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Clinical Studies on the Dental Caries Prevention Effects of the Ability of *Weissella cibaria* CMU to Adhere to the Oral Cavity

Hae-Ryoung Park[1], Hye-Jeong Kim[2], Mi-Sun Kang[3]

[1]Assistant Professor, Division of Liberal Arts and Teacher Training, Kwangju Women's University, South Korea,
[2]Researcher, Department of Pediatric Dentistry, School of Dentistry, Chonnam National University, South Korea,
[3]Head, Department of Research Institute, Oradentics Inc. South Korea

ABSTRACT

The purpose of this study is to investigate the effects of diet containing *Weissella cibaria* CMU (oraCMU) on *Streptococcus mutans* causing dental caries. The m-PHP index was Modified Patient Hygiene Performance Index. As a measuring method, Modified patient hygiene performance index was measured to quantify oral hygiene condition among 2 subject groups consisting of *W. cibaria* CMU (oraCMU), a commercial product containing *W. cibaria* CMU and the same commercial product but removing *W. cibaria* CMU as a placebo control. Each group had 25 subjects. Unstimulated saliva were collected and analyzed by real-time polymerase chain reaction (RT-PCR). The measurement of Modified patient hygiene performance index was performed every 2 weeks starting from the first day of the experiment. And to know the effects of post-diet, its measurements were continued every 2 weeks for 4 weeks of a post-diet period. Data were analyzed by One-way ANOVA was carried out for the significance test between groups. Scheff's post hoc was used to determine variance homogeneity differences between the mean values ($p < 0.05$).

Modified-patient hygiene performance (m-PHP) index were significantly reduced in the *Weissella cibaria* CMU (oraCMU) compared to the removing *W. cibaria* CMU (oraCMU) as a placebo control, with significant differences between groups ($p < 0.05$). Our survey confirms that there are many individual subjects who believed that oral health could be maintained with diet of *W. cibaria* CMU (oraCMU). Specifically, there was a statistical significance difference between *Weissella cibaria* CMU (oraCMU) and placebo control groups during both of the 2-weeks and 4-weeks ($p < 0.05$). *Streptococcus mutans* levels significantly decreased in the *W. cibaria* CMU (oraCMU) compared to the removing *W. cibaria* CMU as a placebo control during the intervention. The oral colonization of *W. cibaria* CMU (oraCMU) was also observed. These results suggest that *W. cibaria* CMU (oraCMU) were serves as oral probiotics, to prevent dental caries and good oral health.

Our results represented that *Weissella cibaria* CMU (oraCMU) diet were significantly suppression of biofilm formation on the surface enamel in terms of oral hygiene. This study would be used as basic data for future research in the development of oral hygiene products.

Keywords: *Biofilm, Dental caries, modified patient hygiene performance (m-PHP) index, Streptococcus mutans, Weissella cibaria CMU (oraCMU)*

Corresponding Author:
Hae-Ryoung Park
Assistant Professor,
Division of Liberal Arts and Teacher Training,
Kwangju Women's University, South Korea
E-mail: hrpark@kwu.ac.kr

INTRODUCTION

Dental caries are one of highly occurring diseases in South Korea, and are developed by oral bacteria that can decompose carbohydrates of food debris in the mouth and synthesize organic acids, which in turn decalcificate inorganic minerals in the enamel tissue of the teeth[1,2].

Weissella cibaria has been found in Korean kimchi fermentation and other sources, including fermented foods such as healthy human infants, Greece salami, and sausages. Generally, *W. cibaria* were abundant in various fermented foods[3,4,5,6].

W. cibaria CMU are isolated strains of *W. cibaria* from the saliva of healthy children[7]. Through various experiments of inhibition of biofilm formation on the enamel surface of oral cavity by using oral lactic acid bacteria *W. cibaria* CMU. Generally, *W. cibaria* were abundant in various fermented foods.

The purpose of this research is to assess to what extent diet including oral probiotics, *Weissella cibaria* CMU affect oral environments through oral examinations including counting the bacterium number, and to find their roles in preventing dental caries.

Current clinical and preventive practices include fluoride coating, oral hygiene management, sealants, etc, and if dental caries are found, typically caries area is removed and synthetic materials such as amalgam, resin or gold are filled in[8,9]. Such treatments are a symptomatic treatment and not a therapeutic treatment against disease-causing oral bacteria, and there are high chances of recurrence[10]. To remove such risky disadvantages, a preventive practice in this study is presented as a new paradigm of using live oral lactic acid bacteria (probiotics) to cure dental caries[11,12,13,14].

Probiotics are one of the various prevention methods to reduce dental caries and oral diseases. The purpose of this study was to evaluate the effects of tablets containing *Weissella cibaria* CMU (oraCMU) on dental caries risk factors. The participants were divided randomly into two groups, and treatment consisted of administration of a daily tablet containing *W. cibaria* CMU (oraCMU) or placebo control.

The clinical and microbiological monitoring was performed during and after the intervention. Unstimulated saliva were collected and analyzed by real-time polymerase chain reaction (RT-PCR). Modified-patient hygiene performance (m-PHP) index were significantly reduction of *Weissella cibaria* CMU (oraCMU) compared to the removing *W. cibaria* CMU (oraCMU) as a placebo control. *Streptococcus mutans* levels significantly decreased in the *W. cibaria* CMU (oraCMU) group compared to the control during the intervention. The oral colonization of *Weissella cibaria* CMU was also observed. These results suggest that *W. cibaria* CMU (oraCMU) are good oral care probiotics to prevent dental caries.

MATERIALS AND METHOD

Subjects

This study was carried out in accordance with Declaration of Helsinki: ethical principles for medical research, and approved by IRB (IRB appraisal number: 1041485-201608-HR-001-02). Study subjects were 60 individuals selected from 70 in total who applied for this study program. They checked the objectives and scope of the study and personal information uses before they signed a research agreement. Through a questionnaire screening, subjects who were in dental braces (n=6) or taking antibiotics (n=4) were eliminated. To perform an oral hygiene test, at least 2 hours before, they were discouraged to do any oral activities (eating, drinking, chewing, brushing, mouth washing), then, questionnaire survey, oral check-up, plaque index and saliva testing were conducted.

Randomly, each half of subjects were assigned into a test group (Oradentics Inc., commercial products) or control group (the same but *W. cibaria* CMU omitted). Each individual was instructed to take a test or control diet for 4 weeks. Every 2 weeks, plaque index, saliva amount, *S. mutans* bacteria in saliva and *W. cibaria* were compared between the test and control group. Also, during 4 weeks of a follow-up, plaque index and saliva test were performed in every 2 weeks. In addition, *W. cibaria* in saliva were counted to assess *W. cibaria* settlement in the mouth for the test group *W. cibaria* CMU (oraCMU).

The dietary food used for this clinical study was in the form of a tablet in identical shape and color and was provided by Oradentics Inc. Depending on the presence or absence of *W. cibaria* CMU (oraCMU) ($1x10^9$ CFU/tablet), it is divided into a test or control group. Applying a double-blind test, both subjects and non-subjects did not know whether it is a test diet or control diet, and each group (test or placebo groups) consisting of 30 individuals was subject to this study.

They were instructed to consume a tablet by slowly melting it in the mouth without swallowing it in the evening before going to bed and after dinner and tooth brushing, once a day. Meanwhile, they were also

instructed to do tooth brushing as usual but not to use mouth wash.

Plaque Index

To monitor the oral hygiene status, we used index modified patient hygiene performance (m-PHP) index. To calculate the index, both sides of six human teeth were equally sectioned into 5 pieces and each section was used to calculate the bacterial biofilm extent and all obtained sum is given as an absolute value from minimal 0 to maximal 60.

Saliva collection

Saliva was collected into a 50 ml sterilized tube between 10-11 am after chewing wax for 2 to 3 minutes. Some of collected saliva was kept at -80℃ until DNA extraction. The 1 ml of each collected saliva was added to 5 ml of 1x PBS buffer, suspended and centrifuged at 1800x g for 5 minutes. DNA was extracted from the pellet after removing the supernatant following manufacturer's instructions (G-Spin genomic DNA extraction kit, iNtRON biotech., Korea). The final eluted volume of DNA was 100ul and 1ul was used for quantitative real-time PCR.

Bacterial strains and culture

W. cibaria CMU (oraCMU) provided by Oradentics Inc.(South Korea) was inoculated in De Man, Rogosa, Sharpe broth (MRS, Difco, USA) and cultured for 16 hours in an aerobic condition. *Streptococcus mutans* (ATCC25175) was purchased from Korean Culture Center of Microorganisms and cultured. *S. mutans* that was kept in the deep-freezer was cultured on the BHI (Brain Heart Infusion, Becton, Dickinson and Company) media.

Extraction of DNA from the bacterial culture

The Genomic DNA was extracted from cultured *W. cibaria* CMU or *S. mutans*. Genomic bacterial DNA was used as a standard DNA template for quantitative RT-PCR assay methods. Genomic DNA was extracted following by vendor's instructions manual (G-Spin genomic DNA extraction kit, iNtRON biotech., Korea). Isolated DNA was quantified by using NanoDrop2000 (Thrmo Scientific, USA) and used as standard DNA for RT-PCR analysis after 10 times dilution.

Real-time PCR

The Real-time RT-PCR assay was conducted by using Rotor- Gene 6000 (Corbett Research, Sydney, Australia). Reaction mixture includes sense primer and antisense primer (100 nM, 1 μℓ each), template DNA 2 μℓ, 2x QuantiTect SYBR Green PCR Kit (Qiagen, CA) 12.5 μℓ, and distilled water 8 μℓ in 25 μℓ total. The sequences of primers were W. cibaria-specific forward primer Wc612-F, 5′- GTGAAAGCCCTCAGCTCAAC -3′ and reverse primer Wc711-R, 5′- CTACGCATTTCACCGCTACA -3′; S. mutans specific forward primer Sm-F, 5′-GCCTACAGCTCAGAGATGCTATTCT-3′ and reverse primer Sm-R, 5′-GCCATACACCACTCATGAATTGA-3′. For RT-PCR, template DNA was denatured at 95℃ for 5 minutes, 40 times of polymerization reaction at 95℃ for 5 seconds, and additionally incubated at 60℃ for 10 seconds. The melting curve from 65℃ to 95℃ was plotted every 0.2 second interval.

Statistical analysis

Using quantitative real-time PCR, oral settlement in which the count of *W. cibaria* CMU in the saliva was monitored was statistically significant. To monitor changes of the m-PHP index in each group during treatments, repeated measure ANOVA was harnessed for validity. Relative coefficients between *W. cibaria* CMU and *S. mutans* were analyzed. SPSS Version 21.0 for Windows was used for all the statistical analysis used in this study at a significance level of 0.05.

RESULTS AND DISCUSSION

m-PHP index of *W. cibaria* CMU(oraCMU)

Many studies on bacteriotherapy, such as caries vaccine, probiotics, etc have been going on by targeting *S. mutans*, principal dental caries-causing bacteria[15], and also as a new paradigm that can replace conventional therapies, many other studies have been harnessing live oral lactic acid bacteria (probiotics) to cure dental caries, peritonitis and bad breath[16,17,18].

The m-PHP index of the oraCMU containing *W. cibaria* and placebo control not containing *W. cibaria* CMU (oraCMU) was measured every 2 weeks during 4 weeks of diet and subsequently 4 weeks after diet. Starting from 2 weeks and thereafter, *W. cibaria* CMU (oraCMU) showed a marked decrease compared to the control, which was statistically significant ($p< 0.05$).

This trend remained the same even for 4 weeks of after-diet in figure 1.

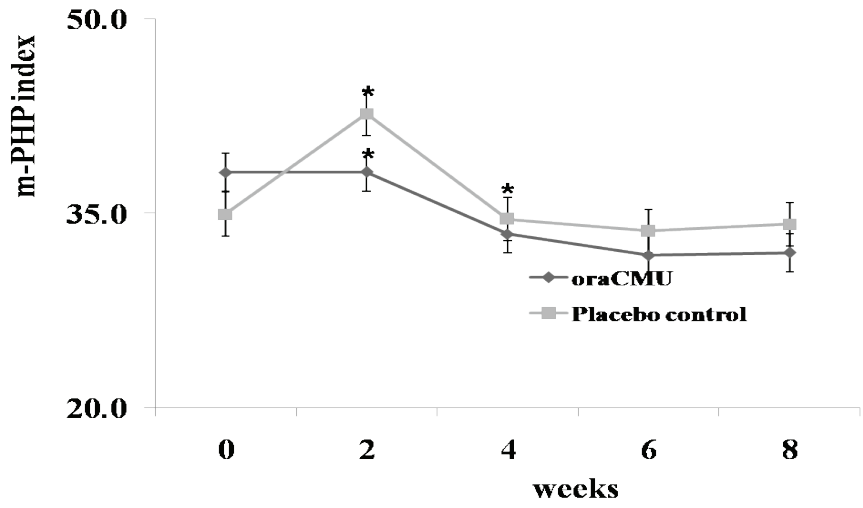

Figure 1. m-PHP index among the oraCMU and Placebo control during the study. *p<0.05

Oral colonization Resistance of *W. cibaria* CMU (oraCMU)

Korean companies have know-how on developing products using lactic acid bacteria and they are good at exports but they do not have oral lactic acid bacteria technology. A variety of lactic acid bacteria candies are available on the market, they are not made of oral lactic acid bacteria but of common lactic acid bacteria for bowel health, or only limitedly available in no sugar lactic acid candy products containing xylitol or other sugar alcohols, and there are no available oral lactic acid products[19,20,21].

While dieting *W. cibaria* CMU (oraCMU), the count of *W. cibaria* CMU was increased as shown in table 1. Even it was higher than 0 week for 4 weeks of after-diet, which suggest that *W. cibaria* CMU (oraCMU) could settle in the oral environment for a while even without daily diet ($p < 0.05$) as shown in table 2.

Table 1: Oral colonization resistance of *W. cibaria* CMU (oraCMU) for 0-4weeks

Cell number of *W. cibaria* CMU/ml, (n=25)

	Mean	**SE**	***p***
0 wks	360.6760	110.66437	.003*
2 wks	1347.7320	286.52665	.000*
4 wks	1492.5800	400.19344	.001*

*$p < 0.05$

Table 2: Oral colonization resistance of *W. cibaria* CMU (oraCMU) for 6-8weeks

Cell number of *W. cibaria* CMU/ml, (n=25)

	Mean	**SE**	***p***
0 wks	360.6760	110.66437	.003*
6 wks	921.6040	176.11997	.000*
8 wks	855.0440	197.10158	.000*

*$p < 0.05$

Adhesion of *W. cibaria* CMU (oraCMU) in Oral Cavity

The effects of probiotics on periodontal disease and the microbial of subgingival plaque in human subjects[22]. After administration probiotics reduced salivary *S. mutans* was found[23]. The analyzed results of *S. mutans* growth in the mouth showed that the growth of *S. mutans* was significantly inhibited during the 4 weeks of oraCMU dieting in figure 2. The number of *W. cibaria* CMU (oraCMU) during the post-oraCMU dieting (6-8 weeks) was higher than that at 0 week, which demonstrated that the growth of *S. mutans* was effectively suppressed in figure 3. It is confirmed that the diet of *W. cibaria* (oraCMU) not only inhibited significantly the growth of caries-causing bacteria, *S. mutans* but its effects continued during the one month of post-oraCMU ($p < 0.05$).

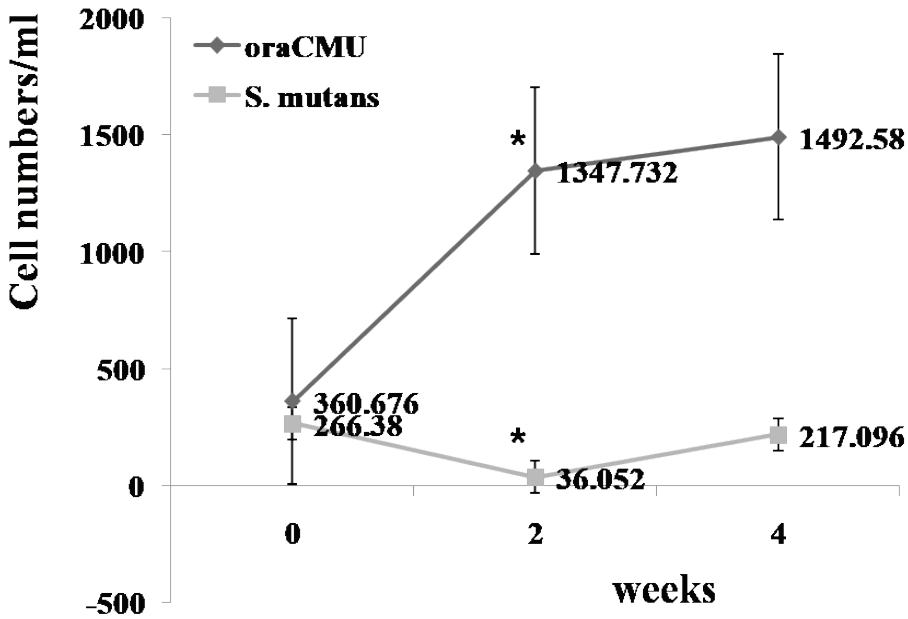

Figure 2. Comparison of cell numbers of *W. cibaria* CMU (oraCMU) and *S. mutans* in saliva for 4 weeks

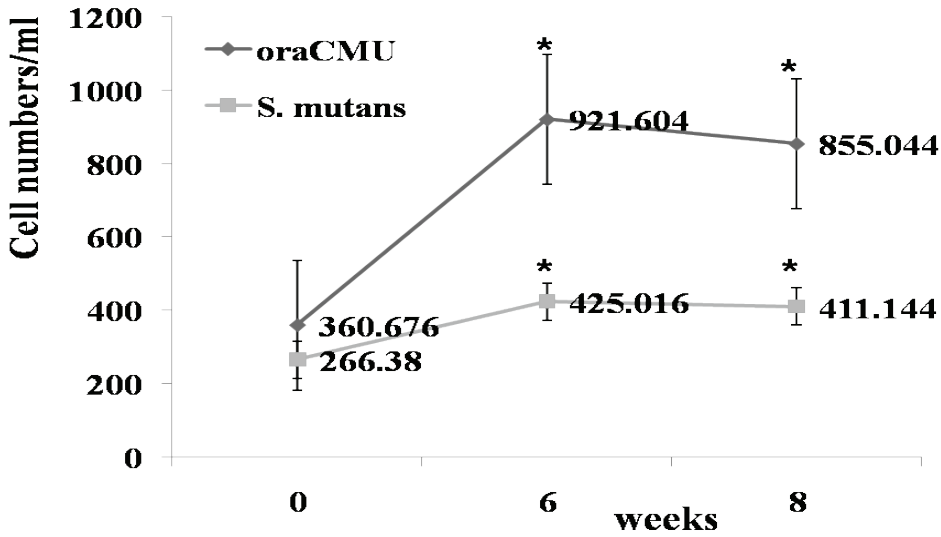

Figure 3. Comparison of cell numbers of *W. cibaria* CMU (oraCMU) and *S. mutans* in saliva for 6 and 8 weeks

CONCLUSION

This study assessed the effects of *W. cibaria* CMU (oraCMU), oral probiotics on the oral environments to prevent dental caries by dieting commercial products containing the selected oral probiotics, conducting oral examinations and measuring bacterial counts.

The count increased in 'after 4-weeks diet' and was maintained more than 50% in 'after diet stop' in a statistically significant way although the count started to decrease after diet stop, which supports the oral cavity of adhesion ability of *W. cibaria.*

By using real-time PCR quantification, the attachment ability in the oral environments estimated by counting *W. cibaria* CMU supported that the count of *W. cibaria* CMU was increased during the 4 weeks of oraCMU diet demonstrating its attachment ability in the oral environments and *S. mutans* growth was inhibited. Meanwhile, after oraCMU diet was stopped, the count of *W. cibaria* CMU started to decrease but it was kept

no less than 50% of 0 week, and the growth of *S. mutans* was suppressed ever post-oraCMU diet period. With oraCMU diet, the growth of dental caries-causing bacteria, *S. mutans* was significantly suppressed, which is statistically significant between two groups ($p < 0.05$).

Ethical Clearance: Kwangju Women's University

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Conflict of Interest: Nil

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