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RESEARCH ARTICLE

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Evaluation of a *Burkholderia ambifaria* strain from plants as a novel promising probiotic in dental caries management

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ABSTRACT

Background: Probiotics serve as a novel preventive or therapeutic approach for dental caries owing to their ability to reverse dysbiosis and restore a healthy microbiota. Here, we identified *Burkholderia ambifaria* AFS098024 as a probiotic candidate isolated from plants.

Methods: The safety of *B. ambifaria* was evaluated by hemolytic activity, D-lactic acid production and antibiotic susceptibility. *In vitro* biofilm model derived from the saliva of caries-free and caries-active donors and *in vivo* rat caries model were used to assess the efficacy of *B. ambifaria* in caries prevention and treatment.

Results: *B. ambifaria* was safe as a probiotic candidate and it could integrate with *in vitro* biofilm model. It significantly reduced the biomass and lactate production of biofilms from caries-active donors and disrupted biofilm structures. *B. ambifaria* effectively reduced the severity of carious lesions in rat molars, regardless of the inoculation sequence. Molars pretreated or treated with *B. ambifaria* demonstrated notably higher enamel volumes. Additionally, colonization of rat molars by *B. ambifaria* persisted for 6 weeks.

Conclusion: The *B. ambifaria* strain used in this study holds promise as a probiotic for inhibiting dental caries, both *in vitro* and *in vivo*.

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Introduction

Dental caries is defined as the gradual loss and breakdown of hard tooth tissues resulting from various factors, with disturbance of homeostasis in the oral microbial community being predominant [1]. With frequent intake of dietary carbohydrates, acidiferous and aciduric bacteria such as *Streptococcus mutans* and *Lactobacillus casei* enhance, leading to significant acid production, an imbalance between demineralization and remineralization in hard tissues, and ultimately the initiation and progression of caries [2].

Currently, existing oral hygiene products used for anticaries typically contain agents with broad-spectrum antibacterial activities, such as chlorhexidine, cetylpyridinium chloride, and alcohol. However, long-term use of these antimicrobials may lead to resistance in oral pathogens such as *S. mutans*, *Streptococcus sobrinus*, and *Porphyromonas gingivalis* [3], and may induce a decline in beneficial bacteria [4], resulting in the overgrowth of pathogenic bacteria or fungi [5]. Hence, it is important to have agents that can modulate the microbiome and reverse the dysbiotic community to a healthy one, rather than eliminating the microbial flora unselectively.

Probiotics, defined as 'live microorganisms which when administered in suitable amounts confer health benefits on the host' [6], have increasingly been applied for human health as promising biological agents for adjuvant therapy in various human diseases, including diarrhea, constipation, caries, periodontal diseases, halitosis, and even cancer [7]. To date, the most widely used commercial probiotics for caries management are *Lactobacillus* and *Bifidobacteria* strains from the human gastrointestinal tract or fermented milk. However, these strains are highly acidogenic and are likely to contribute to the caries process [8,9]. For instance, *Lactobacillus rhamnosus* GG, frequently investigated as a probiotic in the intestines, has been demonstrated *in vitro* to induce dentin demineralization rather than inhibiting cariogenic *S. mutans* [10]. In addition, *Lactococcus lactis* HY 449 has been found to produce a high level of acid in the presence of sugar, reducing the pH of the growth medium to 5.5, a critical value for enamel demineralization [11]. Similarly, a prospective study reported that after supplementation with curd containing *Lactobacillus* in a daily diet for one year in 15 healthy children aged 10–15 years, there was no significant decrease in *S. mutans* compared

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to the pre-use period. In contrast, the number of *Lactobacillus* species increased significantly, potentially increasing the risk of caries [12]. Thus, it is necessary to search for new probiotic strains from alternative sources.

Plants, encompassing a wide variety of species, offer a potential alternative source of probiotics [13]. Furthermore, given the increased demand for health and safety-oriented green foods, there is a strong need for new probiotics originating from natural environments to enhance the genetic diversity and robustness of probiotic bacteria [14]. Several studies have isolated bacteria from fresh or fermented fruits and vegetables, confirming their significant role in disease prevention [15–17]. *Burkholderia ambifaria*, a bacterium isolated from the rhizosphere of various plants including peas, sugarcane, and maize [18], demonstrates the ability to inhibit pathogenic fungi and bacteria by producing diverse antimicrobial substances such as burkholdines [19], cepacin [20], enacyloxins [21], and pyrrolnitrin [22]. In addition, as a diazotrophic microorganism, *B. ambifaria* can convert nitrogen from the air into ammonia or ammonium salts through nitrogenase [23], potentially increasing the pH of the surrounding environment. Moreover, a previous study revealed that *B. ambifaria* exhibits tolerance to low-pH challenges (pH 5.3), enabling it to colonize and survive in acidic environments [24]. Collectively, *B. ambifaria* may withstand the harsh conditions of the oral cavity and possess anticaries effects owing to its acidity and pH-buffering capacity.

Therefore, the aim of this study was to investigate the colonization of *B. ambifaria* in preformed saliva-derived microcosm biofilms from caries-free and caries-active donors and to evaluate its anticaries activity both *in vitro* and *in vivo* as a potential probiotic candidate against dental caries. The *in vitro* biofilm models were established under constant neutral pH and pH cycling conditions. The pH-cycling conditions comprised an 8-hour period at neutral pH followed by a 16-hour period at pH 5.5 [25], simulating the cariogenic conditions commonly encountered in dental plaque.

Materials and methods

Bacterial strains and growth conditions

Two strains were used in this study. *B. ambifaria* AFS098024 was purchased from the China General Microbiological Culture Collection Center (CGMCC, China), while *S. mutans* ATCC 25,175 was obtained from the Guangdong Provincial Key Laboratory of Stomatology. Both strains were preserved in 30% (vol/vol) glycerol broth at – 80°C and regularly cultivated in brain heart infusion (BHI) broth under anaerobic conditions (90%N_2, 5%CO_2, 5%H_2) at 37°C. The medium for biofilm formation comprised BHI broth supplemented with 0.2% sucrose (BHIS) at pH 7.0 or pH 5.5, which was adjusted by the addition of 100 mm acetic acid.

In vitro *safety assessment*

The hemolytic activity, D-lactic acid production, and antibiotic susceptibility of *B. ambifaria* were examined to evaluate its safety as a probiotic according to previous studies [26–28]. Hemolytic activity was assessed by streaking *B. ambifaria* on blood agar plates (HuanKai Microbial, China) for 24 h, observing under transmitted light, and determining the photochromic properties around the colonies. A clear zone surrounding the bacterial colonies represents non-hemolysis or gamma (γ) hemolysis. A clear yellow zone around the colonies indicates beta (β) hemolysis, while a greenish to brown zone surrounding the colonies signifies alpha (α) hemolysis [29,30]. *Porphyromonas gingivalis* ATCC 33,277, a β-hemolytic bacterium, was used as a positive control.

The measurement of D-lactate production was to determine whether *B. ambifaria* produced harmful levels of D-lactate. This arises from the fact that bacteria can produce either D-lactate or L-lactate during carbohydrate metabolism, depending on the environmental conditions they encounter, whereas the human body only possesses enzymes that can metabolize L-lactate [31]. Consuming excessive amounts of D-lactate may result in metabolic disturbances, intestinal discomfort, acidosis, and other adverse health effects [32]. Therefore, to measure the D-lactate production, *B. ambifaria* was grown in BHI broth anaerobically at 37°C for 24 h. The cell-free supernatant obtained by centrifugation (10,000 g for 10 min) was analyzed with the Amplite® Colorimetric D-lactate Assay Kit (AAT Bioquest, USA) following the manufacturer's protocol. Briefly, 50 µL of cell-free supernatant or a serial dilution of 1000 µM D-lactate solution was mixed with 50 µL of the D-lactate assay solution. After incubation at room temperature (20°C ~ 25°C) for 30 min, the absorbances at 575 nm and 605 nm ($A_{575\,nm}$ and $A_{605\,nm}$) were measured using a microplate spectrophotometer (BioTek, USA). Thereafter, the concentration of D-lactate in the bacterial supernatant was quantified according to a standard curve calculated from the ratio of $A_{575\,nm}/A_{605\,nm}$ and D-lactate dilution.

The antibiotic susceptibility of *B. ambifaria* was evaluated by determining the minimal inhibitory concentration (MICs) using a serial two-fold dilution method in BHI broth, according to the criteria of the European Food Safety Authority (EFSA) [33]. In detail, eight commonly used antibiotics (ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline) were individually dissolved in the BHI broth at

a concentration of 1024 μg/mL, then two-fold serially diluted into 96-well plates. The turbidity of bacterial suspensions of *B. ambifaria* was adjusted to 0.5 MacFarland units to ensure uniformity. One hundred microliters of each bacterial suspension were added into each well containing 100 μL of antibiotic solution in various concentrations and incubated for 24 h at 37°C. Thereafter, the MIC value was determined as the minimum concentration of antibiotics that completely inhibited the growth of *B. ambifaria* compared to the antibiotic-free control.

Saliva collection and sample processing

Saliva samples were obtained from healthy volunteers aged 18 to 24, devoid of systemic or oral diseases (i.e. gingivitis, periodontitis, or mucosal diseases, excluding caries) and who had not taken antibiotics for three months prior to collection. Volunteers with a history of orthodontic treatment, smoking, alcohol abuse, or any other habits affecting oral health were excluded. After examination by an experienced dentist, volunteers were divided into two groups based on the Decayed, Missing, and Filled Teeth (DMFT) index: the caries-active group ($n = 5$, DMFT ≥ 6 with at least three untreated carious teeth) and the caries-free group ($n = 2$, DMFT = 0). Participants were instructed to refrain from eating or drinking for at least 2 h or to maintain oral hygiene for at least 12 h before saliva collection. Five milliliters of unstimulated saliva were collected from each volunteer on ice, mixed with an equivalent volume of 60% (vol/vol) glycerol solution, and stored at – 80°C.

In vitro *biofilm model*

To prepare bacterial suspension for subsequent biofilm formation, a single colony of the *B. ambifaria* strain was inoculated into BHI medium for 24 h. Subsequently, it was adjusted to 5×10^7 colony-forming unit (CFU)/mL in fresh BHIS (pH 7.0) and dispensed into a 96-well plate at 200 μL/well. An equal volume of BHIS (pH 7.0) without the *B. ambifaria* suspension was used as the control group.

All biofilms were cultivated using an active attachment model employing the MBEC Assay® biofilm inoculator (Innovotech Inc., Canada), comprising a polystyrene lid with 96 pegs and a corresponding 96-well microtiter plate. Five individual saliva samples from the caries-active group or two individual saliva samples from the caries-free group were mixed, and the pooled sample was diluted 1:20 with BHIS at pH 7.0. Thereafter, 200 μL/well of the diluted sample was dispensed into the 96-well plate and incubated for 24 h. The pegs with 24-h preformed biofilms were initially rinsed with sterile distilled water, then transferred into the plate containing 200 μL/well of *B. ambifaria* suspensions (C-MBa as the caries-active group, and H-MBa as the caries-free group), or BHIS only (C-M and H-M as the controls of the caries-active group and caries-free group, respectively). Two different growth conditions were used in this study based on the pH of the culture medium. Half of the pegs with four groups of biofilms (C-MBa, H-MBa, C-M, and H-M) were inoculated under constantly neutral pH conditions (in BHIS at pH 7.0 for 48 h), whereas the other half were grown under pH-cycling conditions (in BHIS at pH 7.0 for 8 h, and in BHIS at pH 5.5 for 16 h, alternately). Thereafter, the 72-h biofilms formed on the pegs were rinsed with sterile distilled water and collected for biomass assays, lactic acid quantification, biofilm structure observation, and colonization assessment. A flowchart depicting biofilm formation and processing is shown in Figure 1. The pegs were washed with sterile distilled water to remove the unattached bacterial cells before the medium was refreshed. All experiments were repeated thrice, and four replicates were performed for each experiment under each test condition.

Biomass assay

A crystal violet staining assay was performed to examine biofilm biomass [34]. The pegs were inserted into 0.01% crystal violet solution (200 μL/well) for 5 min, and the excess stain was removed by washing twice with sterile water. Next, a 2% sodium deoxycholate solution was used to stain the crystal violet for 5 min. The absorbance of the decolorized solution was measured at 608 nm using a microplate spectrophotometer (BioTek, USA).

Lactic acid quantification

The pegs with biofilms were inserted into buffered peptone water (BPW; HuanKai Microbial, China) with 1% glucose to trigger lactic acid production at 37°C for 1 h. The lactic acid concentration produced by the biofilms was measured using a Lactic Acid Assay Kit (Nanjing Jiancheng Bioengineering Institute, China). In short, 20 μL of the supernatant was mixed with 1 mL of lactate dehydrogenase solution and 200 μL of chromogenic reagent. After incubation at 37°C for 30 min, 2 mL of stop solution was added to terminate the reaction. The absorbance of the mixture, which is proportional to lactic acid production, was measured at 530 nm using a microplate spectrophotometer (BioTek, Winooski, VT, USA). Finally, the concentrations of lactic acid were calculated using a standard curve from the serially diluted lactate solution.

DNA extraction and quantitative real-time PCR of the saliva-derived biofilms

To investigate the colonization of *B. ambifaria* in the saliva-derived microcosms, pegs with biofilms were

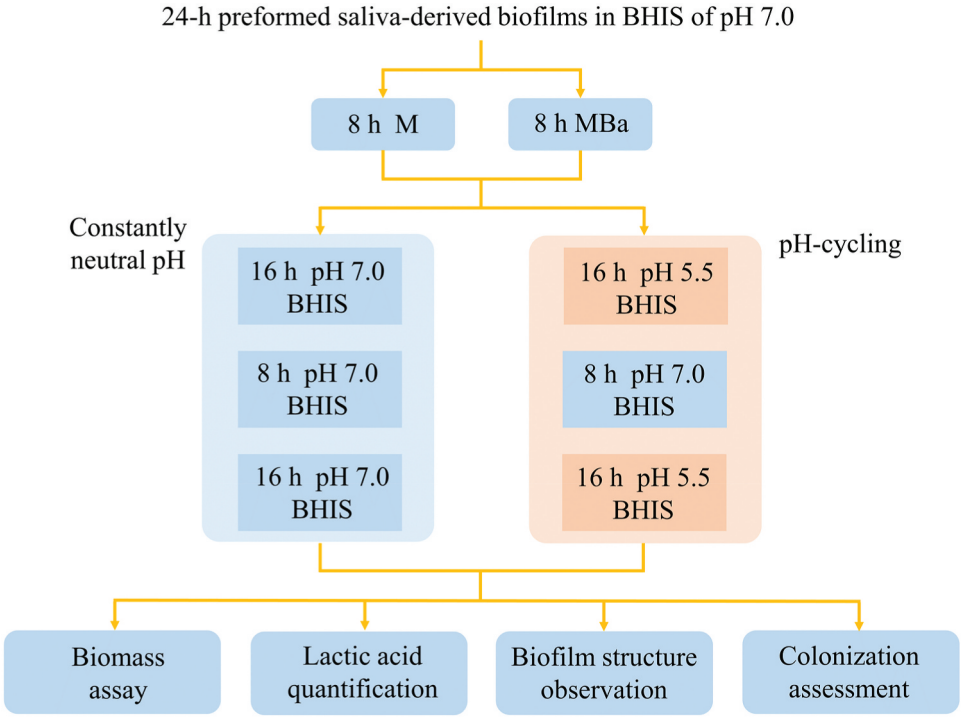

Figure 1. Flow chart of *in vitro* 72-h biofilm model establishment and processing. M: microcosm biofilms without *B. ambifaria*; MBa: microcosm biofilms with *B. ambifaria*; BHIS: brain heart infusion broth supplemented with 0.2% sucrose.

carefully excised using a sterile scalpel without disrupting the biofilms and transferred into 1 mL of phosphate buffer (NaH_2PO_4 0.05 M, Na_2HPO_4 0.05 M, pH 7.0). The biofilms formed on the pegs were dispersed by sonication on ice for 2 min at 1 s pulse at an amplitude of 40 W (Q700 Sonicator®, Qsonica, USA). Genomic DNA was extracted for qPCR analysis using a TIANamp Bacteria DNA Kit (TIANGEN Biotech Co., Ltd., Beijing, China). Amplification and quantification were performed using a Lightcycler 96 system (Roche, Basel, Switzerland). The following primers specific for *B. ambifaria* were used: forward, 5'-AACCCTTGTCCTTAGTTGCT-3'; reverse, 5'-TTGTATGACGTGTGAAGCCC-3' (BGI Genomics, China). The total volume of the qPCR mixture was 20 µL, containing 10 µL of TB Green Premix Ex Taq II (Takara, Japan), 0.8 µL of each forward and reverse primer, 2 µL of the template DNA, and 6.4 µL of nuclease-free water. The thermal cycling conditions for the qPCR assays were as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The genomic DNA extracted from a known concentration of *B. ambifaria* was used as the control to draw a standard curve for quantification as previously described [35,36]. In detail, 24-h *B. ambifaria* suspensions prepared as described above were serially 10-fold diluted. The genomic DNA from each diluted *B. ambifaria* suspension was extracted for the qPCR reaction. Phosphate buffer was used as a negative control. A standard curve was established based on the Cq values and the logarithmic CFU counts of related concentrations using LightCycler® 96 Software v.1.1.0.1320. Subsequently, the concentrations of *B. ambifaria* in the biofilms were calculated according to the standard curve.

Observation of biofilm structure

Scanning electron microscopy (SEM) was used to observe the effect of *B. ambifaria* on biofilm structure. The biofilms (C-MBa, H-MBa, C-M, and H-M) under the two pH conditions were cultivated on sterile slides with a diameter of 14 mm in a 24-well plate according to the procedure described above. After 72 h, the biofilms were fixed in 2.5% glutaraldehyde, dehydrated in increasing grades of ethanol from 30% to 100%, and dried by critical point-drying using a freeze drier (CHRIST, Germany). Finally, the samples were mounted on scanning electron micrograph stubs, sputter-coated with gold, and viewed under a Quanta 400F scanning electron microscope (FEI, USA).

In vivo *rat caries model*

A rat caries model was used to evaluate the efficacy of *B. ambifaria* in caries prevention and treatment. The experiments utilized 21-day-old male specific-pathogen-free Sprague Dawley rats (Laboratory Animal Center of Sun Yat-sen University, China), weighing 100 ± 10 g. Rats were randomly divided into five groups ($n = 5$): (1) the positive cariogenic group (Cario) infected with *S. mutans* for 5 days; (2) the low-frequency treatment

group (L), infected with *S. mutans* for 5 days followed by treatment with *B. ambifaria* for 5 days; (3) the high-frequency treatment group (H), infected with *S. mutans* for 5 days then treated daily with *B. ambifaria* until the rats were euthanized; (4) the prophylactic treatment group (Pro), treated with *B. ambifaria* for 5 days followed by *S. mutans* infection for 5 days; (5) the negative control group (Con), without infection or treatment. Control group rats were provided a normal diet and distilled water throughout the study. Other groups received a cariogenic diet 2000 (Trophic Animal Feed High-tech Co., Ltd., China) and water with 5% sucrose. Bacterial suspensions of *B. ambifaria* or *S. mutans* were prepared as described previously, with a final concentration of 5×10^7 CFU/mL. As showed in Supplementary figure S1, each rat molar was coated with 200 µL of the bacterial suspensions mentioned above using oral swabs with a diameter of 1.5 millimeter for 15 s per quadrant [37]. Dental plaque samples were collected weekly by scraping each molar surface with sterile cotton sticks and stored in 1 mL of phosphate buffer (NaH_2PO_4 0.05 M, Na_2HPO_4 0.05 M, pH 7.0) at −80°C. Rats were weighed weekly. Six weeks after the initial infection, the animals were euthanized, and the maxillae and mandibles were aseptically removed and halved. The left half was utilized for caries scoring, and the right half was used for micro-CT images. The flow chart of rat caries model establishment and processing were shown in Supplementary figure S2.

DNA extraction and quantitative real-time PCR of rat molar dental plaque

Genomic DNA was also extracted from dental plaque samples collected from rat molars for qPCR analysis using the aforementioned method. The amounts of *B. ambifaria* and *S. mutans* were quantified in dental plaque samples via qPCR. The primers used for *B. ambifaria* had been described previously. The following primers for *S. mutans* were used: forward, 5'-GCCTACAGCTCAGAGATGCTATTCT-3'; reverse, 5'-GCCATACACCACTCATGAATTGA-3' (BGI Genomics, China).

Caries scoring

After removing the soft tissues from the teeth and jaws, the left maxillae and mandibles of each rat were stained with 0.4% murexide solution for 12 h, then rinsed with distilled water. The maxillary and mandibular molars were hemi-sectioned in the mesiodistal direction using an ultrathin carborundum disk (0.15 mm in thickness). Molar caries was evaluated and scored according to Keyes' method using a stereoscopic microscope [38]. Caries scoring was evaluated by two expert examiners who conducted blind scoring as the jaws were mixed and randomly assigned to the examiners.

Micro-ct analysis

The right maxillae and mandibles underwent scanning using the Scanco Medical µCT-50 system (Scanco Medical AG, Switzerland) at an operating voltage of 70 kV, a tube current of 200 µA, and a field of 5 µm. Subsequently, all images were imported into Mimics Research software (version 21.0) for the reconstruction of three-dimensional photographs of the maxillae and mandibles. Enamel could be discriminated from dentine using a fixed density threshold of 6,700 hounsfield units, and subsequently, the volume and density of the enamel were calculated to assess the severity of caries.

Statistical analysis

For statistical analysis, SPSS Statistics 25.0 (IBM Corp., USA) was employed. Data conforming to normal distribution and homogeneity of variance are presented as mean ± standard deviation (SD), and differences among groups were evaluated using one-way analysis of variance (ANOVA), followed by the Bonferroni test for multiple comparisons. Conversely, data unfit for the assumptions of normality or homogeneity of variance were analyzed using the nonparametric Kruskal-Wallis test. During the animal experimentation, a two-way ANOVA analysis was conducted to assess both the quantity of *B. ambifaria* in the rat molar plaques of the Pro, L, and H groups, and the quantity of *S. mutans* in the rat molar plaques of the Cario, Pro, L, and H groups. The independent variables utilized in this analysis were days and groups, and was used for multiple comparisons in groups using Bonferroni test. Statistical significance was set at $p < 0.05$.

Results

Safety of B. ambifaria *on the host and the susceptibility to antibiotics*

Figure 2 demonstrates the absence of hemolytic activity surrounding colonies of *B. ambifaria* AFS098024 on blood agar, contrasting with the colorless halos observed around colonies of *P. gingivalis*. Moreover, the concentration of D-lactate generated by *B. ambifaria* was measured at 52.14 ± 1.41 µM (data not shown), significantly lower than the normal plasma concentration (0.01 ~ 0.25 mm) and the threshold causing acidosis (>3 mm) [39]. Antibiotic susceptibility testing revealed that *B. ambifaria* was susceptible to kanamycin, gentamicin, chloramphenicol, erythromycin, and tetracycline, in accordance with EFSA's recommended cut-off values (Table 1). Conversely, it exhibited resistance to ampicillin, clindamycin, and streptomycin.

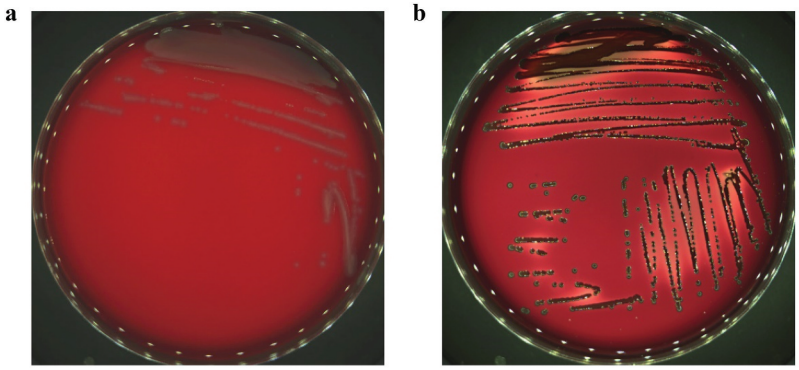

Figure 2.The hemolytic activity of *B. ambifaria* (a) and *P. gingivalis* (b) as a positive control. The two strains were streaked on blood agar plates for 24 h and observed with transmitted light.

Inhibition of *B. ambifaria* on biofilm formation and lactate production *in vitro*

For caries-active donors, the biomass formation of biofilms treated with *B. ambifaria* was dramatically reduced compared to those without *B. ambifaria* treatment under both neutral pH and pH cycling conditions ($p < 0.001$), as presented in Figure 3a,b. Similarly, lactic acid production in biofilms incubated with *B. ambifaria* was significantly lower than those without *B. ambifaria* under neutral pH conditions ($p < 0.001$), whereas there was no significant reduction in lactate in biofilms with *B. ambifaria* under pH-cycling conditions (Figure 3c,d). In caries-free donors, both biomass and lactic acid concentrations in biofilms were significantly lower than those in caries-active donors, regardless of the culture conditions, and were not significantly affected by the addition of *B. ambifaria* (Figure 3a–d).

Moreover, the integration of *B. ambifaria* with saliva-derived biofilms *in vitro* was determined by quantifying the number of *B. ambifaria* using logarithmic CFU counts through qPCR analysis. As shown in Figure 3e,f, *B. ambifaria* was detected in biofilms supplemented with *B. ambifaria*, with counts ranging from 2.40 $\log_{10}$ CFU/mL to 2.76 $\log_{10}$ CFU/mL.

Furthermore, SEM images confirmed the formation of dense biofilms in all saliva-derived microcosms (Figure 4). Interestingly, biofilms from caries-active donors formed network-like structures under the two pH conditions, which were disrupted by the addition of *B. ambifaria*. For biofilms from caries-free donors, there was no significant change in structure with or without *B. ambifaria*, consistent with the results of the biomass assay.

Table 1. Minimum inhibitory concentration (MIC) evaluation of antibiotic susceptibility in *B. ambifaria*.

Antibiotics	cut-off value (mg/L)	MIC (mg/L)
Ampicillin	2	>512 (Resistant)
Clindamycin	4	64 (Resistant)
Kanamycin	1024	32 (Susceptible)
Gentamicin	32	16 (Susceptible)
Chloramphenicol	16	16 (Susceptible)
Erythromycin	4	4 (Susceptible)
Streptomycin	128	>512 (Resistant)
Tetracycline	4	1 (Susceptible)

Anti-caries effect of *B. ambifaria in vivo*

During the animal experimentation, all the rats exhibited a favorable health status, and no significant difference in weight gain was observed among the various groups (Supplementary figure S3). The sulcal carious lesions of each molar were evaluated and scored on four levels based on the Keyes method. Notable carious lesions were observed in the molars of the positive cariogenic group Cario, whereas either prophylactic treatment or treatment with *B. ambifaria* at low and high frequencies remarkably reduced the severity of the carious lesions, as shown in Figure 5. At the level of slight dentinal caries (D_s), a significant reduction in the caries score was observed in the high-frequency treatment group H and the prophylactic treatment group Pro compared to that in Group Cario ($p < 0.05$). At the levels of moderate dentinal caries (D_m) and extensive dentinal caries (D_x), the scores of the prophylactic treatment, the low- and high-frequency treatment groups (Pro, L, and H, respectively) were significantly lower than those in Group Cario ($p < 0.05$), whereas at the level of enamel caries (E), there was no statistical difference between the positive cariogenic group and the groups pretreated or treated with *B. ambifaria*. In addition, the scores at the four levels were similar between the prophylactic treatment and treatment groups with *B. ambifaria*.

To improve the detectability of carious lesions on rat molars, three-dimensional reconstructions of the right mandibular molars were performed using micro-CT. The enamel was stripped from the dentine based on the Hounsfield units for individual analysis, and the corresponding sagittal slice of the same molar was also taken for observation

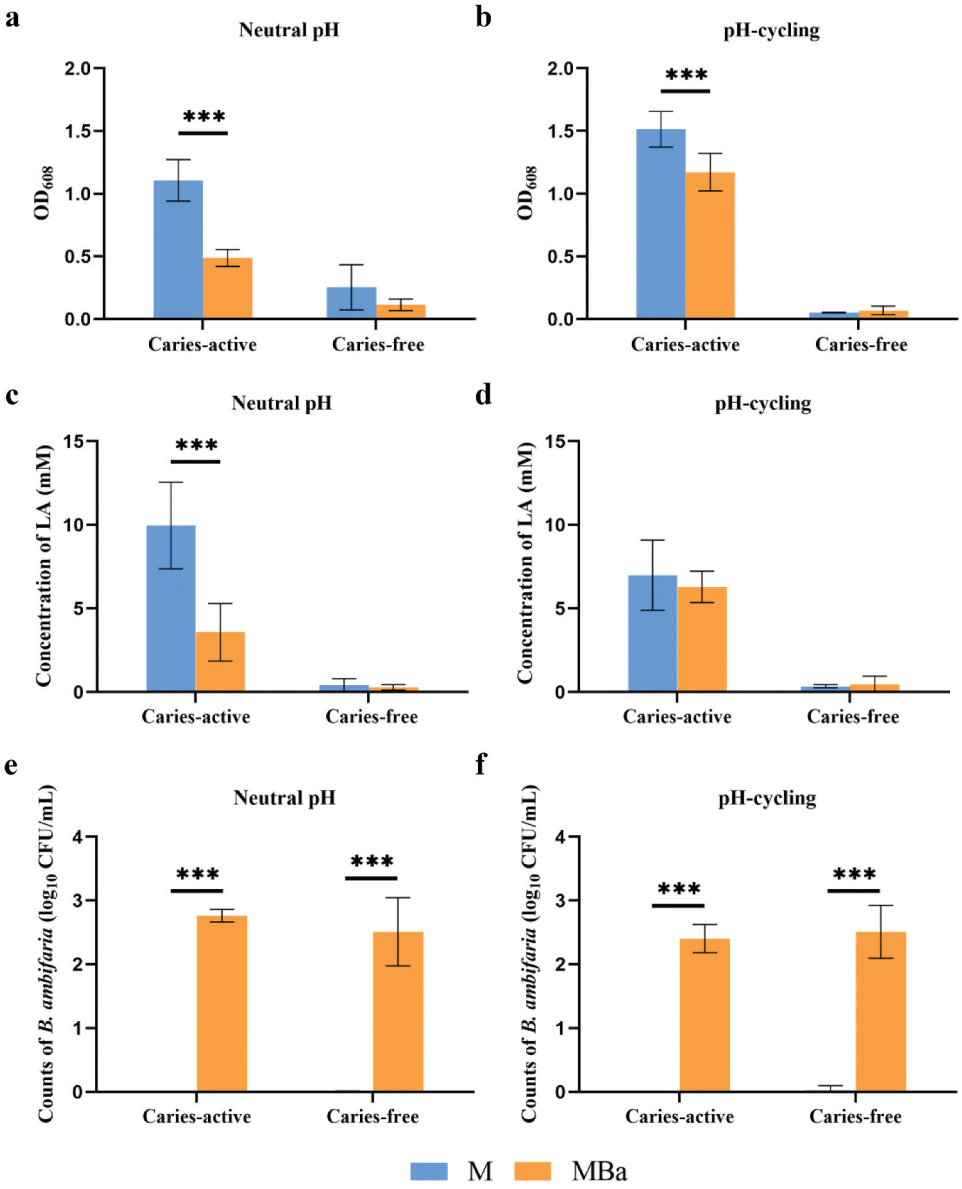

Figure 3. Biomass and lactic acid quantification, and amounts of *B. ambifaria* in 72-h biofilms with or without *B. ambifaria* from the caries-active or caries-free donors under two pH conditions. (a) Biomass formation under constantly neutral pH conditions. (b) Biomass formation under pH-cycling conditions. (c) Lactic acid production under constantly neutral pH conditions. (d) Lactic acid production under pH-cycling conditions. (e) The amount of *B. ambifaria* in biofilms under constantly neutral pH conditions. (f) The amount of *B. ambifaria* in biofilms under pH-cycling conditions. LA: lactic acid. ***$p < 0.001$.

(Figure 6a). The green area highlighted in the left and middle panels of Figure 6a represented the enamel, which was not continuous when caries occurred. Morphometric volume analysis revealed that the molar enamel volumes in the groups pretreated with *B. ambifaria* and treated with *B. ambifaria* at a high frequency were significantly higher than those in the positive cariogenic group ($p < 0.05$, Figure 6b), whereas there was no statistical difference between the positive group and the low-frequency treatment group ($p > 0.05$, Figure 6b). For enamel density, significant differences were detected only between the positive cariogenic group and the negative control group ($p < 0.05$, Figure 6c).

Colonization of *B. ambifaria* and *S. mutans* *in vivo*

The colonization of *B. ambifaria* and *S. mutans* on rat molars *in vivo* was confirmed via qPCR analysis. As presented in Figure 7a, the number of *B. ambifaria* colonizing the dental plaque in the prophylactic treatment group stabilized at around 10^2 CFU/mL until the end of the experiment, after the initial 5-day infection, whereas the counts of *B. ambifaria* in the low- and high-frequency treatment groups exhibited a higher level at the first week after infection, subsequently leveling off to approximately 10^2 CFU/mL. On the other hand, the number of *S. mutans* in the groups pretreated or treated with *B. ambifaria* was

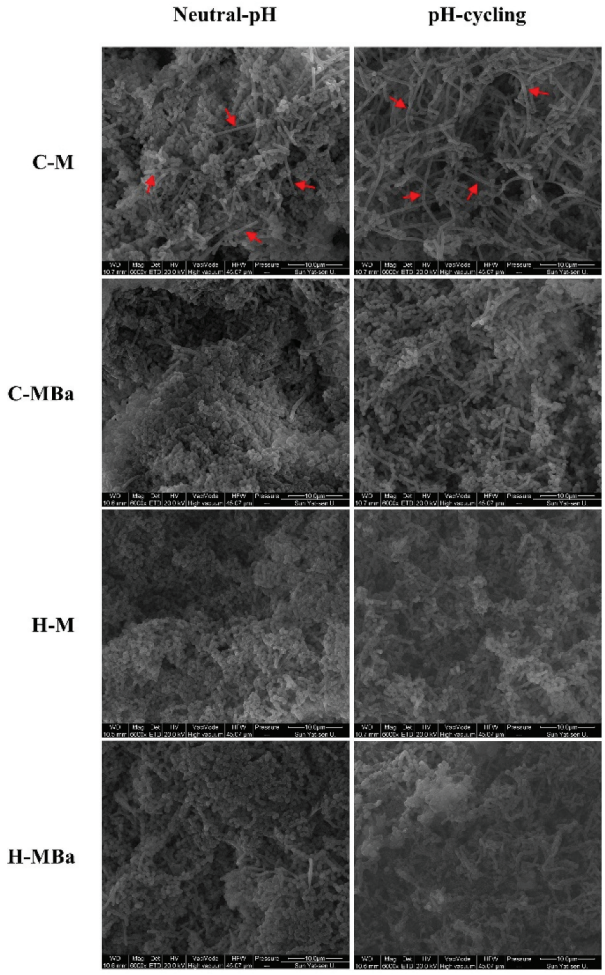

Figure 4.SEM analysis of 72-h biofilms grown under constantly neutral pH conditions and pH-cycling conditions. Images were taken at 6000× magnification. Red arrows point to the network-like structures. C-M: microcosm biofilms from caries-active donors without *B. ambifaria*; C-MBa: microcosm biofilms from caries-active donors with *B. ambifaria*; H-M: microcosm biofilms from caries-free donors without *B. ambifaria*; H-MBa: microcosm biofilms from caries-free donors with *B. ambifaria*.

significantly lower than that in the positive cariogenic group throughout the experiment ($p < 0.05$, Figure 7b).

Discussion

Host safety and non-carcinogenicity are prerequisites for a probiotic strain used in dental caries management. In this study, *B. ambifaria* sourced from the rhizosphere of plants underwent safety assessments as a novel probiotic species against caries. The data showed that *B. ambifaria* exhibited no hemolysis and generated a negligible amount of D-lactate, suggesting its non-toxicity to the human body and non-cariogenic nature due to its low acidogenicity. Antibiotic sensitivity is another essential requirement for probiotic candidates devoid of transferable resistance genes. In this study, we tested the susceptibility of *B. ambifaria* AFS098024 to routine antibiotics and found it to be susceptible to most antibiotics. Although it showed resistance to certain antibiotics, such as ampicillin of the β-lactam group and streptomycin of the aminoglycoside group, The occurrence of horizontal transfer is difficult to achieve because resistance to these antibiotics was intrinsically encoded by chromosomes [40–42]. Previous studies have found a Class A β-lactamase encoded by *penA* located on chromosome 2 is responsible for primary resistance to β-lactam antibiotics in *Burkholderia* species [43]. Furthermore, the efflux pump AmrAB-OprA of the resistance nodulation cell division (RND) family, which is expressed in most *Burkholderia* strains, is responsible for intrinsic resistance to aminoglycosides [44]. Those studies indicated that the resistance to ampicillin and streptomycin was inherent in *B. ambifaria*, and it was difficult to occur due to horizontal transfer. Therefore, the *B. ambifaria* strain used in this study met the prerequisites for serving as a probiotic for dental caries.

At present, numerous *in vitro* studies have introduced novel probiotics for caries management by evaluating their inhibition of caries-associated bacteria or biofilms consisting of one or a few cariogenic species [45–48]; however, few studies have focused on the oral microbiome [49]. In this study, we established an *in vitro* microcosm biofilm model using saliva from caries-active and caries-free donors and found that the integration of *B. ambifaria* reduced biofilm formation and lactate production in the saliva-derived microcosms of caries-active donors under constantly neutral pH conditions. However, under pH-cycling conditions, *B. ambifaria* did not inhibit lactate generation, likely due to the acid tolerance response of the biofilm after prolonged exposure to low pH [50]. Previous reports have indicated that bacterial cells in biofilms better survive acid exposure after pretreatment in a low but non-lethal pH environment [50,51]. Similarly, our previous study observed a delayed reduction in lactate in streptococci biofilms under pH-cycling conditions compared to constantly neutral pH conditions [34]. Thus, a neutral environment seems to favor the probiotic effects of *B. ambifaria*. Moreover, our results showed that saliva-derived biofilms from caries-free donors produced extremely low amounts of biomass and lactate regardless of pH conditions, possibly explaining why *B. ambifaria* had no clear inhibitory effect on these biofilms. Overall, our findings suggest that *B. ambifaria* may prevent the occurrence and progression of dental caries by reducing biomass and acid generation in microcosms of caries-active subjects.

Another significant finding in our study was that *B. ambifaria* was capable of overcoming colonization resistance and establishing itself in preformed microcosm biofilms. Colonization of the oral cavity

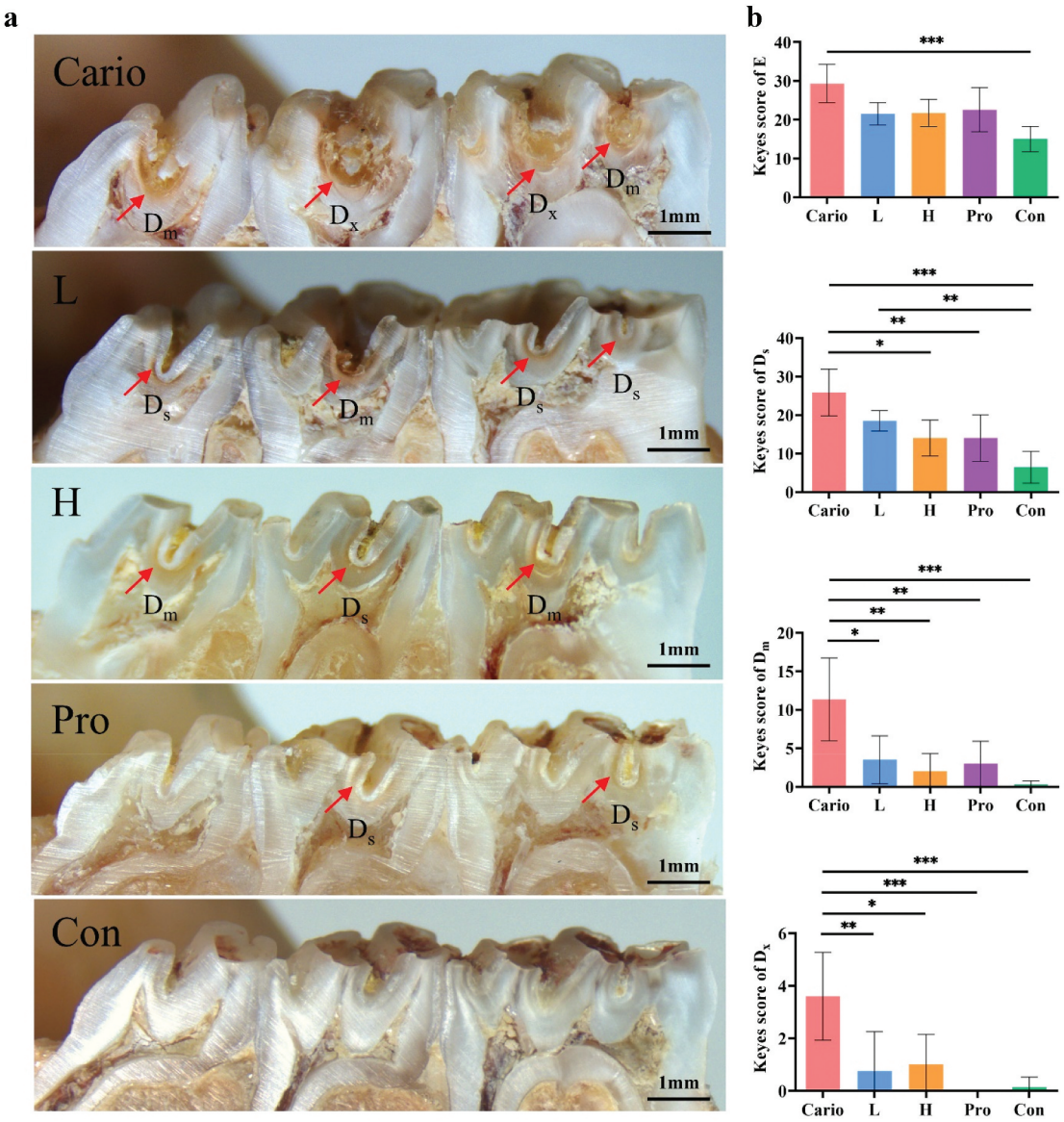

Figure 5. Anticaries effect of *B. ambifaria* on rat molars *in vivo* as evaluated by caries scoring of Keyes' method. (a) Representative images of hemisectioned molars from each group under stereoscopic microscopy. Red arrows indicate the carious lesions that have been dyed orange. (b) Caries scores for the four lesion levels in rat molars across the five groups. E: enamel caries; D_s: slight dentinal caries with involvement of approximately one-fourth of the dentin; D_m: moderate dentinal caries with dentin involvement between one-fourth and three-fourths; D_x: extensive dentinal caries with dentin involvement beyond three-fourths. Cario: the positive cariogenic group; L: the low-frequency treatment group; H: the high-frequency treatment group; pro: the prophylactic treatment group; con: the negative control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

is vital for prevention and treatment of oral diseases [52]. An existing microbial community in the oral cavity or intestines can impose selective pressure to prevent the colonization or integration of exogenous bacteria to maintain community stability, which is called colonization resistance [53,54]. An early *in vitro* study demonstrated that the intestinal probiotic *Lactobacillus salivarius* failed to integrate with the microbial community of 24-h or 48-h preformed saliva microcosm biofilms [55]. Other probiotic bacteria from the gut, such as *Lactobacillus casei* and *Lactobacillus rhamnosus* GG, are unable to incorporate or persist in dental biofilms or saliva [56]. However, despite originating from plants, *B. ambifaria* succeeded in colonizing the preformed saliva-derived microcosm in our study, making it a probiotic candidate against dental caries. Bacteria can outcompete indigenous microbes by producing inhibitory compounds or engaging in nutritional competition and modifying the niche to counteract colonization resistance [54]. Hence, the potential mechanism by which *B. ambifaria* integrates into the human salivary microcosm may involve microbiome modulation or niche construction, which should be explored in future studies.

A rat caries model was established to verify the inhibitory effects of *B. ambifaria*. The results illustrate that both prophylactic treatment and treatment

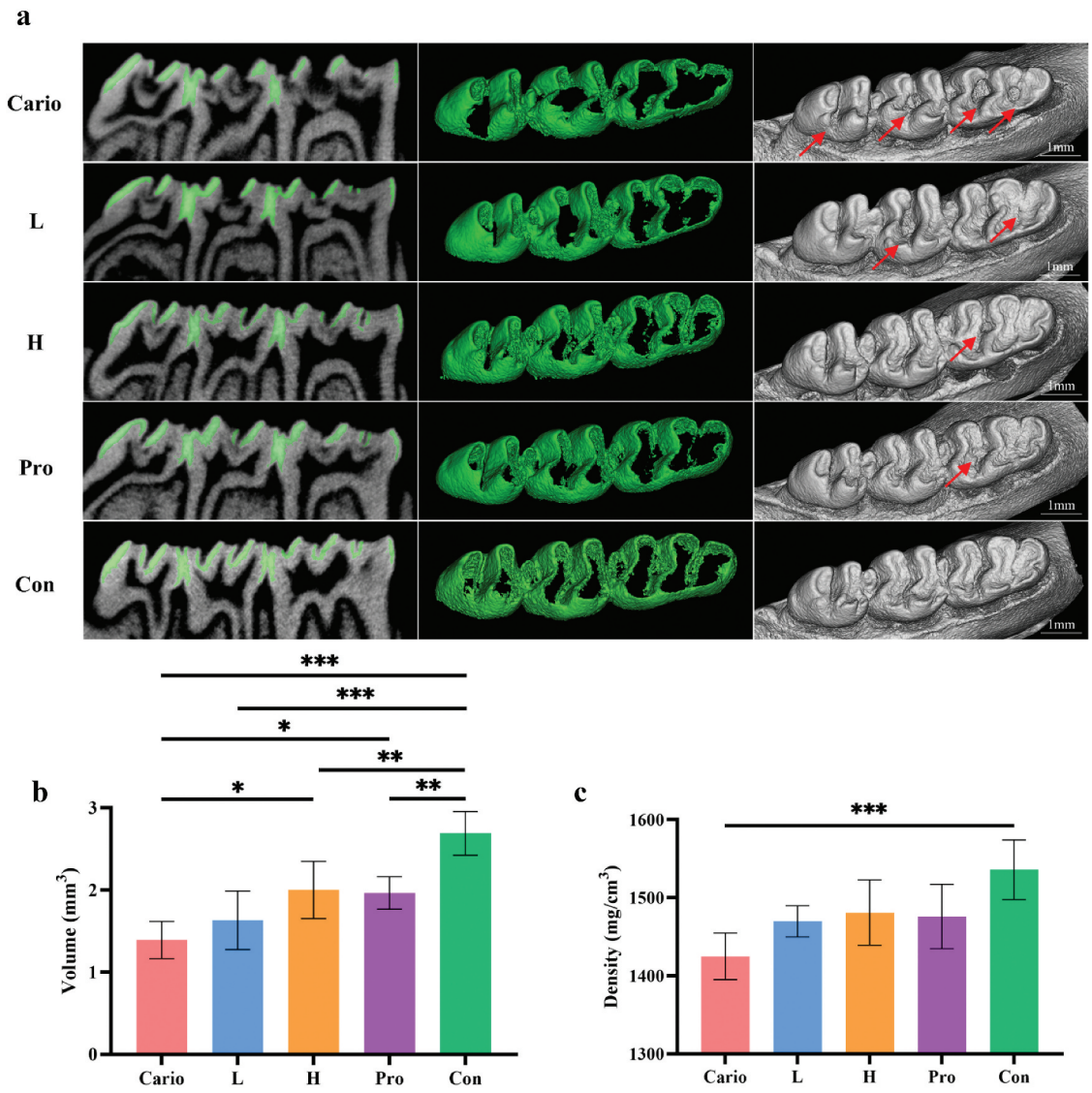

Figure 6.Micro-ct analysis for the inhibition of *B. ambifaria* on the enamel lesion of rat molars *in vivo*. (a) Representative images of sagittal slices, divided enamel (green), and corresponding 3D images of the right mandibular molars in the five groups. Red arrows indicate the caries lesion site. Cario: the positive cariogenic group; L: the low-frequency treatment group; H: the high-frequency treatment group; pro: the prophylactic treatment group; con: the negative control group. (b) The volume of molar enamel. (c) The density of molar enamel. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

with *B. ambifaria* alleviated carious lesions. Likewise, *B. ambifaria* was shown to decrease the demineralization volume of enamel and still colonize the rat molars 6 weeks after inoculation, implying that *B. ambifaria* could exert long-term inhibition of caries in the rat oral cavity. Another study [57] measured the dental colonization of 12 probiotic *Streptococcus* strains (e.g. *S. sanguinis* BCC23, *S. mitis* BCC45, *S. cristatus* BCA6) in a mouse model; however, the experimental period was only 20 days, which was much shorter than that in this study. In addition, previous studies have shown that the sequence of inoculation between probiotics and their counterparts influences the probiotic effect. For instance, pretreatment with the probiotic strain *L. plantarum* CCFM8724 exhibited a better effect on mitigating the severity of sulcal caries in rats, compared to treatment with it after exposure to cariogenic *Streptococcus mutans* and *Candida albicans* [58]. Similarly, early inoculation of health-associated *S. sanguinis* on enamel followed by *S. mutans* displayed reduced cariogenicity compared to the opposite sequence [59]. Nevertheless, our data showed no clear difference in the effectiveness between the prophylactic treatment and treatment groups with *B. ambifaria*, indicating that its probiotic effect was not affected by the sequence of inoculation. Moreover, our results uncovered that the number of *S. mutans* in the groups pretreated or treated with *B. ambifaria* was significantly lower than that in the positive cariogenic group without *B. ambifaria* treatment. This suggests that *B. ambifaria* exerts the anticaries effect in rat molars by inhibiting the growth of *S. mutans*. Unexpectedly, despite the high-frequency treatment group receiving daily inoculations of *B. ambifaria*, the detected level of bacteria colonizing

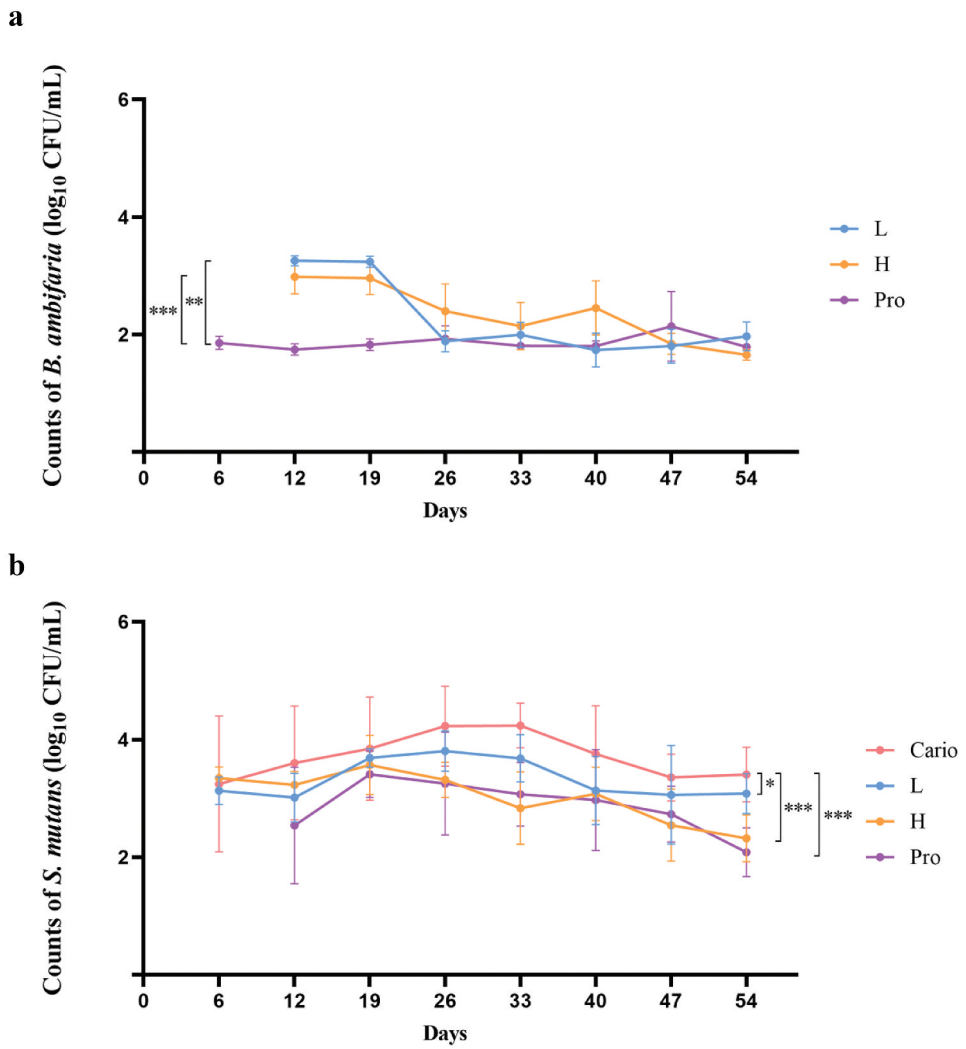

Figure 7. Colonization of *B. ambifaria* and *S. mutans in vivo* rat molars by qPCR analysis. (a) The amount of *B. ambifaria* in the dental plaques of rat molars pretreated or treated with *B. ambifaria*. (b) the amount of *S. mutans* in the dental plaques of rat molars pretreated or treated with *B. ambifaria*. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

rat molars did not stay significantly higher than that of the low-frequency treatment or prophylactic treatment groups. This finding implies that the colonization level of *B. ambifaria* may be limited and does not increase due to daily inoculations.

A limitation of this study is the lack of investigation into the specific mechanisms of *B. ambifaria* in combating caries. As mentioned above, *B. ambifaria* can suppress pathogens through the production of antimicrobial compounds and buffer pH by nitrogen conversion, the role of which needs to be clarified in its anticaries activity. Additionally, for *B. ambifaria* to become a probiotic candidate, it must modify the microbiota to reverse dysbiosis of the microbial community or maintain homeostasis [60,61]. From the SEM images in our study, the network-like structures in the biofilms of caries-active donors were disrupted by the integration of *B. ambifaria*, leading to a decrease in biomass, as observed in the crystal violet assay. These network-like architectures often act as scaffolds to promote biofilm formation [62]. Thus, future research may focus on investigating the molecular mechanisms by which *B. ambifaria* suppresses cariogenic biofilms and exploring its interactions with other oral microorganisms.

Conclusions

Overall, our data confirm the safety, non-carcinogenicity, and colonization of the *B. ambifaria* strain AFS098024 isolated from plants. It indicates that its administration both *in vitro* and *in vivo* serves an anti-caries function. *B. ambifaria* integrated into human saliva-derived microcosms *in vitro* and suppressed biomass and lactic acid accumulation in biofilms from caries-active subjects, simultaneously affecting their biofilm structure. In addition, it continued to colonize rat molars *in vivo* for a long period and attenuated the severity of molar caries. Therefore, *B. ambifaria* strain AFS098024 emerges as

a promising probiotic strain for the prevention or treatment of dental caries.

Disclosure statement

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

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Author contributions

YC and XW: Conceptualization and Project administration. ZC, YL and YC: Methodology. ZC, ZX and LW: Investigation, Data curation and Writing – original draft preparation. YC, XW and YL: Writing – review & editing and Funding acquisition.

Ethical approval

All research on human were conducted in accordance with the principles stated in the Declaration of Helsinki, and were approved by the Ethics Committee of the College of Life Sciences at Sun Yat-sen University (No. KQEC-2024-16-01). Informed consent was obtained from all volunteers. All procedures of animal experiment were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University (No. SYSU-IACUC-2024-001337).

Key messages

- The *B. ambifaria* strain AFS098024 isolated from plants is safe as a probiotic candidate to inhibit dental caries.
- This *B. ambifaria* strain is capable of integrating into the human saliva-derived biofilms and depressing the cariogenicity of biofilms from caries-active individuals.
- The *B. ambifaria* strain can also colonize the rat molars and reduce the severity of molar caries.

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