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Effects of D-Psicose on Growth and Hyphal Development of *Candida albicans*

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Abstract

D-Psicose ($C_6H_{12}O_6$), also referred to as D-allulose, is a well-known rare sugar that exists as a monosaccharide in nature and has approximately 70% of the sweetness of sucrose and no calories. In recent years, D-psicose has been reported to have a variety of physiological effects in animals and humans, such as anti-inflammatory and antioxidant effects. In this study, we investigated the effect of D-psicose on the growth of *Candida albicans* (Ca) - a heterotrophic fungus that is commonly found in humans and which is a frequent etiological agent of candidiasis. We observed morphological changes using two microscopic techniques. Furthermore, we analyzed the expression of virulence-related genes in Ca when cultured in liquid medium containing 4% D-psicose at 37°C under aerobic conditions using quantitative reverse transcription-PCR (qRT-PCR) methods. Compared to culturing in liquid Sabouraud's medium containing 4% D-glucose (SM), Ca took longer to reach the stationary growth phase in liquid peptone medium containing 4% D-psicose (PM). Further, in the methyl thiazolyl tetrazolium (MTT; 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay, Ca strain ATCC 10261 cultured in liquid PM showed a significant reduction in metabolic activity (approximately 80%) when compared culturing in liquid SM, suggesting that D-psicose reduces Ca viability. Interestingly, microscopic analysis revealed the inhibition of hyphal development in Ca cultured in liquid PM at 37°C. In addition, qRT-PCR showed downregulation of the expression of virulence-related genes *HWP1* and *PLB1* in Ca cultured in liquid PM compared to Ca cultured in liquid SM. Taken together, these results show that D-psicose weakens the growth and inhibits hyphal development of Ca, and strongly suggest that D-psicose suppresses Ca morphogenesis and biofilm formation. By substituting sugar (glucose) with D-psicose in the daily diet, it may be possible to delay the onset of candidiasis in the body and oral cavity of the aged and immunocompromised patients, and thus, to reduce the frequency of dental visits for maintaining the oral cavity and dental prosthesis. This is the first report regarding the impact of D-psicose, a rare sugar, on Ca.

Keywords: *D-Psicose; Rare Sugar; Candida albicans; Growth; Hyphae; HWP1; PLB1*

Abbreviations

Ca: *Candida albicans*; SM: Sabouraud's Medium; PM: Peptone Medium Containing 4% D-Psicose; PBS: Phosphate Buffered Saline; EFB: Elongation Factor B; ECE: Extent of Cell Elongation; HWP: Hyphal Wall Protein; PLB: Phospholipase B; SAP: Secreted Aspartic Protease

Introduction

D-Psicose ($C_6H_{12}O_6$), also referred to as D-allulose, is a C-3 epimer of D-fructose, which is a monosaccharide existing in nature. It is well-known as a rare sugar that has approximately 70% of the sweetness of sucrose but has a caloric value close to zero [1]. Following the discovery of the enzyme D-tagatose 3-epimerase, which converts D-fructose to D-psicose [2], D-psicose has been biosynthesized widely using the Izumoring strategy [3]. Currently, D-psicose biosynthesis has been attempted industrially using a variety of microorganisms to produce this sugar on a large scale and at a low cost [4,5]. In addition, D-psicose was, in 2014, approved for use as a food sweetener by the Food and Drug Administration (FDA) in the US (GRAS Notice No. GRN 000498) [6] where it is used as an ingredient in a variety of foods and as a substitute sweetener.

Interestingly, animal and human experiments have shown that D-psicose plays a role in the regulation of both lipid metabolism [7,8] and carbohydrate metabolism [9-12]. In addition, D-psicose has been shown to have additional physiological functions, such as anti-inflammatory effects through the suppression of tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) in serum [13], as well as antioxidant effects by scavenging reactive oxygen species (ROS) [14]. Of particular interest are recent studies which showed that D-psicose can alter serum cholesterol levels, partly by reducing proprotein convertase subtilisin/kexin type 9 (Pcsk9) levels in hamsters [15] and ameliorating systemic and muscle insulin sensitivity in rats [16].

The genus *Candida* contains heterotrophic commensal and opportunistic fungal pathogens that colonize the mucosal surfaces and skin of healthy [17] and ill humans [18]. In an aging society, the incidence of nosocomial candidiasis has trended upward [19,20]. *Candida albicans* (Ca) is the predominant cause of invasive candidiasis, accounting for more than 50% of all nosocomial candidiasis cases [20,21]. Importantly, Ca is a common cause of oral candidiasis, typically manifesting as explosive growth and biofilm formation in immunocompromised patients or older people with unbalanced oral ecosystems [22].

The morphological transition from budding yeast to filamentous forms of Ca is commonly associated with an increase in virulence and Ca infectivity [23]. In addition, phenotypic plasticity is firmly under the control of environmental factors. For example, the production of hyphae can be induced efficiently when Ca is cultured in liquid culture medium with neutral pH or in the presence of serum at 37°C [24,25]. Conversely, blocking hyphal growth diminishes damage to host tissue, and produces mutants that have defective filaments or that are locked in the yeast form and are avirulent in systemic candidiasis [26,27]. In other words, the development of hyphae assists in the formation of biofilms, which are highly resistant to standard antifungal treatments and which play a role in resistance to host defenses and increased pathogenicity [28].

In the present study, we investigated the *in vitro* effect of D-psicose on the growth and hyphal development of planktonic Ca by using liquid peptone medium containing 4% D-psicose (PM) and 4% D-glucose in Ca standard medium (Sabouraud`s medium; SM). Our results showed that D-psicose reduced the growth and the hyphal development of the fungus, Ca, and this is the first report regarding the impact of D-psicose, a rare sugar, on Ca.

Materials and Methods

Microorganism strains and culture conditions

The *Candida albicans* (Ca) strain ATCC 10261 used in this study was kindly provided by Professor Toshinori Okinaga from the Department of Bacteriology, Osaka Dental University and Ca was cultured in liquid medium (pH 5.6) or on agar medium (pH 5.6) containing Bacto peptone (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 4% D-glucose (Sabouraud's medium; SM) or 4% D-psicose (Chem-Impex International, Inc., Wood Dale, IL, USA) (PM) without glucose at 37°C under aerobic conditions.

Growth curve of Ca in liquid SM and PM

Ca strain ATCC 10261 was cultured in liquid SM for 24h at 37°C when the optical density was adjusted to 1.2 at 600 nm (OD_{600}). A total of 100 µL of the culture was transferred to 15 mL of SM containing 4% D-glucose or 15 mL of PM 4% D-psicose instead of 4% D-glucose and incubated for 60h at 37°C under aerobic conditions. The absorbance of the culture medium was determined at 6h intervals at OD_{600}.

Sizes of Ca colonies on SM/PM agar plates

Ca strain ATCC 10261 was cultured in liquid SM for 24h at 37°C until the optical density was 1.2 at OD_{600}. An aliquot of one hundred mL of the fungal culture was then plated on an SM agar plate or a PM agar plate (diameter 10 cm) and incubated under aerobic conditions for 48h at 37°C. The diameter of all colonies was then measured under a stereoscopic microscope.

Metabolic activities of Ca cultured in liquid SM/PM

The viability of Ca cells was determined using an methyl thiazolyl tetrazolium (MTT) Cell Count Kit (Nacalai Tesque, Kyoto, Japan). After culturing Ca strain ATCC 10261 in liquid SM or liquid PM at 37°C until the optical density was 1.2 at 600 nm, an aliquot of 100 µL of the fungus liquid adjusted to optical density 0.1 at 600 nm with PBS was mixed with 10 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution on a 96-well plate and incubated at 37°C in the dark for 3h. Then, 100 µL of solubilization solution was added to the wells, the plates were incubated at 37°C overnight, and the absorbance of the solutions was measured at 570 nm using a microplate reader (SpectraMax M50s, Molecular Devices, San Jose, CA, USA).

Morphological analysis with the light microscope and scanning electron microscope (SEM)

To investigate the effect of the morphological forms of planktonic Ca strain ATCC 10261 in liquid media, the strain was cultured in liquid SM or liquid PM medium for 24h at 37°C and then observed under an All-in-one microscope (BZ-X800; Keyence Corporation, Tokyo, Japan) and scanning electron microscope (SEM) (S-4800; Hitachi High-Tech Corporation, Tokyo, Japan). For the light microscope analysis, an aliquot of 10 µl of Ca strain ATCC 10261 was observed in the bright field mode (magnification: ×200). For the SEM analysis, Ca strain ATCC 10261 was cultured in liquid SM at 37°C until the optical density was 1.2 at OD_{600}. Then, 100 µL of the fungus liquid was transferred into 10 mL fresh liquid SM or liquid PM. Subsequently, 1 mL of the fugus liquid was then placed on glass coverslips in 24-well plates and incubated for 24h at 37°C. Following incubation, CA strain ATCC 10261 was fixed in 2.5% (vol/vol) glutaraldehyde at 4°C overnight and washed with sterile phosphate buffered saline (PBS). Then, CA strain ATCC 10261 was dehydrated in an ethanol series (30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%, vol/vol) for 15 minutes at each concentration, and the coverslips were dried. After gold spraying, the samples were examined under the SEM. In each sample, three points were randomly selected for investigation at two magnifications (×3,000, ×5,000).

Quantitative real-time (qRT)-polymerase chain reaction (PCR) analysis of Ca virulence-related gene expression

Ca strain ATCC 20161 was cultured in liquid SM or liquid PM for 24h at 37°C and total RNA was extracted with NucleoSpin RNA Plant and Fungi kit (Takara Bio USA, Inc., San Jose, CAL, USA). The purity and concentration of the RNA was determined using a Nanodrop™ 2000 (Thermo Fisher Scientific Inc.). The cDNA was synthesized using a ReverTra Ace® qPCR RT Master Mix (Toyobo Co. Ltd, Osaka, Japan). Primers (Table 1) were designed to generate amplicons with sizes ranging between 291 - 891 bp with a T_m between 50 - 60°C in order to detect gene expression of *ECE1*, *HWP1, PLB1,* and *SAP1* [29-32]. The reactions were run on a Step One Real time PCR System (Version 2, Applied Biosystems®) using a Thunderbird® Next SYBR® qPCR Mix (TOYOBO Co.) according to the manufacturer's instructions. The PCR reaction was first denatured at 95°C for 30s, followed by 40 cycles of 95°C for 5s and 60°C for 10s. Relative gene expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method, using *EFB1* as an internal standard [29,33]. Gene expression was expressed using relative values, with the expression level of the control samples set to 1.

Gene	Sequences	T_m (°C)	Product length (bp)	References
ECE1	Forward: ATCGAAAATGCCAAGAGAG Reverse: AGCATTTTCAATACCGACAG	58	291	Moyes., *et al*. 2016 [29]
HWP1	Forward: CCATGTGATGATTACCCACA Reverse: GCTGGAACAGAAGATTCAGG	58	572	Naglik., *et al*. 2006 [30]
PLB1	Forward: GGATTTGACAATGCTGGGTT Reverse: TTTCACCTAATGGCTCACCC	60	808	Samaranayake., *et al*. 2005 [31]
SAP1	Forward: AGGGAAAGGTATTTACACT Reverse: GATTTGCTTACATAGTAAGTAC	50	891	Schaller., *et al*. 1998 [32]
EFB1	Forward: ATTGAACGAATTCTTGGCTGAC Reverse: CATCTTCTTCAACAGCAGCTTG	60	526	Tsang., *et al*. 2012 [33]

Table 1: *Primers used in this study.*

Statistical analysis

Experiments were performed at least three times and the significance of comparisons of treated samples versus control samples was analyzed by a Student's t-test using the GraphPad Prism version 7 (GraphPad Software, Inc., La Jolla, CA, USA), with p values less than 0.05 or 0.01 considered to be significant.

Results

D-Psicose weakens growth of planktonic Ca

To examine the effects of the Ca growth using D-psicose as a substitute for sucrose, Ca strain ATCC 10261 in the logarithmic growth phase was transferred to liquid SM or liquid PM without D-glucose and cultured for 60h at 37°C under aerobic conditions. Ca strain ATCC 10261 in liquid SM entered the logarithmic growth phase at 12 to 36h after Ca inoculation, followed by the stationary growth phase. Conversely, sluggish and prolonged growth was observed for 60h from the Ca inoculation, when the Ca was cultured in liquid PM (Figure 1). In the liquid PM culture, the Ca strain ATCC 10261 only entered the stationary growth phase (i.e. optical density: 1.5) after 120h from the start of culture. These findings showed that D-psicose slows the growth of Ca strain ATCC 10261 in liquid cultivation medium.

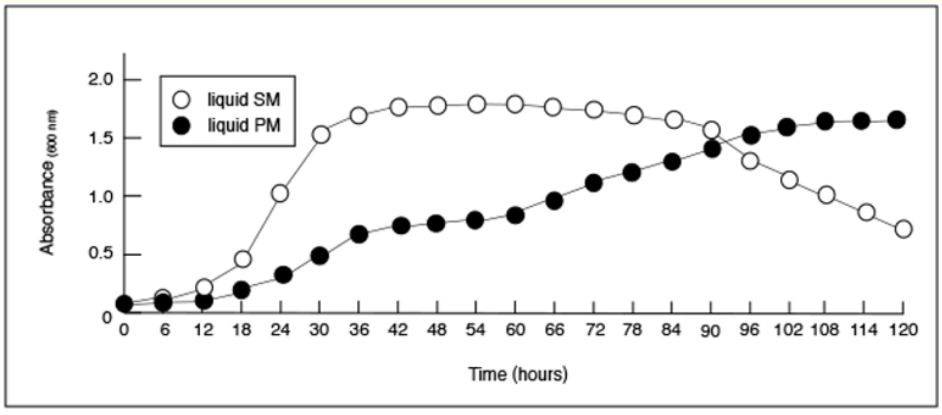

Figure 1: *Growth curve of Ca strain ATCC 10261 in liquid SM or in liquid PM. Ca strain ATCC 10261 in the logarithmic growth phase was cultured for 60h at 37°C under aerobic conditions, and the absorbance of the culture medium was measured at 600 nm every 6h.*

In addition, when the same number of Ca strain ATCC 10261 was cultivated on solid SM and PM agar for 24h at 37°C, the colonies on PM agar were smaller in size than those on SM agar (Figure 2A and 2B). This result implies that D-psicose inhibits the grows of Ca strain ATCC 10261.

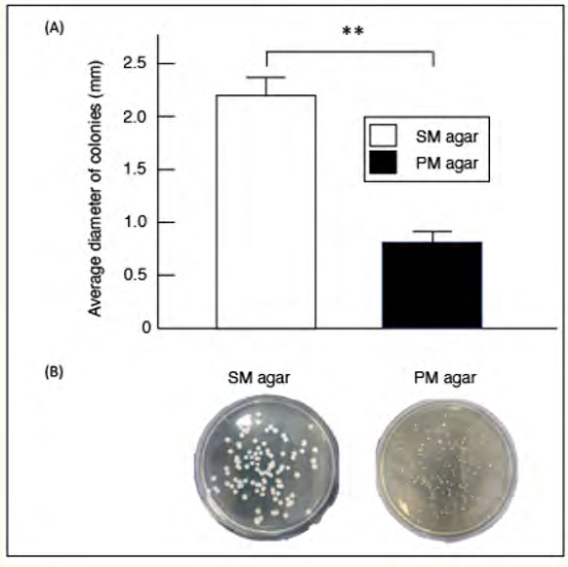

Figure 2: *Measurement of Ca strain ATCC 10261 colony size on SM and PM agar. Ca strain ATCC 10261 was grown on SM agar and PM agar for 48h at 37°C following cultivation for 24h at 37°C in liquid SM (OD600 = 1.2), and the diameter (A) of all colonies was measured. The photographs (B) show colonies on each type of agar plate after cultivation for 48h. The values in the graph (A) are shown as means ± SD. **p < 0.01, Student's t-test.*

D-Psicose impairs the metabolic activity of Ca

Further, we examined the changes in the concentrations of Ca strain ATCC 10261 cultured in SM and PM liquid medium using the MTT assay. Ca strain ATCC 10261 grown in liquid PM showed an approximately 80% reduction in viability compared to Ca strain ATCC 10261 cultured in liquid SM (Figure 3) ($p < 0.01$).

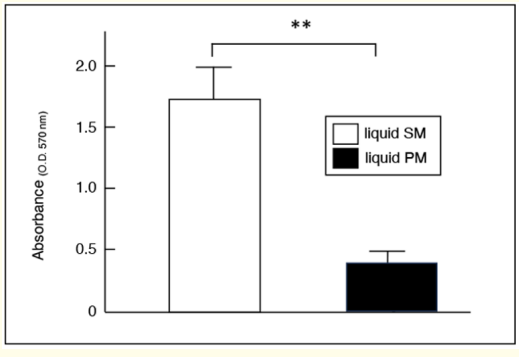

Figure 3: *Measurement of Ca strain ATCC10261 viability after culturing in liquid SM and liquid PM. Ca strain ATCC 10261 was cultured at 37°C in each liquid medium until the optical density at 600 nm was 1.2. The fungus in the liquid SM or liquid PM was subjected to the MTT assay, which was performed according to the manufacturer's instructions. The optical density of the fungal cultures was measured at 570 nm. The values in the graph are means ± SD. **p < 0.01, Student's t-test.*

Morphological changes in mycobionts using D-psicose

To investigate virulence, we evaluated changes in gross morphology and architecture of Ca cultured in liquid PM or SM using SEM and the light microscopy. Interestingly, Ca cells grown at 37°C in the liquid SM consisted of stacked and filamentous cells that had the appearance of a developing biofilm (Figure 4A). Conversely, fewer filamentous cells were observed in the liquid PM, which contained scattered yeast cells (Figure 4A). In addition, SEM images clearly showed that the cells of Ca cultures grown in liquid PM did not possess hyphae and consisted entirely of aggregates of blastospores (Figure 4B). Taken together, the microscopic findings showed that D-psicose significantly suppressed Ca biofilm formation and hyphal growth.

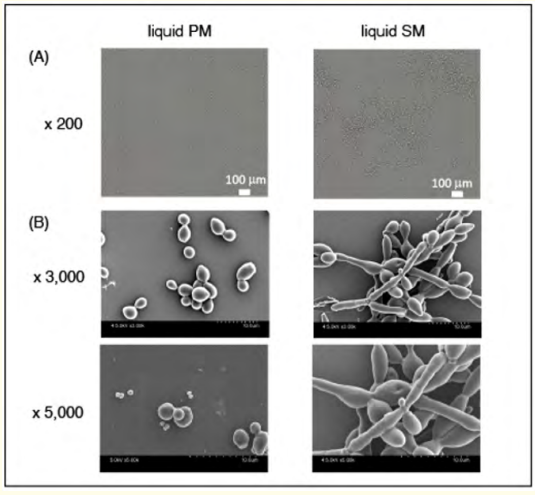

Figure 4: *Gross morphology and architecture of Ca cells cultured in liquid SM or PM. Ca strain ATCC 10261, was cultured in the liquid medium for 24h at 37°C and examined under (A) a light microscope (magnification: × 200) and (B) by SEM (magnification: × 3,000, × 5,000).*

Effects of D-psicose on the expression of virulence-related genes

To clarify the underlying relationship between D-psicose and Ca morphogenesis, we examined the expression of four virulence-related genes (*ECE1*, *HWP1, PLB1,* and *SAP1)* including two hyphae-associated genes (*ECE1*, *HWP1*) in Ca exposed to D-psicose. The expression of the four virulence-related genes in Ca strain ATCC 10261 cultured in liquid PM was downregulated, albeit to different extents (Figure 5). For example, the transcription levels of *HWP1* and *PLB1* were reduced significantly by 34% and 28%, respectively (Figure 5), implying that D-psicose attenuates the virulence of Ca strain ATCC 10261.

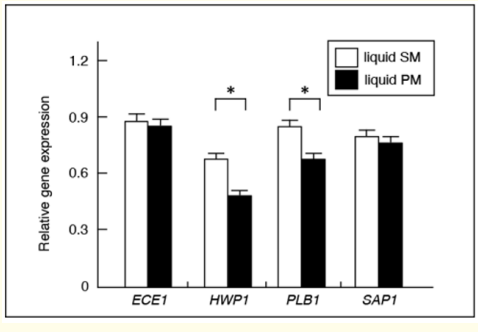

Figure 5: *Expression of virulence-related genes (ECE1, HWP1, PLB1 and SAP1) in Ca strain ATCC 10261 in the presence of D-psicose. The relative expression levels of genes were measured by qRT-PCR, and EFB1 was used as an internal control. Statistical data are presented as the mean ± standard deviation (SD) from at least three independent experiments (*p < 0.05).*

Discussion and Conclusion

D-Psicose is a rare sugar that exists as a monosaccharide in nature [1]. Previous studies have shown that D-psicose has wide variety of potentially beneficial physiological effects, including increasing lipid [7,8] and carbohydrate metabolism [9-12] in animals and humans. Further, recent studies have demonstrated that D-psicose also has anti-inflammatory and antioxidant effects [13], that it may reduce serum cholesterol levels [15], and that it may improve insulin sensitivity [16]. However, to the best of our knowledge, the effects D-psicose on microorganisms have only been investigated in *Trichomonas foetus* Inui, in which D-psicose reinforced the action of metronidazole on this parasite [34]. Thus, in this study, we investigated how D-psicose affects both the growth and hyphal development of Ca. Ca is the most prevalent fungus in the body and oral cavity, and is known to cause systemic diseases [17] as well as oral candidiasis [18,35]. Our findings showed for the first time that D-psicose attenuates the growth and hyphal development of this fungus.

Since these findings in Figure 1-3 indicate that D-psicose has a deleterious effect on the rate of planktonic growth in Ca strain ATCC 10261, it was suggested that Ca was reduced to a state of starvation as the culture lacked nutrients for growth as D-psicose has no caloric value. A previous study reported that fermentable dietary sugars, including sucrose and glucose, promoted the growth, adhesion, and biofilm development of Ca, and that the presence of these sugars could promote the development of, and can aggravate, oral candidiasis [36]. Our findings also suggest that D-psicose may contribute to postponing the onset of oral candidiasis by the delay of Ca growth among the elderly and immunocompromised people. Since salivary secretion is often reduced in the elderly and immunocompromised people and they are in a state of xerostomia, it is considered that oral care with toothpastes and oral care gel [37,38] is important. Sucrose is often included in toothpastes and oral care gels as moisturizing and flavoring ingredients [39]. Therefore, replacing the sugar in oral care products with D-psicose might provide us with a great benefit by inhibiting the growth of oral Ca.

Ca is unique in that it is capable of dimorphic switching (i.e. it can transition from budding yeast to the hyphal form); the hyphal form plays a key role in the invasion of host tissues and biofilm formation [24,26,40]. These findings in Figure 4 showed that D-psicose significantly suppressed Ca biofilm formation and hyphal growth, and suggest that D-psicose may affect hyphal formation in Ca strain ATCC 10261. Several genes have been reported to be either directly or indirectly involved in the regulation of hyphal morphology [24,41]. We therefore examined the effect of D-psicose on the expression of four virulence-related genes (*ECE1*, *HWP1*, *PLB1*, and *SAP1*), including two genes associated with hyphal formation in Ca. Significant reductions were observed in the transcription levels of *HWP1* and *PLB1* when incubated for 24h at 37°C (Figure 5). Since *HWP1* is involved in cell adhesion and invasion of host tissues [42,43], and *PLB1* controls the main enzymes involved in cell adherence, invasion, and damage of host tissue [44,45], the obtained results suggest that D-psicose may attenuate the virulence of Ca strain ATCC 10261. Although it is known that *ECE1* is essential for Ca cell elongation and biofilm formation [46] and *SAP1* contributes to tissue damage in Ca mucosal infection [47], it is suggested that D-psicose may have no impact on *ECE1* and *SAP1* gene expression.

In summary, our results showed that D-psicose reduced the growth of, and inhibited the hyphal development of Ca. To the best of our knowledge, this is the first study to show the effects of D-psicose on Ca growth and morphology. Ca is the most common fungus in the oral cavity and is known to cause oral candidiasis [48]. It has a high affinity for the surface of the mucosal membrane as well as the resin components of dentures, especially in the elderly [49]. Our findings suggest that replacing sugar (glucose) with D-psicose in the daily diet could not only delay the onset of candidiasis both in the body and in the oral cavity of the elderly and immunocompromised individuals, but also reduce the frequencies of oral care. In this respect, further studies will be essential to demonstrate the utility of D-psicose, especially in the development of oral flora biofilm.

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QL mainly conducted this study, collected data, and performed the statistical analysis. KK participated in the study design and data interpretation and drafted the manuscript. HY helped perform fungal assays and statistical analyses. TM helped draft the manuscript. All authors commented on draft of the manuscript and approved the final manuscript.

Finally, the authors thanks FORTE Science Communications (http://www.forte-science.co,jp/) for English language editing.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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