown that the pattern and number of microbes and the type of microbial communities found on the surface of different shoe types are considerably varied. This is a preliminary approach that obviously has its own limitations such as the small sample size used for enumeration and mere primary level identification of microbes on the footwear surface, without exploring into the generic and specific level of the organisms. Also in this study the foot microflora of owners of the footwear were not collected which would have possibly helped in the identification of the source of these microorganisms. Furthermore, studies are also needed to determine how far the microbiome community is practically useful in individual identification.

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