Obesity in School: Association of Gum Bacteria Distribution and Adolescent Obesity

Kim Yujin
Anderson Junior College
Singapore
hannahk0331@gmail.com

Tao Zhen Yuan
Anderson Junior College
Singapore
taozy98@gmail.com

Harsha Prem
Anderson Junior College
Singapore
lbharsha98@gmail.com

Abstract— **The aim of the experiment is to identify association between body weight and gum bacteria distribution, which might be helpful to develop early screening approaches. The relationship between gut microbiota and obesity has been established in rodent models where mice fed with high-fat, high-sugar western diet has different gut microbiota as compared to mice fed with low-fat diet when housed in germ-free environment. There has also been accumulating evidence showing that periodontal disease, caused mainly by gum bacteria, is associated with multiple metabolic disorders including obesity in certain populations. This has led us to hypothesize that there can be particular gum bacteria that is accountable for obesity. To investigate, we have collected saliva sampled from 54 junior colleague students aged 16-19, with age, height, weight, body mass index (BMI), as well as hip to waist ratio through filling of survey forms. Upon collection of the saliva samples, samples were processed in Singapore Bioimaging Consortium (SBIC), A*STAR, where bacterial DNA were extracted, amplified, purified and classified. We analyzed the body weight distribution and compared different obesity criteria, which can be applicable to Singapore adolescents. And no specific bacteria were identified in current study, which could be due to limited qPCR screen power**

Keywords-obestiy; gum bacteria; sample collection; qPCR; gel electrophoresis

I. INTRODUCTION (BACKGROUND AND PURPOSE)

Obesity is defined as abnormal or excessive fat accumulation that may impair health. It is becoming a global pandemic influenced by sedentary lifestyle and increasing affluence in our society. Obesity is associated with a low-grade chronic inflammation that contributes to the development of insulin resistance, Type II diabetes and cardiovascular diseases (Hotamisligil, 2008; Shoelson and Goldfine, 2009).

Various literature reports (Le Chatelier, 2013) have shown the relationship between gut microbiota and obesity. This was previously demonstrated in rodent models, where mice fed with high-fat, high-sugar western diet has different gut microbiota, as compared to mice fed with low-fat diet when housed in a germ-free environment. It is evident that the shift in composition of gut microbiome and their functional properties can be elicited by diet and affect energy homeostasis.

In addition, there are accumulating evidence showing that periodontal disease which is a chronic inflammatory disease caused mainly by gum bacteria[1] is associated with multiple metabolic disorders including obesity in certain population[2].

The most efficient way to fight against obesity is early prevention, which requires early diagnostics. The main aim of this research is to identify the association of metabolic abnormalities to periodontal diseases, which may result in the development of a probe specific bacteria species that can be used for preliminary screening for obesity. To achieve this, we will identify the link between the type of bacteria and the body metabolic parameters.

II. HYPOTHESIS

We propose that gum bacteria profile can, at least partly, reflect one's inflammation status and risk of onset of obesity. Figure 1 shows a flowchart that explains how previously established information has been used to derive our hypothesis. As shown in Figure 1[3], we hypothesize that gum bacteria's effect on one's appetite or energy metabolism may lead one to having metabolic disorder such as obesity. If one of our hypotheses is proven to be correct, the result can be used to develop detection probe for early screening of a certain type of gum bacteria which leads to metabolic disorder hence preventing further development of disorders. We will be addressing our hypothesis through a two-step approach. First, we will collect saliva specimen from 60 student volunteers in Anderson Junior College. The specimen will be stored for DNA isolation and quantitative real time Polymerase Chain

[1] Is Obesity an Oral Bacterial Disease- http://www.ncbi.nlm.nih.gov/pubmed/19587155,
[2] Microbiota in the oral subgingival biofilm is associated with obesity in adolescence- http://www.ncbi.nlm.nih.gov/pubmed/21996660)
[3] Goodson, J. M., et al. "Is obesity an oral bacterial disease?" Journal of dental research 88.6 (2009): 519-523.

Sponsor: Singapore Bioimaging Consortium (SBIC), A*STAR

Reaction (qPCR) on 16S rDNA. This is used to identify the association between the type of bacteria and body metabolic parameters. Next, we will screen a bacteria-specific fluorescent molecule against the identified bacteria, which can be used as a detection probe for quick test for obesity risk.

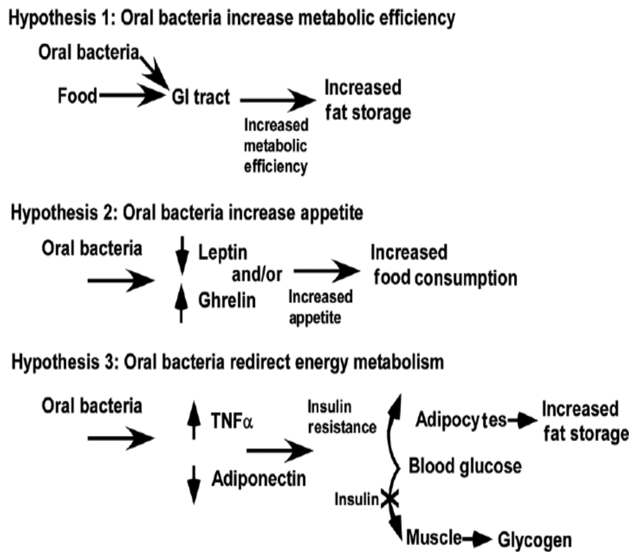

FIGURE 1: Flowchart to illustrate hypothesis (cited from J.M.Goodson, 2009)

III. METHOD AND MATERIALS

A. *Method of obtatining saliva samples*

A total of 54 students, of which 46 were normal weight and 8 were overweight, were recruited on voluntary basis to provide us their mouthwash of no more than 50 ml containing their saliva. There was 1 obese male, 3 overweight males, 3 overweight females, 22 normal weight females, 18 normal weight males, 5 underweight females and 1 underweight male with WHO criteria[4]. Volunteers had to get a consent form signed by their parents to approve them of participating in our research. They were told not to eat or brush 30 mins before the sample collection.

During the sample collections, the volunteers had to complete a questionnaire (attached in Appendices) that collects the following information: hip and waist measurements, age, ethnicity, height, and weight, personal and family medical history if available. The height, weight, hip and waist measurements will be used to calculate the Body Mass Index (BMI) and Waist-to-Hip ratio (WHR). They would then be provided with sterile water to rinse their mouth and spit no more than 50 ml into a falcon tube. These falcon tubes are labelled with a code number which corresponds to that of the questionnaire. This is to maintain the anonymity of our subjects.

At the end of each day, these collected samples in 50ml distilled water capped in 50ml falcon tubes, kept in ice will then be delivered to Laboratory of Metabolic Medicine (LMM) in Singapore Bioimaging Consortium (SBIC), A*STAR for analysis. The hardcopy of data will be stored in secured areas, including teacher's office and SBIC. The softcopy of data will be stored in secured computers guarded by AJC and A*Star IT department. Only involved researchers and mentors have access to the data. The data will be kept for record purpose for 12 months after presentation and/or publications.

B. *Experimental Procedures*

1. All falcon tubes containing the samples are placed in a centrifuge to spin at 4000rpm at 4ºC for 20 mins to pellet the bacteria in the samples.
2. The supernatant is discarded and the pellet is re-suspended in 200 µl ddH_20[5]. The re-suspended pellet is transferred to a 1.5ml tube.
3. 20µl of suspension was then transferred to a 500µl tube where 40µl of freezing buffer was added. Vortex the suspension till it is mixed well. Pipette the mixture several times to ensure is mixed well.
4. Leave the mixture at room temperature or ice for approximately 15 minutes to let the glycerol get into the cells. Vortex the mixture again before freezing it at -80ºC.
5. 5 1% and 0.1% of the mixture are plated on LB plates and incubated for 2 days at 37°C. Bacteria colonies are classified and counted.
6. Spin the remaining 180µl at 4000rpm at 4ºC for 20 mins.
7. Discard the supernatant. Proceed to spin column protocol.
8. Extract DNA, elute to no more than 50µl final volume.
9. Measure the DNA concentration and purity with a Nanodrop.
10. Amplify the 16s rDNA with universal primer pairs 27F and 1492R. In brief, PCRs with HotStart polymerase (Bioatalas) were conducted at 95 °C for 10min, followed by 35 cycles at 95 °C for 30 s, at 56 °C for 30s, 65°C for 30s, and a final extension step at 72 °C for 5 min using an Eppendorf PCR machine. Primers used are: 27fA AGAGTTTGATCATGGCTC; 27fC AGAGTTTGATCCTGGCTC; 63f CAG GCC TAA CAC ATG CAA GTC; 1387rt GGG CGG TGT GTA CAA GGC and 1492r TACCTTGTTACGACTT.
11. PCR products of 1.4kbps are purified with Gel purification kit (Qiagen) after electrophoresis in 1% agarose gels.

[4] World Health Organisation (WHO) Growth Reference 2006- http://www.who.int/growthref/en/

[5] Volume of 200µl could be increased depending on pellet size, but always kept 10% of volume with 2 times freezing buffer, capped at 300µl.

12. qPCRs were carried out in a final reaction volume of 10 µl with 5 µl SYBR green PCR master mix (Life science) 0.5 µM of primers. Two-microliter aliquots of diluted samples (approximately 5 ng) were added to the reaction tubes. The step-one thermocycler (ABI) was used for the amplifications with default program settings.
13. Primers used for qPCR are: SNF1: GCCTGCAATCCGAACTGAGA; SAF: CAAGTAGGACGCACAGTTTA; SAR: TGTGTTACATACTGTTATGCGGT. Primer 1492r and reversed 1387r are used as general bacteria control.
14. Chi-square test was used to analyse the difference among four obesity criteria. Student-t test was used to analyze the significant differences among datasets (e.g., 16S rRNA gene copy numbers and BMI or Waist/Hip ratio). Differences of $P<0.05$ were regarded as statistically significant.

IV. RESULTS AND DISCUSSION

A. *Characteristics of the subjects.*

Variables	underweight (n=6)	normal (n=40)	overweight (n=6)	obese (n=1)
	Means(sd)			
female/male	5/1	22/18	3/3	0/1
age	17.7(1.4)	17.4(0.8)	17.7(0.8)	17
weight	46.2(3.8)	61.2(10.3)	76.5(12.3)	104.5
height	1.61(0.05)	1.69(0.10)	1.67(0.11)	1.79
waist(cm)	65.3(7.3)	73.3(8.0)	85.4(11.1)	104.5
hip(cm)	79.8(3.5)	87.1(6.8)	99.4(6.4)	112
BMI	17.9(0.6)	21.4(1.7)	27.2(1.9)	32.6
WHR	0.82(0.08)	0.85(0.07)	0.86(0.07)	0.93

TABLE 1: Characteristic of subjects

An individual was deemed as obese if their Body mass index (BMI) ⩾30, Waist-to-hip ratio(WHR) ⩾0.8(females) and WHR ⩾1.0(males) WHO and Z-score classification system by the World Health Organisation. The Z-score classification system refers to the anthropometric values of the weight-for-height, height-for-age and weight-for-age that is expressed as a number of standard deviations. A fixed Z-score interval implies a fixed height or weight difference for adolescents of a given age. WHO Global Database on Child Growth and Malnutrition uses a Z-score cut-off point of >+2 SD classifies high weight-for-height as overweight in adolescents. [6]

As we noticed the overweight/obesity ratio is low, and it is well known that ethics and criteria play a role to define a proper cut-off. Therefore we analysed and compared the data of waist and waist-to-hip ratio with additional criteria from International Obesity Task Force (IOTF) and International Diabetes Federation (IDF). The categories and reading are listed in table 2.1 and 2.2 respectively.

[6] http://www.who.int/nutgrowthdb/about/introduction/en/index5.html

	underweight		normal		overweight		obese	
	number							
criteria	female	male	female	male	female	male	female	male
BMI/Z-score	5	1	22	18	3	3	0	1
BMI/IOTF-asian	5	0	18	14	4	5	3	4
WHR(WHO)	nil	nil	14	12	9	nil	7	11
waist (IDF)	nil	nil	30	18	nil	nil	0	5

TABLE 2.1: Illustrates the data set by WHO and IDF

	underweight		normal		overweight		obesity	
	means(sd)							
criteria	female	male	female	male	female	male	female	male
BMI/Z-score	17.7(0.5)	18.8	21.0(1.7)	21.9(1.6)	27.4(2.2)	26.9(1.9)	nil	32.6
BMI/IOTF-asian	17.7(0.5)	nil	20.4(1.3)	20.9(1.0)	23.5(0.4)	24.0(0.6)	27.4(2.2)	28.3(3.2)
WHR(WHO)	nil	nil	0.77(0.02)	0.84(0.07)	0.82(0.01)	nil	0.87(0.04)	0.95(0.04)
waist (IDF)	nil	nil	68.6(5.6)	77.4(6.0)	nil	nil	nil	97.6(7.0)

TABLE 2.2: Illustrates the data set by WHO and IDF

There are great differences between criteria; therefore we Chi-Square Goodness of Fit Test among each pair of them. The P value outcome for total, girl or boys only is listed in table 2.3-2.5 respectively. It is interesting to find out that other than z-score and waist pair, all other comparisons are significantly different. It suggests that it will be helpful to consider more parameters as well as more criteria when the school assigns students for FUN programme in order to reduce the false negative ratio.

Total	Z-score	IOTF	WHR	waist
Z-score	-	0.007	0.000	0.34
IOTF	0.007	-	0.003	0.0000
WHR	0.0000	0.003	-	0.0000
waist	0.34	0.0000	0.0000	-

TABLE 2.3: Overall results of the Fit Test

Female	Z-score	IOTF	WHR	waist
Z-score	-	0.084	0.000	nil
IOTF	0.084	-	0.001	nil
WHR	0.0000	0.001	-	nil
waist	nil	nil	nil	-

TABLE 2.4: Results for the females in the Fit Test

Male	Z-score	IOTF	WHR	waist
Z-score	-	0.032	0.003	0.61
IOTF	0.032	-	0.40	0.043
WHR	0.003	0.40	-	0.002
waist	0.61	0.043	0.002	-

TABLE 2.5: Results for the males in the Fit Test

B. Analysis of bacteria culture

The most straight forward way to differentiate the bacteria profile of each subject is to culture the samples. Even though there are limitations of culture condition availability and most of gum bacteria can't grow well under culture condition. To our surprise, we still observed diverse growth from subjects as shown in Figure 2.

control	#2	#15	#21	#51
Ow/Ob	#12	#44	#51	#54

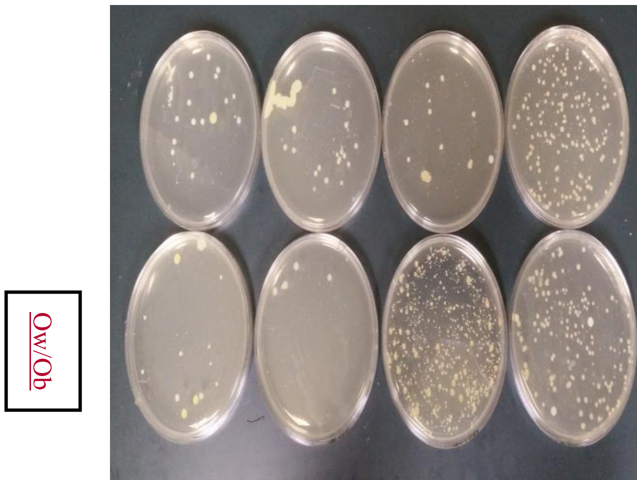

FIGURE 2: Diagram illustrating the bacteria cell culture plates

Based on colony color, transparency, size, texture and edge shape, we classified them into 11 types as shown in Figure 3.

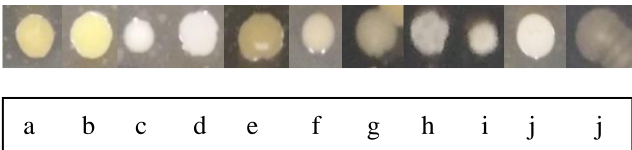

FIGURE 3: Classification of bacteria species

Although we have not identified the association and specification with limited data, it could be the foundation to prepare bacteria culture for probe screening.

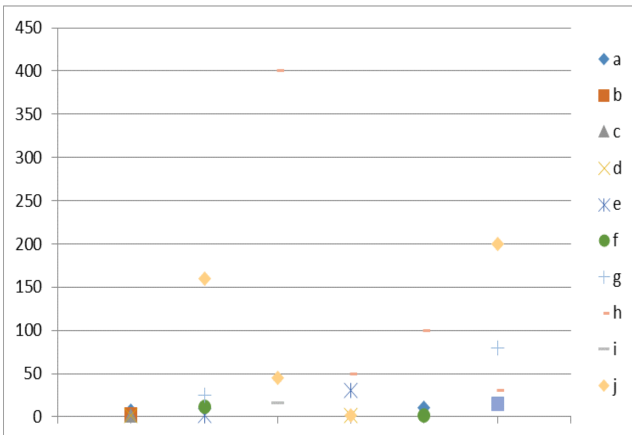

CHART 1: The counts of major colony types

C. Analysis of qPCR

1st PCR: 16S rDNA amplification with indicated primer pairs. The yield and specificity were good as shown in Figure 4.

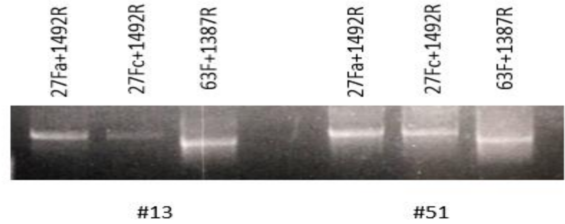

FIGURE 4: Picture of Gel Electrophoresis with illustration of 16S rDNA amplification

2nd qPCR with primer pairs for Selenemonas noxia (SN), Streptococcus anginosus (SA) and general bacteria (All). The two reported bacteria species were examined in 5 normal subjects (control) and 8 overweight or obese subjects met with either WHO or 2 of any criteria (Ow/Ob). The normalized result is shown in chart 2. Although there was slight trend of increase of SN and SA in agreement with the literature, there was no significance.

mean	control	Ow/Ob	sem			t-test	
SN	0.388533	0.45806		0.031333	0.113941		0.648843
SA	0.295553	0.577665		0.031439	0.230313		0.362943
SN/SA	0.81652	1.236323		0.174926	0.194812		0.167938

TABLE 3: Results of the 2nd qPCR

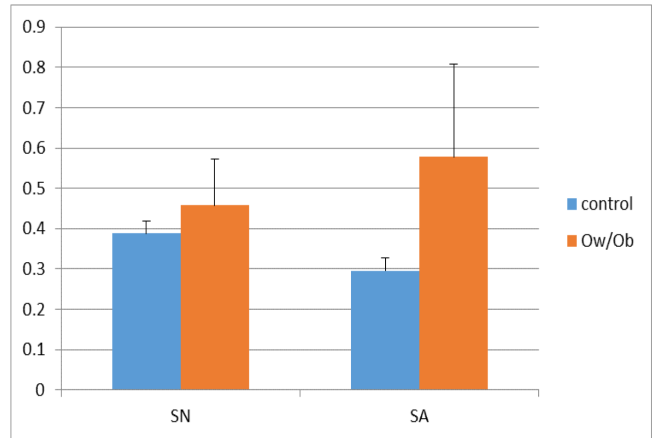

CHART 2: The normalized result of two bacteria species

V. CONLUSION AND RECOMMENDATIONS FOR FUTURE WORK

In current report, we collected body weight, height, waist and hip measures from 54 AJC students and analyzed with four well known criteria to classify the obesity condition. The analysis shows that the coverage of individual criteria varies a lot, and the overlap between criteria was poor. As we know the obesity is not simply equal to absolute value of weight, height or fat depot. It raises the concern that to evaluate the obesity risk with individual criteria would lead to high chance of false negative. Based on our result, the combination of BMI/IOTF-Asian and WHR is the most stringent criteria which cover all the subjects identified by WHO Z-score or waist measure. In

the effort to prevent obesity in school students, FUN program shall consider adoption of these criteria to enroll more students that are at risk of obesity.

Our search for obese specific bacteria was not carried out fully due to various limitations. We then attempted to verify whether the most positive hits in the literature occurs in our cohort. Our results from the experiment have shown that there are no significant relationship between those two bacteria and a person's obesity status. Hence, the results could not beckon our hypothesis. It could have been due to limited sample size to achieve significance, or non-specific species for our targeted cohort, if there is possible association. Therefore, in the future, we need to increase the qPCR target coverage and sample size in next step. Also more general primers should be used to screen positive targets in our cohort other than species specific primers so that we can detect a wide range of bacteria present in one's mouth. Provided above mentioned improvements, we look to screen a bacteria-specific fluorescent probe as a handy prescreening kit. Hence, students with positive bacteria present in their mouth can be alerted and pay more attention to a healthier life style.

REFERENCES

[1] Hotamisligil GS (2008) "Nutrient sensing and inflammation in metabolic diseases"

[2] Shoelson and Goldfine (2009) "Getting away from glucose: fanning the flames of obesity-induced inflammation."

[3] Le Chatelier E (2013) "Richness of human gut microbiome correlates with metabolic markers."

[4] Goodson JM (2009) "Is Obesity an Oral Bacterial Disease" http://www.ncbi.nlm.nih.gov/pubmed/19587155,

[5] Zeigler CC (2012) "Microbiota in the oral subgingival biofilm is associated with obesity in adolescence" http://www.ncbi.nlm.nih.gov/pubmed/21996660

[6] World Health Organisation Growth Reference 2006

[7] Goodson, J.M, et al. "Is obesity an oral bacterial disease?" Journal of dental research 88.6 (2009): 519-523