Senior Thesis Project

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A Comparative Analysis of Fluoride's Impact on Oral Microbiome Diversity

1. Abstract

The oral microbiome is a complex ecosystem made up of hundreds of different microorganisms, including bacteria and fungi. This ecosystem plays a crucial role in maintaining dental health, which, in the long run, helps maintain overall bodily health. Fluoride is a mineral commonly found in dental hygiene products, including toothpaste, mouthwash, and dental floss. While fluoride is widely used in dentistry and oral health care, the precise effects on the microorganisms of the oral cavity are still currently being researched and studied. The aim of this study is to better understand these effects and the level of concentration at which these effects occur. Through a combination of in vitro experiments on two subjects and an extensive literature review, I experimented and examined how different concentrations of fluoride affects both the beneficial and potentially harmful bacteria which exist in the oral cavity. It was found that the higher the concentration of fluoride, the least amount of bacterial growth was observed. Interestingly the medium concentration conditions raised a few unexpected questions due to the severe differences in bacterial growth between the two subjects. One subject showing an increase in coverage, while the other showing a significant decrease.

2. Introduction

2.1 Bacteria and other Microorganisms of the Oral Cavity

The oral cavity, or mouth, is home to over 600 different species of microorganisms. The majority of these microorganisms are bacteria. The human oral cavity contains a number of different habitats, including the teeth, gingiva, tongue, buccal mucosa, hard and soft palates, and the tonsils, all of which are colonised by bacteria (Dewhirst et al., 2010). Previous studies have shown that these different oral structures and tissues are colonised by distinct microbial communities, with approximately 280 bacterial species that have been isolated, cultured and formally named (Aas et al., 2005, Paster et al., 2001).

The bacteria found in the oral cavity is among the most diverse of any of the resident flora associated with humans which is due to the many habitats able to support the growth and colonisation of the microorganisms. In particular, the non-shedding surfaces of the teeth (tooth enamel), support complex bacterial biofilm communities (Bowden and Hamilton, 1998).

Streptococcus mutans are among the most abundant bacterial species which call the oral cavity 'home'. This particular species of bacteria play a huge role in the process and formation of dental plaque biofilms and which eventually creates tartar, and produces dental caries (tooth decay), invading the enamel (outermost layer) of the tooth structure. Caries, periodontitis (gum

disease), and other infections are now being recognised to be caused by a whole range of different organisms which are present in a biofilm environment, rather than a single pathogen (Jenkinson and Lamont, 2005).

Microorganisms within the oral microbiome have been shown to cause a number of oral infectious diseases, including caries, periodontitis, endodontic infections, alveolar osteitis, and tonsillitis. Present studies are collecting evidence that bacterial colonisation within the different habitats of the oral cavity are linked to certain systemic diseases as well, including cardiovascular disease (Beck and Offenbacher, 2005), pneumonia, diabetes (Genco et al., 2005), and some cancers. For example, if found present in the mouth, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* can be used as an effective biosensor for early diagnosis of pancreatic cancer.

2.2 Biofilm Formation

Bacteria in the mouth can either exist as free floating or as part of a biofilm. Plaque biofilms are formed through a series of stages which involve the growth and colonisation of complex bacterial communities (Bowden et al., 1979; Brecx etal.,1983; Gilbert etal.,1993; BowdenandLi,1997). The first stage of biofilm formation starts with the deposition of a conditioning film called the acquired pellet onto the outermost layer of the tooth, otherwise known as tooth enamel. The acquired pellet is made up of the proteins and other molecules found in the saliva of the host. This film or ‘pellet’ provides a favourable thin layer on the enamel for the bacteria to adhere to.

The paper “Survival of Oral Bacteria” authored by George H.W Bowden and Ian R. Hamilton explains that the ability of bacteria to adhere to certain surfaces within the oral cavity is essential for their survival in various environments, not only limited to the oral cavity itself. These bacteria possess specific surface molecules and appendages known as adhesins which allow the bacteria to recognise and bind to specific biological receptors on mucosal cells and surfaces, other bacterial species, and the acquired pellicle on solid surfaces.

Adhesins are molecules that facilitate the attachment of bacteria to host tissues. Bacterial adherence usually occurs in one of two ways; Specific and non-specific adhesion. Specific adhesion refers to the ability of bacteria to adhere to these surfaces and host tissues through specific interactions between the surface molecules present on the bacteria (adhesions) and particular receptors on the target surface. For example *Streptococcus oralis* and *Streptococcus sanguis* are often the first colonisers as they are able to recognise and bind to the molecular components of the acquired pellicle.

Non-specific adhesion refers to the bacterial ability to attach to surfaces through pure physical or chemical interactions rather than through specific molecular recognition, meaning these interactions DO NOT rely on the acquired pellicle binding site, or the receptors. The paper suggests that during the early stages of biofilm development, non-specific adhesion plays a more significant role than originally thought for developing a biofilm with a diverse array of microorganisms to attach to surfaces, including free-floating bacterial species, subsequently creating complex bacterial communities.

The next stage of a biofilm formation further enhances the bacterial diversity of the bacterial communities found with the biofilm itself. Co-aggregation (Bos et al., 1994, 1995, 1996) refers to the aggregation of different bacterial species allowing the microorganisms with non-specific adhesins or organisms of which cannot adhere directly to the tooth surface or acquired pellicle and which may hold other metabolic or ecological functions within the biofilm, to be a part of that biofilm (Kolenbrander and London, 1992).

Once adhered to the tooth surface or host tissue, the microorganisms begin to form bacterial colonies and the biofilm begins to mature (Bowden and Hamilton, 1998). As these bacterial colonies grow and begin to mature, they construct an extracellular matrix made up of proteins, nucleic acids and polysaccharides (Bowden and Hamilton, 1998). This matrix protects the bacteria from environmental stresses such as a change in pH levels of the oral cavity, antimicrobial agents, as well as the shear mechanical force produced by the maxillary and mandibular functions. It also functions as a waste removal and facilitates the retention of nutrients.

During maturation, a dental plaque biofilm takes up a three-dimensional form, otherwise known as calculus or tartar. Over time, the extracellular matrix creates chambers within the structure to allow for the flow of the nutrients and removal of waste. Biofilms on teeth are ordered structures with cells embedded in the extracellular matrix that resists removal and serves as a very effective habitat for oral bacteria (Bowden and Hamilton, 1998).

These biofilms can spread rapidly across all surfaces of the oral cavity, and if left alone, can mature and form calculus or tartar which can then eventually lead to tooth decay and demineralisation of the tooth enamel and the rest of the tooth structure. The bacteria which have colonised in the plaque biofilm feed on the food and drinks that the human host consumes on a daily basis. The bacteria metabolise the sugars and carbohydrates and in return produces an acid which has the ability to demineralise the hard tissues of the tooth (enamel, dentin, etc).

2.3 Fluoride

Fluoride is the negatively charged ion of the element fluorine. It is one of the most commonly used treatments across the world for the prevention of tooth decay or caries. The promotion of fluoride in the dental community as a protection against tooth decay has always faced the high concerns regarding the risk of dental fluorosis (Do et al., 2020). Dental fluorosis is a developmental condition of enamel which occurs when the enamel is exposed to a too high amount of fluoride while the tooth is still developing. Fluoride itself can be found in the earth's soil, resulting in small amounts of fluoride being found in our everyday foods. Fluoride is also applied to almost all of Australia’s water supply. As of 2021, 90% of Australians have access to fluoridated drinking water which includes the areas that have naturally occurring fluoride concentrations (NHMRC, 2017).

2.4 Enamel Formation

The outermost layer of a tooth is called the enamel. It is the strongest substance in the human body and is primarily composed of hydroxyapatite, a crystalline calcium phosphate mineral. In addition to hydroxyapatite, tooth enamel contains calcium, phosphate, hydroxyl (contributes to

the hydroxyapatite), magnesium (small amounts), carbonate (small amounts), and fluoride which can be incorporated into the molecular structure of tooth enamel (Robinson et al., 2004). The overall composition of tooth enamel varies from person to person, depending on outside factors such as diet, individual biological differences and fluoride exposure, varying from person to person.

2.6 Aim

The aim of this study is to understand if the concentration of fluoride affects the growth of bacterial cultures.

2.7 Hypothesis

The higher the concentration of fluoride exposure, the fewer the amount of growth of bacterial cultures will be observed.

3. Materials and Methods

3.1 Experimental Design

This study prompted an in vitro experimental design to investigate the effects of different fluoride concentrations on the microorganisms which inhabit the human oral cavity. This particular design allowed for any effects to be observed without the risk of harm to any individuals working within the experiment. The experiment utilised saliva and plaque samples from two subjects which were then exposed to different concentrations of fluoride. The samples were kept in an incubator with set conditions to echo those of the human oral environment and observed. Followed by culturing and analysis of microbial growth.

3.2 Subjects and Sampling

Two subjects, one male and one female, both within the ages of 17 and 18 years old, were chosen for this experiment. Female is a vegetarian, male is a generalist omnivore. Ethical approval was obtained from the Central Coast Sports College to carry out this experiment, and informed consent was obtained from all participants. Prior to sample collection, subjects were given and instructed to complete a diet and oral hygiene diary, to be completed and filled out for the 48 hours before sample collection. In addition, subjects were also instructed to avoid food intake other than water for 2 hours prior to sample collection. Saliva samples were collected using sterile cotton swabs, with subjects swabbing the gums behind their second or third molars (depending on whether or not the subject has wisdom teeth) to ensure controlled sampling across both subjects.

3.3 Preparation of Fluoride Samples

Three fluoride concentrations were used to simulate different exposure levels:

1. **Low Concentration:**
   Fluoridated water collected from Kariong, Central Coast, NSW, Australia
   1 ppm fluoride

2. **Medium Concentration:**
   Colgate Toothpaste (based off survey results)
   1450 ppm Fluoride (0.32% w/w)

3. **High Concentration:**
   Enamelast Fluoride Varnish
   22,600 ppm Fluoride (2.26% w/w)

These concentrations were chosen to represent different fluoride levels using drinking water, standard colgate toothpaste, and a prescription-strength fluoride treatment. The exact procedure for the fluoride solution preparation was as follows:

- Low concentration: 2 millilitres of water was mixed with 2 millilitres of deionised water.
- Medium concentration: 1 gram of toothpaste was mixed with 2 millilitres of deionised water.
- High concentration: Fluoride varnish treatment was mixed with 2 millilitres of deionised water.

3.4 Sample Treatment and Plating

For each subject, five conditions of treatment were prepared:

1. Control Group 1 - Deionised water exposure
2. Control Group 2 - Sample with no fluoride treatment
3. Low Fluoride Concentration
4. Medium Fluoride Concentration
5. High Fluoride Concentration

Equal volumes (1ml) of saliva and treatment solution were mixed together and allowed to interact for 2 minutes to simulate the same exposure time as a fluoride treatment conducted in a dental practice, in the oral cavity. Following this interactive time period, roughly 0.1ml of each solution were swabbed and plated onto nutrient agar plates using sterile and aseptic technique.

3.5 Incubation and Observation

All the petri dishes were incubated at 37 degrees celsius to simulate the environmental conditions of the oral cavity. The incubation period lasted for approximately 1 Week (168 hours). Any factors that could potentially affect the microbial growth in this time period, for example

rises or drops in temperature within the incubator during this time period, were monitored closely.

3.6 Data Collection

After the incubation period, the following data were observed and collected for each plate.

- Size of varying colonies
- Unique characteristics of colonies (colour, texture, shape, size, etc.)

Photographs were taken of each plate for further analysis and documentation.

3.8 Safety and Ethical Considerations

Strict safety protocols were followed for handling all samples and bacterial cultures throughout the experiment. All procedures were completed in an isolated laboratory-like environment, and personal protective equipment (PPE) including gloves, protective face mask and safety glasses were worn at all times. Disposal of biological samples were carried out in accordance with the *NSW EPA's Protection of the Environment Operations Act 1997*.

3.9 Standardisation and Limitations

It is important to outline the limitations to this study and the approaches of standardisation. Diet is an important factor that has the ability to significantly influence the composition and activity of oral bacteria present in the oral cavity. Certain compounds and minerals found in foods may have the potential to interact with fluoride. These foods also may affect the pH of saliva, making the oral cavity either more basic or acidic. Specific techniques of standardisation were chosen with careful consideration and tailored to help isolate the effect of fluoride, which is the primary focus of the investigation.

To create consistent conditions across all participants and minimise the amount of variables, certain protocols were put in place. Both participants avoided eating for 2 hours before the sample collection, only allowed to consume water. Both participants were also asked to complete a food and oral hygiene diet for 48 hours prior to sample collection.

4. Results

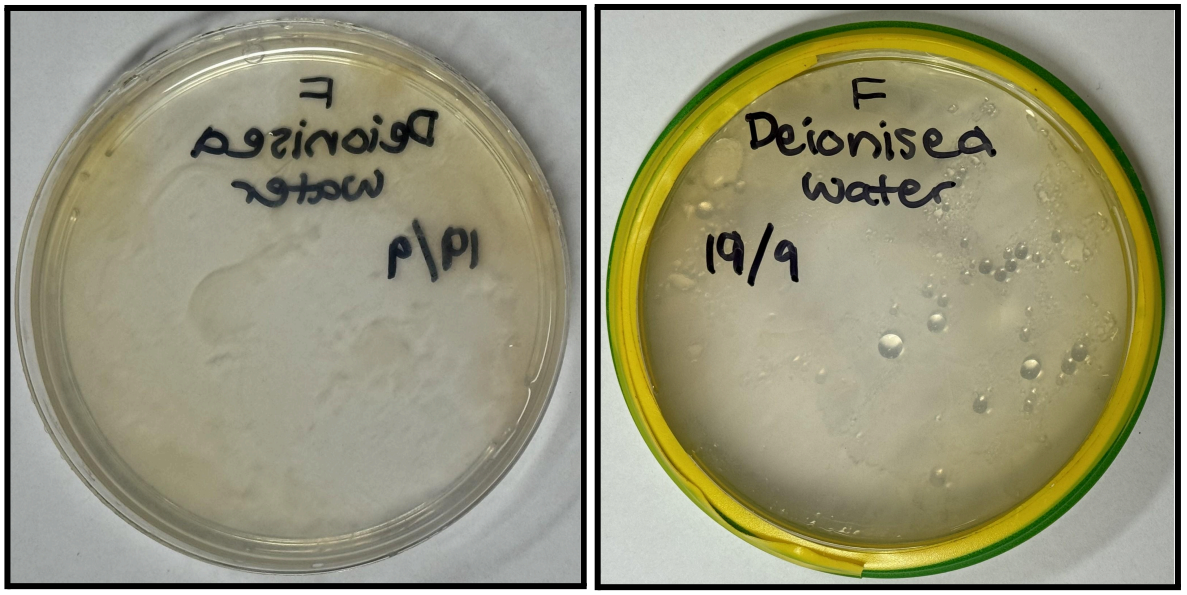
After being placed in the oral cavity simulation incubator for approximately two weeks, a considerable amount of bacterial growth was reached in all dishes.

Contaminated Cultures

Three petri dishes were pre-contaminated upon arrival at the lab. All three were kept, cultivated and observed as a third control group within the experiment. Bacterial colonies that did continue to grow are very different compared to all the other samples, with no similarities observed at all.

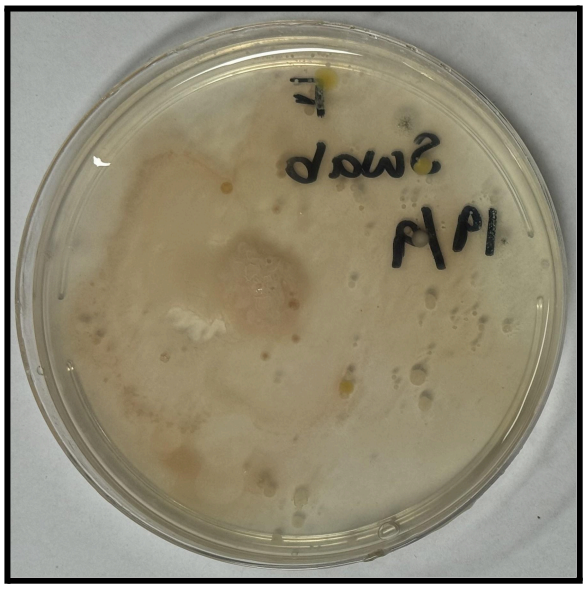
Subject 1. Female. 17 Years of Age.

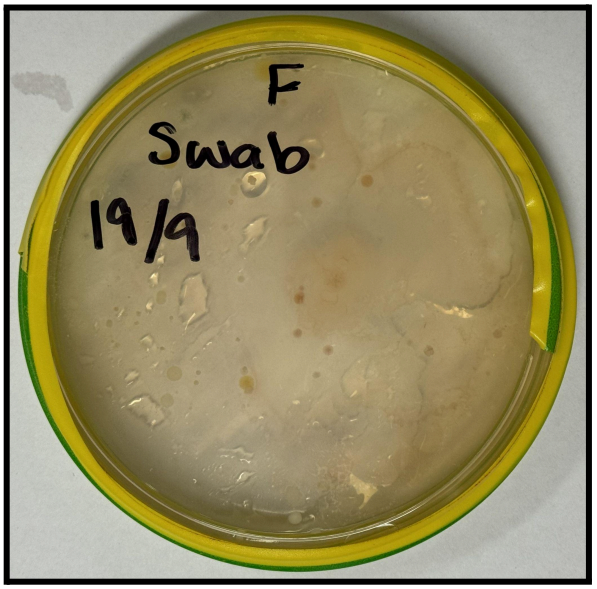
Control Group 1 - Deionised water exposure



10% growth coverage, almost fuzzy colonies, yellow in colour. Splotchy in pattern, does not follow swab.

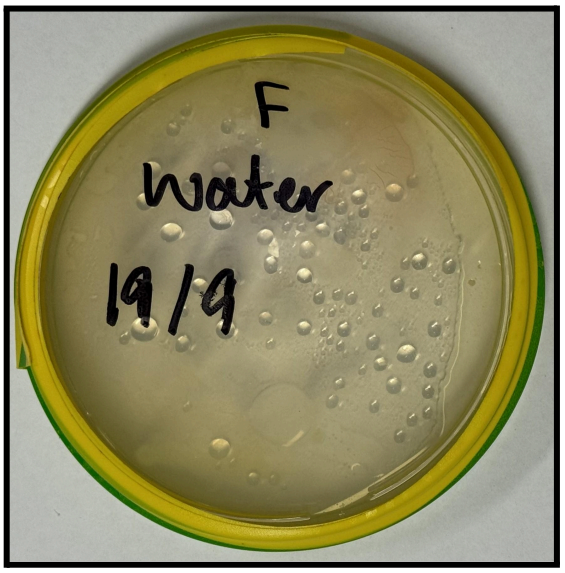
Control Group 2 - Sample Swab





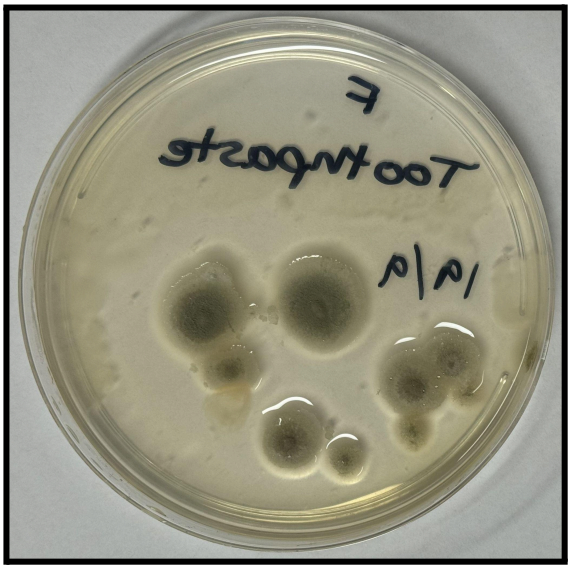
45-50% growth coverage, growth does not follow the swab pattern, growth comes from the centre spot and expands, each colony varies in colour, shape and size, ranging from orange to beige with some darker orange patches.

Low Concentration - Fluoridated Water (1 ppm)



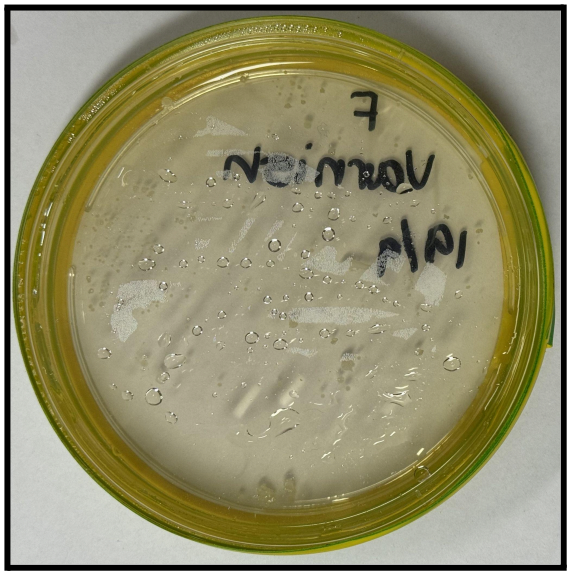
20% growth coverage, dark orange in colour, fuzzy, did not grow in the swab pattern.

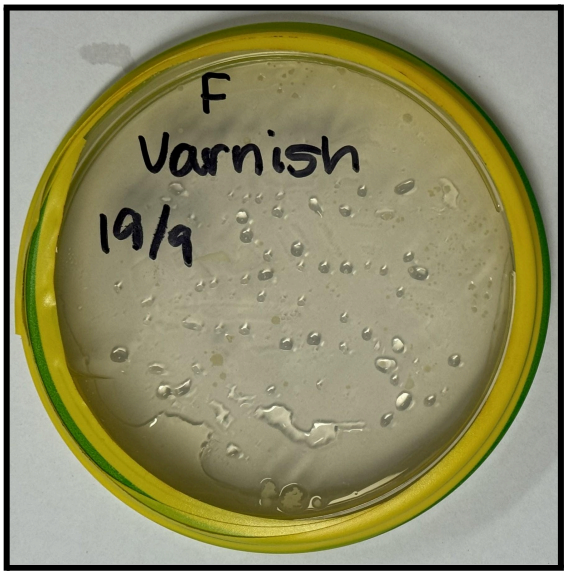
Medium Concentration - Toothpaste (1450 ppm)



25% growth coverage, did not grow in the swab pattern, dark green in colour, growth comes from a centre spot and expands growth. Size of colonies varies from a diameter of 0.5cm - 2cm.

High Concentration - Fluoride Varnish (22,600 ppm)





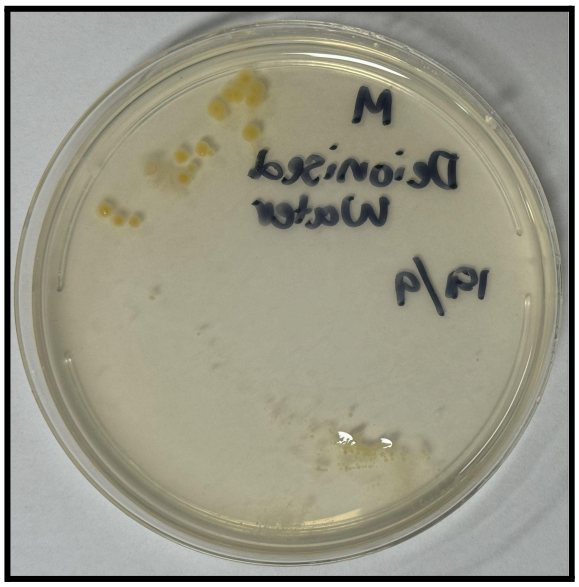
5% growth coverage, light beige in colour, follows the swab pattern.

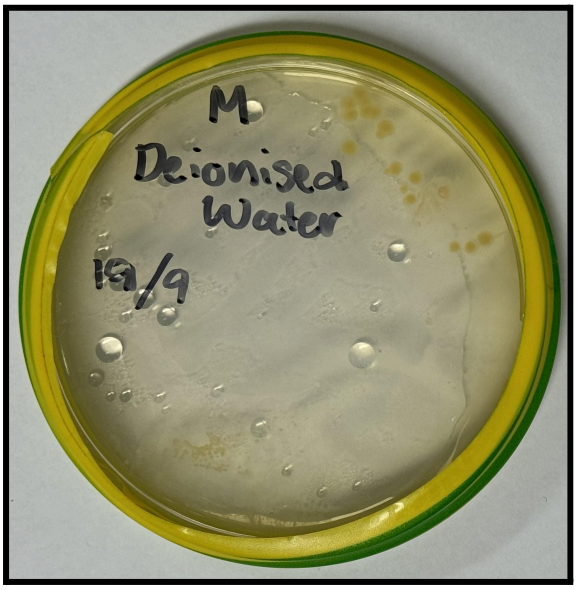
Subject 1 Summary:

Colonies of bacteria of which are orange in colour can be observed in the control group 2 and the low concentration petri dishes. Colonies of bacteria of which are beige in colour can be observed in all petri dishes excluding the medium concentration sample. Medium concentration sample had the most bacterial growth, as well as the most stand out growth, having its colour be a dark green, almost black.

Subject 2. Male. 18 Years of Age.

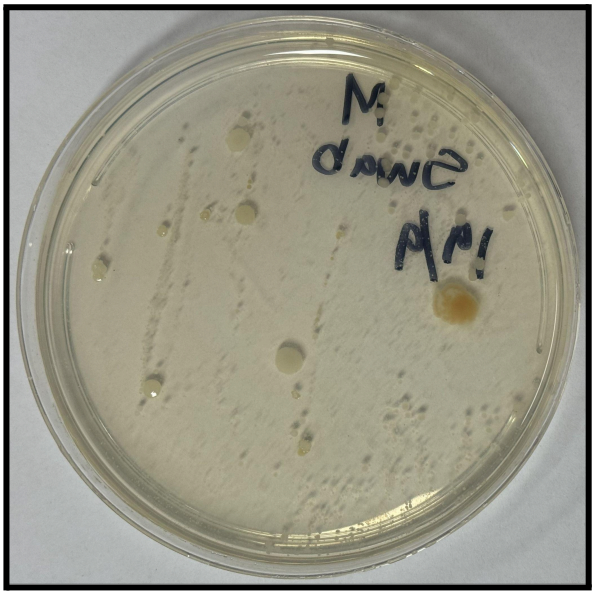
Control Group 1 - Deionised water exposure

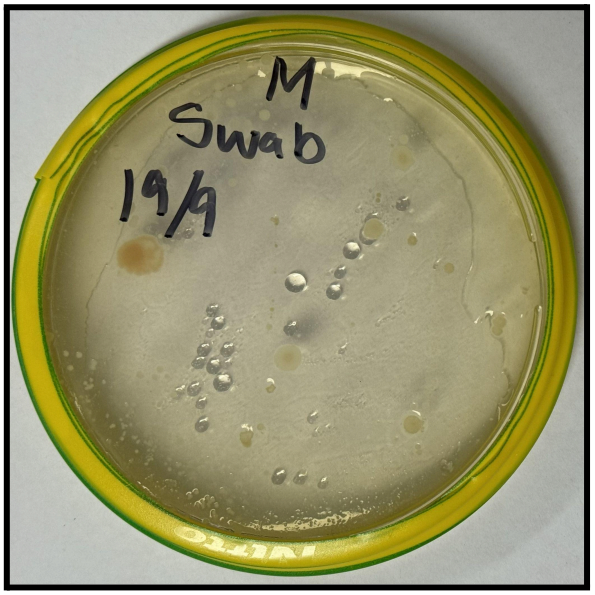




10% coverage, yellow in colour, follows the swab pattern, small colonies of bacterial growth.

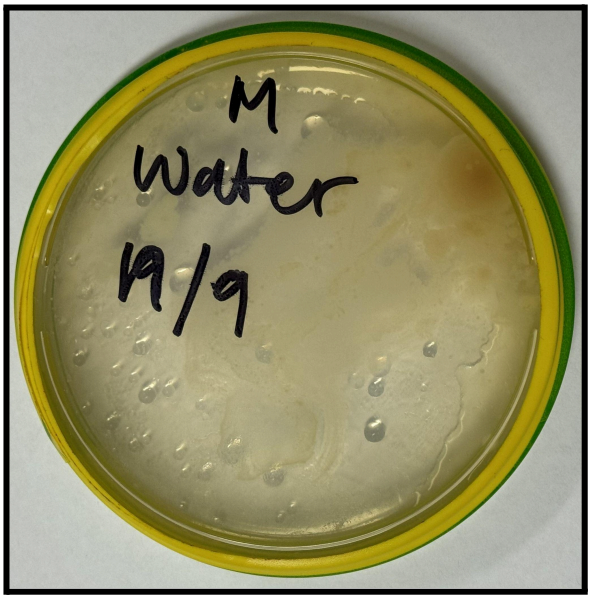
Control Group 2 - Swab Sample





10% coverage, orange and beige colonies, small bacterial growth, follows the swab pattern.

Low Concentration - Fluoridated Water (1 ppm)

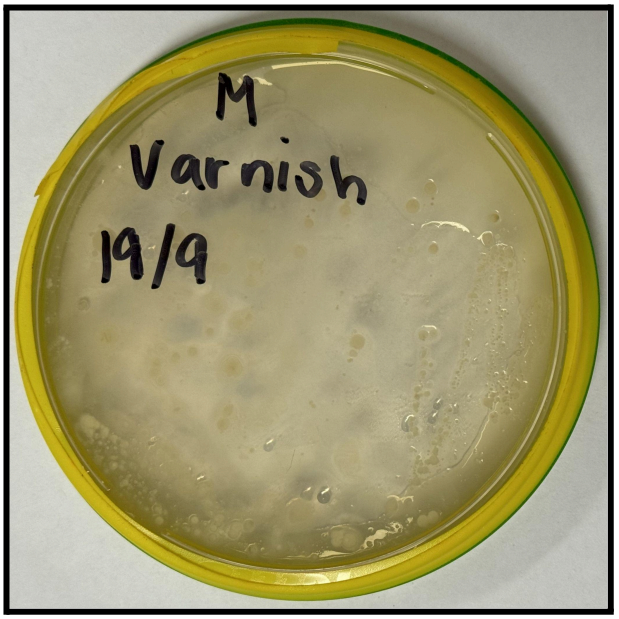


45-50% coverage, darker orange in colour, fewer but larger colonies present, does not follow swab patterns.

Medium Concentration - Toothpaste (1450 ppm)

5% coverage, beige, almost white in colour, smaller colonies so fewer bacterial growth, does not follow the swab pattern.

High Concentration - Fluoride Varnish (22,600 ppm)



5% coverage, beige, almost white in colour, smaller colonies, follows the swab pattern.

Subject 2 Summary:

Colonies of bacteria of which are orange in colour can be observed in the control group 2 and the low concentration petri dishes. Colonies of bacteria of which are beige in colour can be observed in all petri dishes excluding the Control group 1, where the bacterial colonies are observed to be quite small and yellow in colour. Medium concentration sample had the least bacterial growth out of all of the dishes.

5. Discussion

This pilot study investigated the effects of different fluoride concentrations on the microorganisms of the oral cavity, revealing several significant patterns and raising important considerations about the relationship between fluoride exposure and oral bacterial growth.

Looking at the results, it is hard to determine a conclusion on the exact effects that varying concentrations of fluoride have on the microorganisms of the oral cavity. With this, the outcome of the high concentration of petri dishes from both subjects has the least amount of bacterial growth, suggesting that higher concentrations of fluoride have a stronger antimicrobial effect.

Both control groups for both subjects worked effectively. Both of the deionised water control groups came out with similar characteristics having yellow, splotchy bacterial colonisation. Each of the subjects' control swabs were each to their own. Subject 1 seemingly having a greater diversity of bacterial colonies. Each generally followed the original swabbing pattern, especially Subject 2.

However, there was quite a remarkable variation between the two subjects regarding the bacterial response to the medium fluoride concentration sitting at 1450 ppm (parts per million). Subject 1 had the most unexpected increase in bacterial growth with approximately 25% coverage and distinct dark green colonies. Whereas subject 2 had the least amount of growth with approximately 5% coverage and beige, almost white-ish small colonies in one.

The fact that no bacterial characteristics are observed from the pre-contaminated dishes within both subjects samples across all conditions, suggests that the results gathered from this experiment are not due to that contamination, and are purely based on the different concentrations of fluoride.

This divergence between the two subjects may be because of:

1. Individual variations in the composition of the subjects oral microbiome
2. Dietary differences between subjects (vegetarian vs generalist omnivore)
3. Sex/Gender

The difference in diet between Subject 1 (vegetarian) and Subject 2 (omnivore) raises a lot of interesting questions about the role of diet when it comes to the composition of microorganisms in the oral cavity, as well as its interaction with specific fluoride treatments. Subject 1 generally showed higher bacteria growth across all conditions when compared to Subject 2. This may be because of the different pH levels in the oral cavity due to dietary requirements and/or patterns.

There are several aspects/factors of which need to be addressed and considered when looking at the results from this experiment. The first factor is that the pre-contaminated plates may still have had some sort of effect on the subject samples, which may not be visible when it comes to observing the characteristics and appearances of bacterial growth. The second factor is that the incubation period of 1 week, while effective for observing the actual growth of bacteria, may not have been as effective when trying to simulate the environment of the human oral cavity. The third factor is that the standardisation protocols and time period may not have been sufficient enough to completely 100% wipe out any dietary influences.

Conclusion

As said above, it is hard to determine a conclusion on the exact effects that different fluoride concentrations may have on the microorganisms of the oral cavity, which may also be due to the small sample size of this investigation. Seeing such large and distinct colonies in toothpaste raises more than enough questions regarding dietary patterns, ingredients within toothpaste, basic oral hygiene habits, etc, and suggests that further investigation expanding the sample size may need to occur in order to get precise results.

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