Efficacy of BSafe HOCl® Facial Sanitiser with Ag^+ on the Skin of the Face Report

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1. ADMINISTRATIVE INFORMATION

Key Study Roles	Responsible Individual
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4. LIST OF ABBREVIATIONS

Ag^+	Silver ions
$AgNO_3$	Silver nitrate
AgNP	Silver nanoparticles
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CFU	Colony forming units
DNA	Deoxyribonucleic acid
ECHA	European Chemicals Agency
FDA	Food and Drug Administration
G3PD	glyceraldehyde-3-phospate dehydrogenase
GSH	Glutathione
GSSG	Glutathione disulphide
H_2O	Water
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
HOCl	Hypochlorous acid
IP	Investigational product
N	Population size
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
OH^-	Hydroxide
ROS	Reactive oxygen species
TSA	Tryptone soy agar

5. RESPONSIBLE PARTIES

Key Study Roles	Responsible Individual
Investigators/Study Statisticians	Dr. Aman Nowbuth MBChB, BSc (Human Biology) aman@bsafehocl.co.za Dr. Josh Armstrong MBChB, BSc (Hons) (Human Physiology), BSc (Human Biology) josh@bsafehocl.co.za
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6. ABSTRACT

Hypochlorous acid (HOCl) is a secondary reactive oxygen species (ROS) produced by phagocytic cells of the immune system. This molecule is produced during the respiratory burst during phagocytosis. HOCl has a well-documented antimicrobial spectrum and safety profile. Silver is a historical antimicrobial metal, which is currently used for domestic, commercial, and medical uses. The use of silver ions (Ag^{+}) is considered to be the safest modality of nanoscale silver to be used. Although silver is a highly efficacious antimicrobial molecule, safety precautions are to be taken into consideration. It is due to this that silver ions are being implemented in the product, in comparison to other nanoscale silver particles. However, there remain some safety concerns with the use of silver ions internally, thus, the product is only advocated for external use.

In this study, the efficacy of BSafe HOCl® Face Sanitiser was evaluated. This was done by first evaluating a baseline of colony forming units (CFUs) on a set of 26 participants. Thereafter, the same group of participants were applied BSafe HOCl® Face Sanitiser with a periodicity of two hours. This allowed for the immediate efficacy (measured after a period of 20 minutes), as well as the efficacy of multiple exposures of BSafe HOCl® Face Sanitiser, and finally, evaluation of the residual two-hour efficacy of BSafe HOCl® Face Sanitiser.

The results demonstrated a steady state in CFU counts over the first four-hours in the control group, followed by a 102.23% increase over the second four-hours. Comparison of the final counts to the initial counts revealed a 104.93% increase on average (N=18). Growth trends identified in the control study presented with the most frequent growth trend being a decrease followed by a rapid increase in CFU counts. The average rate of growth identified in this dataset was an increase of 110,26% over the eight-hour period. Over the second four-hour period, an increase of 811.11% was identified with the average values (N=6). The increase in the rate of bacterial proliferation over the final four hours of the workday may be attributed to increased levels of face touching, as well as the fact that the swab was taken well after the participants had lunch, thus increasing the potential for the deposition of nutrients for bacterial colonies to proliferate. It is of note however, that further research into this regard should be carried out for more accurate deductions.

The results obtained with application of BSafe HOCl® Facial Sanitiser showed an average decrease of 72.22% reduction after one application, with a final reduction of 98.13% after five applications (two-hourly apart) (N=18). This translated to a cumulative efficacy with multiple exposure. Review of the two-hourly residual efficacy demonstrated a 77.35% reduction with two applications, and a final 88.48% reduction calculated with four applications (N=11).

The most frequent trendlines seen with application of BSafe HOCl® Facial Sanitiser for both the multiple exposures and residual efficacy was a continuous decrease (Multiple exposure N=7; residual efficacy N=6). A comparison of control to the residual efficacy was done, as these values were obtained at the same time on the same participants over the two-day period. This comparison showed that the control CFU levels remained relatively similar through the day, with the residual efficacy demonstrating a rapid reduction over the eight-hour period (N=10). Additionally, the residual efficacy followed near the calculated exponential trendline.

From the results obtained, P-values calculated were 0.000058 for multiple exposure testing, and 0.023185 for residual efficacy. With these values in mind, it can be concluded that the results obtained are statistically significant (as $P < 0.05$). Furthermore, the calculated F-ratios were 9.18838 and 4.57066, respectively. As these ratios are >1.0, it can be said that the reductions in CFU levels for the multiple exposure testing and residual efficacy are not coincidental, thus, are due to the use of BSafe HOCl® Facial Sanitiser.

7. BACKGROUND AND RATIONALE

7.1. Product(s)

7.1.1. Active Ingredient(s)

- Hypochlorous Acid (HOCl)
    - FDA CAS Number: 7790-92-3
    - ECHA EC/List No. 232-232-5

- Nano Silver in a complex form
    - FDA Cas Number: 14701-21-4

7.1.2. Mechanism of Action

7.1.2.1. Hypochlorous Acid

Hypochlorous acid (HOCl) is a secondary reactive oxygen species (ROS) produced by the immune system, through the respiratory burst occurring during phagocytosis. Cells producing HOCl within the immune system are:

- Neutrophils
- Mast cells
- Macrophages

Macrophages have various names depending on the tissue in which they are found. As such, the following cells can be said to produce HOCl:

- Lung: Alveolar macrophages
- Bone: Osteoclasts
- Brain: Microglia
- Connective tissue: Histiocytes
- Liver: Kupffer cells
- Skin: Langerhans cells

The pathway of production is as follows:

During phagocytosis, NADPH is reduced to NADP+. This results in the formation of hydroxide (OH^-) or hydrogen peroxide (H_2O_2) depending on the enzyme involved. H_2O_2 is catalysed by myeloperoxidase to produce HOCl. An alternate pathway exists, whereby H_2O is oxidised to form H_2O_2 by being catalysed alongside GSSG, producing 2GSH.

Production of HOCl is dependent on mitochondria within the cell – it is seen that cells with increased mitochondrial activity produce more HOCl. Additionally, it has been shown that HOCl is produced through a chloride shift by non-myeloid cells of the oral and nasal mucosa, upon contact with normal saline.

HOCl is a secondary reactive oxygen species. Thus, the mechanism of action is the same as that of other ROS. The mechanism of cellular damage is as follows:

a. Hydroxyl radicals react with heterocyclic DNA bases and result in a cascade of reactions, leading to destruction of DNA. The reaction of hydroxyl radicals with DNA bases result in abnormal mutations and cross-linkages.

b. Hydroxyl radicals or superoxide anions exposed to membrane lipids resulting in peroxidation of the polyunsaturated fatty acids. This causes a chain reaction of peroxidation of membrane lipids, ultimately leading to loss of membrane fluidity.

c. Inhibition of glyceraldehyde-3-phospate dehydrogenase (G3PD), an enzyme required for cellular glycolysis, causes cellular dysfunction. The presence of free radicals results in a change in intracellular redox potential. This further inhibits glycolysis. The end result is depletion of adenosine triphosphate (ATP) and complete disruption of essential cellular functions.

The end result from all the mechanisms mentioned above is activation of enzymes, such as phospholipases, endonucleases, and calcium-dependent proteases. This causes cell lysis.

The mechanisms of action affect all organisms – i.e., bacteria, fungi, virions, and mammalian cells.

7.1.2.2. Nano Silver

Nano-silver has biological properties which are significant for consumer products, food technology (e.g., food processing equipment, packaging materials, food storage), textiles/fabrics (e.g., antimicrobial clothing), and medical applications (e.g., wound care products, implantable medical devices). In addition, nano-silver has unique optical and physical properties that are not present in bulk silver, and which are claimed to have great potential for medical applications (e.g., diagnostics, drug delivery, and imaging).

Antibacterial properties

Nano-silver is an effective killing agent against a broad spectrum of Gram-negative and Gram-positive bacteria, including antibiotic-resistant strains. Gram-negative bacteria include genera such as Acinetobacter, Escherichia, Pseudomonas, Salmonella, and Vibrio. Acinetobacter species are associated with nosocomial infections, i.e., infections which are the result of treatment in a hospital or a healthcare service unit, but secondary to the patient's original condition. Gram-positive bacteria include many well- known genera such as Bacillus, Clostridium, Enterococcus, Listeria, Staphylococcus, and Streptococcus. Antibiotic-resistant bacteria include strains such as methicillin-resistant and vancomycin-resistant Staphylococcus aureus, and Enterococcus faecium.

Recently, it has been shown that silver nanoparticles enhance the antibacterial activity of various antibiotics. The antibacterial activities of penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin against Staphylococcus aureus and Escherichia coli were

increased in the presence of silver nanoparticles. Size-dependent antimicrobial activity of silver nanoparticles has been reported with Gram-negative bacteria and Gram-positive bacteria. Small nanoparticles with a large surface area to volume ratio provide a more efficient means for antibacterial activity even at very low concentration. Nano-silver is an effective killing agent against the majority of bacterial microorganisms.

Antifungal properties

Nano-silver is an effective and a fast-acting fungicide against a broad spectrum of common fungi including genera such as Aspergillus, Candida, and Saccharomyces. Moreover, silver nanoparticles are effective against yeast isolated from bovine mastitis.

Antiviral properties

Size-dependent antiviral activity of silver nanoparticles has been shown with HIV-1 virus. Interaction of silver nanoparticles with HIV-1 was exclusively within the range of 110 nm.

In 2011, Galdiero et al published a study that advocates silver nanoparticles as potential antiviral agents, indicating the inhibition or inactivation of for example HIV-1, HSV- 1, RSV, Influenza virus, HBV, and other virus types. A recent study (2020) by Nikaeen et. al., demonstrates nano silver formulations to be effective against coronaviruses, as did an earlier study from Lv et. al., (2014).

7.1.3. Safety of HOCl

HOCl has been used widely in wound care and healing and has been shown to be of great value. Furthermore, use of HOCl as a sanitising product has also proven to be safe to skin contact.

J. Q. Del Rosso and N Bhatia state that both in-vitro and in-vivo studies have supported antimicrobial, anti-inflammatory and other biological properties of HOCl, thus leading to the use of HOCl for treatment of skin wounds, diabetic ulcers, pruritis, and some inflammatory skin conditions. (Del Rosso & Bhatia, 2018). Furthermore, they state that HOCl appears to be safe and well tolerated for this use, without any major adverse effects noted.

As can be assessed from the above mechanisms, the potential to damage mammalian cells appears to be present. However, intracellular protective mechanisms are in place in order to prevent cellular damage, as mentioned above. Additionally, from a production standpoint, the balance of product pH and control of concentration (ppm) would be required to ensure a safe to use product.

The physiological protective mechanism is based on the antioxidant defence mechanism. As HOCl is a ROS (oxidant), the antioxidant defence mechanism of human cells protects against the oxidative stress caused by HOCl. This specific quality is what renders HOCl safe for use in humans while making it toxic to microbes.

SkinSafe, developed by Mayo Clinic, designates HOCl as hypoallergenic, irritant free, eyelid and lip safe, safe for teens and safe for babies.[1]

[1] (SkinSafe, n.d.)

Antioxidants are molecules which slow down or prevent oxidation of other molecules. The mechanism is through which antioxidants work is as follows:

1. Removal of radical intermediate species
2. Blocking secondary production of toxic metabolites and inflammatory mediators
3. Converting free radicals into less toxic compounds
4. Blocking chain propagation of secondary radicals
5. Repairing molecular injury
6. Enhancing the endogenous antioxidant system (function of exogenous antioxidants)
7. Inhibiting other oxidation reactions by being oxidised themselves

As such, there are two classes of antioxidants – enzymatic and non-enzymatic antioxidants.

Enzymatic antioxidants include:

1. Superoxide dismutases
2. Catalases
3. Glutathione system
4. Thioredoxin system

Non-enzymatic antioxidants include:

1. Ascorbic acid (vitamin C)
2. Glutathione and thiocyanate
3. Tocopherols and tocotrienols (Vitamin E)
4. Beta-carotene (Carotenoids – provitamin A)

This Antioxidant Defence System is what protects the body from oxidative damage. The presence of this system alone makes HOCl safe for use in humans and other species that make use of this kind of system.

7.1.4. Safety of Ag^+

Widespread use of silver nanoparticles for domestic, commercial, and medical use has led to concerns about the safety and potential toxic effects. Toxic effects of AgNPs can be attributed either to direct effects from the AgNPs and by oxidation of AgNPs to Ag^+ ions.

AgNPs interact with membrane proteins and activate signalling pathways, which result in inhibition of cell proliferation. Nanosilver particles also enter the cell through diffusion or endocytosis, which results in mitochondrial dysfunction, generation of ROS, and damage to proteins and nucleic acids.

Ag^+ causes cellular damage largely due to the induction of ROS intracellularly. As the levels of ROS increase, the capacity of the antioxidant defence system is overwhelmed and thus, oxidative stress occurs.[2]

Uptake of AgNPs through the skin keratinocytes was studied by W. Lu et. al., and identified that a rise in absorption of AgNPs rapidly increases over the first 5–10-hour period, whereafter, the intracellular particle count reaches a steady state. Particles absorbed reached a count of near 8000 x 10^4 particles. The particulate forms of the AgNPs tested

[2] (McShan, Ray, & Yu, 2014)

were silver nitrate ($AgNO_3$ salt), colloidal silver, dried silver nanoparticles, and nanoparticle prisms.[3]

Hadrup et. al., mention that silver can deposit as particles in the body, causing a blue-grey discolouration of the skin known as argyria. Localised argyria has been reported with exposure to silver ions, metallic silver and nanocrystalline silver. Generalised argyria was observed with ionic and nanocrystalline silver in humans with cumulative doses between 70-1500mg/kg body weight.[4]

A normal human body contains approximately 1mg of silver. The smallest amounts of silver which have been reported to cause generalised argyria has been 4-5g to 20-40g. As per Padlewska et. al., silver at 50-500mg/kg is a lethal toxic dose.[5]

As silver (and nanosilver specifically) comes in multiple forms, silver salts demonstrate the strongest link to argyria.

[3] (Lu, et al., 2010)
[4] (Hadrup, Sharma, & Loeschner, 2018)
[5] (Padlewska & Schwartz, 2017)

8. OBJECTIVES

8.1. Primary Objectives

Objective	Endpoints
Identify the antibacterial efficacy of BSafe HOCl® Face Sanitiser	Reduction in levels of colony forming units (CFUs) of bacteria on one site on the face.
Identify the benefits of multiple exposures of BSafe HOCl® Face Sanitiser	Stepladder reduction in CFU levels on one site on the face
Identify two-hourly residual exposure antibacterial efficacy of BSafe HOCl® Face Sanitiser	Reduction in CFU levels following two-hourly exposure

8.2. Secondary Objectives

Objective	Endpoint
Identify normal bacterial growth levels on the face	Increase or changes in CFU counts on the face throughout the day

9. RESEARCH METHODS

9.1. Study Design

An evaluation of the efficacy of the investigational product on bacteria will be done, in a real-world environmental simulation. Healthy volunteers will be taken on as participants on this study. The respective investigational product (IP) will be provided.

The study will be using the same participant group in two parts:

1. Day 1 – control
    a. No IP will be provided, and bacterial levels will be identified throughout the day, whereby growth rates will be identified.
    b. Three swabs will be taken, four-hourly apart. The swabs will be labelled S_{01}, S_{02}, and S_{03}.
2. Day 2 – Investigational Product
    a. IP Will be provided, whereby the following will be carried out:

As this investigation is following a simulation of real-world testing, participants will be applying the IP individually. A self-demonstration of application will be done by the investigators, in order to show participants how adequate/correct application of the IP should be carried out.

Adequate/correct application is defined as:

- Application of the IP from a distance of 20cm from the face
- 8 sprays, moving from the lateral left to right side of the face
- Full coverage of the face, without dripping of IP from the face

Variation of application can be expected, although the study aims to simulate real world usage.

Before the first exposure event (E_1), swabs will be taken in order to identify a baseline CFU value(t_0).

Sites from which the swabs will be taken are as follows:

I. Lateral to the nasal alar crease

Swabs will be taken in the following manner, in order to maintain accuracy:

I. 3 swipes in a caudo-rostral direction, and 3 swipes in a mediolateral direction
II. b. Each swipe will be 10 mm in length
III. Distance between each swipe will be 3 mm apart

Baseline swab readings will be referred to as SB_{01}.

Following the swab, the respective IP will be applied (E_1).

Thereafter, the participants are to rest for 20 minutes, during which faces are not to be contaminated, and the IP will be given time to dry. After 20 minutes, the swabs will be repeated in the same manner as above (T_{0+20}). This swab result will be referred to as SE_{01}.

A period of two hours from SB_{01} will pass, during which time participants are to continue with their daily activities. A second exposure (E_2) of the respective IP will be applied thereafter, in the same manner as above, and the participants will continue with their daily activities.

After a period of two hours from the second exposure, another swab will be recorded. This will be deemed SB_{02}. Thereafter, a third exposure (E_3) of the respective IP will be applied thereafter, in the same manner as above. After 20 minutes have passed following the third exposure event, swabs will be repeated (T_{0+4h20}). This will be referred to as SE_{02}.

Following SB_{02}, another period of two hours will pass during which time participants are to continue with daily activities, after which the participants will apply the IP once again. This will be the fourth exposure (E_4). Thereafter, another two-hour period will pass, whereby the next swab will be recorded. This will be termed as SB_{03}.

A fifth exposure event will occur (E_5), as above. Following 20 minutes, swabs will be repeated (T_{0+8h20}). This will be referred to as SE_{03}.

Swabs taken will be streak-cultured on agar plates, whereby they will be incubated for 48 hours at 33°C. Thereafter, the CFU counts will be manually counted using a Quebec Colony Counter.

9.2. Participant Population Characteristics

Healthy volunteers will be invited to participate in this study. The informed consent form will be reviewed with the potential participant in order to include them within the study. Written obtained informed consent is required in order for the participant to be included in the study. Any questions regarding the study and informed consent from the participant are to be addressed and recorded by the investigators.

Inclusion Criteria

- Age 18 years or older
- Male or female participants
- Healthy participants
- Willing and able to participate in the study

Exclusion Criteria

- Diagnosed upper respiratory tract infection by qualified physician (with letter of diagnosis)
- Any serious medical conditions affecting the upper respiratory tract or oral cavity, diagnosed by a qualified physician (with letter of diagnosis)
- Participants, who may be at risk of developing any medical complications, in the opinion of the investigators and supervisor

9.3. Variables

9.3.1. Exposure

Product exposure defined as product contact as per the respective products the participant will be given. As such, this will be defined as the exposure event. Exposure events will be carried out by the investigators. Each exposure event will consist of the following volumes and sprays:

Product	Volume	Sprays
BSafe Electrosaline® Facial Sanitiser	2.7 ml	8 sprays

9.3.2. Safety

Product safety is well documented. The external use of HOCl has been authorised by international regulatory bodies including the FDA. No systemic absorption of the product occurs, due to product volatility. There are no documented reports of adverse events occurring with the use of HOCl. Safety aspects for respective constituents of BSafe Electrosaline® Facial Sanitiser are documented above.

9.4. Data Sources

Data will be collected from participants by investigators. This will be done by swabbing participants on respective contact sites for measurement of CFU counts. The swabs used will be manually counted using a Quebec Colony Counter. The swabs will be cultured on tryptone soy agar (TSA). Total colony counts of all bacterial species present will be counted. The data collected will be recorded on data management sheets on excel for analysis.

9.4.1. Study Size

A study sample size of 26 participants will be recruited.

9.4.2. Data Management

Data collected will be tabulated in excel for management and analysis.

9.4.3. Data Collection

9.4.3.1. Control Arm

Investigators will be collecting data three times during the day, four hourly apart. Thus, data will be collected at T_0, T_{0+4h}, T_{0+8h}, and respectively labelled as S_{01}, S_{02}, S_{03}.

9.4.3.2. Investigational Product

Investigators will be collecting the data before exposure (T_0), labelled as SB_{01}, at 20 minutes after the first exposure event (T_{0+20}; SE_{01}), before the third exposure (T_{0+4h}), labelled as SB_{02}, 20 minutes after the third exposure event (T_{0+4h20}), labelled as SE_{02}, before the fifth exposure (T_{0+8h}), labelled as SB_{03}, and finally 20 minutes after the fifth exposure event (T_{0+8h20}), labelled

as SE_{03}. The investigators are required to sign off the data collection sheet in order to verify that the data collected is complete and accurate.

9.5. Data Analysis

Data collected on the data collection sheet will be tabulated on excel.

9.5.1. Control Arm

Data will be analysed to identify CFU levels throughout the day for participants, which can be interpreted as the levels of bacterial growth or changes throughout the day. This is by comparison of S_{01}, S_{02}, and S_{03}. The changes between these values can be compared to identify trends.

9.5.2. Investigational Product

Data will be analysed to compare CFU levels before any IP has been applied, and changes in CFU counts thereafter. Comparison of the initial counts (SB_{01}) with the counts recorded after the first exposure event (SE_{01}) can define IP efficacy.

Multiple exposures are done throughout this arm during the course of the day. Comparison of SB_{02} and SB_{03} with SB_{01} will demonstrate latent efficacy of IP after a period of 2 hours. Comparison of SE_{02} and SE_{03} with other datapoints demonstrates IP efficacy following multiple exposures. Trends from all datasets can be identified and compared to the control arm. Comparison with the control arm allows for a direct demonstration of efficacy of multiple exposures throughout the day and compounded efficacy from multiple exposures. This may also demonstrate justification of use of IP and frequency of use.

9.6. Visualisation of Methodology

9.6.1. Control Arm

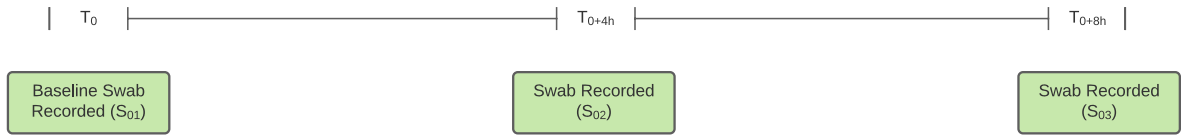

Figure 1: Control Arm Visualisation

9.6.2. Investigational Product

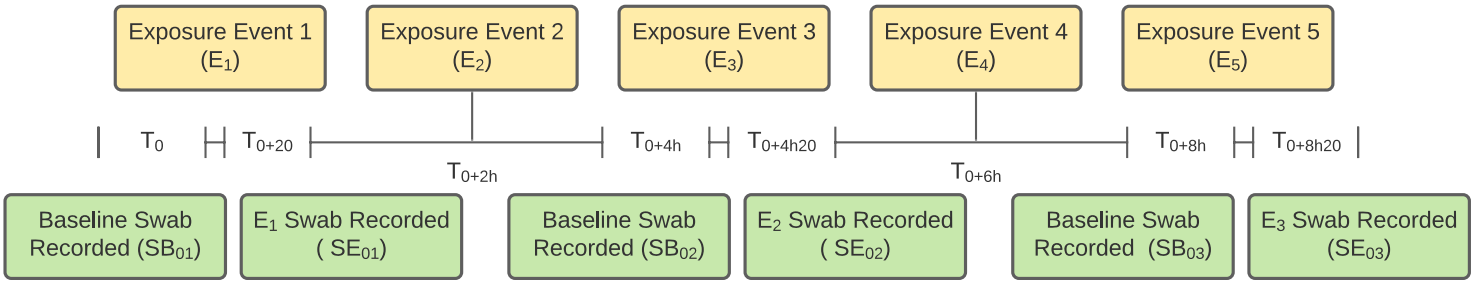

Figure 2: Investigational Product Visualisation

10. RESULTS

The results obtained were as follows:

10.1. Control

Raw data obtained is as follows:

Table 1: Control Raw Data (N=26)

Participant No.	CFU Counts		
	S01	S02	S03
1	1041	1640	1141
2	34	7	452
3	20	5	50
4	70	68	18
5	47	21	71
6	630	120	411
7	643	264	338
8	93	82	49
9	127	45	219
10	19	32	181
11	49	920	113
12	54	61	7
13	35	127	216
14	712	1194	537
15	25	57	68
16	147	10	25
17	352	540	534
18	160	120	17
19	15	2	3
20	11	22	2
21	83	170	984
22	124	48	3
23	182	170	NA
24	3	21	9
25	256	232	1239
26	77	230	945

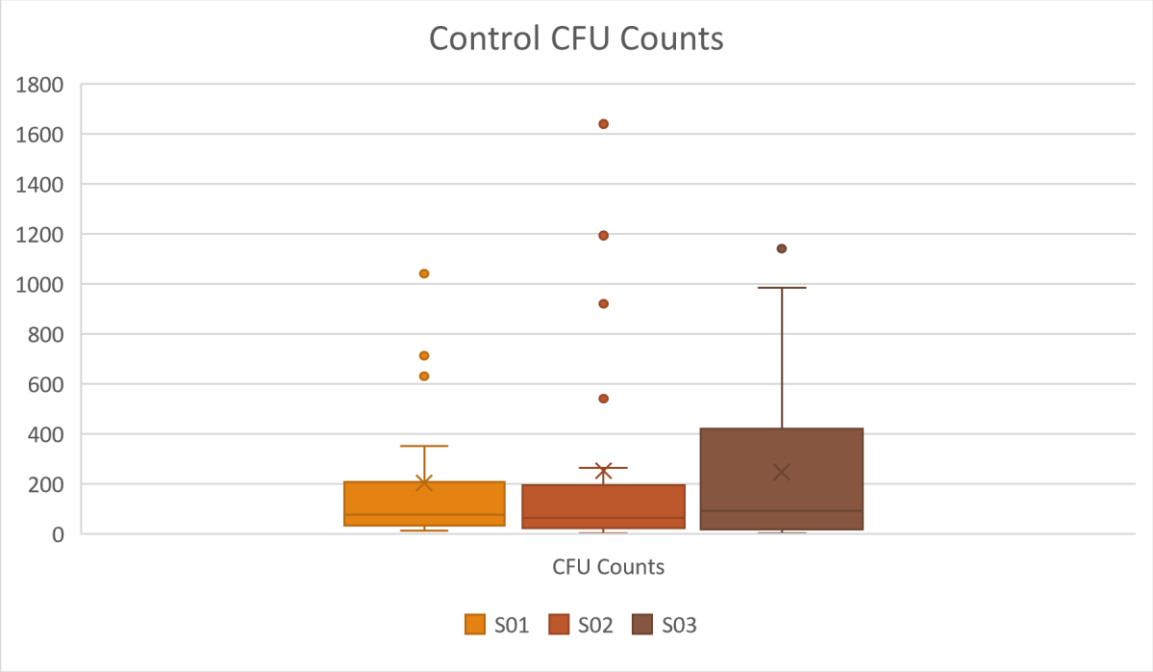

Figure 3: Box and Whisker Chart - Control CFU Counts (N=26)

Outliers:

CFU counts which were either 1.5 times the length of the box away from either the lower or upper quartiles are as follows:

S01: 630, 643, 712, 1041
S02: 920, 1194, 1640
S03: 1239

Thus, data can be cleaned to remove outliers and incomplete data. The data is as follows:

Table 2: Control CFU Counts with Outliers and Incomplete Data Removed

Participant No.	CFU Counts		
	S01	S02	S03
2	34	7	452
3	20	5	50
4	70	68	18
5	47	21	71
8	93	82	49
9	127	45	219
10	19	32	181
12	54	61	7
13	35	127	216
15	25	57	68
16	147	10	25
17	352	540	534
18	160	120	17
19	15	2	3
20	11	22	2
21	83	170	984
22	124	48	3
24	3	21	9

This results in a final participant size of 18. (N=18)

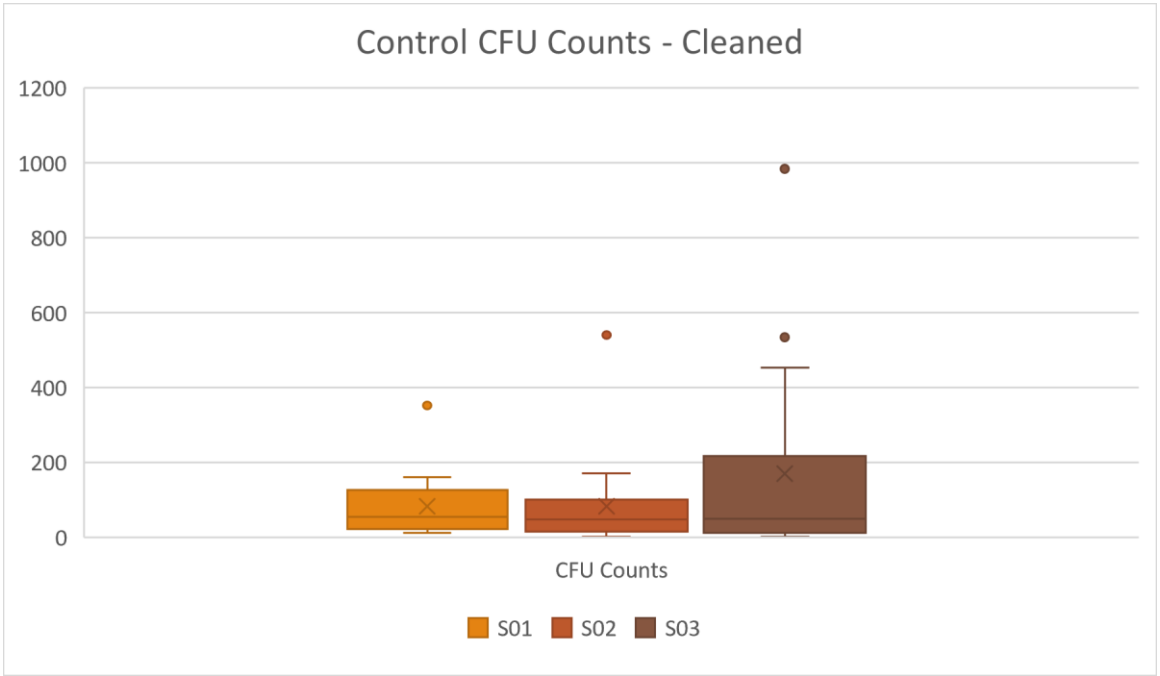

Figure 4: CFU Counts - Outliers and Incomplete Data Removed (N=18)

10.1.1. Analysis of Results

After removal of outliers, an analysis of variance (ANOVA) calculation was done which identified an F-ratio of 2.13399 and a P-value of 0.133948. This can be interpreted as an associated increase in the CFU counts throughout the measures. The mean values obtained for the samples above are as follows:

S01: 78.83
S02: 79.89
S03: 161.56

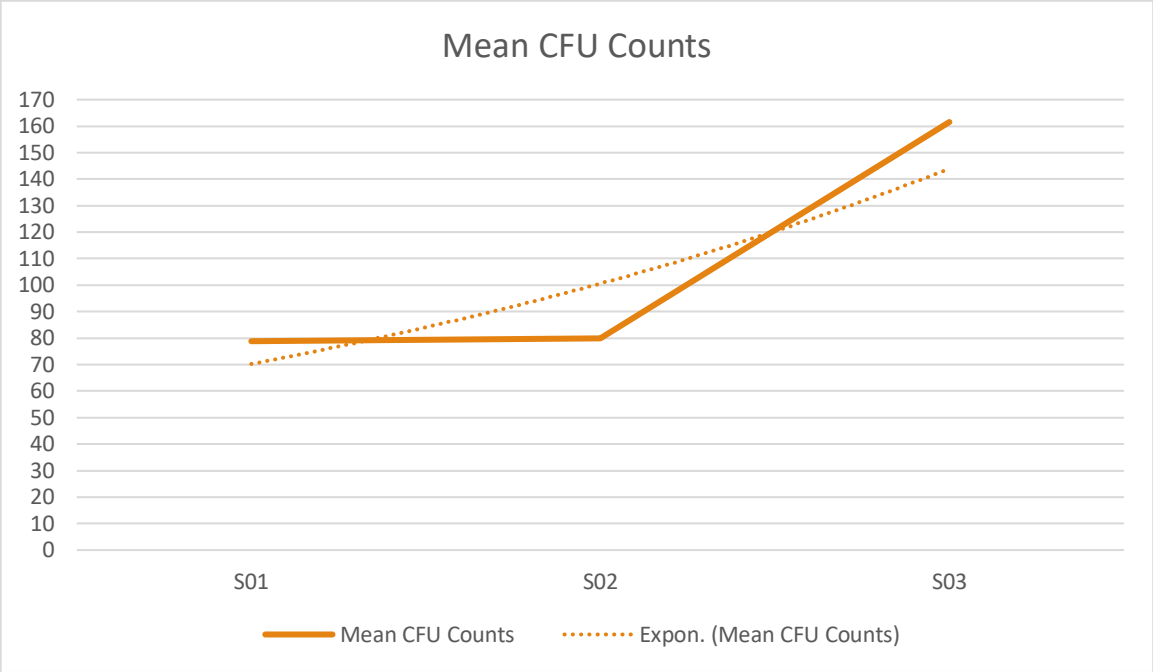

Figure 5: Mean CFU Counts – Control (N=18)

With the above graph comparing the mean values obtained, it can be seen that there is a general steady state of bacterial levels with an increase towards the end of the day. With the changes between S01 and S02 average values, an increase of only ~1 CFU was seen. Thus, between these two datapoints, it can be said that the change is within margin of error and no clear increase identified. However, between S01 and S03, an increase of ~83 CFUs is seen. This is a 104.93% increase. Thus, it can be said over an eight-hour period, the CFU counts doubled on average. However, considering the comparison between S02 and S03, the percentage increase was 102.23%. Therefore, the increase throughout the day was mostly seen over a four-hour period.

Specific Participant Examples

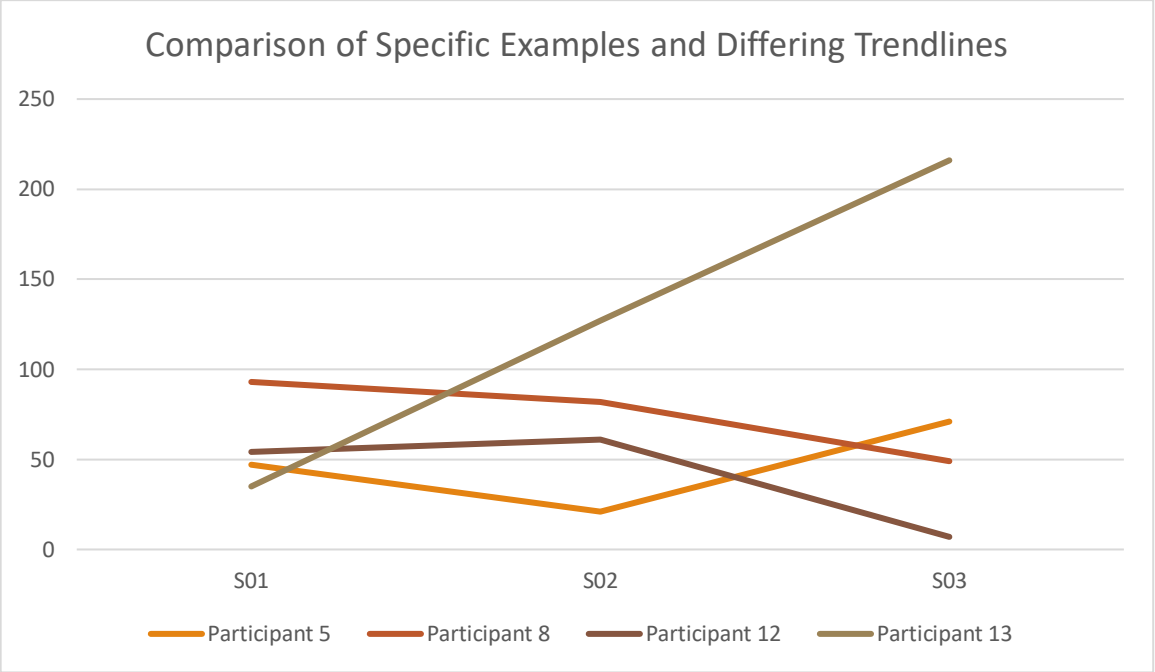

Figure 6: Comparison of Specific Examples and Differing Trendlines (N=4)

As seen in the graph above, four different trends of changes in CFU levels were observed. The trends seen are the following:

1. Continuous decrease in CFU counts
2. Increase followed by a decrease in CFU counts
3. Decrease followed by an increase in CFU counts
4. Continuous increase in CFU counts

This can be due to changes in circumstances and cleanliness practices throughout the day, such as face washing. In addition to this, it can be considered that secretions in sweat may lower levels of bacterial counts, which may be attributed to two possible factors:

1. Physical dispersion of bacteria away from the face
2. Activity of immunoglobulins present in sweat

Due to this, reviewing averages is not the only important measure, but the frequency of trend patterns too. The table below demonstrates the frequency of the 4 trends mentioned above:

Table 3: CFU Growth Trends (N=18)

Participant No.	CFU Growth Trend
2	Decrease-increase
3	Decrease-increase
4	Continuous decrease
5	Decrease-increase
8	Continuous decrease
9	Decrease-increase
10	Continuous increase
12	Increase-decrease
13	Continuous increase
15	Increase-decrease
16	Decrease-increase
17	Increase-decrease
18	Continuous decrease
19	Decrease-increase
20	Increase-decrease
21	Continuous increase
22	Continuous decrease
24	Increase-decrease

Using this dataset, we can identify the most frequent trend pattern is a decrease followed by increase.

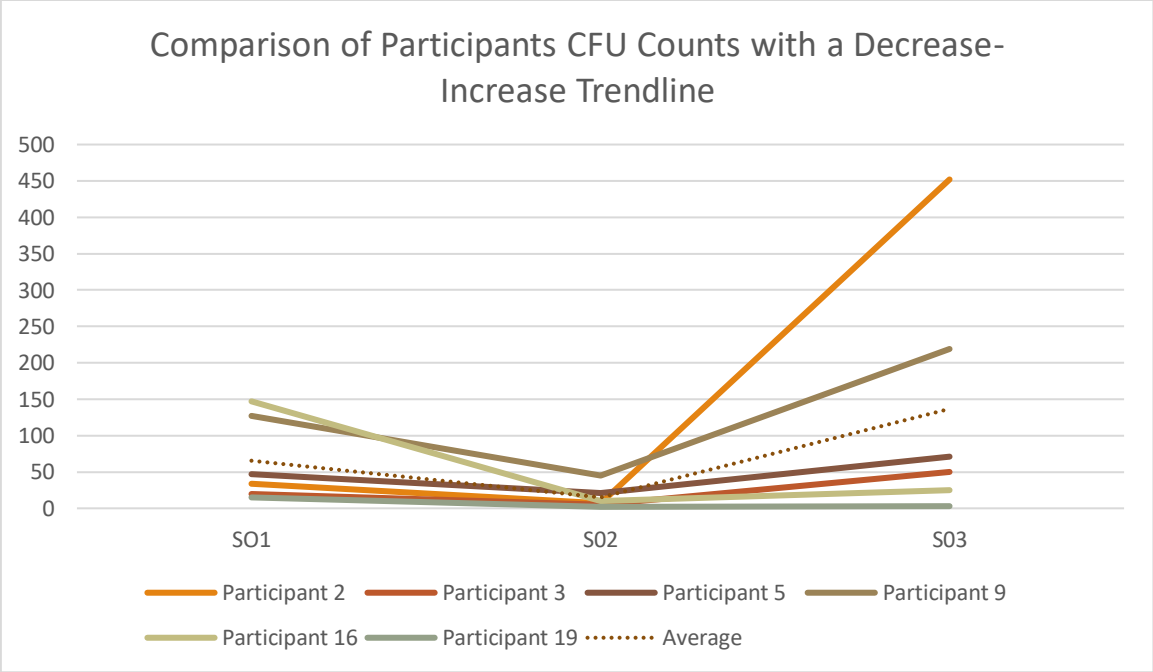

Figure 7: Comparison of Participants with a Decrease-Increase Trendline (N=6)

Upon comparison of the decrease-increase trendlines with that of the overall mean trendline, the presentation demonstrates similarities which show a little differences between S01 and S02, followed by a sharper rise between S02 and S03.

From the data obtained above, it can be said that there is an increased rate of proliferation of bacterial colonies in the final four hours of the workday compared to the first four hours.

10.2. Investigational Product

Raw data obtained was as follows:

Table 4: Raw Data Obtained of Participants Applying IP (N=26; SB02 N=16)

Participant No.	CFU Counts					
	SB01	SE01	SB02	SE02	SB03	SE03
1	958	822	25	483	436	36
2	86	24	248	4	418	0
3	8	1	50	17	21	5
4	116	62	12	4	18	6
5	227	56	50	44	89	10
6	62	12	-	0	44	1
7	480	310	-	53	72	42
8	410	25	-	65	5	0
9	113	13	-	5	64	1
10	417	108	-	187	98	9
11	871	45	104	12	44	2
12	93	2	85	25	5	0
13	174	99	50	17	12	6
14	141	27	32	0	61	28
15	38	0	9	0	3	1
16	375	147	16	0	0	1
17	134	48	60	9	38	14
18	8	7	18	2	2	1
19	20	0	-	10	19	3
20	5	3	10	0	4	8
21	620	274	-	28	166	6
22	822	186	-	26	20	0
23	94	99	-	16	56	4
24	14	0	-	4	5	2
25	228	328	442	91	134	10
26	845	200	46	15	252	0

As seen above, SB02 data is incomplete, as multiple participants had applied at the time of testing. Therefore, SB02 will not be included for the majority of analysis, however, the data obtained is still viable.

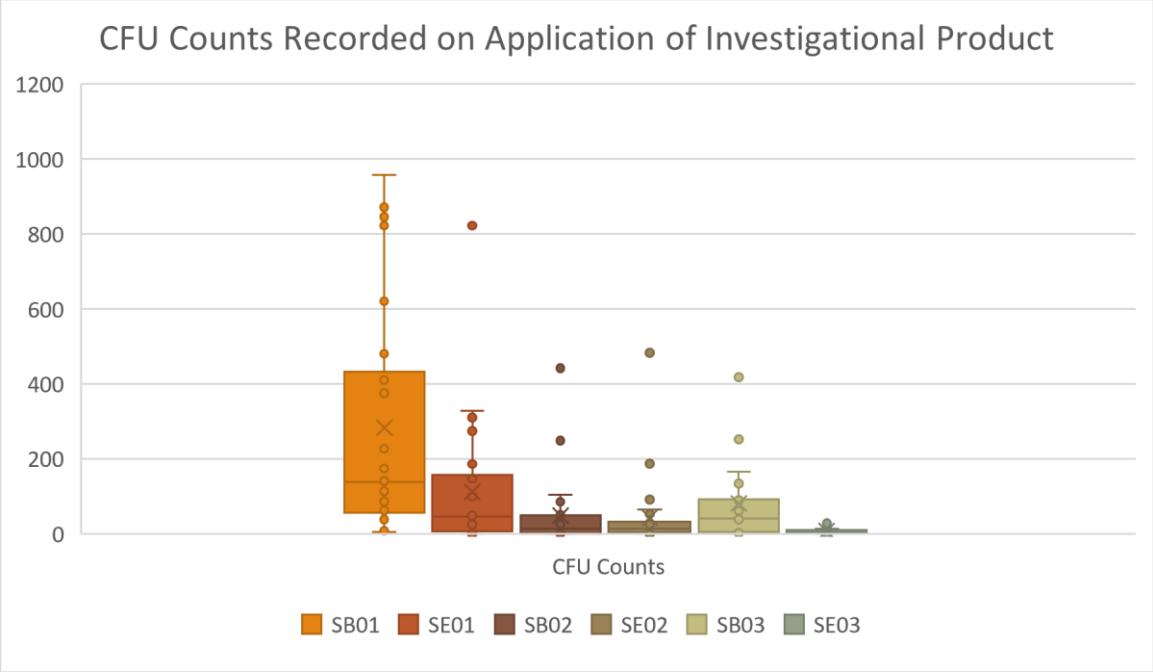

Figure 8: Box and Whisker Chart of CFU counts recorded on Application of Investigational Product (N=26; SB02 N=16)

Outliers:

CFU counts which were either 1.5 times the length of the box away from either the lower or upper quartiles are as follows:

SB01: 958
SE01: 822
SB02: 248, 442 (N=16)
SE02: 65, 91, 187, 483
SB03: 252, 418, 436
SE03: 28, 36, 42

Thus, removal of outliers provides the remaining participants:

Table 5: CFU Counts of Participants Utilising IP with Removal of Outliers (N=18; SB02 N=11)

Participant No.	CFU Counts					
	SB01	SE01	SB02	SE02	SB03	SE03
3	8	1	50	17	21	5
4	116	62	12	4	18	6
5	227	56	50	44	89	10
6	62	12	-	0	44	1
9	113	13	-	5	64	1
11	871	45	104	12	44	2
12	93	2	85	25	5	0
13	174	99	50	17	12	6
15	38	0	9	0	3	1
16	375	147	16	0	0	1
17	134	48	60	9	38	14
18	8	7	18	2	2	1
19	20	0	-	10	19	3
20	5	3	10	0	4	8
21	620	274	-	28	166	6
22	822	186	-	26	20	0
23	94	99	-	16	56	4
24	14	0	-	4	5	2

The final participant count with the removal of outliers is 18 (N=18), with SB02 having a participant count of 11 (N=11).

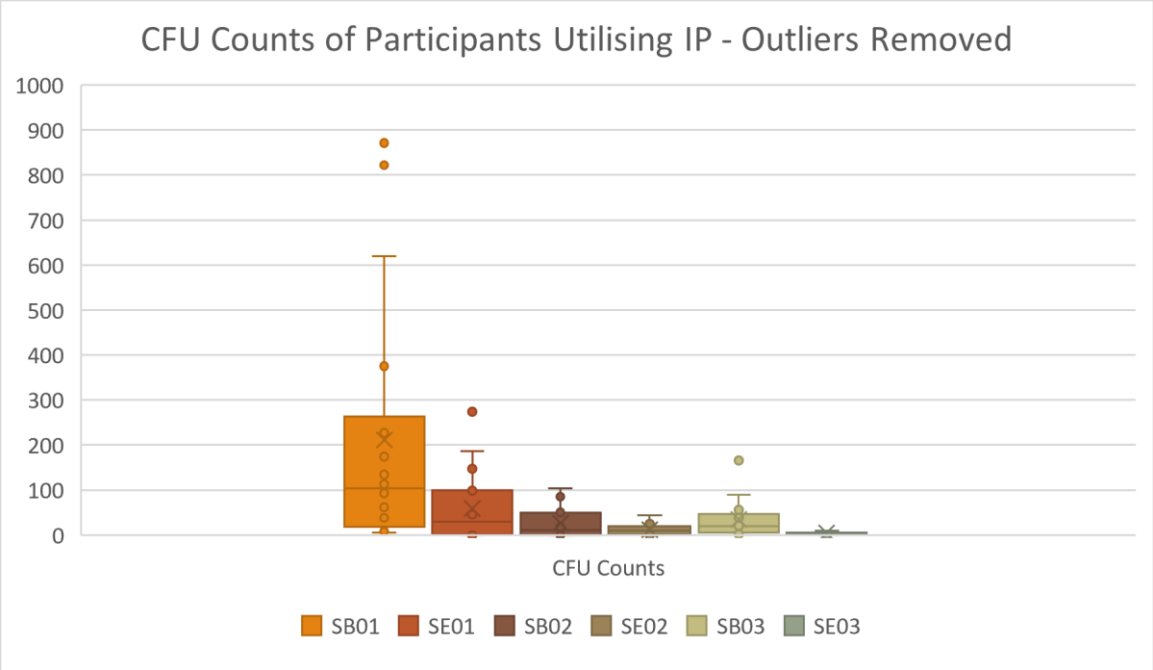

Figure 9: Box and Whisker Chart of CFU Counts of Participants Utilising IP with Outliers Removed (N=18; SB02 N=11)

10.2.1. Analysis of Results

With the removal of outliers, analysis of variation was calculated (ANOVA). In order to analyse the variance, SB01, SE01, SE02 and SE03 were used. These values were used as SB01 was taken at T_0, thus a baseline before any application of the IP, and SE01, SE02, and SE03 were used as they were taken 20 minutes after application of IP, thus demonstrating the efficacy of the IP, and cumulative effect. The P-value was 0.000058, and the F-ratio calculated was 9.18838. With these values in mind, it can be said that there is a strong correlation with the reduction in CFU counts, and thus, the cumulative effect of the usage of the IP throughout the day can be said to be highly efficacious. The P-value of 0.000058 is descriptive of the significance – thus, it can be said that the reductions in CFU counts demonstrated are not coincidental and due to the usage of the IP.

The counts noted at SB02 and SB03 demonstrated IP efficacy after 2 hours of contact. As the data for SB02 is incomplete, the number of participants for analysis was limited to 11 (N-11). As such, with use of the ANOVA calculation, the following values were calculated:

P-value: 0.023185
F-ratio: 4.57066

The P-value is <0.05, therefore the correlation in reductions is of significance and can be said to be due to the residual (2-hourly) efficacy of the IP. The F-ratio is descriptive of a strong correlation between the reductions and the use of the IP.

Mean Values:

SB01: 210.778 (N=18)
SE01: 58.5556 (N=18)
SB02: 42.1818 (N=11)
SE02: 12.1667 (N=18)
SB03: 33.8889 (N=18)
SE03: 3.9444 (N=18)

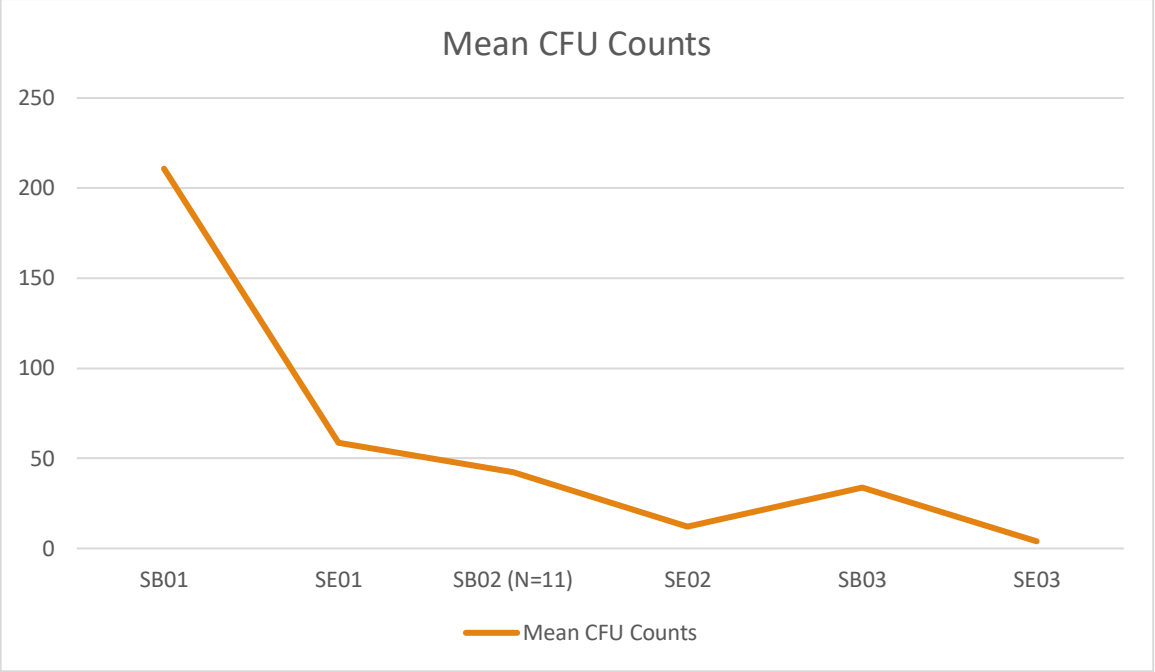

Figure 10: Mean CFU Counts following IP Application (N=18; SB02 N=11)

In order to accommodate for the SB02 values, the following mean values with N=11 were calculated:

SB01: 186.2727 (N=11)
SE01: 42.7272 (N=11)
SB02: 42.1818 (N=11)
SE02: 11.8181 (N=11)
SB03: 21.4545 (N=11)
SE03: 4.9091 (N=11)

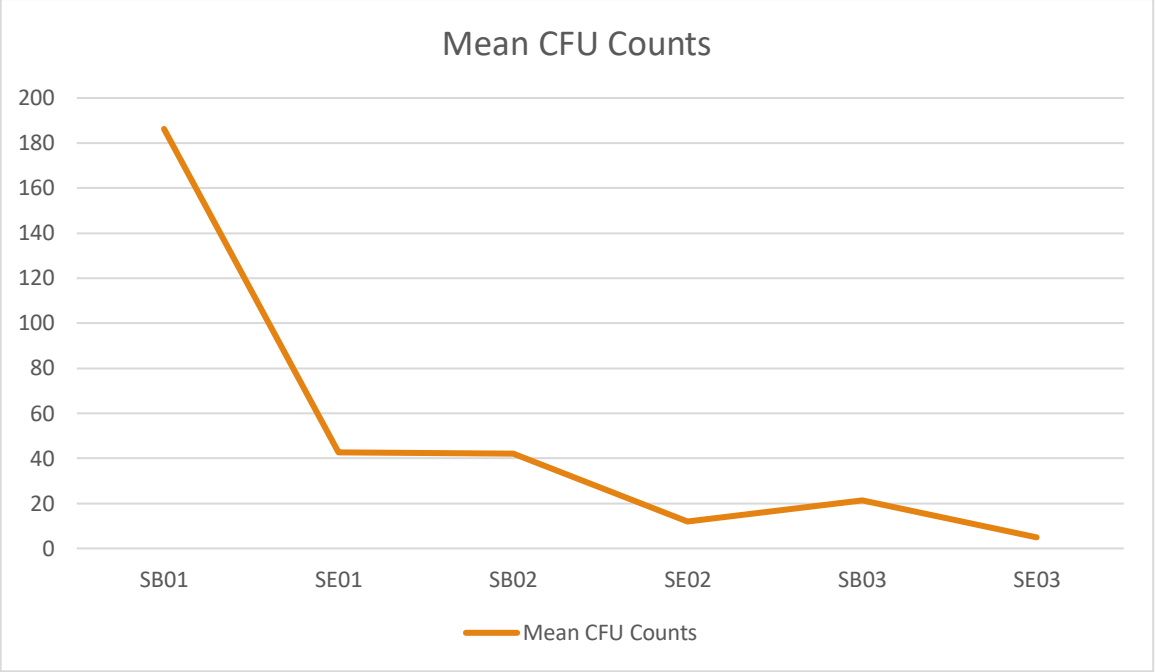

Figure 11: Mean CFU Counts adjusted for SB02 (N=11)

As visualised in the two graphs above, there is a rapid decrease in CFU counts following the initial application of the IP (SB01 reduction to SE01). In order to better visualise the efficacy of the IP and cumulative efficacy, exclusion of SB02 and SB03 should be done. Likewise, to better visualise the 2-hour residual efficacy, exclusion of SE01, SE02 and SE03 should be done. As such, the following can be seen:

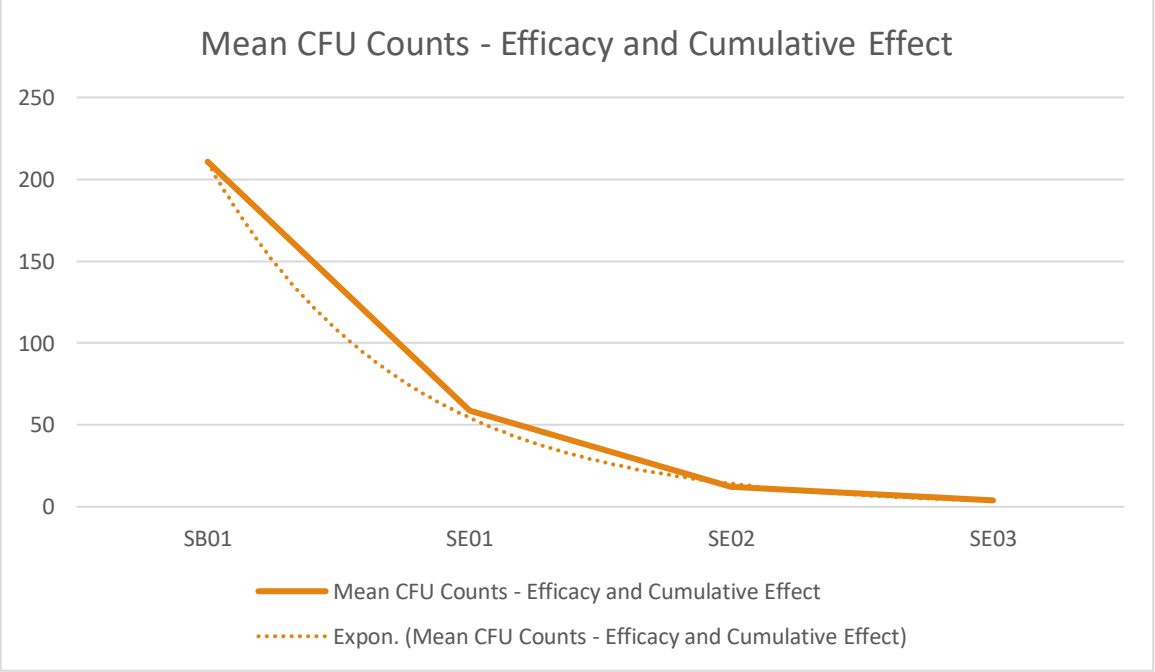

Figure 12: Mean CFU Counts Demonstrating Efficacy and Cumulative Effect of IP Usage (N=18)

From this, we can see a rapid reduction from SB01 to SE01, calculated as a 72.22% reduction after one application. After 5 applications (i.e., reduction from SB01 to SE03), a 98.13% was demonstrated. This translates to a cumulative effect with multiple exposure of the IP. As demonstrated in the graph above, this was close to the exponential reduction trend calculated.

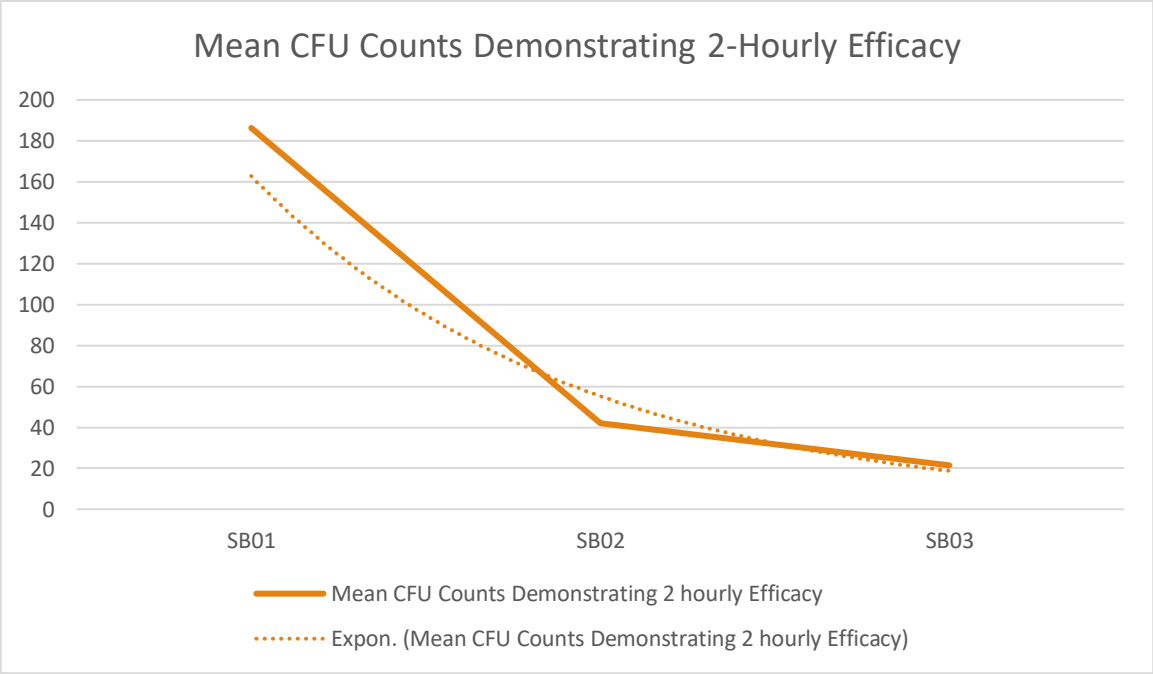

Figure 13: Mean CFU Counts Demonstrating 2-hourly Efficacy (N=11)

Review of the two-hourly residual efficacy of the IP is demonstrated above. With this, the two-hourly reduction percentage was calculated as 77.35%, following two applications. With four applications, the percentage reduction was calculated at 88.48%.

With these results, it can be said that there is a residual two-hourly efficacy, with a compounded effect seen with multiple exposures.

Specific Participant Examples

Identification of the trendline patterns with the participants demonstrates the following possible trend patterns:

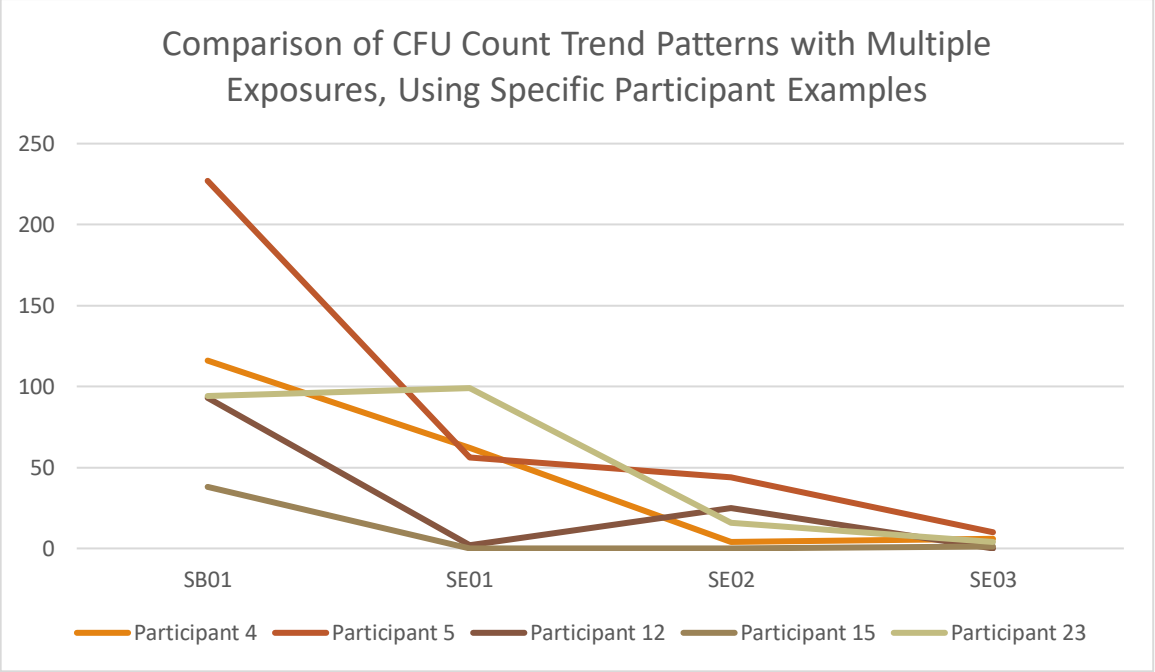

Figure 14: Trendlines of CFU Counts following Multiple Exposures, Using Specific Participant Examples (N=5)

Thus, there are five trend patterns demonstrated. These are as follows:

1. Decrease followed by an increase and subsequent decrease
2. Decrease followed by increase
3. Continuous decrease
4. Decrease, no change, with subsequent increase
5. Increase, followed by subsequent decrease

Below is a tabulation of the trend patterns seen for all participants:

Table 6: Comparison of Trendlines Seen with Participants following Multiple Exposures (N=18)

Participant No.	Trendline
3.	Decrease, increase, decrease
4.	Decrease, decrease, increase
5.	Continuous decrease
6.	Decrease, decrease, increase
9.	Continuous decrease
11.	Continuous decrease
12.	Decrease, increase, decrease
13.	Continuous decrease
15.	Decrease, no change, increase
16.	Decrease, decrease, increase
17.	Decrease, decrease, increase
18.	Continuous decrease
19.	Decrease, increase, decrease
20.	Decrease, decrease, increase
21.	Continuous decrease
22.	Continuous decrease
23.	Increase, decrease, decrease
24.	Decrease, increase, decrease

From the data above, the most frequent trendline presentation is a continuous decrease (N=7).

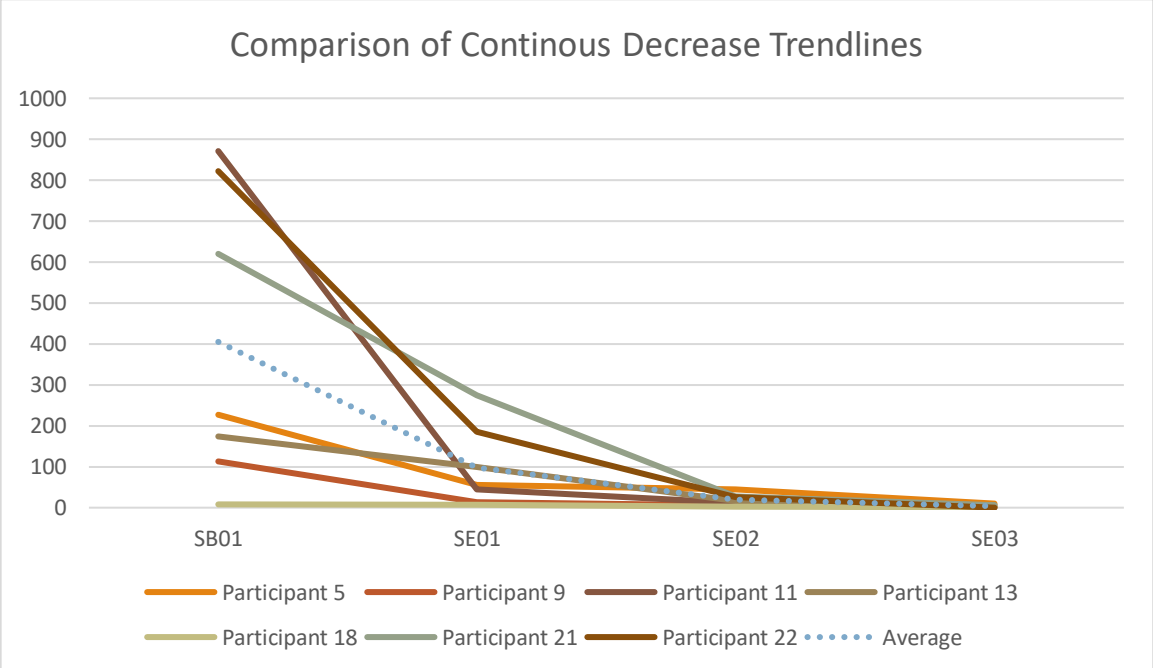

Figure 15: Comparison of Continuous Decrease Trendlines (N=7)

The 2-hourly exposure CFU count trendlines were examined and demonstrated the following:

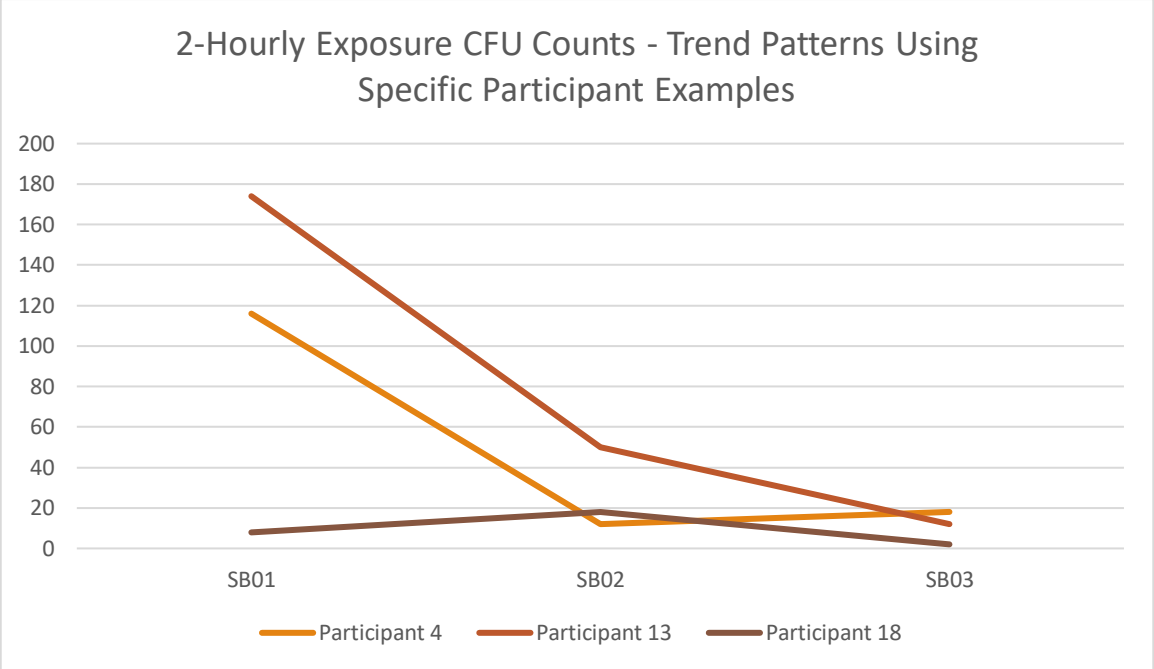

Figure 16: Comparison of Trend Patterns Seen with 2-Hourly IP Exposure (N=3)

Three trendlines are demonstrated with 2-hourly exposure of IP. These trendlines are as follows:

1. Increase followed by a decrease
2. Decrease followed by an increase
3. Continuous decrease

All participant trendlines are tabulated below:

Table 7: Comparison of Trendlines Seen with 2-Hourly Exposures (N=11)

Participant No.	Trendline
3.	Increase, decrease
4.	Decrease, increase
5.	Decrease, increase
11.	Continuous decrease
12.	Continuous decrease
13.	Continuous decrease
15.	Continuous decrease
16.	Continuous decrease
17.	Continuous decrease
18.	Increase, decrease
20.	Increase, decrease

From the data obtained above, the most frequent trendline presentation demonstrated was a continuous decrease (N=6).

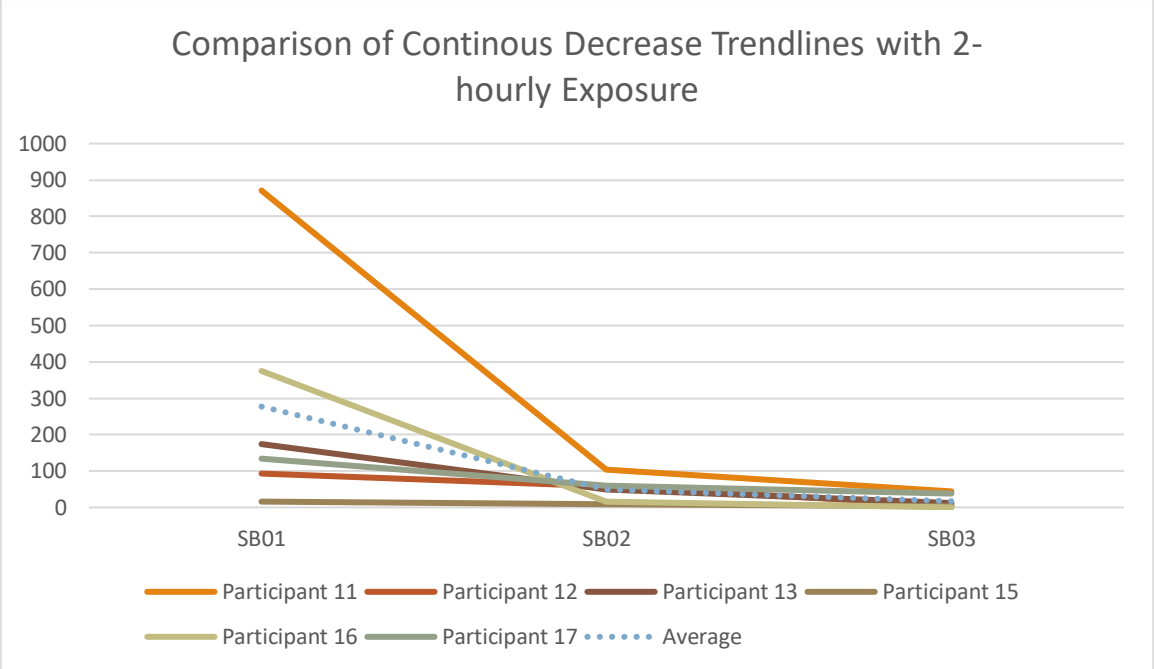

Figure 17: Comparison of Continuous Decrease Trendlines with 2-hourly Exposure (N=6)

With the most frequent trends in efficacy and residual effect both being continuous decrease trends, it can be shown that the IP tested in the study reduces bacterial colony counts consistently and there is a residual effect seen over a 2-hour period. Application of the IP every 2 hours is highly efficacious, and the compounded effect of multiple applications is more beneficial than a single application.

A direct comparison of the averages of the data obtained at the same times for the control arm with the exposure events demonstrates the following data (N=10):

S01: 92.1
S02: 103.1
S03: 100.8

SB01: 117.8
SB02: 36
SB03: 19.2

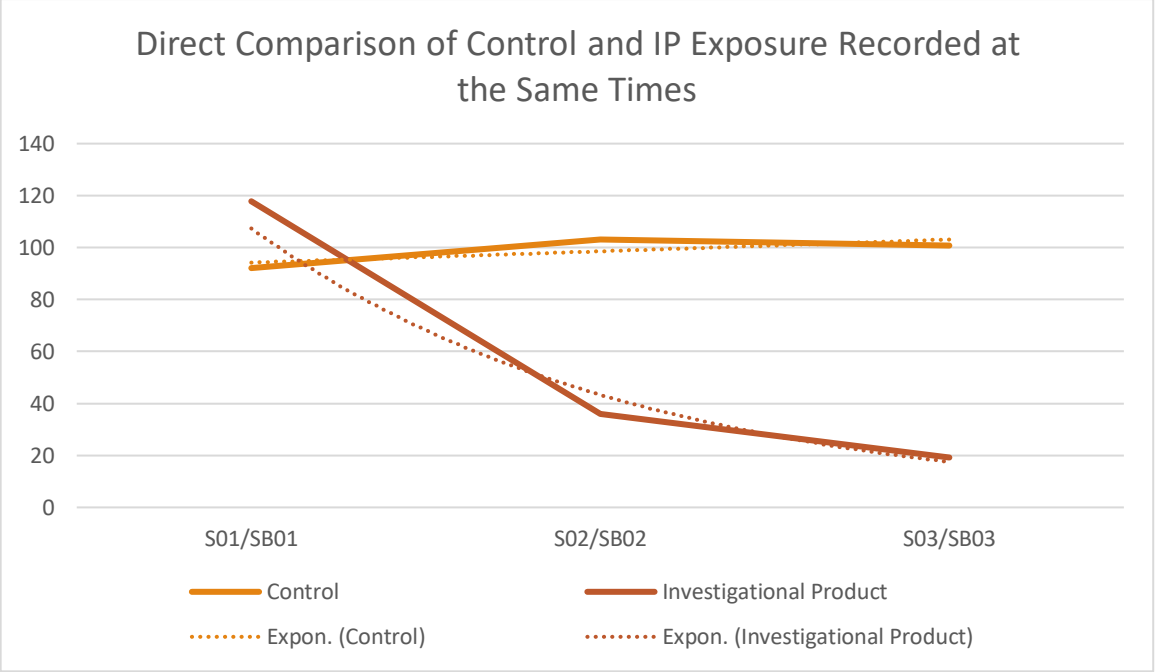

Figure 18: Direct Comparison of Control and IP Exposure Recorded at the Same Times (N=10)

From this, it can be seen that control CFU levels remain relatively the same throughout the day, while the CFU levels of the IP reduce with an exponential trend. Comparison of this data, however, is limited due to the removal of outliers resulting in a smaller participant size. This is also the reason the means and trendlines differ from previous data.

Although the dataset is limited, it still provides insight as it demonstrates efficacy of the IP directly.

11. DISCUSSION

From the results, we can see that without exposure of the IP (control testing), there is a rise in CFU counts as the day progresses. The changes in the first four-hour period were minimal (average change of ~1 CFU; within margin of error). Thereafter, the following four-hour period demonstrated an increase in 102.23%. In comparison from the start of the day and eight hours later demonstrated a 104.93% increase. Four different trends in growth appeared (continuous decrease, increase followed by a decrease, decrease followed by an increase, and a continuous increase). It was hypothesised that a continuous increase would be the most frequent presentation, however, testing showed that a decrease followed by an increase was the most frequent trend (N=6). Reasons for the variations in the growth trends may be attributed to the occupational variances and activity levels. Increased levels of sweat provide immunoglobulins which reduce the bacterial levels and may present with a mechanical action in removal of the bacteria.

Reasons for an increased rate over the latter four-hour period of they day may be attributed to an increased rate of face touching during that period of time, which may occur during the participants' lunch break. With this, there is also an addition of increased levels of nutrients for bacterial growth.

Upon testing of the investigational product, the results demonstrated a mean reduction of 72.22% following one application. Following five two-hourly applications however, a 98.13% reduction was seen when compared to the baseline reading (N=18). These findings can be interpreted as a cumulative effect of multiple applications. The reduction curve is similar to the projected exponential curve.

Results of the swabs examining the two-hourly residual efficacy of the IP demonstrated a mean reduction percentage of 77.35% following two applications. With four applications, the reduction was 88.48% compared to the baseline (N=11). Thus, it is proven that the IP possesses a residual efficacy over a two-hour period.

Furthermore, the residual efficacy compounded with multiple applications further strengthens the findings of the efficacy of multiple exposures.

On examination of the frequency of the trendlines of the CFU counts, five trend patterns were demonstrated with the multiple exposure. These were a decrease followed by an increase and subsequent decrease, a decrease followed by an increase, a continuous decrease, a decrease with no change and a subsequent increase, and an increase followed by a decrease. The most frequent trendline seen was a continuous decrease (N=7).

The frequency of the trendlines of the CFU counts demonstrated with residual efficacy presented three trends. These trends were an increase followed by a decrease, a decrease followed by an increase, and a continuous decrease. The most frequent trendline presentation was a continuous decrease (N=6).

Comparison of the average CFU counts of the control to the residual efficacy was done. In order to do this, further removal of participants was required in order to compare results directly. The results showed a slight increase in CFU counts in the control study, with an increase of only ~10 CFUs seen over the eight-hour period. Therefore, the CFU counts in

the control study can better be described as remaining relatively the same throughout the day. The residual efficacy demonstrated a consistent decline which was similar to the exponential trend. (N=10)

Statistical significance of this study was evaluated which showed a P-value of 0.000058 for multiple exposures, and a P-value of 0.02385 for residual efficacy. Both P-values are <0.05 and thus statistically significant. The F-ratio of the multiple exposure study was calculated to be 9.1883, and the F-ratio for the residual efficacy was calculated to be 4.57066. As the F-ratios drift further from 1.0, it can be concluded that the reduction in CFU counts is not attributed to chance and are due to a direct effect of the IP.

12. CONCLUSION

From the results obtained above, it can be concluded that the product tested showed efficacy with both single exposure and multiple exposure. With this, the product demonstrated two-hourly residual efficacy. The efficacy was further compounded with multiple exposures. Statistical significance was analysed, and the study demonstrated this. Additionally, the reduction in CFU counts is a direct consequence of the usage of the product and not coincidental. The most frequent trendlines seen with multiple exposures and residual effects were a continuous decrease, thus demonstrating the compounded efficacy of multiple applications of the product and demonstrating the residual effect.

Although every precaution was taken to prevent experimental error, slight variances may be present due to such, however, with the participant size and removal of outliers, the data presented remains accurate.

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