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Understanding the Role of Anaerobic Microbes in an In Vitro Skin Model

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EXECUTIVE SUMMARY

Military personnel that have sustained extremity injuries can develop infectious complications with substantial morbidity. Approximately 80% of bacterial infections and infection persistence are associated with microbial biofilms, as they significantly prevent the penetration of antimicrobial drugs, resulting in the development of resistance towards antibiotics. The microbial bioburden of chronic wounds, specifically the aerobic microbial population, has been extensively investigated; however, few studies regarding the anaerobic component (approximately 38% of the microbial population) have been performed. Chronic wounds have poor blood flow and are hypoxic in nature; delineating the role of anaerobic bacteria in chronic wound infection is of growing interest, but is limited by the challenges posed in both in culturing and characterization. In this study, three commercially available anaerobes (namely *Anaerococcus murdochii, Bacteroides vulgatus*, and *Fusobacterium necrophorum)* were investigated for the first time for their ability to form biofilms, influence motility of skin cells, and adhere to the in vitro skin wound model (Human Epidermal Keratinocyte Cells (HEK)). It was observed that all three anaerobes may have different roles to play in a chronic wound infection as (a) they form biofilms of varying adherences, (b) do not affect the motility of the cells in the given experimental conditions, and (c) all the three anaerobes were able to stimulate the formation of vesicular structures in the nucleus of HEK cells upon 15 min of incubation, suggesting that the anaerobes induce a response in the skin cells upon exposure. Therefore, this study further validates the important role of anaerobes in persistent chronic wound infections.

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UNDERSTANDING THE ROLE OF ANAEROBIC MICROBES IN AN *IN VITRO* SKIN MODEL

1. INTRODUCTION

Military personnel that have sustained extremity injuries can develop infectious complications with substantial morbidity. Approximately 80% of bacterial infections and infection persistence are associated with microbial biofilms, as they significantly prevent the penetration of antimicrobial drugs, resulting in the development of resistance towards antibiotics. The microbial bioburden of chronic wounds, specifically the aerobic microbial population, has been extensively investigated; however, few studies regarding the anaerobic component (approximately 38% of the microbial population) have been performed. Chronic wounds have poor blood flow and are hypoxic in nature; delineating the role of anaerobic bacteria in chronic wound infection is of growing interest, but is limited by the challenges posed in both in culturing and characterization. In this study, three commercially available anaerobes (namely *Anaerococcus murdochii, Bacteroides vulgatus*, and *Fusobacterium necrophorum)* were investigated for the first time for their ability to form biofilms, influence motility of skin cells, and adhere to the in vitro skin wound model (Human Epidermal Keratinocyte Cells (HEK)). It was observed that all three anaerobes may have different roles to play in a chronic wound infection as (a) they form biofilms of varying adherences, (b) do not affect the motility of the cells in the given experimental conditions, and (c) all the three anaerobes were able to stimulate the formation of vesicular structures in the nucleus of HEK cells upon 15 min of incubation, suggesting that the anaerobes induce a response in the skin cells upon exposure. Therefore, this study further validates the important role of anaerobes in persistent chronic wound infections.

2. BACKGROUND

The pathogenic role of anaerobic bacteria in chronic wounds has generated a lot of interest with the perspective of developing novel wound treatment regimens and materials. Treatment failure and wound infection result in increased trauma, medical costs, and demands for better resources and understanding of the infection [1]. Chronic wounds are wounds that have failed to heal after 3 months of appropriate wound care; it is postulated that a diverse collection of bacteria may contribute towards delayed healing [2]. Chronic wounds are characterized by decreased blood flow and oxygen levels, emulating an environment that supports the proliferative growth of anaerobes. Research groups performed 16S ribosomal RNA gene sequencing to determine the microbial community present in these environments; it was observed that the wound microbiome is composed of obligate and facultative anaerobes. Some of the commonly identified anaerobes include strains from genera *Anaerococcus* [2], *Bacteroides* [3], and *Fusobacterium* [4]. The primary goal of this study was to use three commercially available anaerobic bacterial strains commonly found in chronic wounds and determine a) biofilm development, b) effect on motility of skin cells, and c) adherence to the wound model. The three anaerobes used in this study are *Anaerococcus murdochii*, *Bacteroides vulgatus,* and *Fusobacterium necrophorum*. A commercially available epidermal keratinocyte cell line was used as the wound model because keratinocytes constitute the major cell type of the

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epidermis (the outermost layer of the skin). Developing an understanding of the adherence and biofilm formation ability of the anaerobic microbial component can pave way to minimizing chronic wound infection. The data obtained from this study can be used to identify critical anaerobic bacterial biofilm development factors, which are essential to the persistence of chronic wounds.

3. MATERIALS AND METHODS

3.1 Cell Culture

The normal adult Human primary Epidermal Keratinocyte (HEK) cell line used in this study was obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in a Dermal Cell Basal Media (ATCC PCS-200-030) supplemented with a Keratinocyte Growth Kit (ATCC PCS-200-040) that contained the following growth supplements: Bovine Pituitary Extract (BPE), rh TGF-α, L-glutamine, hydrocortisone hemisuccinate, insulin, epinephrine, and apo- transferrin. Phenol red (ATCC PCS-999-001) was added as a pH indicator. The cells were maintained in a 37˚C, 5% CO_2 incubator. The media was replenished every other day after washing the cells twice with room temperature 1x PBS (ATCC).

3.2 Microbial Growth

The anaerobic bacteria used in this study (*Anaerococcus murdochii* (ATCC BAA-1385), *Bacteroides vulgatus* (ATCC 8482), and *Fusobacterium necrophorum* (ATCC 25286)) were obtained from ATCC in a lyophilized vial and stored at room temperature. Microbes were reconstituted according to the ATCC protocol in TSB media. The strict anaerobes were cultured in an anaerobic chamber (Coy Laboratory Products Inc, Grass Lake, MI) with an 80% nitrogen, 10% hydrogen, and 10% CO_2 gas mixture at room temperature. The microbes were propagated either on a solid support, Tryptic Soy Agar (TSA) plate (DF0369-17-6 Fisher Scientific, Pittsburg, PA); TSA with defibrinated sheep blood (B21239X , Fisher Scientific, Pittsburg, PA); or as liquid culture in Tryptic Soy Broth (TSB) media (DF0370-17-3 Fisher Scientific, Pittsburg, PA). For maintenance, the microbes were subcultured every 2 weeks and the cultures were subcultured 72 hr prior to assay performance in TSB.

3.3 Biofilm Formation Assay

The biofilm formation assay was performed using two different protocols. The first protocol was adapted from Patterson et al [5]. The cultures were diluted 1:10 with fresh TSB media; 200 µl of the diluted culture was added to each well of a 96 well plate (One row (9 wells) per microbe). Three additional rows with 200 µl of media and no microbes served as a negative control. The plate was incubated for 72 hr at room temperature in the anaerobic chamber. After 72 hr, the spent medium was pipetted out and the wells were washed once with 200 µl of 1x PBS at room temperature and air dried for 1 hr at room temperature. The plates were stained for 5 min with 200 µl of 2% crystal violet (Sigma-Aldrich, St. Louis, MO) and then rinsed with water to remove the excess stain, then allowed to dry for a few hours at room temperature. Glacial acetic acid (Fisher Scientific, Pittsburg, PA) was added to each well (150µl of 33% (v/v)) to solubilize the bound crystal violet. The optical density (OD) of the plate was read at 590 nm using a microplate reader (BioTek, Winooski, Vermont). This assay was performed in triplicate.

The second protocol was adapted from Donelli et al [6].

On the day of the assay, the OD of the cultures were measured; the study used cultures of 0.5 MCF (McFarland standards). Twenty microliters of the culture were pipetted into each well of the 96 well plate (USA Scientific, Ocala, FL) for a total of three rows (27 wells) for each microbe. Fresh TSB media (180 µl) was added to each well, bringing the total volume in each well to 200 µl. Three additional rows with just 200 µl media and no microbes served as a negative control. The plates were placed in the anaerobic chamber at room temperature for 72 hr. After 72 hr, the plates were removed from the anaerobic chamber and the contents of each well were pipetted out. The wells were rinsed with 200 µl of 1x PBS twice and left to dry overnight at room temperature.

The rest of the protocol was similar to the previous biofilm protocol. This assay was performed in triplicate. The degree of biofilm adherence was calculated as described in [7].

The cut-off OD (ODc) is calculated as three standard deviations above the mean OD of the negative control. According to the defined ODc, the anaerobes were classified on the basis of their adherence ability into the following categories:

- Nonadherent: (OD ≤ ODc)
- Weakly adherent (ODc < OD ≤ 2 ODc)
- Moderately adherent (2ODc < OD ≤ 4 ODc)
- Strongly adherent (4 ODc < OD)

3.4 Biofilm-conditioned Medium

The OD of the cultures were measured at 600 nm and the cultures were diluted to 0.05. Tissue culture inserts (27.85 mm diameter, 0.4 µm pore size; USA Scientific, Ocala, FL) were inoculated with 10 µl of the microbial cultures. Three inserts for each microbe were used. The inserts were then placed in a 6-well plate containing 2 ml of TSB media in the anaerobic chamber at room temperature. The TSB media in the 6-well plates were collected after 24 hr and fresh media was added to the plate. This was repeated for a total of 72 hr of incubation. A total of three time points (24 hr, 48 hr, and 72 hr) were collected and were used as Day 1, 2, and 3 Biofilm-conditioned Medium (BCM) respectively for the scratch assay. TSB media not exposed to the bacterial biofilm was collected as negative control.

3.5 Scratch Assay

HEK cultures were grown in 6-well plates (240,000 cells/well) for 2 days to achieve 80-90% confluency. The cells were rinsed once with 1x PBS at room temperature. A 200 µl pipette tip was used to scratch the culture, following which the cells were rinsed with 1x PBS at room temperature to remove any cellular debris. Two milliliters of Dermal Cell Basal Media (DCBM) were added to the wells and the scratched cultures were imaged on the Nikon Eclipse TS100 microscope using the NIS Elements Imaging software (Nikon Instruments Inc. Melville, NY) to obtain the initial scratch area. This was considered Day 0. Soon after, Day 1 BCM was placed in a 6-well insert and placed above the scratched cell culture. The plate was then placed in an anaerobic chamber at room temperature. After 24 hr, Day 1 BCM was replaced with Day 2 BCM. Representative images were taken. After 48 hr of Day 2 BCM co-culture, Day 2 BCM was replaced with Day 3 BCM and was allowed to incubate for an additional 72 hr, and representative images were taken. All images were taken using a 4X Objective on the Nikon Eclipse TS100 microscope. This assay was done in triplicate.

3.6 Microbial Adherence Assay using Differential Interface Contrast and Atomic Force Microscopy

HEK cells (1.5 x 10^5 cells) were seeded in a fluorodish coated with poly-D-lysine and incubated for 48 hr until a monolayer of cells formed. The media was removed and the cell layer was rinsed with 1x PBS at room temperature twice, after which 2 ml of fresh DCBM were added to the dish. The microbial cultures were subcultured 72 hr prior to the assay. The OD of the cultures were measured at 600 nm and the cultures were diluted to obtain an OD_{600} of 0.5. The microbial cultures (10 µl) were pipetted on to the fluorodish and wrapped using parafilm, and then spun down in a centrifuge at 1200 rpm for 30 sec. The fluorodishes were placed back in the anaerobic chamber for 15 min to facilitate microbial adherence. After the incubation, the fluorodishes were rinsed with 1x PBS at room temperature twice and fixed with 2% glutaraldehyde (Sigma - Aldrich, St. Louis, MO) in 1x PBS for 30 min. The cells were rinsed with 1x PBS twice, and 2 ml of 1x PBS were added to the dishes prior to analyzing the samples using Differential Interference Contrast (DIC) and Atomic Force Microscopy (AFM).

3.7 Differential Interface Contrast and Atomic Force Microscopy

The bacterial and HEK cells used in this study were imaged using both the Nanowizard 4a AFM (JPK Instruments AG, Berlin, Germany) mounted on an Eclipse Ti-E inverted microscope (Nikon Instruments Inc. Melville, NY) for AFM, and the Eclipse Ti-E inverted microscope for DIC. The samples were analyzed at room temperature using qp-BioAC-CB3 AFM probes (NANOSENSORS, CH-2000 Neuchatel, Switzerland) in Quantitative Imaging (QI) mode with a set point of 400pN. The DIC images were obtained at 100X magnification.

4. RESULTS AND DISCUSSION

4.1 Culturing of Bacterial and Mammalian Cells

For this exploratory study three anaerobes were investigated for (a) their ability to form microbial biofilms, (b) influence HEK cell motility upon infection and (c) adhere to the surface of HEK cells. These processes facilitate bacterial colonization and could result in failure of wound treatment strategies. The three anaerobes were *Anaerococcus murdochii*, *Bacteroides vulgatus* and *Fusobacterium necrophorum*. *Anaerococcus murdochii* is a gram positive, coccus shaped, obligate anaerobe that was isolated from a foot ulcer and is 0.7 µm in size. *Bacteroides vulgatus* is a gram negative, rod-shaped anaerobe and is 0.5–15 µm in size, while *Fusobacterium necrophorum* is a gram negative, rod-shaped, obligate anaerobe that is 0.7–10 µm in size. All three anaerobes are slow growing microbes and have an incubation period of 72 hr. This was a challenge for this particular study as optimum culturing conditions (an anaerobic environment with ambient room temperature) for extended periods were difficult to achieve. To delineate the pathogenic role of the anaerobes, the HEK cell line was identified to perform pathogen-cell interaction studies. The HEK cells used in this study were derived from normal human adult skin (ATCC). The cells were well-rounded and had a cobblestone appearance. They had a high mitotic index; therefore, when the cultures reached approximately 80% confluency, the cells needed to be subcultured, since keratinocytes begin to terminally differentiate once they become 100% confluent and do not serve as a good cell line model to perform microbial studies. The most challenging part of this study was interfacing anaerobes with the HEK cells, as the HEK cells cannot withstand an environment devoid of oxygen for an extended period of time. Minimal real-time interaction studies were performed to explore the pathogenic role of the

anaerobes. However, assays that addressed the overall aim of this study were successfully executed in conjunction with the HEK cells.

4.2 *A. murdochii, B. vulgatus, and F. necrophorum* Form Biofilms

Recurrent infections by several pathogens are characterized by their ability to form biofilms; they are surface associated microbial communities shown to contribute towards antibiotic resistance and oxygen stress [8, 9]. There are limited studies that have evaluated biofilm formation by pathogenic anaerobes; therefore, it was imperative to determine if the selected anaerobes for this study could generate biofilms. For this study, two different protocols were identified to evaluate biofilm formation. This first protocol, adapted from Patterson et al [5] confirms the formation of biofilms and the second protocol adapted from Donelli et al [6] determines the degree of adherence for each of the biofilms formed. The anaerobes were seeded in 96-well plates, as mentioned in the Materials and Methods section, and the assay was performed. All three anaerobes formed biofilms and were adherent in nature (Fig 1A). No biofilm formation was observed on the surface of the culture medium. It was observed that *F. necrophorum* had a higher OD590 value than *A. murdochii* and *B. vulgatus*. All anaerobes exhibited different degrees of biofilm development in the wells. This indicates the potential of clumping of cells in liquid culture, which could lead to different degrees of biofilm formation. The ability of an anaerobe to form a biofilm validates its ability to colonize and contribute towards an infection [10]. This is the first report to show that anaerobes *A. murdochii*, *B. vulgatus,* and *F. necrophorum* can form biofilms; this adds credence to the idea that these microbes are essential to the sustainability of chronic wounds. To further quantify this observation, the biofilm assay was performed again using a modified protocol adapted from Doneli et al [6] (Fig 1B).

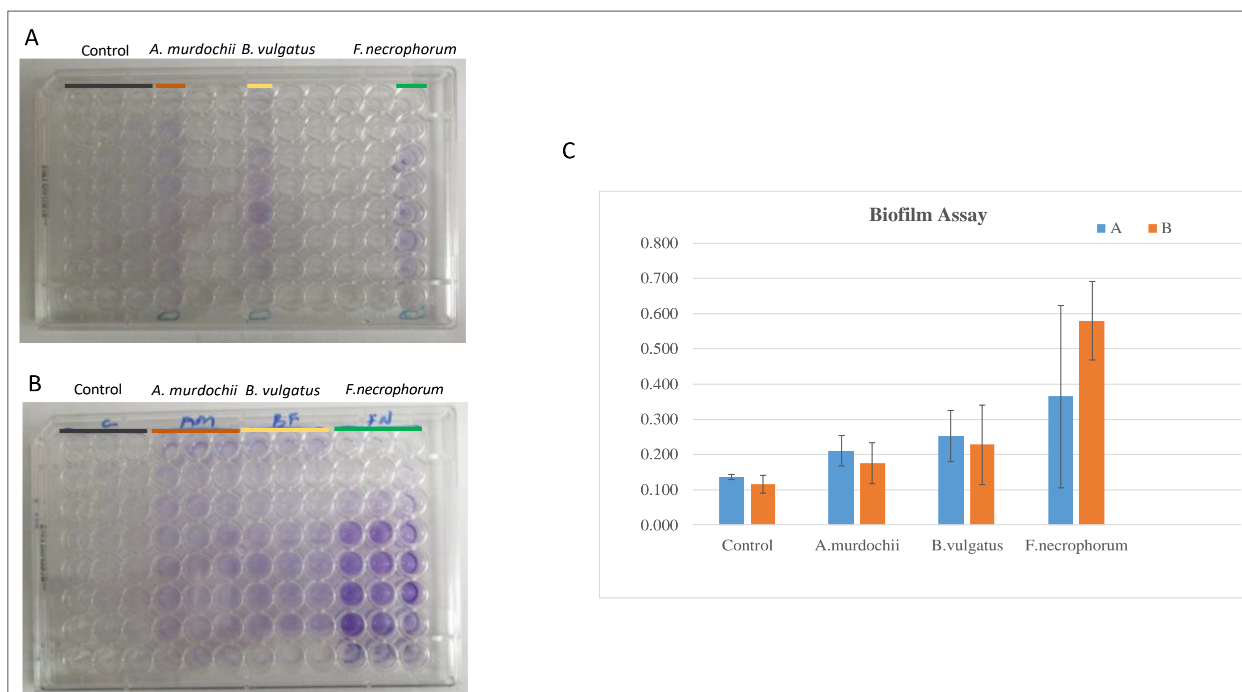

Fig. 1—Biofilm formation assay: Microbes were analyzed for their ability to form biofilms using two different protocols. (A): All three anaerobes form biofilms that are adherent at the base of the well but are not of uniform thickness. (B): The degree of adherence was determined by measuring the cut-off ODC. (C): The absorbance values (OD590) from both assays were plotted together; it is evident that *F. necrophorum* formed a stronger adherent biofilm as compared to *A. murdochii* and *B. vulgatus*.

The absorbance values were tabulated and the ODc value was determined to be 0.117 (Table 1). The 2 ODc and 4 ODc were calculated, and it was determined that *F. necrophorum* was strongly adherent while *A. murdochii* and *B. vulgatus* were weakly adherent. This assay thus corroborated with the previous observation that *F. necrophorum* might have a stronger ability to colonize to an *in vivo* wound environment when compared to *A. murdochii* and *B. vulgatus*.

Table 1—Quantification of Biofilm Assay: Biofilms Formed by the Three Anaerobes were Measured for Degree of Adherence Based off of the Cut-off OD (ODc)

	Control			***A. murdochii***			***B. vulgatus***			***F. necrophorum***		
A	0.115	0.128	0.126	0.245	0.245	0.264	0.211	0.317	0.253	0.123	0.13	0.129
B	0.106	0.12	0.125	0.172	0.304	0.165	0.217	0.163	0.496	0.374	0.418	0.432
C	0.107	0.115	0.127	0.174	0.155	0.154	0.232	0.189	0.23	0.649	0.528	0.401
D	0.114	0.116	0.121	0.153	0.132	0.139	0.171	0.209	0.179	0.573	0.541	0.636
E	0.111	0.115	0.119	0.151	0.144	0.157	0.176	0.193	0.201	1.12	0.592	0.7
F	0.108	0.116	0.121	0.15	0.139	0.147	0.159	0.223	0.257	0.643	0.728	0.736
G	0.111	0.114	0.121	0.156	0.146	0.171	0.152	0.207	0.281	0.949	0.819	0.714
H	0.109	0.115	0.119	0.174	0.165	0.167	0.174	0.26	0.313	0.948	0.565	0.481
Mean	0.110	0.117	0.122	0.171	0.178	0.170	0.186	0.220	0.276	0.672	0.540	0.528
Mean of triplicates			0.117			0.174			0.228			0.580
SD			0.006			0.044			0.073			0.259

ODc = 0.117 + (3*.006) = 0.135
2 ODc = 0.27
4 ODc = 0.5

Upon plotting the absorbance values obtained from both the biofilm assays (Fig 1C), it is evident that *F. necrophorum* formed a stronger biofilm as 4 ODc < OD (0.5 < 0.58) as compared to the weakly adherent *A. murdochii* and *B. vulgatus,* where the ODc < OD ≤ 2 ODc (0.174 and 0.228 respectively). Because the microbiomes analyzed from various chronic wounds comprise of a milieu of microbes, we can hypothesize that *A. murdochii* and *B. vulgatus* might play a different role in addition to adherence during wound infections. They might be responsible for providing other secreted factors/biomolecules to support colonization and invasion of microbes associated with chronic wounds, as both the anaerobes form weakly adherent biofilms. Because *F. necrophorum* has a strong biofilm formation capability *in vitro*, one might conclude this microbe is required for wound colonization. Further studies are required to explore the pathogenic role in chronic wounds, which involves identifying the key genes that facilitate adhesion and colonization.

4.3 *A. murdochii, B. vulgatus, and F. necrophorum* Biofilm-conditioned Mediums Do Not Affect Cell Motility

The scratch assay mimics a simple wound model system devoid of an extracellular matrix and immune components. It is most commonly used to investigate the direct role of a foreign agent on cells that

are involved in wound healing [11, 12]. In a controlled environment, the cells move into the region that was displaced by the scratch, and the rate of cell movement determines the impact of the foreign agent on cell motility. In this assay, direct anaerobe-cell interaction could not be performed because optimum environments for both could not be achieved simultaneously. Therefore, BCM was used for this assay. The BCM was placed above the scratched HEK cells, and the effect of the BCM exposure was analyzed over a period of 6 days. We observed no significant difference in the motility of the cells (Fig 2). HEK cells in the control well also did not migrate, which is indicative of the cell line characteristic where cells begin to terminally differentiate upon 70-80% confluency and therefore do not efficiently migrate. Other factors that include longer incubation times for BCM generation, such as presence of certain microbes that might activate various downstream signaling pathways that facilitate or inhibit cell motility, must be investigated. Additional assays involving cell death markers will also need to be performed as elevated cell death is observed in the BCM exposed wells. Therefore, this assay involved no significant difference in motility, and cell displacement was observed for HEK cells exposed to *A. murdochii, B. vulgatus* and *F. necrophorum* BCM when compared to the control.

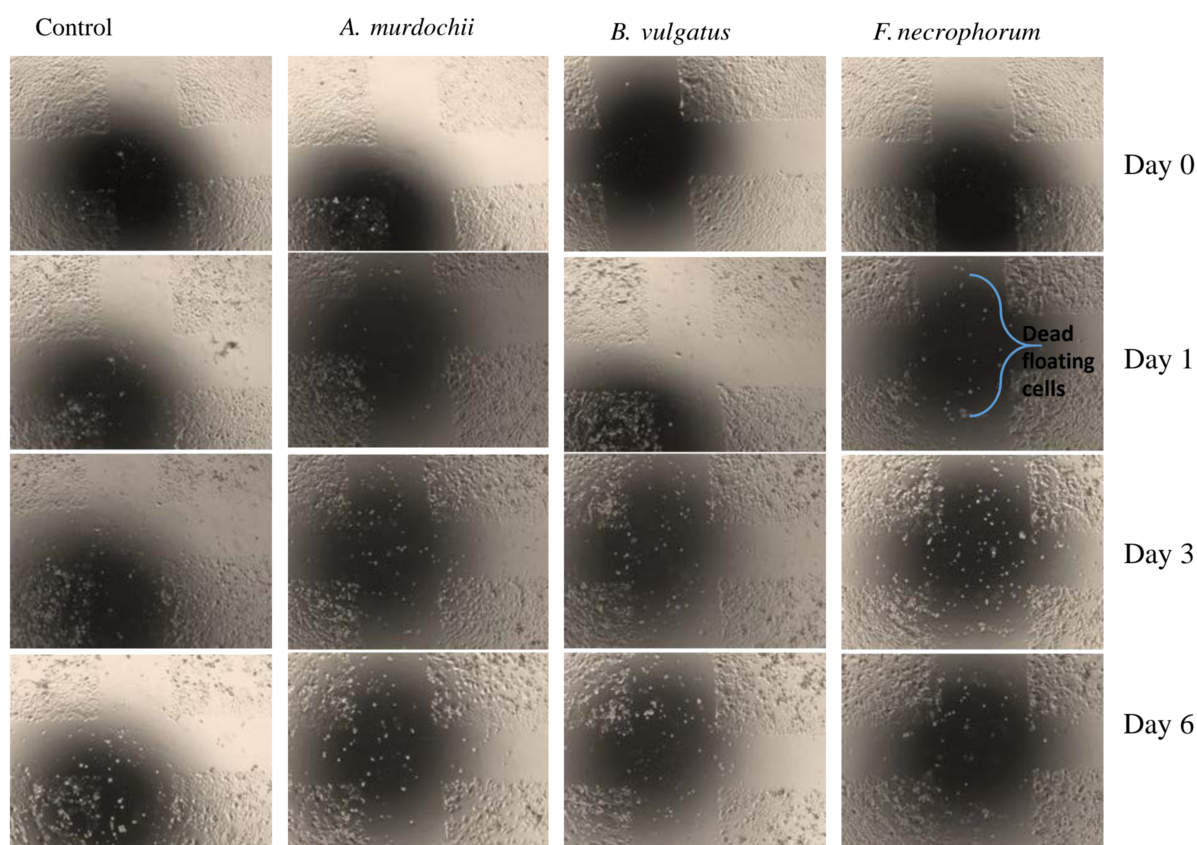

Fig. 2—Scratch assay: The anaerobes were analyzed for their ability to affect cell motility of HEK cells. This assay used BCM. No significant difference was observed in the rate of motility between the BCM exposed HEK cells and the negative control.

4.4 Incubation of HEK cells with the Individual Anaerobes Stimulates a Morphological Change in the Nucleus

The ability of *A. murdochii*, *B. vulgatus,* or *F. necrophorum* to bind to the HEK cells was analyzed using the microbial adherence assay; the cells were imaged using AFM. The OD of the microbial cultures

were measured and standardized to ensure that equal number of microbial cells were added to the HEK cells for 15 min. At the end of the timed experiment, the cells were fixed and analyzed using DIC & AFM. The DIC images (Fig 3B-D) indicate there were vesicular structures in the nucleus of the HEK cells that were incubated with the anaerobes, and these structures were not observed in the control cells (Fig 3A). The anaerobes, however, were not detected on the surface of the HEK cells (Fig 3F-H), indicating that the anaerobes did not bind to the cells and were washed away during the 1x PBS rinses. This assay suggests that a) longer incubation times might be required for the anaerobe to bind to the HEK cells (more than 15 min), and b) the presence of certain microbes or the biomolecules secreted by them might be required to facilitate increased adherence. The AFM images demonstrate that the control cells (Fig 3E) display increased roughness and are further spread out in nature than the anaerobe exposed cells that exhibit decreased roughness. The control cells are more circular than the anaerobe exposed cells, especially the nuclear region.

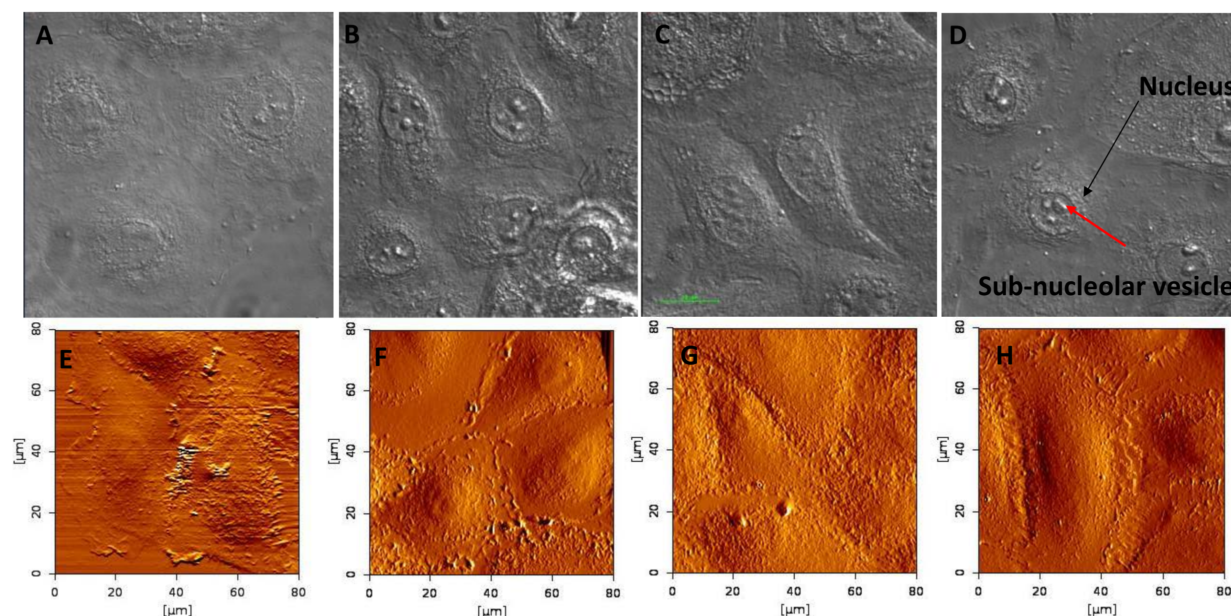

Fig. 3—Microbial adherence using DIC & AFM: The HEK cells were incubated with each of the anaerobes for 15 min and the cells were fixed after incubation. DIC images (A-D) and AFM images (E-H) are shown above. (A & E): Control cells that have not been incubated with any anaerobe. The cells appear flat with no vesicular structures in the nucleus. (B & F): Cells were incubated with *A. murdochii,* and the sub-nucleolar vesicles are observed in the DIC image. (C & G): Cells were incubated with *B. vulgatus,* and sub-nucleolar vesicles are also observed in the DIC image; however, these are not as prominent as cells incubated with *A. murdochii* and *B. vulgatus*. (D & H): Cells were incubated with *F. necrophorum,* and several sub-nucleolar vesicles are observed in the DIC image.

The appearance of sub-nucleolar vesicles in the HEK cells after 15 min of incubation with an anaerobic microbial culture suggest that the HEK cells were responding to the presence of the anaerobe in their immediate vicinity. Its speculated that the anaerobes could be releasing biomolecules into the media, priming the HEK cells for adhesion or colonization. Further studies are required to identify and investigate the biomolecules.

In conclusion, it was observed for the first time that all three anaerobes (*A. murdochii*, *B. vulgatus,* and *F. necrophorum)* previously determined to be a part of the chronic wound microbiome can form biofilms. Of the three anaerobes studied, *F. necrophorum* formed the strongest adhering biofilm, indicating this anaerobe had a higher colonization ability when compared to *A. murdochii* and *B. vulgatus*. This suggests that *F. necrophorum* may contribute towards drug resistance and oxygen stress, which are

features commonly observed in chronic wounds. None of the three anaerobes affected the motility of the cells, but further studies are required to determine if longer incubation periods or the presence of certain biomolecules are required to induce changes in motility. When performing the microbial adherence assay, the appearance of sub-nucleolar structures in the nucleus of the HEK cells suggests that the anaerobes are able to induce a response (morphological change) in the HEK cells within 15 min of incubation, even though we detected no binding. The observations from all the assays performed in this study suggest that all three anaerobes may have different roles to play in a chronic wound infection as (a) they form biofilms of varying adherences, (b) they do not affect the motility of the cells in the given experimental conditions, and (c) all the three anaerobes were able to stimulate the formation of vesicular structures in the nucleus of HEK cells upon 15 min of incubation, suggesting that the anaerobes induce a response in the skin cells upon exposure. Therefore, this study further validates the important role of anaerobes in persistent chronic wound infections.

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