JOURNAL OF ORAL MICROBIOLOGY
2025, VOL. 17, 2473938
https://doi.org/10.1080/20002297.2025.2473938

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RESEARCH ARTICLE

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Candida albicans and NCAC species: acidogenic and fluoride-resistant oral inhabitants

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ABSTRACT

Objective: Although *Candida* species are thought to contribute to dental caries, their acid production under anaerobic conditions and susceptibility to fluoride have not been thoroughly studied. We therefore investigated the growth, acid production, and effect of fluoride on *Candida* species.

Methods: Aerobic growth, acid production from glucose and its end-products under aerobic and anaerobic conditions, and enolase activity were measured in *C. albicans* and non-*Candida-albicans-Candida* (NCAC) species (*C. tropicalis*, *C. parapsilosis*, *C. maltosa*, and *C. glabrata*), and the effect of fluoride on these abilities was evaluated.

Results: All *Candida* species produced acids under aerobic and anaerobic conditions, and acetate and TCA cycle metabolites were detected. However, these organic acids only accounted for 1.9–57.6% of the acids produced. Up to 80 mM fluoride hardly inhibited growth and did not inhibit acid production except for *C. glabrata*, despite the low 50% inhibitory fluoride concentration of 0.19–0.34 mM for enolase.

Conclusion: *Candida* species produced acids under aerobic and anaerobic conditions, indicating their significant cariogenicity. Their growth and acid production were highly fluoride-resistant, whereas their enolase was fluoride-sensitive, suggesting mechanisms for maintaining low intracellular fluoride. The mechanisms underlying the fluoride resistance remain underexplored. Approaches other than fluoride may be needed to control *Candida*-associated caries.

KEY MESSAGES

Candida species produced acids from glucose under both aerobic and anaerobic conditions, although most of the acids produced could not be identified as organic acids. Except for *C. glabrata*, *Candida* species were highly fluoride-resistant in growth and acid production, despite their enolase being fluoride-sensitive

ARTICLE HISTORY
Received 24 December 2024
Revised 5 February 2025
Accepted 19 February 2025

KEYWORDS
Acid production; *Candida* species; candidal growth; dental caries; enolase; fluoride; metabolic profile; Non-*Candida-albicans-Candida* (NCAC) species

Introduction

Candida species is a common potentially pathogenic microorganism inhabiting the oral cavity of almost all healthy individuals [1]. Despite being usually endosymbionts of humans, *Candida* species can cause serious infections, especially in immunosuppressed individuals [2]. Wearing removable dental appliances, poor oral hygiene, smoking, xerostomia, carbohydrate-rich diet, and certain diseases such as AIDS have been among the common factors predisposing to oral candidal infections in humans [3]. Among *Candida* species, *C. albicans* has always been the most commonly isolated species both in infections and healthy individuals [3]. Recently, infections due to non-*Candida-albicans-Candida* (NCAC) species are rising, especially in cases of immunosuppression or relatively older patients [4].

For a long time, it has been well known that caries-associated bacteria such as *Streptococcus mutans* play the major role in dental caries development and progression [5]. However, it has been also postulated that *C. albicans* specifically plays a supporting role in caries, and it has been frequently isolated from carious lesions, especially in children [6,7]. Very limited information exists about whether NCAC species play a similar role.

Possible mechanisms by which *C. albicans* promotes dental caries include inducing the oral dysbiosis and increasing *S. mutans* [6], promoting the growth of *S. mutans* [8], promoting the production of extracellular polysaccharides by *S. mutans* [9], and even acting as '*S. mutans* reservoir' that mediates *S. mutans* adhesion to the oral mucosa [10,11].

However, because *C. albicans* produces acid from sugars [12–15], it may not only assist cariogenic bacteria but also itself shift the demineralization/remineralization balance toward demineralization of tooth surfaces, inducing dental caries [16,17], but its acid production has not been fully studied. In addition, although *Candida* species are often referred to

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as facultative anaerobes, limited evidence exists regarding their acid production under anaerobic conditions. This is particularly important considering that as dental plaque matures, it becomes more anaerobic and more cariogenic [18,19].

Furthermore, limited evidence exists regarding the effect of fluoride – a well-known caries control agent – on candidal acid production, especially when it comes to NCAC species despite their rising clinical importance. Meurman et al. [20] reported that topical application of amine fluoride-stannous fluoride (AmF/SnF_2) for 8 months did not result in a clinically significant reduction in *Candida* counts, although a previous study [21] reported that the same combination (contained in Meridol®) showed antifungal activity in vitro against seven *Candida* species. Similarly, Flisfisch et al. [22] confirmed growth inhibition with the AmF/SnF_2 combination but failed to observe a similar effect with NaF. Other studies found that conventional NaF toothpastes were not particularly effective against *C. albicans* [23,24].

Therefore, in this study, we investigated the glucose metabolism, acid production potential, and fluoride sensitivity of *C. albicans*, as well as human NCAC species *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* under aerobic and anaerobic conditions. Additionally, for comparison, we also used *Candida maltosa*, a non-pathogenic NCAC species commonly isolated from food such as cheese or soil. Despite its inability to cause oral disease, *C. maltosa* can effectively ferment carbohydrates such as glucose [25], and its inability to cause oral disease despite being phylogenetically closely related to the other pathogenic *Candida* species could provide valuable insights about *Candida*'s pathogenicity as previously discussed by Chávez-Tinoco et al. [26]. Moreover, although *C. maltosa* is not a regular inhabitant of the oral cavity, transient fungal species acquired from the environment are commonly found viable in the oral cavity [27]. As its role has not yet been fully elucidated, we included this *Candida* species in this study.

Materials and methods

Candida strains, and growth conditions

Five representative *Candida* species *C. albicans* JCM1537, *C. tropicalis* JCM1541, *C. parapsilosis* JCM1618, *C. maltosa* JCM1504, *C. glabrata* JCM3761 were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. *Candida* species were grown at 37°C for 24 h on TYG agar plates (1.7% tryptone (Becton Dickinson, Franklin Lakes, NJ, USA), 0.3% yeast extract (Becton Dickinson), 0.5% NaCl, 50 mM potassium phosphate buffer solution (PPB, pH 7.0), and 1% glucose, under aerobic conditions (atmospheric gases in the air) and then maintained at 4°C. *Candida* species were precultured at 37°C for 24 h in a TYG medium, under aerobic conditions. All incubations were performed in static conditions. The candidal culture was transferred to TYG medium (final glucose concentration: 1%) and grown in the same conditions. In the logarithmic growth phase (optical density at 520 nm [$OD_{520\ nm}$]: 1.0–1.2), the candidal cells were harvested by centrifugation (15,000 g, 7 minutes, 4°C). Then, they were washed three times with washing buffer (2 mM PPB containing 150 mM KCl and 5 mM $MgCl_2$, pH 7.0) [28]. The pelleted cells were resuspended in the same buffer and stored on ice until use.

Growth and fluoride sensitivity

Candida species were grown at 37°C for 24 h on TYG agar plates under aerobic conditions. Then, colonies of *Candida* species were collected and suspended in TYG medium (pH 7.0). Ten milliliters of TYG medium (pH 7.0) were mixed with 1 mL of the candidal suspension ($OD_{520\ nm}$ = 1.0) in the absence or presence of potassium fluoride (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at final concentrations of 80 mM (pH 7.0) (1 mM = 19.1 ppm F). The cultures were incubated at 37°C for 72 h under aerobic conditions. The $OD_{520\ nm}$ was measured during the growth process, while the pH of the medium was measured at the start and end of the experiment [29,30].

Acid production and fluoride sensitivity

A pH-stat system (aerobic conditions: AUTO pH-stat, model AUT-501; Toa Electronics, Tokyo, Japan, anaerobic conditions: AUTO pH-stat, model AUT-211S; Toa Electronics, Tokyo, Japan) was used to monitor and quantify the acid production at pH 7.0 and 5.5, as described previously [29–31]. Anaerobic experiments were performed in the NH-type anaerobic glove box (N_2, 90%; H_2, 10%; NH-type; Hirasawa Works, Tokyo, Japan). For the anaerobic experiments, the pelleted *Candida* were kept in the NH-type anaerobic glove box at 4°C for 60 minutes to remove oxygen.

The pH-stat system was calibrated before each usage using standard pH solutions of pH 6.86 and pH 4.01 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The candidal suspensions ($OD_{520\ nm}$ = 3–3.5) were preincubated at 37°C for 3 minutes and incubated with fluoride (final concentration: 0–80 mM (0–1528 ppm F)) for a further 4 minutes. Glucose was then added to the reaction mixture at a final concentration of 10 mM

and acid production was monitored for 10 minutes. The pH was adjusted prior to the measurement by adding HCl or KOH to the reaction mixture [32]. When the pH of the reaction mixture began to decrease due to acid production by the cells, KOH was automatically dispensed to the mixture to maintain the desired pH, and the amount of acid produced was estimated from the amount of KOH consumed [29,32].

Analysis of acidic end-product

During the pH-stat assay, 0.45 mL of the reaction mixture was collected before and 10 minutes after the addition of glucose. Shortly, 0.05 mL of 6N perchloric acid were added to the samples to halt further metabolism and filtered through a polypropylene membrane (pore size: 0.20 µm; Toyo Roshi Ltd., Tokyo, Japan) [32]. The filtrates were quantitatively analyzed by high-performance liquid chromatography (HPLC; Shimadzu Prominence LC-20AD, Shimadzu Co. Ltd., Kyoto, Japan). Metabolic profile analysis was done using Shimadzu LabSolutions software V 5.42 SP3, and target acidic end-products included succinate, citrate, oxaloacetate, pyruvate, malate, fumarate, lactate, formate, and acetate as described previously [33,34]. Succinate was not determined in the presence of fluoride because the succinate peak overlapped with the fluoride peak.

Enolase assays and fluoride sensitivity

The pelleted *Candida* species were prepared as described above and stored at −80°C until used. After thawing the pellets, the cells were suspended in wash buffer, containing 1 mM dithiothreitol. The cell suspension ($OD_{520\,nm}$ = 10) was then disrupted by sonic oscillation for 10 minutes at 4°C (200 W, 2 A; Insonator, Kubota, Japan). Cell debris was removed by centrifugation (15,000 g, 10 minutes, 4°C) and resultant cell-free extracts (CFE) were assayed for enzymatic activity. The assay mixture for enolase (EC 4.2.1.11) contained 20 mM 2-phosphoglycerate (Sigma-Aldrich, St. Louis, MO, USA), CFE and potassium fluoride (final concentration: 0.02–2 mM) in wash buffer [31]. The enzymatic activity of enolase was measured by monitoring the absorbance of phosphoenolpyruvate produced from 2-phophoglycerate at 240 nm and 37°C using a spectrophotometer (UV-1800, Shimadzu Co., Ltd., Kyoto, Japan). Moreover, the 50% inhibitory concentration (IC_{50}) of fluoride was calculated as below.

$IC_{50} = 10\hat{}(\log[A/B] \times [50 - C]/[D - C] + \log[B])$, where A is a higher concentration across 50%, B is a lower concentration across 50%, C is an inhibition rate (%) at B and D is an inhibition rate (%) at A.

Statistical analysis

The significance of differences was evaluated using unpaired t-test for growth experiments, and Dunnet's test was used to compare the effect of fluoride on acid production and acidic end-products. JMP Pro version 17.2 software was used for all analyses, and P-values of < 0.05 were considered significant.

Results

The growth of candida species and the effect of fluoride

All five *Candida* species grew well on TYG medium. The addition of 80 mm fluoride did not ultimately inhibit the growth of all *Candida* species, except *C. parapsilosis* (Figure 1). Significant growth inhibition occurred by the 10-h time point for *C. tropicalis* and *C. glabrata*, and by the 24 h time point for the slowly growing *C. parapsilosis*. An almost complete growth recovery was observed at 24 h for all species except *C. parapsilosis* (Figure 1).

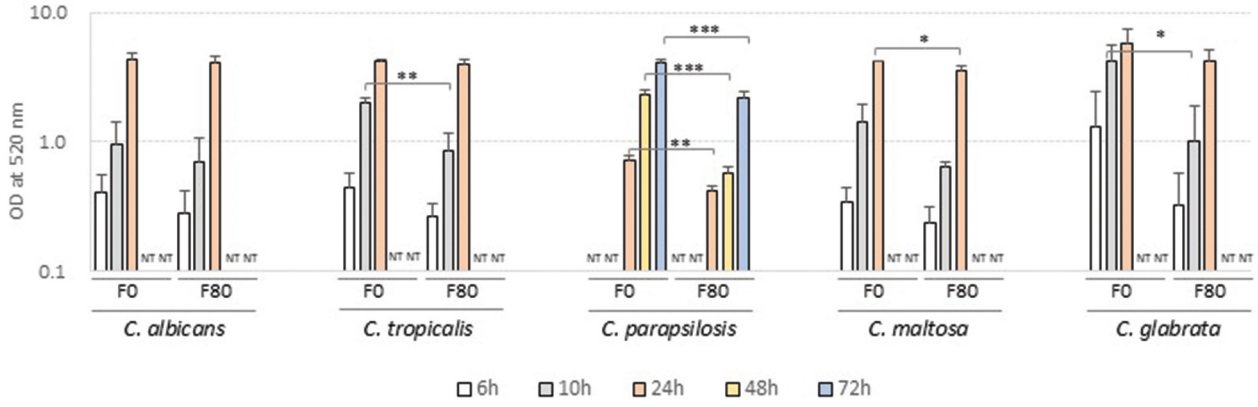

Figure 1. Growth of *Candida* species and the effect of fluoride under aerobic conditions. The data are shown as the mean ± standard deviation (SD) of three independent experiments. Unpaired t-test. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. F0 and F80 represent fluoride concentrations of 0 and 80 mM, respectively.NT, not tested.

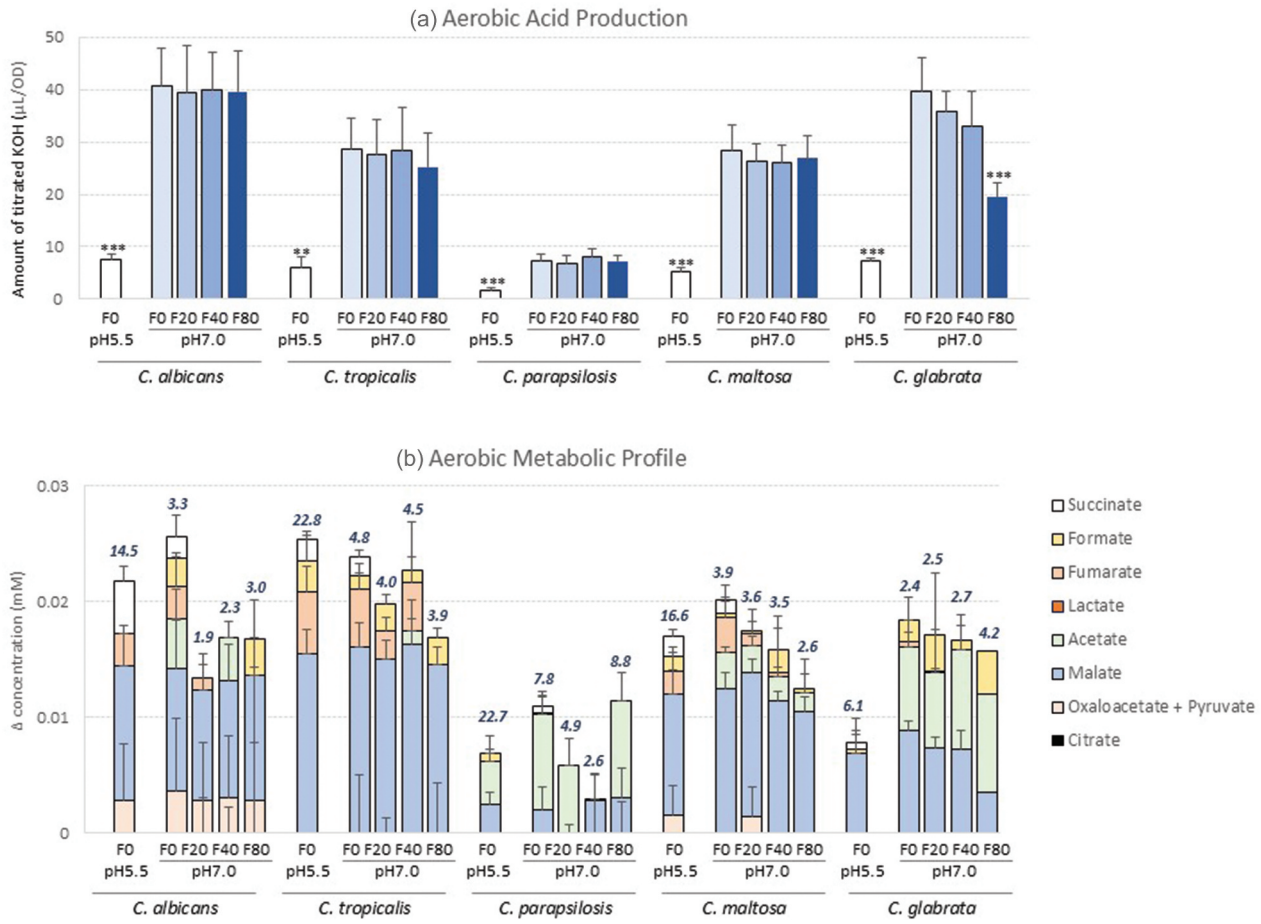

Figure 2. Acid production of *Candida* species and the effect of fluoride under aerobic conditions at pH 7.0 and pH 5.5. The data are shown as the mean ± standard deviation (SD) of three independent experiments. Dunnet's test. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. The numbers on the top of the column indicate the ratio of identified metabolites through HPLC analysis to total acid production. NA, not applicable. F0, F20, F40, and F80 represent fluoride concentrations of 0, 20, 40, and 80 mM, respectively. Succinate was not shown in the presence of fluoride because the succinate peak overlapped with the fluoride peak and could not be quantified.

The acid production of candida *species and the effect of fluoride*

Under aerobic conditions, all *Candida* species produced acid from glucose (Figure 2). The acid production was significantly lower at pH 5.5, compared to pH 7.0 for all species. These acid productions were not inhibited by fluoride for all species except *C. glabrata* (Figure 2(a)), whose acid production was significantly inhibited by 80 mM fluoride.

Under anaerobic conditions, *Candida* species also produced acid; however, the amount of acids produced was less than that produced under aerobic conditions (Figure 3 (a)). The acid production was also significantly lower at pH 5.5 compared to pH 7.0 for all species. Moreover, 80 mM fluoride significantly inhibited the acid production of *C. albicans*, *C. parapsilosis*, and *C. maltosa*, while *C. tropicalis* showed some degree of inhibition (Figure 3 (a)). Only *C. glabrata* was almost completely inhibited upon the addition of 80 and 40 mM fluoride and was significantly inhibited by 20 mM fluoride.

The end products of Candida *species and the effect of fluoride*

Under aerobic conditions, the total amount of end products was not significantly different at different pHs or in the presence of different concentrations of fluoride (Figure 2(b)). *C. albicans*, *C. tropicalis*, and *C. maltosa* mainly produced malate and fumarate, while *C. parapsilosis* and *C. glabrata* produced malate and acetate. The total amount of these end products was only 1.9–8.8% (pH 7.0) and 6.1–22.8% (pH 5.5) of the acid production determined by the pH stat (Figure 2(b)).

Under anaerobic conditions, fluoride significantly inhibited the production of end products by *C. glabrata*, but no significant differences were observed otherwise (Figure 3(b)). All species produced mainly malate and succinate. The total amount of these end products was only 2.2–34.9% (pH 7.0) and 14.0–57.6% (pH 5.5) of the amount of acid produced by the pH stat (Figure 3(b)).

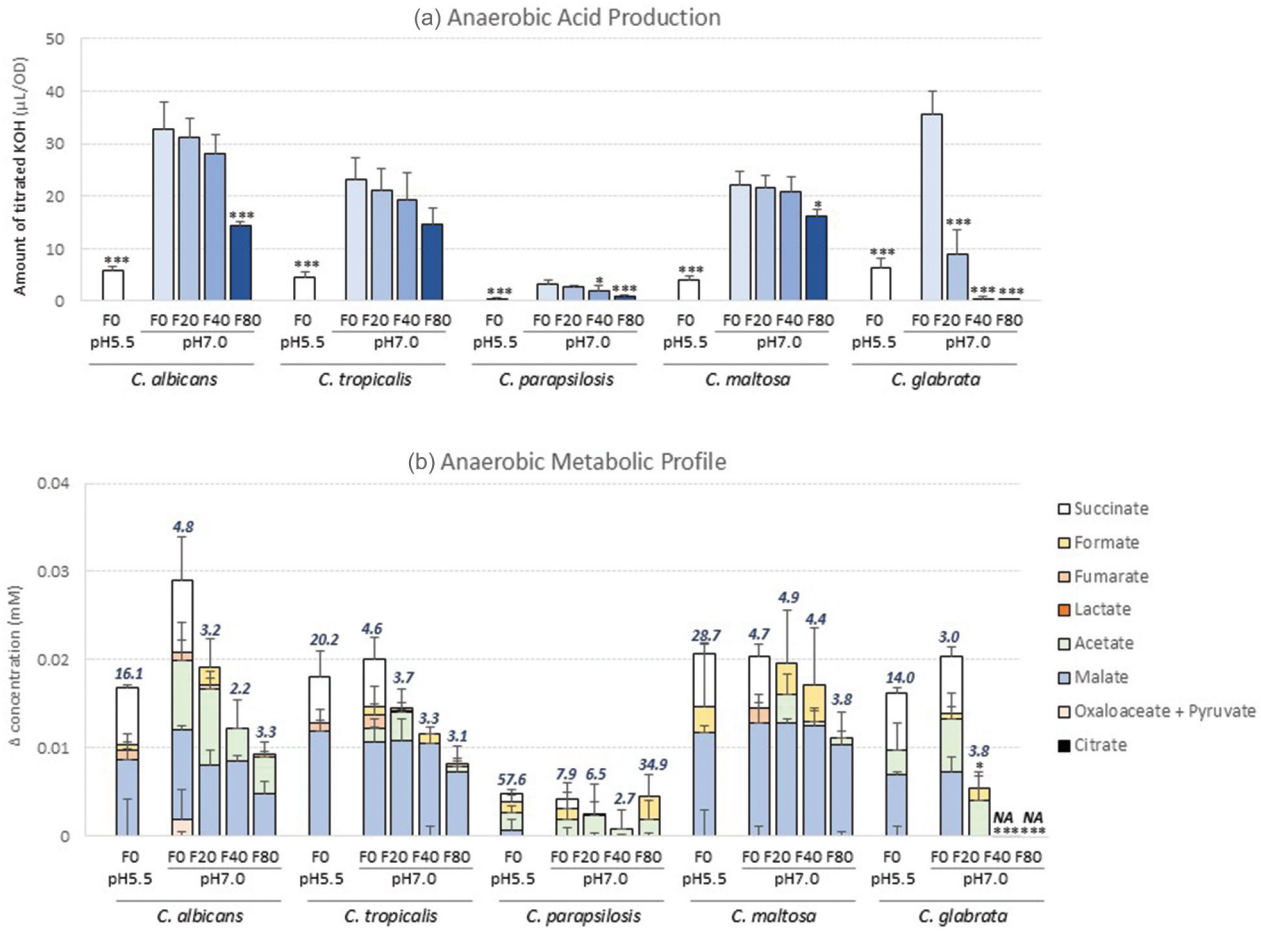

Figure 3. Acid production of *Candida* species and the effect of fluoride under anaerobic conditions at pH 7.0 and pH 5.5. The data are shown as the mean ± standard deviation (SD) of three independent experiments. Dunnet's test. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. The numbers on the top of the column indicate the ratio of identified metabolites through HPLC analysis to total acid production. NA, not applicable. F0, F20, F40, and F80 represent fluoride concentrations of 0, 20, 40, and 80 mM, respectively. Succinate was not shown in the presence of fluoride because the succinate peak overlapped with the fluoride peak and could not be quantified.

Table 1. The 50% inhibitory concentration (IC_{50}) of enolase enzyme by fluoride.

	C. albicans	C. tropicalis	C. parapsilosis	C. maltosa	C. glabrata
Fluoride concentration (mM)	0.19 ± 0.06	0.19 ± 0.03	0.34 ± 0.12	0.29 ± 0.08	0.21 ± 0.02

Data are shown as the means ± standard deviation (SD) of three independent experiments.

The enolase activity of Candida *species and the effect of Fluoride*

Fluoride inhibited the enolase activity of all *Candida* species. (Table 1), with a low IC_{50} of 0.19 to 0.34 mM.

Discussion

Growth and fluoride resistance of candida species

All five *Candida* species were able to grow aerobically in the presence of a high concentration of fluoride (80 mM) that is almost equivalent to that present in most toothpastes, with only a slight growth delay that caught up as time passed (Figure 1). This is in stark contrast to *S. mutans*, whose growth is completely inhibited by 8 mM fluoride [32]. This slight inhibitory effect of fluoride was evident in *C. glabrata* and *C. parapsilosis*. It is noteworthy that *C. parapsilosis* is the most slowly growing out of the five species in the current experimental conditions, and this could explain why it was easier to observe the growth delay attributed to fluoride. This study is the first to demonstrate that the growth of NCAC species is fluoride-resistant, similar to that of *C. albicans*, suggesting the possibility that *Candida* species may survive fluoride stress in the oral cavity and exert cariogenic potential.

Acid production and fluoride resistance of candida species

The results of the pH-stat experiments showed that all *Candida* species were able to utilize glucose and produce acid at neutral pH under both aerobic and anaerobic conditions. It is surprising that *Candida* species

can produce acid from sugars not only under aerobic conditions but also under anaerobic conditions, indicating the high cariogenic potential of *Candida* species (Figures 2(a) and 3(a)). However, despite the well-known ability of *Candida* species to survive and grow in acidic culture medium, its acid production was substantially reduced at pH 5.5, possibly reflecting an attempt to maintain a preferable environmental pH. Control samples without glucose showed a slight increase in pH at pH 5.5 over time (data not shown). This may be due to pH regulation by *Candida* cells, but further studies are required to clarify it.

Due to the small acid production at pH 5.5, fluoride sensitivity was only assessed at pH 7.0 (Figures 2(a) and 3(a)). All *Candida* species, except *C. glabrata* were notably resistant to fluoride, consistent with the results of growth experiments. These results indicate that the sugar metabolism of *Candida* species, which is known to begin with glycolysis [35], is highly fluoride-resistant, but the enolase activity of the glycolytic pathway in *Candida* species was inhibited by low concentrations of fluoride (Table 1), similarly to that of *S. mutans* [32]. This discrepancy implies that the fluoride export mechanism of *Candida* species, FEX transporters [36,37], may keep the intracellular fluoride concentration low (Figure 4) or that fluoride has poor penetration into *Candida* cells. Another possibility could be the presence of an alternate metabolic pathway that skips the enolase enzyme pathway, but no evidence supporting this hypothesis exists. More studies are needed to explore this. Acid production in *C. glabrata* was inhibited by fluoride, suggesting that *C. glabrata* is taxonomically closer to baker's yeast than to the other four species [38], and that differences in its cell structure may influence the sensitivity of its sugar metabolism to fluoride. Overall, these results are the first to show that sugar-

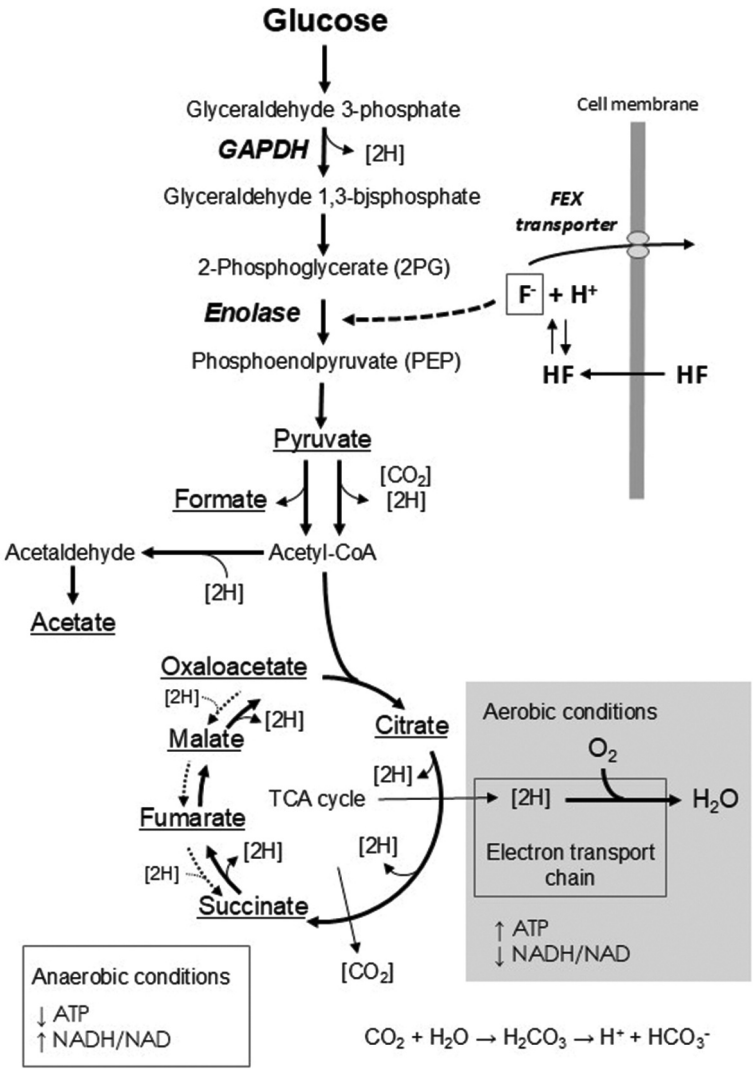

Figure 4. Proposed glucose metabolism in *candida* species and its inhibition by fluoride. Dashed line indicates inhibition by fluoride. Dotted lines indicate predicted metabolic pathways. See text for details.

induced acid production in *C. albicans* and NCAC species as well as nonpathogenic environmentally derived *Candida* species is fluoride-resistant and suggest that, unlike *S. mutans*, whose acid production was completely inhibited by 6 mM fluoride [32], *Candida* species exhibited cariogenic acid production in the presence of much higher fluoride concentrations.

Under anaerobic conditions, fluoride inhibited the acid production of almost all species more effectively than under aerobic inhibition (Figures 2(a) and 3(a)). This is presumably due to the unfavorable metabolic conditions for *Candida* species under anaerobic conditions, namely, (1) the inability to efficiently produce ATP through oxidative phosphorylation and (2) the tendency for intracellular NADH/NAD to increase because NADH produced during metabolism is not reoxidized by oxygen (Figure 4). The former is thought to reduce sugar uptake and fluoride excretion, while the latter results in a high NADH/NAD ratio and inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in glycolysis [39, 40], thereby enhancing the inhibitory effect of fluoride.

End products from glucose and possible pathways for glucose metabolism of candida species

Candida species were found to produce various organic acids from sugar metabolism (Figures 2(b) and 3(b)). Under aerobic conditions, *C. albicans*, *C. tropicalis*, and *C. maltosa* mainly produced malate and fumarate, while *C. parapsilosis* and *C. glabrata* produced malate and acetate, whereas under anaerobic conditions all species produced mainly malate and succinate, suggesting that *Candida* species may use different metabolic pathways depending on atmospheric conditions, as well as differences among the *Candida* species. Fluoride reduced the production of acidic end products under both aerobic and anaerobic conditions, but the profile of end products did not change significantly except for *C. glabrata* (Figures 2(b) and 3(b)). *C. glabrata* decreased malate and increased acetate, indicating the possibility of metabolic shift by fluoride.

It was unexpected that metabolic intermediates of the TCA cycle, which is thought to function under aerobic conditions, were detected even under anaerobic conditions (Figures 2(b) and 3(b)). However, the detected succinate, fumarate, malate, and oxaloacetate are all metabolites in the latter stage of the TCA cycle, suggesting that *Candida* species may reverse the TCA cycle to consume reducing power produced by sugar metabolism (Figure 4). Such anaerobic succinate production by reverse TCA cycle has been observed in various bacteria, including oral *Actinomyces* species [41].

Even more surprising is that the amount of organic acids measured by HPLC was 1.9–57.6% of the amount of acids measured by pH stat (Figures 2(b) and 3(b)), which is consistent with the results reported previously [13], suggesting the possibility that inorganic acids (not detected by the HPLC method) were produced. Under aerobic conditions, it is assumed that carbon dioxide produced by pyruvate dehydrogenation and the TCA cycle (Figure 4) dissolves in water and produces acids through the reaction $CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^-$. The reducing power produced by the glycolysis, pyruvate dehydrogenation, and TCA cycle is used for ATP production via the electron transport chain, and then oxidized by oxygen to water. On the other hand, under anaerobic conditions, it is thought that acids were produced from carbon dioxide in the same way, but the reducing power must be oxidized by something other than oxygen. The higher production of succinate and acetate compared to aerobic conditions (Figures 2(b) and 3(b)) suggests the disposal of reducing power through the operation of the reverse TCA cycle as mentioned above and the acetate production as described above (Figure 4).

Limitations of this study and future research direction

Since only standard *Candida* strains were used in this study, more research should be done to compare the metabolic behavior of different clinical isolates and their sensitivity to fluoride. The static culture method used in this study to compare anaerobic and aerobic conditions could be further improved. Biochemical and molecular mechanisms behind fluoride resistance also need to be explored in more depth, to understand the mechanisms behind *Candida*'s fluoride resistance, and the cause behind the fluoride sensitivity of *C. glabrata*. Moreover, future studies should test different approaches to identify the unrecognized portion of the acid production.

Conclusion

C. albicans and NCAC species were shown to produce acid from glucose both under aerobic and anaerobic conditions, demonstrating their cariogenic potential. For all *Candida* species except *C. glabrata*, fluoride did not inhibit their growth and acid production, despite being effective in inhibiting their enolase enzyme. These results suggest that fluoride alone may not be sufficient to prevent *Candida*-associated caries, such as caries in children. In the future, it will be necessary to develop not only oral care agents that inhibit *Candida* species but also methods to control the cariogenicity of *Candida* species from an oral ecological perspective through clarifying its biological properties, such as its sugar metabolism in the oral cavity. This may also help to elucidate and prevent *Candida*-associated diseases, such as oral mucositis.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Grants-in-Aid for Scientific Research B (21H03151) and Grants-in-Aid for Scientific Research C (23K09475) from the Japan Society for the Promotion of Science (JSPS), and JST SPRING, Grant Number JPMJSP2114.

Author contributions

HRF. Mousa: Contributed to data acquisition and analysis, performed all statistical analyses, drafted and critically revised the manuscript; Y. Abiko: Contributed to design, data interpretation, drafted and critically revised the manuscript; J. Washio: Contributed to data interpretation and critically revised the manuscript; S. Sato: Contributed to data interpretation for acid end-product analysis; N. Takahashi: Contributed to conception, design, data interpretation, drafted and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

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