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Effects of *Streptococcus thermophilus* on volatile sulfur compounds produced by *Porphyromonas gingivalis*

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ABSTRACT

Halitosis as oral malodour is an unpleasant odour caused by volatile sulfur compounds (VSCs). VSCs are produced primarily by anaerobic bacteria that abundantly produce proteinase as trypsin-like enzyme. General therapies, such as mouthwash and plaque control, do not provide a continuous effect on oral halitosis. *Streptococcus thermophilus* is a probiotic bacterium that is beneficial for human health. The aim of this study was to evaluate the effect of *S. thermophilus* on *Porphyromonas gingivalis*-producing VSCs and to analyze the inhibitory mechanism of halitosis. *P. gingivalis* was cultured with or without *S. thermophilus*, and the emission of VSCs from the spent culture medium was measured by gas chromatography. In order to analyze the inhibitory effect, the antibacterial activity of *S. thermophilus* against *P. gingivalis* was assessed. After the spent culture medium or whole bacterial of *S. thermophilus* was mixed with the spent culture medium of *P. gingivalis*, VSCs were again measured by gas chromatograph. When *S. thermophilus* and *P. gingivalis* were co-cultivated, VSCs were present at a lower level than those of single-cultured *P. gingivalis*. *S. thermophilus* inhibited growth of *P. gingivalis*, and the whole bacteria and the spent culture medium of *S. thermophilus* reduced emission of VSCs gas. *S. thermophilus* may reduce oral malodour by inhibition of *P. gingivalis* growth and neutralizing VSCs with their metabolites or themselves.

1. Introduction

Halitosis is known as bad breath, oral malodour or fetor oris. Temporary halitosis is caused by consumption of foods or drinks. Persistent malodour is mainly due to metabolites of oral microorganisms.[1] Persistent malodour occurs mainly by production of volatile sulfur compounds (VSCs) from putrefaction of proteinaceous substrates.[2,3] In the oral cavity, putrefaction of proteins is mainly due to anaerobic gram-negative bacteria, such as *Porphyromonas gingivalis*, *Tannrella forsythia* and *Treponema denticola*.[2,4,5] These three bacteria as periodontopathogens have a characteristic of benzoyl-D,L-arginine-naphthylamide (BANA)-positive bacteria by secretion of trypsin-like enzyme[6], by which the periodontopathogens can produce large amount of hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH) and dimethyl sulfide ($(CH_3)_2S$) from methionine and cysteine in serum protein.[7] Until now, halitosis has typically been treated with mechanical therapy like dental floss and mouth rinse with chemical agents for reduction of VSC.[1,3] Recently, improvement of halitosis using *Streptococcus salivarius* K12 has been explored.[8,9]

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Streptococcus thermophilus is a probiotic, gram-positive and facultative anaerobe. It is used in preparation of homemade yogurt.[10] *S. thermophilus* is beneficial on gastrointestinal health by producing its exopolysaccharide or bacteriocin.[11,12] *S. thermophilus* showed antibacterial activity against oral Streptococci such as *S. mutans*, *S. oralis* and *S. sobrinus*.[13] Furthermore, *S. thermophilus* adheres to tooth-like surfaces such as hydroxyapatite with casein-containing dairy products, and comparatively inhibits the attachment of *S. mutans* and *S. sobrinus*. Thus, *S. thermophilus* has been considered to prevent dental caries.[14] However, the effect of *S. thermophilus* on provocation of oral malodour by periodontopathogens has not been investigated.

The purpose of this study was to evaluate the effect of *S. thermophilus* on emission of *P. gingivalis*-producing VSCs and to analyze the inhibitory effect on reduction of VSCs emission.

2. Materials and methods

2.1. Bacterial strain and cultivation

P. gingivalis ATCC 33277 was used for generation of halitosis-related VSCs and cultivated in Brain Heart Infusion broth (BHI; BD bioscience, San Jose, CA, USA) supplemented with hemin (1 μg/ml) and vitamin K (0.2 μg/ml) at 37 °C anaerobically. *S. thermophilus* strain HY2, HY3 HY9012 were donated from Yakult (Korea yakult Com, Gyeonggi, Korea) and cultured with BHI broth at 37 °C in anaerobic chamber (H_2 5%, CO_2 10% and N_2 85%).

2.2. Measurement of volatile sulfur compounds

Since halitosis is mainly caused by VSCs such as hydrogen sulfide, methyl mercaptan and dimethyl sulfide, the level of the compounds was measured from the spent culture medium of *P. gingivalis* after treating or non-treating with various cell densities of *S. thermophilus*. *P. gingivalis* was cultivated in the presence or absence of *S. thermophilus* at 37 °C for 36 h. Each bacterial suspension (1 ml) was transferred to a new 50 ml conical tube and then vortexed for 30 s followed by measurement of VSCs with Oral Chroma™ gas chromatograph (FIS Inc., Itami, Hyogo, Japan). In order to investigate the inhibitory mechanism of VSCs, each *P. gingivalis* and *S. thermophilus* were cultivated and then the spent culture medium and whole bacteria were separated by centrifugation at 7000 × *g* for 10 min at 4 °C. The supernatants (1 ml) were transferred into fresh 15 ml conical tubes. The spent culture medium of *P. gingivalis* was mixed with various volume of the spent culture medium or whole bacteria of *S. thermophilus* in a 50 ml conical tube and incubated at room temperature for 5 min. VSCs were collected in 5 ml of gas above the mixed solution using 10 ml syringe and measured the level by gas chromatograph.

2.3. Antibacterial activity of S. thermophilus against P. gingivalis

Since *S. thermophilus* produces a bacteriocin, the antibacterial activity of *S. thermophiles* against *P. gingivalis* was evaluated. Susceptibility assay was performed according to the methods of Clinical Laboratory Standard Institute (CLSI). Briefly, 180 μl of fresh BHI broth including hemin (1 μg/ml) and vitamin K (0.2 μg/ml) was dispensed in each well of a 96-well polystyrene plate (SPL Lifescience, Gyeonggi, Korea), and then 180 μl of the spent culture medium of two species was added to the first row of the plate. Two-fold serial dilutions were made using a multi-channel micropipette. *P. gingivalis* was counted by Petroff–Hasser bacteria counter (Hausser Scientific, Horsham, PA, USA) and then diluted to 3×10^6 cells/ml with BHI broth including hemin (1 μg/ml) and vitamin K (0.2 μg/ml). The bacterial suspensions (20 μl; 6×10^4 cells) were inoculated in each well. The plates were incubated at 37 °C in anaerobic chamber for 36 h and the optical density was measured at 660 nm using an ELISA reader.

2.4. Co-cultivation of P. gingivalis and S. thermophilus

Bacterial co-cultivation was carried out according to the method described by Lee and Baek [15] *P. gingivalis* and *S. thermophilus* were co-cultured using Millicell cell culture insert (Millipore, Billerica, MA, USA). BHI broth was mixed with hemin and vitamin K and then dispensed in two new tubes. *P. gingivalis* and *S. thermophilus* were inoculated into each tube. After hanging Millicell cell culture insert in a well of 12-well plate, *P. gingivalis* and *S. thermophilus* were inoculated in the apical and basolateral side, respectively. Contamination of each bacterium in the separating chamber was investigated by observation using a microscope, and *P. gingivalis* colonies were counted after plating on BHI agar plate.

2.5. Statistical analysis

Statistically significant differences were analyzed by Mann–Whitney *U*-test using SPSS ver. 10 (SPSS Inc., Chicago, IL). *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Effect of S. thermophilus on the VSCs-producing P. gingivalis

P. gingivalis produces halitosis-associated VSCs using proteolytic enzyme. The effect of *S. thermophilus* on VSC production of *P. gingivalis* was investigated. When *P. gingivalis* was co-cultured with *S. thermophilus*, the level of hydrogen sulfur, methyl sulfide and dimethyl sulfide were reduced in the presence of *S. thermophilus* (Fig. 1). Especially, methyl mercaptan was decreased by 90% in the present of *S. thermophilus* (Table 1).

3.2. Antibacterial activity of S. thermophilus against P. gingivalis

To investigate the correlation of VSCs reduction and growth inhibition of *P. gingivalis*, antibacterial activity of *S. thermophilus* against *P. gingivalis* was examined according to CLSI. The spent culture medium of *S. thermophilus* were prepared and tested the antibacterial activity against *P. gingivalis*. The spent culture medium of *S. thermophilus* strain HY2, HY3 and HY9012

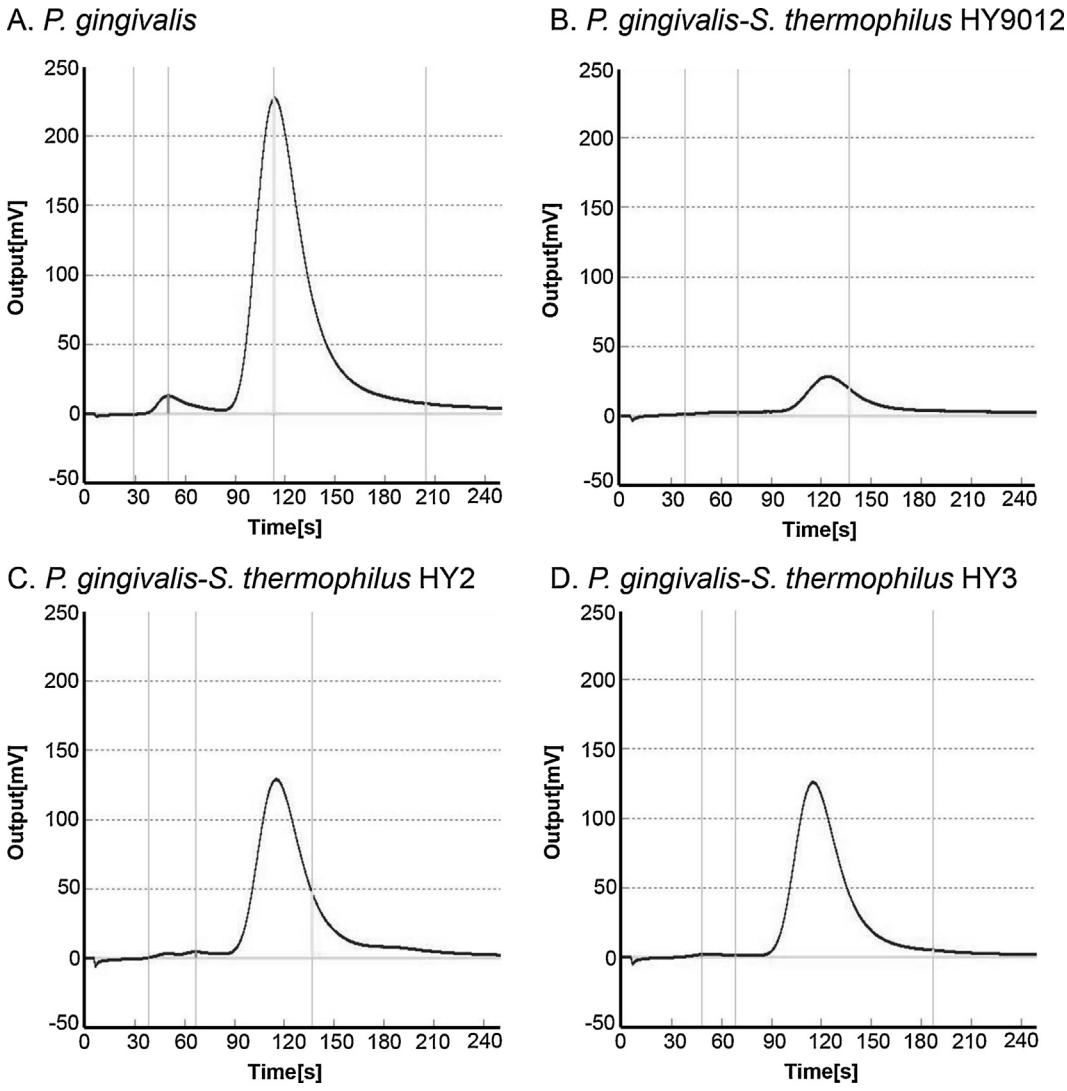

Fig. 1 – Reductive effect of *S. thermophilus* on oral malodour by *P. gingivalis*. *P. gingivalis* **(1 × 10^7 cells) was cultivated in the presence or the absence of** *S. thermophilus* **(1 × 10^7 cells), and then gaseous VSCs was collected using syringe above the bacterial suspensions. The level of gaseous VSCs was measured by gas chromatograph (Oral chroma™). The level of VSCs was expressed with histogram.**

exhibited antibacterial activity at the concentration of 25%, 50% and 50%, respectively (Fig. 2A). Furthermore, when *S. thermophilus* and *P. gingivalis* were co-cultivated using Millicell culture insert, *S. thermophilus* significantly inhibited growth of *P. gingivalis* (Fig. 2B).

3.3. *Inhibition of VSCs emission by whole bacteria and metabolites of S. thermophilus*

Finally, it was investigated whether the reduction of VSCs was derived by growth inhibition of *P. gingivalis* or by other

Table 1 – Production of VSCs by *P. gingivalis* with or without *S. thermophilus*.

Samples		Volatile sulfur compounds (ppb)		
		H_2S	CH_3SH	$(CH_3)_2S$
P. gingivalis	None	52.83 ± 3.48	2150.17 ± 118.15	47.67 ± 10.19
	+*S. thermophilus* HY2	17.83 ± 4.11	136.17 ± 28.50	20.67 ± 5.27
	+*S. thermophilus* HY3	20.17 ± 4.16	213.17 ± 41.89	24.50 ± 3.44
	+*S. thermophilus* HY9012	9.67 ± 1.03	40.33 ± 6.21	15.83 ± 2.78

The data value are expressed as mean ± SD.

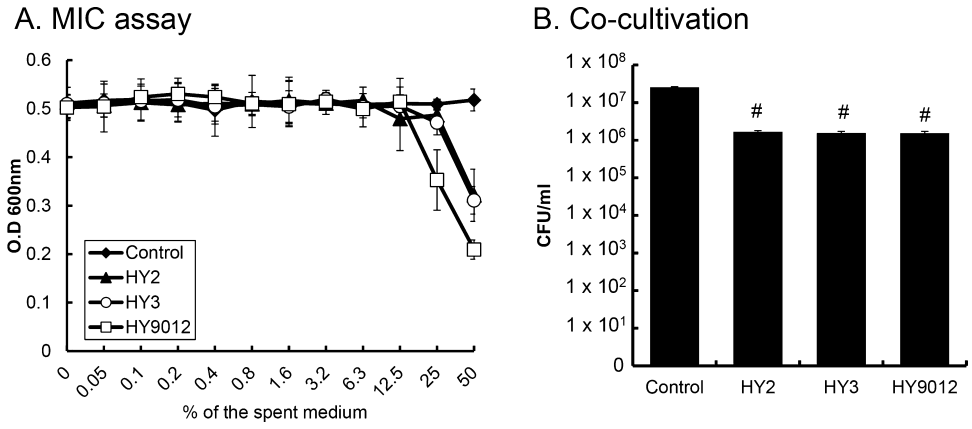

Fig. 2 – Antibacterial activity of *S. thermophilus* against *P. gingivalis*. After cultivation of probiotics, the spent culture media were collected by centrifugation. *P. gingivalis* was incubated with the spent culture medium of *S. thermophilus* in the various concentrations (A). In another experiment, *P. gingivalis* (1×10^7 cells) was co-cultivated with or without *S. thermophilus* (1×10^7 cells) using Millicell culture inert (B). The growth of *P. gingivalis* were measured by spectrophotometer at 600 nm. The experiments were performed in triplicate and the representative data are shown. * Statistically significant compared with untreated control bacteria ($p < 0.05$). Control group was treated with fresh BHI medium.

mechanism. After cultivating *S. thermophilus*, the spent culture media and whole bacteria of *S. thermophilus* were separated, and then each preparations was mixed with the spent culture medium of *P. gingivalis*. Gaseous VSCs were significantly lower in the mixture with two conditions as the spent medium and whole bacteria of *S. thermophilus* than the spent culture medium of *P. gingivalis* (Fig. 3 and 4).

4. Discussion

Temporary oral malodour is caused by consumption foods or drinks, and is improved by mouth rinse and tooth brushing. However, persistent malodour can be induced by metabolites of oral bacteria and is not easily remedied by mechanical or chemical treatment. Furthermore, approximately 80% of halitosis is caused by microbial putrefaction of oral substrates.[16] The mechanical or chemical means of persistent halitosis are often transient improvement because of continuous growth of oral bacteria. For the reason, probiotics have been explored for the relief halitosis. Probiotics can stimulate host immune systems and inhibit host-pathogen contact by occupying potential pathogen colonization sites and antibacterial activity.[17,18] In oral bacteria, periodontopathogens such as *P. gingivalis*, *T. denticola* and *T. forsythia* produce trypsin-like proteases, by which the periodontopathogens produce volatile sulfur compounds (VSCs) including hydrogen sulfide, methyl mercaptan and dimethyl sulfide.[4,6] These VSCs contribute to persistent halitosis.

S. thermophilus is a probiotic bacterium, which provides beneficial effect on gastrointestinal health by its exopolysaccharide or bacteriocin, and which protects dental caries by growth inhibition and binding inhibition of cariogenic bacteria.[9,11,13] However, their roles have not completely been

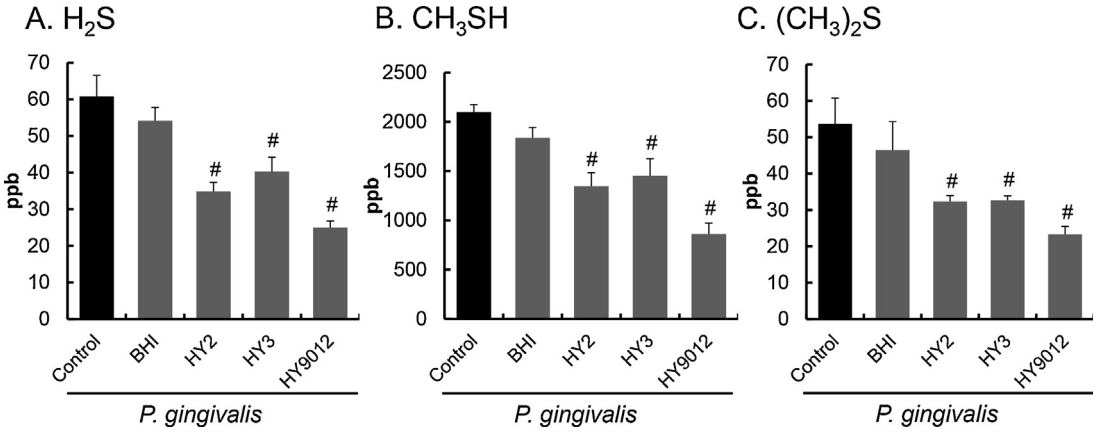

Fig. 3 – The effect of the spent culture medium of *S. thermophilus* on gaseous VSCs emission. The spent culture medium of *P. gingivalis* was mixed with the spent culture medium of *S. thermophilus*. The level of VSCs was measured by gas chromatograph (Oral chroma™). The experiments were performed in triplicate and the representative data are shown. # Statistically significant compared with untreated control bacteria ($p < 0.05$).

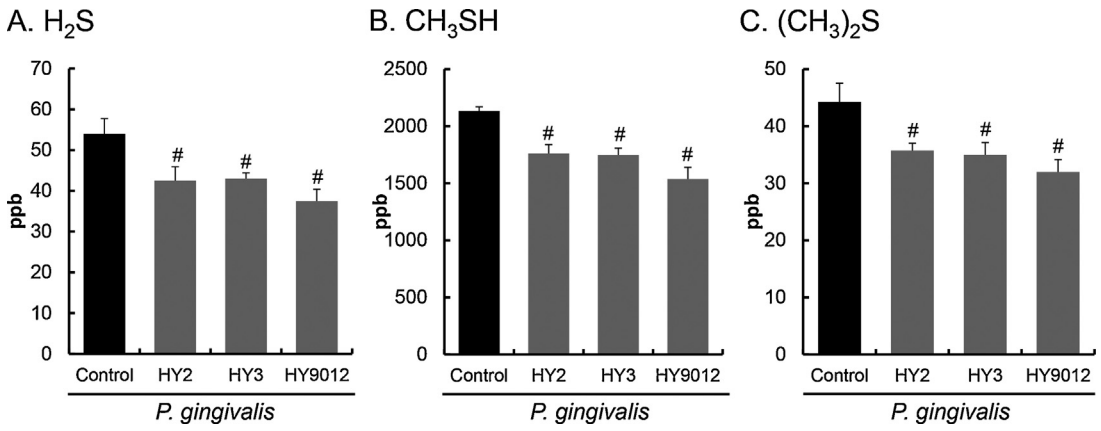

Fig. 4 – The effect of whole bacteria of *S. thermophilus* on VSCs emission. The spent culture medium of *P. gingivalis* was mixed with whole bacteria of *S. thermophilus* (1×10^7 cells). The level of VSCs was measured by gas chromatograph (Oral chroma™). The experiments were performed in triplicate and the representative data are shown. # Statistically significant compared with untreated control bacteria ($p < 0.05$).

understood in oral health, especially in halitosis. This study evaluated the effect of *S. thermophilus* on emission of *P. gingivalis*-producing VSCs and analyzed the inhibitory mechanism.

When *P. gingivalis* and *S. thermophilus* were co-cultivated, the level of VSCs was reduced in the presence of *S. thermophilus*. Therefore, to analyze the inhibitory effect on reduction of VSCs emission, antibacterial activity against *P. gingivalis* and capturing VSCs of *S. thermophilus* were investigated. In study using the spent culture medium of *S. thermophilus*, antibacterial activity against *P. gingivalis* showed at high concentration of the spent culture medium with strain differences. Also, *S. thermophilus* inhibited growth of *P. gingivalis* in a co-culture system. The difference of the antibacterial activity that was evident may reflect differences of culture-conditions. Another reason may be that *P. gingivalis* can inhibit the activity of antibacterial agents of *S. thermophilus* or has weak resistance for the antibacterial agent.

We evaluated whether the reduction of VSCs was derived from growth inhibition of *P. gingivalis* or by other mechanism. After the spent culture medium of *P. gingivalis* and *S. thermophilus* were mixed, the level of emitted VSCs was measured using Oral Chroma™ gas chromatograph. The mixed spent medium exhibited lower level of VSCs than that of *P. gingivalis*. Since the removal of VSCs is effective for halitosis reduction, various studies have focused on VSC removal using mouth rinse including herbal extract or *S. salivarius* K12 and on antibacterial activity against VSC-producing bacteria.[9,19–21] Halitosis may be improved by reduction of the bacterial count through antibacterial activity of mouth rinse or *S. salivarius* K12. Presently, *S. thermophilus* inhibited the growth of *P. gingivalis* and the spent culture medium reduced the emission of gaseous VSCs from the spent culture medium of *P. gingivalis*. Moreover, *S. thermophilus* also reduced emission of gaseous VSCs from the spent culture medium in the test of whole bacteria. These results are the first report that whole bacteria and the metabolites of *S. thermophilus* reduce the level of VSCs.

In case of chemical mouth rinse, halitosis cannot be continuously improved because chemical agents quickly flow into oesophagus by saliva. Thus, *S. salivarius* K12 has been studied for oral health. *S. salivarius* K12 is a normal flora in oral cavity and a probiotic bacterium.[22] *S. salivarius* K12 has antibacterial activity for cariogenic bacteria and periodontopathogens and can integrate into oral biofilm.[8,9,23] Mouthwash with *S. salivarius* K12 was reported to be able to reduce oral malodour by *P. gingivalis*.[24]. For this reason, *S. salivarius* K12 constantly helps to treat and prevent halitosis. *S. thermophilus* is also a probiotic bacterium and has the characteristic of *S. salivarius* K12. It was considered that the difference of two probiotics in application for halitosis may neutralize gaseous VSCs by metabolites or whole bacteria of *S. thermophilus*. Thus, *S. thermophilus* may immediately affect oral malodour.

5. Conclusions

Although this study has a simple design, it is the first and meaningful outcome that using *S. thermophilus* for halitosis treatment is capable of reducing oral malodour by inhibition of *P. gingivalis* growth and neutralizing VSCs with their metabolites or themselves.

Conflict of interest statement

None declared.

Funding

The present research was conducted by the research fund of Dankook University in 2012.

Competing interests

None declared.

Ethical approval

Not applicable.

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