The Impact of Lifestyle Factors on Periodontal Disease

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Introduction

Periodontal disease affects gum health. Gums become unhealthy when bacteria cause inflammation and infection in the gums and bones that surround and support teeth [1]. There are two different forms of periodontal disease, gingivitis and periodontitis. Gingivitis is the most common form of periodontal disease. Gingivitis irritates the gums because of swelling and redness occurring in the gingiva, the part of the gum around the base of the tooth [2]. If gingivitis goes untreated it can progress becoming a more serious form of periodontal disease known as periodontitis. Periodontitis can cause bone and tooth loss as the gums and connective tissue that hold your teeth in place deteriorate because of the proliferation of periodontal bacteria [3].

In 2012 the Centers for Disease Control and Prevention (CDC) published a study on the prevalence of periodontal disease in America. They found that 47.2% of adults older than 30 had periodontal disease [1]. They also saw that periodontal disease prevalence increases with age as 70.1% of adults older than 65 had periodontal disease [1].

Periodontal disease is polymicrobial [4]. In 1998 Dr. Sigmund Socransky identified all microorganisms that cause periodontal disease and classified them based on the microorganism's association with the severity of periodontal disease (Fig. 1) [5]. These microorganisms were classified into a set of complexes based on the role they played in the onset and progression of periodontal disease [5]. The green and yellow complexes include microorganisms that are considered early colonizers and result in periodontal disease in the form of gingivitis [5]. The orange complex are microorganisms that result in the progression of periodontal disease [5]. These orange complex microorganisms create the bridge between gingivitis and periodontitis where the gum line recedes allowing bacteria to proliferate around the bone level of the tooth [5]. The red complex and Aa complex are compiled of the microorganisms that cause periodontitis where bone and tooth loss may occur [5].

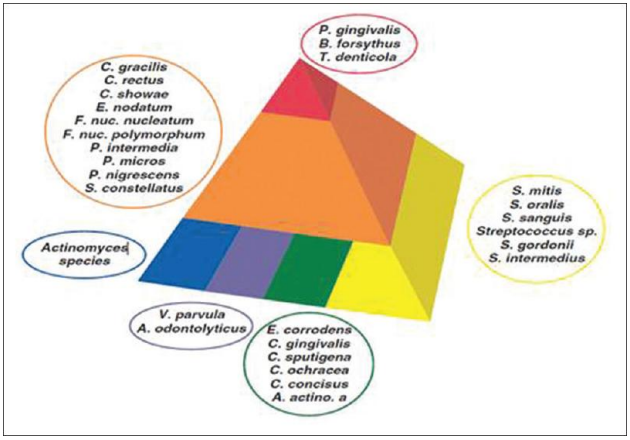

Figure 1: A visual representation of Sigmund Socransky's classification of microorganisms associated with periodontal disease [6].

Periodontal disease is a multifactorial disease existing with intrinsic and extrinsic factors [4]. There is significant evidence that these intrinsic factors increase the risk or progression of periodontal disease these being underlying conditions such as diabetes, immune-deficiencies, heredity, female hormonal changes, and crooked or missing teeth [1]. There is also some significant evidence that certain extrinsic factors increase the risk or progression of periodontal disease, these being smoking, poor oral hygiene, stress, and improper or defective dental procedures [1]. However, there is very little research on the relationship that diet has on periodontal disease [7].

This experiment aims to investigate periodontal disease's relationship with lifestyle factors consisting of certain intrinsic and extrinsic factors that characterize one's lifestyle. Lifestyle factors include diet, hygiene, tobacco use, heredity, body mass index (BMI), and underlying conditions. Some of these factors such as tobacco use, and underlying conditions are known to increase the risk of periodontal disease. However, these factors are still important to investigate as a relationship or correlation to another lifestyle factor may be discovered. Certain factors such as diet have insufficient evidence to prove a relationship to periodontal disease but have been shown to cause cavities in teeth [7]. Carbohydrates, sugars, and starchy foods have been proven to not only cause decay in teeth but accelerate the process as the bacteria proliferates on the surfaces of the teeth [7]. The consumption of vitamin D and calcium reduces the risk of dental caries [7]. Cavities and Gum disease are both different diseases but share the same environment. Therefore, it is important to note these findings as they may pertain to periodontal disease as well.

It was hypothesized that a correlation between lifestyle factors and periodontal disease would occur in two forms. First, all factors that have been proven in the past to cause or accelerate periodontal disease such as smoking, underlying conditions, and poor hygiene would be confirmed by the study. Secondly a new association between diet and periodontal disease would be discovered, mirroring the relationship between diet and cavities.

Materials and Methods

Before research could begin the project had to be approved by the Hampden-Sydney Human Research Committee as the experiment involved human subjects. The research project was approved after the committee deemed the experiment ethical.

A survey was created for participants to fill out that would accurately depict one's lifestyle. Participants were told all survey data was confidential and anonymous as they would be assigned a random number. The survey included three sections. The first section was heredity, it asked the participant's age, height, weight, gender, and underlying conditions. The height and weight were used later to calculate participants' BMI. The second section was diet. This consisted of asking a participant how much of a certain food they ate in a normal week. Fruits, vegetables, proteins, and carbohydrates were listed, so that the full food pyramid was accurately represented. The third section was the lifestyle factors section, this included questions about tobacco use, hygiene, and stress. Tobacco use was determined by asking the participant if they smoked, dipped, or vaped and, if so, how many times a week. Hygiene representation came from asking an array of questions ranging from dental visits, to nightly hygiene routines, to the use of gum and/or toothpicks, and if one chewed their nails. Stress levels were determined by having the participant rate their stress level as well as rate their quality of sleep and how many hours of sleep a participant got per night on average. Ratings of stress level and quality of sleep were done on a scale 1-10, 1 being the worst and 10 being the best. All survey questions were asked in a way where the answer was either a "yes" or "no" or a numerical value. This was important as survey data would be analyzed later and all answers needed to be in the same metric so that the data could be tested.

Prior research had been done to determine the best growth media for optimal growth among periodontal bacteria. A study found that three media were best at growing periodontal microorganisms, one being tryptic soy broth (TSB) [8]. Tryptic soy broth was chosen out of the three found in the article because it was a generic medium that was relatively inexpensive and could be made in large quantities in the lab. Roughly 25 TSB agar growth plates were made using 500 ml of deionized water and 20g of TSB agar. A saline solution was also made using 100 ml of deionized water and 0.9g of sodium.

Once the survey, growth media, and saline had been created the experimentation started. Participants were given a survey with direction on how to complete the survey. Once participants had finished filling out the survey, they were assigned a random number which was written on their survey and corresponding TSB agar plate. Then participants were instructed on how to swab their own mouths. They would unwrap a sterile swab and dip it in saline so that the tip was moist. Then the participant would swab the inside of their mouth focusing along the gum line and make a full circle around their mouth, the swab pressing between the cheek and gums on the top and bottom of the mouth. Next the participant would hand the person conducting the experiment (wearing sterile nitrile gloves) the swab which would then be taken and streaked onto a TSB agar plate that was numbered corresponding to the participants survey. The TSB agar plates were then incubated at 37°C for 96 hours to allow for maximum growth.

Once a plate had fully grown it was taken from the incubator and evaluated for growth sites of interest. Growth sites of interest were marked and then using a sterile pipette tip transferred into an isolated tube containing 5ml of liquid broth (LB). After the growth sites had been removed from the TSB plate, the plate would then be moved to an incubator set to 4°C, where it was kept for the remainder of the experiment in case it was needed again. The isolated tube was then marked corresponding to the growth site. Once labeled the tube was then placed in an agitator at 30°C, the caps on the tubes were loosely on to ensure an aerobic environment. The tubes were then left to incubate in the agitator for at least 24 hours.

After a sample tube had incubated aerobically in the agitator for at least 24 hours the DNA was extracted by following QIAGEN's DNeasy ® UltraClean ® Microbial Kit Quick-Start Protocol. Once a sample's DNA had been extracted polymerase chain reaction (PCR) was run to exponentially increase the DNA found in the sample. Following the polymerase chain reaction gel electrophoresis was run to confirm the sample had yielded enough DNA. QIAGEN's QIAquick PCR Purification Kit procedure was then used to further purify the DNA before being sequenced. After being purified each DNA sample was assigned to a specific SimpleSeq Tube pertaining to the original growth site of interest. These SimpleSeq tubes were then sent to Eurofins Genomics, Inc. located in Louisville, Kentucky. Once received this lab would sequence a section of 16s Ribosomal DNA, the results of this sequencing were shown through chromatogram data.

Using the chromatogram data each bacterial growth site was identified. Identification was found through the National Center for Biotechnology Information (NCBI). Using NCBI's Basic Local Alignment Search Tool (BLASTn) chromatogram data was matched with various bacterial species in the database. Bacterial species were identified with confidence because of the combination between the

percent of similarity between bacterial species and its E value.

Once the bacterial species making up the growth sites on the TSB agar plates had been identified, they could begin to be classified. Research was done to classify and assign the identified bacterial species to Dr. Sigmund Socransky's set of microbial complexes.

All data was then entered into a Microsoft Excel spreadsheet. All survey and identified bacteria data corresponded to the participant in which it belonged too. A data analysis regression was then run to determine the relationship between the survey data and periodontal bacteria.

Once the experiment was completed all tubes and plates were autoclaved before being discarded.

Results

The experiment had a total of 46 participants. Participants varied in age, gender, ethnicity, and BMI. These 46 participants' swabs produced 106 growth sites that were identified and analyzed. Growth sites

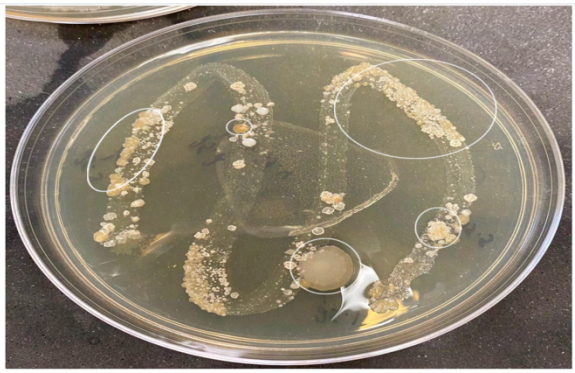

Figure 2: A picture taken of TSB agar plate. The white circles identify the 5 bacterial growth sites identified on this plate.

were identified based on appearance in shape and color in which the bacterial species grew (Fig. 2).

The bacterial species from the growth sites DNA was then isolated, purified, and multiplied exponentially. These processes were confirmed by running gel electrophoresis that proved bacterial yield was successful (Fig. 3).

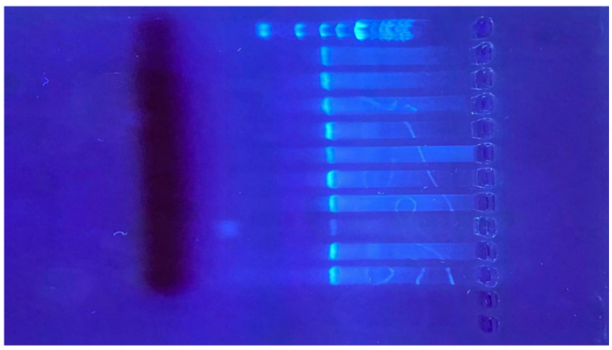

Figure 3: Gel Electrophoresis. The illuminated lanes prove that bacterial yield was successful. Lanes that are not illuminated indicate no bacterial yield. Ladder bands are shown at the top of the gel.

Once confirmation was made that the bacterial DNA had been yielded successfully, the 16s Ribosomal DNA was then sent for sequencing. Sequencing results came back in the form of chromatogram data, the peaks show the computers prediction of the sequence by determining the DNA base pairs A,C,G,T (Fig. 4). The entire of sequence for the bacteria of interest's 16s Ribosomal DNA was then used to determine what bacteria was found with the use of NCBI BLAST search tool (Fig 5).

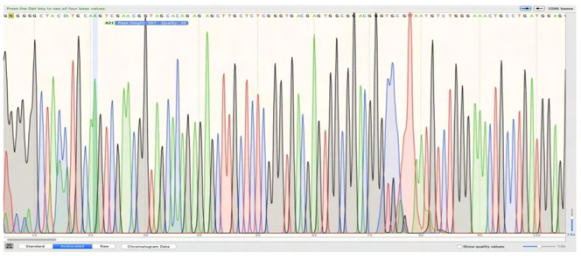

Figure 4: Chromatogram: the horizontal axis above the graph shows the chromatogram data. The vertical axis shows qualitative probability of a specific nucleotide present at each segment of DNA. Each bacterial species produced an individual chromatogram from which its sequence was determined.

Figure 5: Chromatogram results: This is an example of a 16s Ribosomal DNA sequence for a bacterial growth site. This sequence was then used to determine what bacteria species was present.

Out of the 106 growth sites that were identified 98 were successfully identified through sequencing, a 92% yield. Species from the *Streptococcus* genus were the most commonly identified as 18 *Streptococcus* species were found, making up 18% of the total number of bacteria. 14 *Bacillus* and *Neisseria* species were found; therefore, both of these species made up 14% of the bacteria identified in the study. The *Granulicatella* genus represented 9% of the samples identified as it was identified 9 times. *Staphylococcus* species were found 8 times making up 8% of the total sample. The remaining 34% were among the following 14 bacterial genera, all of which were not identified more than 3 times. These species include: *Agromyces, Citrobacter, Digitaria, Enterococcus, Enterobacter, Fusobacterium, Gemella, Humibacter, Morococcus, Mus, Oryzins, Pantoea, Pelomonas,* and *Rothia.* Figure 6 gives a visual representation of the amount each genus was found (Fig 6). *Streptococcus* was classified by Dr. Socransky as a yellow complex periodontal bacterium [6]. *Fusobacterium* is classified as an orange complex periodontal bacterium [6]. Therefore, out of all the bacteria identified in the study, 22.5% were made up of periodontal bacteria (Fig 7).

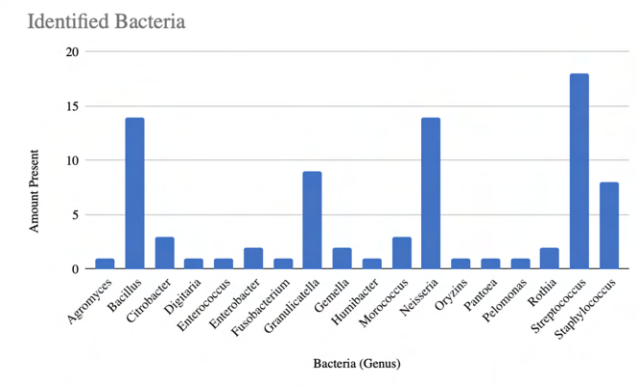

Figure 6: Bar graph representing how much of a certain genus of bacteria was identified in the study.

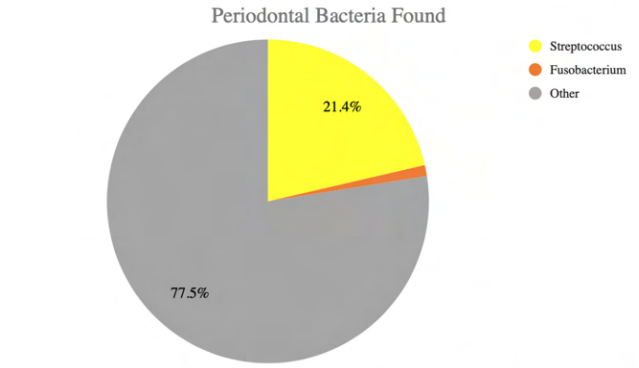

Figure 7: Pie Chart showing the percentage (22.5%) of periodontal bacteria found in the study. All other bacteria were not classified as periodontal bacteria according to Dr. Sigmund Socransky.

The raw data analysis regression in which the response variable was total number of bacterial genera only found one statistically significant variable that being average sleep, this was a negative relationship denoted by the negative t-statistic (Fig 8). There were no statistically significant results on the raw regression run where the response variable was *Streptococcus* spp. (Fig 9).

Response Variable: Number of Streptococcus spp. per subject

Variables	*t Stat*	*P-value*
Body Mass Index	0.588086923	0.560875391
Underlying Conditions Total	-0.69591999	0.491835523
Meat Total	0.171635247	0.864876826
Nuts	1.113072958	0.274516596
Fruit Total	-0.148556559	0.882897638
Vegetable Total	-0.042205922	0.9666142
Processed Carbohydrates Total	-0.156987682	0.876306343
Dairy Total	-0.101979782	0.919451312
Alcohol Total	0.003484019	0.997243225
Tobacco Total	-0.209460005	0.835505067
Artificial Drinks Total	0.447320501	0.657856846
Dentist	1.166049388	0.252781265
Grinding Teeth	1.519606621	0.13908
Chewing Gum	-0.695769339	0.49192853
Average Sleep	-1.667053047	0.105911833

Figure 9: Results of a data analysis regression. Regression was raw as it included variables with missing values. N=46.

Explanatory variables with either high P-values or participants who were missing information were then removed and another set of regressions were run with the response variables staying the same. The refined

Response Variable: Number of bacterial genera per subject

Variables	*t Stat*	*P-value*
Body Mass Index	2.928236229	0.01175312
Underlying Conditions Total	-2.611167381	0.021544475
Nuts	3.884228564	0.001881493
Dairy Total	0.159200559	0.875957613
Alcohol Total	-3.147887348	0.007702903
Artificial Drinks Total	-3.127577098	0.008010056
Dentist	0.420653987	0.680878347
Grinding	0.928836582	0.369899891
Chewing Gum	0.730124748	0.478265493
Average Sleep	-1.14896971	0.271269853
Stress	-1.583401461	0.137345133
Chewing Nails	-1.193467843	0.254011801
Toothpicks	1.919705756	0.077110153
Daily Brushing (# of times)	-2.207916912	0.045826056
Flossing	2.192372438	0.047153191
Mouthwash	2.357376979	0.034744866

Figure 10: Results of a data analysis regression. Variables with highest p-values were removed and replaced with variables that were not included in raw regression. N=30.

data analysis regression where the response variable was total number of bacteria genera found many statistically significant variables. There was a positive relationship with BMI, nuts, flossing, and mouthwash causing a greater number of bacterial genera (Fig 10). There was a negative relationship found with underlying conditions, alcohol, artificial drinks, and daily brushing causing a fewer number of bacterial genera (Fig 10). When the response variable was changed to number of *Streptococcus* spp. there were slightly fewer relationships present. BMI and nuts were found to cause a greater amount of *Streptococcus* spp. (Fig 11). Underlying conditions, alcohol, and daily brushing were proven to cause fewer *Streptococcus* spp. to be present (Fig 11).

Response Variable: Number of Streptococcus spp. per subject

Variables	*t Stat*	*P-value*
Body Mass Index	2.073896786	0.058515875
Underlying Conditions Total	-3.274468691	0.006037136
Nuts	2.30295648	0.038447319
Dairy Total	-1.125569023	0.280699337
Alcohol Total	-2.314383359	0.037640076
Artificial Drinks Total	-0.35789645	0.726164845
Dentist	1.779224462	0.098579522
Grinding	1.411713662	0.181519688
Chewing Gum	-0.284102586	0.780806455
Average Sleep	-2.26481089	0.041262619
Stress	-0.85503039	0.408016374
Chewing Nails	0.370231896	0.717171723
Toothpicks	0.925499427	0.371568338
Daily Brushing (# of times)	-3.281525707	0.005955706
Flossing	0.453231755	0.65785438
Mouthwash	1.162662711	0.265865757

Figure 11: Results of a data analysis regression. Variables with highest p-values were removed and replaced with variables that were not included in raw regression. N=30.

Discussion

Out of all the bacteria identified 22.5% were classified as periodontal bacteria. This percentage is slightly lower than expected. The lower volume of periodontal bacteria may be related to the significant number of college students that participated in the study. Roughly a third of the participants were under the age of 22, therefore the presence of periodontal bacteria is less likely as periodontal disease becomes more prevalent as one ages. The most commonly found bacteria in the study was of the *Streptococcus* genus, which is classified as a yellow complex bacteria. *Fusobacterium pseudoperiodonticum* was

identified once but is significant as it is classified as an orange complex bacteria. One of the more commonly identified bacteria *Granulicatella adiacens* was not classified by Dr. Socransky as a periodontal bacterium. However, upon doing further research it was found in a study to be associated with aggressive periodontitis [9]. Dr. Socransky died in 2011, as a result no further bacteria have been added to the periodontal complex. Therefore, *Granulicatella adiacens* was not included or categorized as a periodontal bacterium. The other statistically significant bacterial genera found included *Bacillus, Neisseria, and Staphylococcus. Staphylococcus* species are commonly found in the mouth as a study found that 94% of adults carry some form of the species but these species have no connection to periodontal disease [10]. It was also found that many *Bacillus* species are commonly found in dried foods and dairy [11]. The fact that *Bacillus* species are commonly present in the foods and drinks of many people's diet explains the high volume found in this study, however these species do not cause or contribute to periodontal diseases. A study examining saliva samples found that *Neisseria* species are highly prevalent in the human oral microbiome and upper respiratory tract but there is no evidence in these species related to periodontal diseases [12]. All other bacteria identified were researched but no connections or relationships were found to classify or mention these genera as periodontal bacterium.

A regression was run to determine the relationship between the lifestyle factors and periodontal bacteria. Results with a p-value less than 0.05 and a t-statistic greater than 2.00 are statistically significant. The t-statistic also determines the nature of the relationships. Results that are statistically significant from this experiment are likely to be seen in a larger population as well. The raw analysis run (Fig. 8) found one statistically significant relationship, the more one sleeps fewer bacteria species will be present, this relationship was also seen in the refined analysis (Fig.11). A different study pertaining to periodontal bacteria found a similar relationship, as they found that less than 6 hours of sleep can increase your risk of periodontal disease [13]. Interestingly there was no statistical significance between tobacco use and periodontal disease, despite the fact that tobacco is known to cause periodontal disease [3]. The lack of correlation between tobacco and periodontal bacteria in this study may be because few participants used tobacco products. Those participants who did were relatively young thereby, decreasing their risk of decreasing periodontal disease.

A new regression was run after removing participants with missing information as well as the variables that had the highest p-values as the maximum number of variables to test in one regression is 16. This refined regression found more statistically significant relationships. In the regression run corresponding to the overall number of bacteria present in the mouth found 8 relationships. Interestingly the regression found that the more one flosses and uses mouthwash the more bacterial species will be present in the mouth (Fig. 10). This positive relationship is not supported as flossing and mouthwash are encouraged by many dentists as a means to prevent periodontal disease. It was also seen that artificial drinks had a negative relationship with oral bacteria (Fig. 10). This relationship may seem misleading at first however, one dentist states that artificial products (sweeteners) are damaging to the enamel on teeth but the components of the drinks themselves do not feed bacteria [14]. All other relationships found in the regression corresponding to the overall bacterial species present in the mouth were also seen in the regression studying the relationship between these variables and periodontal bacteria. BMI had a positive relationship in both regressions (Fig. 10&11). The positive relationship between BMI and oral bacterial specifically periodontal bacteria is not surprising as obese people are much more likely at developing periodontal disease [15]. The only other positive relationship the two regressions shared was the relationship between bacteria and nuts. This was a peculiar relationship as nuts are often thought of as healthy, however the relationship was statistically significant in both of the regressions, so further research may be needed to further investigate this relationship. Surprisingly alcohol consumption was seen in both studies to have a negative relationship with bacterial growth (Fig. 10&11). Research has found that alcohol consumption has a positive relationship with periodontal disease as it increases the risk of developing periodontal disease as well as accelerating and aggravating existing periodontal disease [16]. Underlying conditions were also seen to have a negative relationship with all bacterial growth in the mouth (Fig. 10&11). Although this relationship is statistically significant in this study it may be misleading, as only 4 out of the 31 participants included in the refined analysis had an underlying condition. Its also well known that underlying conditions increase the risk of periodontal disease [1]. Unsurprisingly it was found that the amount one brushes their teeth had a negative relationship with bacterial growth. This is unsurprising because it is readily known that brushing your teeth removes bacteria therefore inhibiting bacterial growth and promoting oral health.

Conclusion

Given the results from the statistical analysis while considering the sample size of participants, it can be suggested that there is correlation between certain

lifestyle factors and periodontal disease. Lifestyle factors that had already shown correlation to the onset and progression of periodontal disease such as BMI, amount of sleep, and hygiene were confirmed. However, underlying conditions and alcohol consumption in this study showed a negative correlation to periodontal disease, this contradicted prior research that concordantly showed a positive relationship between these factors and periodontal disease. The only relationship found between diet and periodontal disease in this study was the positive correlation between nuts and periodontal disease. Carbohydrates, proteins, fruits, and vegetables had no correlation to periodontal disease in this study.

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