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Levilactobacillus brevis CD2 as a multifaceted probiotic to preserve oral health: results of a double-blind, randomized, placebo-controlled trial in healthy adults

Serena Altamura[1,2,3†], Francesca Lombardi[1†], Francesca Rosaria Augello[1], Antonella Barone[1,3], Mario Giannoni[1,3], Benedetta Cinque[1] and Davide Pietropaoli[1,3*]

Abstract

Background A growing number of in vitro and in vivo studies suggest the application of probiotics as a natural approach to maintaining oral health. This double-blind, randomized controlled trial aimed to evaluate the efficacy of *Levilactobacillus brevis* CD2 (CNCM I-5566), a multifunctional probiotic frequently used in oral medicine, in preserving or improving several recognized oral health indicators.

Methods Thirty consenting healthy adults were randomized to receive four lozenges per day of *L. brevis* CD2 probiotic ($n=15$) or placebo ($n=15$) over four weeks. Clinical parameters (full-mouth bleeding on probing (BoP) and plaque index (PI) scores) were recorded. Unstimulated saliva was collected to measure salivation rate, pH, and buffer capacity. Salivary biomarkers were analyzed, including glucose, D-lactate, and secretory immunoglobulins A (sIgA). Clinical and salivary parameters were assessed at baseline, after four weeks of intervention, and two weeks post-intervention. Wilcoxon rank-sum test and robust regression analysis were used for statistical comparisons. The possible mediating effect of PI on BoP changes was assessed.

Results After four weeks, the probiotic group showed significant improvements in BoP and PI compared to baseline and placebo. The probiotic group had a higher salivation rate than baseline and placebo after four weeks of treatment and washout. While changes in salivary pH were not significant, buffering capacity increased in the probiotic group after four weeks of treatment and washout. Salivary glucose and D-lactate levels were lower in the probiotic group post-treatment and after washout. sIgA values increased and remained stable after washout in the probiotic group. No adverse effects were reported.

Conclusions The treatment with *L. brevis* CD2 significantly improved clinical and salivary parameters, supporting its efficacy as a probiotic for oral health.

†Serena Altamura and Francesca Lombardi contributed equally to this work.

*Correspondence:
Davide Pietropaoli
davide.pietropaoli@univaq.it

Full list of author information is available at the end of the article

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Keywords Probiotics, *Levilactobacillus brevis*, Oral health, Randomized clinical trial, Clinical salivary parameters, Salivary biomarkers

Background

The oral microbiome, a crucial element in maintaining oral health, is a delicate dynamic balance between mutualistic and opportunistic microorganisms. These microorganisms thrive in the oral cavity's microenvironment through a symbiotic relationship with the host [1]. This complex ecosystem is second only to the gut microbiome in terms of abundance and diversity, including bacteria, fungi, viruses, archaea, and protozoa [2]. It is well established that these commensal microorganisms contribute to host health by resisting pathogens, maintaining homeostasis, and modulating the immune system [1]. However, multiple factors affect the oral microbiome, including aging, genetic predisposition, an unhealthy diet, poor oral hygiene, certain medications (i.e., antibiotics), smoking, and systemic and immune conditions [3–7]. When the balance of the oral microbiome is disturbed, it can lead to an overgrowth of potentially harmful organisms, which may contribute to the development of several oral diseases, such as dental caries, periodontitis, and endodontic infections [8]. Saliva plays a fundamental role in maintaining oral homeostasis [9]. This complex biofluid has multiple functions, including aiding in digestion, lubricating oral tissues, neutralizing acids, and removing unwanted substances. When saliva loses its normal characteristics, it can negatively impact oral health and contribute to the development and progression of dental caries [10, 11].

One of the emerging strategies to preserve oral health and prevent dental caries, among other oral diseases, assumes that selected probiotics can restore equilibrium in the oral microbial ecosystem [12]. According to the United Nations Food and Agricultural Organization and the World Health Organization definition, probiotics are living microorganisms that provide health benefits to the host when administered in adequate amounts [13]. The human health benefits associated with the intake of probiotics are mainly attributed to their ability to modulate and rebalance the gut microbiome, particularly in the presence of dysbiosis, antagonize pathogens, favorably modulate the host's immune response, and strengthen the intestinal barrier [14–17]. Over the past two decades, the scientific community's interest in applying probiotics in oral medicine has surged exponentially, underscoring the great importance of this field [18]. So, an increasing number of studies revealed the beneficial effects of probiotics in various oral conditions, including dental caries, periodontal and peri-implant diseases, halitosis, oral candidiasis, and oral mucositis induced by chemoradiotherapy [19–21]. Probiotics can help prevent and treat oral disorders through mechanisms similar to those in the gastrointestinal tract. These mechanisms include the release of active metabolites, such as short-chain fatty acids and antimicrobial peptides, which strengthen the mucosal barrier, inhibit pathogenic biofilms, and compete with pathogens for adhesion and colonization. Additionally, probiotics can coaggregate with pathogens, exert immunomodulatory and anti-inflammatory effects, and improve the overall microecology of the oral cavity [22]. Some probiotics may also reduce the risk of tooth decay by increasing salivary flow, thereby reducing the time potential pathogens remain in contact with the tooth surface [18]. Additionally, certain probiotics have been shown to raise salivary levels of secretory immunoglobulins A (sIgA) [23–25], an important component of mucosal immunity. sIgA play a pivotal role in maintaining oral health by counteracting bacterial adhesion, protecting the host from antigen absorption through mucosal surfaces, reducing inflammation, enhancing phagocytosis, and neutralizing microbial toxins and invasive pathogens [26–28].

Probiotics more commonly used in oral medicine are from the *Lactobacillus* and *Bifidobacteria* genera. In particular, *Lactobacillus brevis* CD2, recently reclassified as *Levilactobacillus brevis* CD2 (*CNCM I-5566*) [29], is a multifunctional probiotic that has been assessed in various oral conditions, i.e., caries [30, 31], gingivitis and periodontitis [32–36], oral mucositis [37, 38], Behçet's disease, and aphthous oral ulcers [39, 40]. *L. brevis* CD2, other than competing with oral pathogens, has been reported to impact the host inflammatory response by inhibiting the activity of the nitric oxide synthase 2 (NOS2) pathway, thus reducing the synthesis of nitric oxide (NO) [31, 32], a known potent inflammatory agent. The beneficial effects of oral intake of *L. brevis* CD2 are largely attributed to its high level of arginine deiminase (ADI) [31, 32, 41, 42], the enzyme that converts arginine to citrulline and ammonia (NH_3). In an aqueous solution, NH_3 forms ammonium ions (NH_4^+), which are crucial to maintaining the physiological salivary pH, thus preventing pH acidification and enamel decalcification [43–46]. Moreover, by metabolizing arginine, due to its higher chemical affinity, ADI prevents the use of arginine by both arginase and NOS2, thus inhibiting, respectively, the synthesis of polyamines (i.e., putrescine and spermidine) involved in halitosis and tumor cell proliferation

and NO that is also associated with periodontal inflammation [32, 42]. Our group recently published findings from an experimental study using a pH cycling model that simulates mineral loss. The results show that *L. brevis* CD2 can effectively protect the dental enamel surface from damage caused by a demineralizing agent. Additionally, it enhances the enamel's resistance to demineralization, demonstrating its potential to prevent or counteract carious lesions by safeguarding the tooth surface during chemical challenges that imitate the caries process [47]. However, despite the numerous and growing studies and trials supporting the efficacy of *L. brevis* CD2 in oral medicine, there is a current need for robust evidence concerning the impact of this probiotic on oral health in terms of prevention.

Therefore, the present study aimed to verify the impact of the probiotic *L. brevis* CD2 treatment (four lozenges daily for four weeks) on clinical parameters (full-mouth bleeding on probing (BoP) and plaque index (PI)), which were considered as primary outcomes. Moreover, co-primary outcomes such as salivation rate, pH, and buffering capacity of unstimulated saliva samples, as well as salivary levels of glucose, D-lactate, and sIgA, were also evaluated in both probiotic and placebo groups. All clinical and salivary parameters were analyzed at baseline, after four weeks of treatment, and again after a two-week washout.

Methods

Study design and ethical approval

This double-blind, randomized, controlled trial (RCT) was conducted from November 2022 to November 2023 to determine whether a four-week intervention with the probiotic *Levilactobacillus brevis* CD2 (CNCM I-5566) was effective in reducing the full-mouth BoP score in a population of otherwise healthy adults with at least localized gingivitis (BoP ≥ 10%). The full-mouth BoP score served as the primary outcome of the study, while the full-mouth PI score and salivary indices (including salivation rate, pH, buffer capacity, salivary glucose, D-lactate, and sIgA) served as co-primary outcomes.

The study was approved by the Internal Review Board (Protocol n. 48/2022, 22.11.2022) of the University of L'Aquila (Italy) and registered on the clinicaltrials.gov registry (NCT06457724). This research received no external funding, and the trial was designed following the ethical principles of Helsinki's Declaration for Human Clinical Studies [48] and the CONSORT 2010 guidelines for reporting randomized clinical trials [49]. General visit descriptions and study schedules are reported in Table 1.

Questionnaire and sampling criteria

Subjects visiting the Dental Clinic of the University of L' Aquila were invited to participate in the study. They were informed about the study's aim, procedures, and the possibility of participating in the study. Data regarding subjects' age, general health status, smoking habits, and oral hygiene habits were collected through a questionnaire and stored in an electronic password-protected archive. Thirty healthy adult volunteers, aged 20 to 75, were enrolled and assigned to interventions by one of the study authors. During the screening visit, a full-mouth clinical examination was performed to assess the number of decayed, missing, and filled teeth (DMFT), PI, and BoP scores (yes/no) at each of six sites per tooth for 28 teeth, resulting in 168 measurements per subject. Written informed consent was obtained from all subjects to participate in the study and to publish the data for research and educational purposes. Participants did not receive any compensation, and no recommendations regarding personal home oral care were provided during the trial period.

Subjects were required to be between 20 and 75 years old, have permanent dentition, and have more than 20 teeth. They were required to have a full-mouth BoP score of ≥ 10% and to be free from systemic and chronic

Table 1 Study schedule

Elegibility Assessment	Screening Visit	Baseline Visit (T0)	Four Weeks (T1)	Six Weeks (T2)
Inclusion	X			
Exclusion	X			
DMFT	X			
Unstimulated saliva collection		X	X	X
BoP	X	X	X	X
PI	X	X	X	X
Salivation rate		X	X	X
Salivary pH		X	X	X
Salivary buffering capacity		X	X	X
Salivary glucose		X	X	X
Salivary D-lactate		X	X	X
sIgA		X	X	X

DMFT-Decayed, missing, and filled teeth; PI – plaque index; BoP - Bleeding on probing; sIgA- secretory immunoglobulins A

diseases. Individuals were excluded if they refused to sign informed consent, had used supplements or lozenges containing probiotics or prebiotics within three weeks prior to the study, had used antibiotics within one month before the study, were undergoing orthodontic or prosthetic treatment, or had allergies to any components of the study treatments. Additionally, participants were excluded if they used other hygiene products, immunostimulants, antibacterials, probiotics, or prebiotics during the study, refused to take the assigned lozenges, or failed to attend scheduled check-ups.

Sample size calculation

The sample size for this RCT was determined based on data from a prior exploratory case-control study (unpublished data). The primary outcome measure was the full-mouth bleeding on probing (BoP) score. To detect a reduction in the BoP score from 25.2% in the placebo group to 11.6% in the *L. brevis* CD2 group (SD = 12.1), with 80% power and a 5% significance level, a total of 26 subjects was required. This corresponds to 13 subjects per group. The calculation ensured that the study was adequately powered to detect a statistically significant difference between the treatment and placebo groups if such a difference existed. The sample size was computed using standard formulas for comparing continuous outcomes in clinical trials, taking into account the expected effect size and variability [50, 51].

Randomization procedures and interventions

Eligible subjects were randomized in a 1:1 ratio using a block randomization approach (sealedenvelope.com, block size: 4, 6, 8; list length: 36). Allocation concealment was maintained through sealed opaque envelopes containing information about the allocation (intervention or placebo). After a participant had given his/her consent to participate in the trial, an envelope was unsealed, revealing the assigned treatment regimen. To ensure blinding for both participants and clinical experimenters, an external investigator —who was not involved in clinical or statistical procedures — performed the unsealing and delivered the treatments. Out of 36 individuals screened, 30 met the eligibility criteria. Thus, these 30 subjects (15 females and 15 males) were assigned to one of the following study groups: the *L. brevis* CD2 group ($n = 15$, females = 7 and males = 8), which received lozenges containing the probiotic, and the placebo group ($n = 15$, females = 8 and males = 7), which received placebo lozenges. An enrolment CONSORT diagram is provided as Fig. 1. The probiotic and placebo lozenges were identical in taste, color, texture, and size, but the placebo lozenges did not contain active bacteria. Each participant received a container of lozenges upon enrolment. Neither the subjects nor the researchers were aware of the type of lozenges provided. All 30 participants were able to complete the entire study protocol. No adverse effects were registered.

Interventions

All participants took four lozenges daily of active intervention (*L. brevis* CD2, CNCM I-5566 $\geq 1 \times 10^9$ CFU/lozenge, Mucomixx Lot. N° 2301401; EOS2021 Srl, Rome, Italy)/placebo for four weeks. The composition of both the intervention and placebo is detailed in Table 2. Following this period, there was a two-week washout period, during which participants did not taken any lozenges. This period was implemented to assess the stability of the results achieved.

Saliva sample collection and flow rate measurements

To collect unstimulated salivary samples, participants were instructed to refrain from eating, drinking, smoking, or conducting oral hygiene procedures for at least 90 minutes prior to the collection. All measurement were conducted between 9:00 and 12:00 a.μ. to minimize diurnal variations in saliva output. Participants were coμfortably seated in the dental chair and, after five minutes of relaxation, were trained to avoid swallowing saliva and asked to lean forward and expectorate all saliva produced during the 5 minutes into a pre-labeled, preweighed 50 mL sterile polypropylene tube. The voluμe of saliva collected was recorded. The salivary flow rate was calculated using the following formula: Salivation rate (mL/min) = saliva volume (mL)/saliva collection time (min). Each vial of saliva was then thawed and centrifuged at 500 x g for 10 minutes, and the supernatants were aliquoted into 1.5 mL tubes and stored at 80 °C until further processing.

Clinical evaluations

Clinical examinations were performed by one of the study authors. Full-mouth BoP, calculated as the percentage of bleeding sites from a total of six sites per tooth (excluding the third molars), was used to assess the gingival condition. The presence or absence of BoP was recorded using a millimeter probe (UNC-15 probe, PCPUNC15, Hu-Friedy, North Carolina, USA). The probe was gently inserted between the tooth and the gum at four sites per tooth (buccal, mesial, distal, and palatal/lingual) with a pressure of approximately 20 N [52]. The extent of BoP was determined by dividing the number of bleeding sites by the total number of probed sites and expressing it as a percentage. The dichotomous status of non-bleeding or bleeding gums was defined according to BoP (non-bleeding gums: BoP in < 10%; bleeding gums: BoP in ≥ 10%) [53]. PI was defined as the percentage of sites with dental plaque, considering four sites per tooth and excluding the third molars [54]. Each surface (buccal,

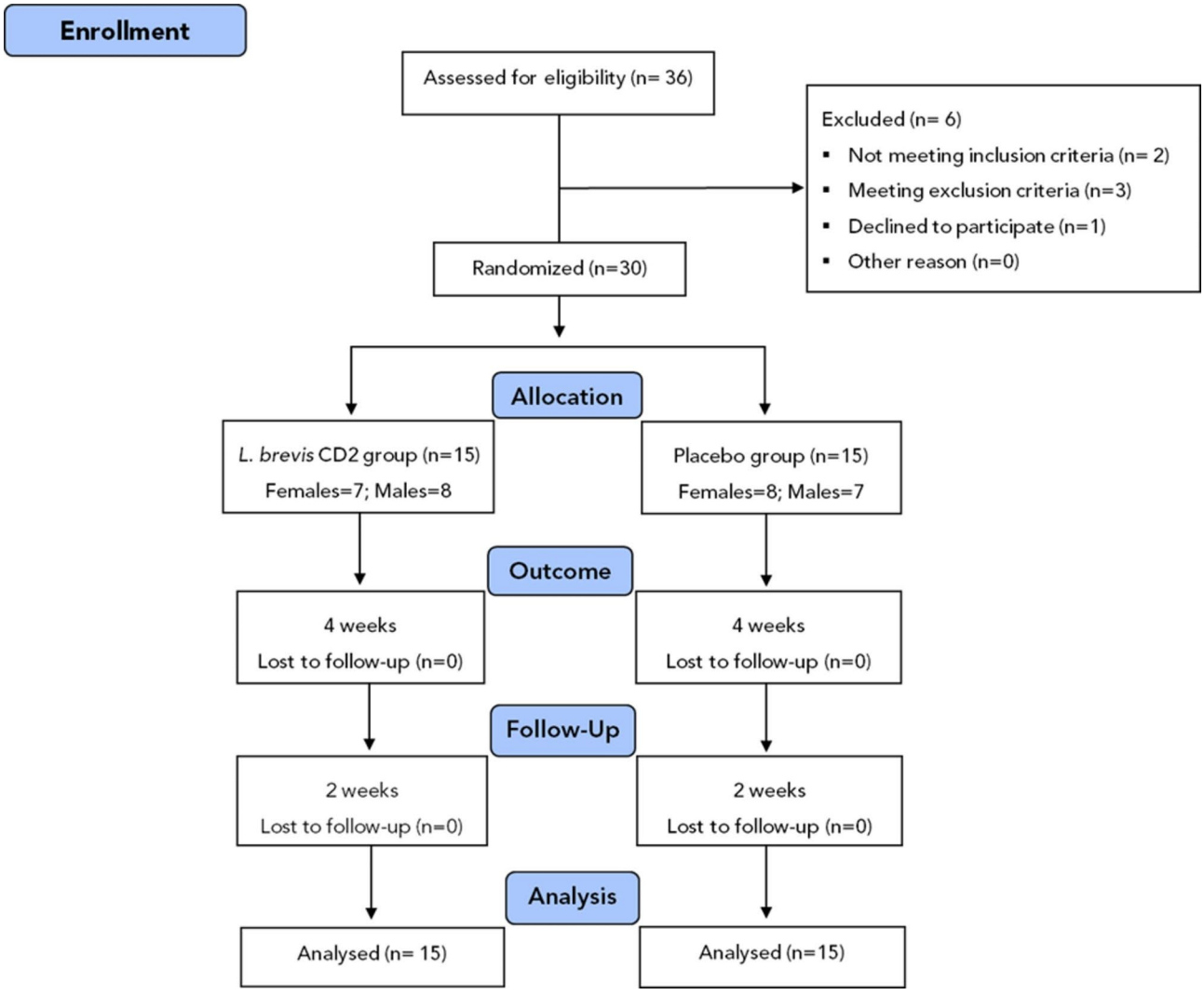

Fig. 1 Consort flowchart

Table 2 Dietary supplement composition

L. brevis CD2 ($\geq 1 \times 10^9$ CFU/lozenge): sorbitol, hydroxypropyl cellulose, xylitol, magnesium stearate, talc, silicon dioxide, flavoring - strawberry Placebo lozenge: sorbitol, hydroxypropyl cellulose, xylitol, magnesium stearate, talc, silicon dioxide, flavoring – strawberry	Dissolve in the mouth four daily lozenges for four weeks

lingual, mesial, and distal) was scored as follows: 0 = no plaque; 1 = a film of plaque adhering to the free gingival margin and adjacent area of the tooth, visible only with the probe; 2 = moderate accumulation of soft deposits on the tooth or gingival margin visible to the naked eye; 3 = abundant soft matter on the tooth and gingival margin. The scores of each examined surface were summed and divided by the total number of surfaces examined .

Estimation of pH and buffering capacity of saliva

Immediately after collection, 0.5 mL of each saliva sample was placed in a sterile tube. The pH was measured using a digital pH meter (Mettler Toledo, Columbus, Ohio, USA). The buffering capacity of saliva was evaluated using the Ericsson method [55]. For this evaluation, 0.5 mL of saliva was added to 1.5 mL of 5 mmol/L hydrochloric acid (HCL). The mixture was vigorously shaken (Corning Incorporated, Corning, NY, USA) and allowed to stand for 10 min. Finally, the pH reading was recorded using digital pH meter.

Quantification of salivary biomarkers

Glucose The quantitative concentration of salivary glucose levels was carried out using a glucose assay colorimetric Kit (Cell Biolabs, San Diego, CA, USA), according to the manufacturer's instructions. The absorbance was

measured using a spectrophotometric reading (microplate reader; Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 550 nm. Glucose concentration was calculated based on a standard curve and expressed in mg/dL.

D-lactate assay Salivary D-lactate levels were measured using a D-lactic acid/lactate colorimetric assay kit (Immunological Sciences, Roma, Italy), according to the manufacturer's instructions. The absorbance was assessed using the spectrophotometric reading at 530 nm (microplate reader; Bio-Rad Laboratories). The D-lactate concentration was calculated according to the standard curve values and expressed in mmol/dL.

Secretory immunoglobulins a (sIgA) sIgA were quantified using an ELISA assay kit (Immunological Sciences) according to the manufacturer's instructions. The absorbance reader was evaluated by an ELISA reader set to a wavelength of 570 nm. The sIgA concentration was expressed in µg/mL.

Statistical analysis

Data preparation and descriptive statistics

The primary outcome measure was the change in full-mouth BoP from baseline (T0) to four weeks post-treatment (T1). The change score (ΔBoP) was calculated as the difference between the follow-up scores (T1 and T2) and the baseline (T0) score for each participant. Summary statistics were computed for each group, including means and standard deviations (SD). Bar plots and scatterplots were primarily used to visualize the distribution of all variables across the treatment and placebo groups. Mean and standard errors (SE) were reported unless specified otherwise.

Non-parametric testing

Due to the non-normal distribution of the data, as confirmed by the Shapiro-Wilk test of the residuals from an ANCOVA model ($W = 0.9438$, $p < 0.001$), we employed the Wilcoxon rank-sum test to compare the change scores between the *L. brevis* CD2 group and placebo group.

Alternative transformations and normality assessment

To further assess the robustness of our findings, we applied the inverse hyperbolic sine transformation (ASINH) to the change scores. The ASINH transformation can handle negative values, unlike the logarithmic transformation. The normality of the transformed data was re-evaluated using the Shapiro-Wilk test, ensuring that the assumptions for parametric tests were met as closely as possible.

Robust regression analysis

In addition to the non-parametric tests, a robust regression analysis was performed. This approach is less sensitive to outliers and provides more reliable estimates when the data do not meet the assumptions of ordinary least squares regression [56, 57].

Bootstrapping for confidence intervals

To provide robust estimates of the treatment effect and associated confidence intervals, we employed bootstrapping with 1000 replications. This non-parametric resampling technique allows for the estimation of the sampling distribution of the statistic and provides more accurate confidence intervals, particularly in small sample sizes or non-normally distributed data.

Software used

The statistical analyses were performed using R software (version 4.3.2) for ARM M1 processors. The complete R Markdown file, which included the code and detailed steps of the analysis, was provided as supplementary material accompanying this manuscript.

Results

Baseline characteristics

The baseline characteristics of the study participants, stratified by the *L. brevis* CD2 and placebo groups, are summarized in Table 3. The sample consisted of 30 participants, with 15 individuals in each group. The gender distribution was similar across the groups, with 46.7% females in the *L. brevis* CD2 group and 53.3% in the placebo group. Among the 30 enrolled subjects, 24 were aged between 24 and 43 years, while six participants—three from each group—were aged between 51 and 70 years. The average age was nearly identical in the two groups: 37.9 years (SD = 14.2) for the *L. brevis* CD2 group and 38.1 years (SD = 13.2) for the placebo group. Smoking status was also balanced, with 46.7% non-smokers and 53.3% smokers in both groups. No statistically significant differences were found between the groups in terms of age, sex, smoking status, and other baseline parameters, including full-mouth BoP and PI scores, salivation rate, pH, buffer capacity, and glucose, D-lactate, and sIgA levels ($p > 0.05$ for all comparisons). Considering the impact of smoking on oral health [58–60], we further stratified the clinical and demographic characteristics of the enrolled individuals at baseline by smoking status (non-smokers and smokers) (Supplementary file Table S1).

Effects of *L. brevis* CD2-containing lozenges on full-mouth BoP and PI scores.

The four-week *L. brevis* CD2 treatment resulted in a highly significant reduction in BoP score compared to baseline ($p < 0.0001$). The values remained significantly

Table 3 Clinical and demographic characteristics of enrolled individuals at baseline

Parameters	L. brevis CD2	Placebo	*p*-value
N	15	15	
Female (%)	7 (46.7)	8 (53.3)	1
Male (%)	8 (53.3)	7 (46.7)	
Age (mean (SD))	37.93 (14.16)	38.13 (13.21)	0.968
Nonsmokers (%)	7 (46.7)	8 (53.3)	1
Smokers (%)	8 (53.3)	7 (46.7)	
Full-mouth BoP score (%) (mean (SD))	21.37 (6.70)	20.42 (4.32)	0.649
Full-mouth PI score (%) (mean (SD))	36.33 (4.08)	35.47 (4.36)	0.578
Salivation rate (*mL/Min*) (mean (SD))	0.37 (0.06)	0.36 (0.07)	0.691
pH (mean (SD))	7.10 (0.27)	7.07 (0.21)	0.786
Buffer capacity (mean (SD))	5.03 (0.35)	5.09 (0.38)	0.682
Glucose (*mg/dL*) (mean (SD))	0.28 (0.17)	0.22 (0.10)	0.303
D-lactate (*uM*) (mean (SD))	5.20 (2.10)	4.76 (1.79)	0.546
sIgA (*µg/mL*) (mean (SD))	8.10 (3.83)	9.93 (4.36)	0.232

lower even after washout ($p < 0.0001$), demonstrating the lasting effect of the probiotic on this parameter (Fig. 2A). In contrast, no significant changes occurred in the placebo group after treatment or following the washout period (Fig. 2B). Regarding the differences between the groups, the *L. brevis* CD2 group exhibited a significantly lower BoP score than the placebo group at both T1 and T2 ($p < 0.001$) (Fig. 2C). Similar to BoP, after four-week treatment with *L. brevis* CD2, PI values were significantly lower than baseline ($p < 0.0001$), and the values remained significantly lower even at washout ($p < 0.0001$), demonstrating the stability of the probiotic's effect (Fig. 2B). No significant changes were observed in the placebo group at either T1 or T2. When comparing the groups, the probiotic group showed significantly lower PI scores than the placebo, both at post-treatment and after washout ($p < 0.0001$) (Fig. 2D). When the data was analyzed by dividing participants into smokers and non-smokers, it was found that the effects of *L. brevis* on BoP and PI scores were not influenced by smoking habits (Supplementary file Fig. S1).

Data reported in Fig. 2E and F show a significant positive correlation between changes in BoP and PI scores after four weeks of treatment ($R = 0.72$) and two weeks of washout ($R = 0.67$), with an improvement in both BoP and PI scores for the *L. brevis* CD2 group (dots in the green area) as shown in the quadrant analysis.

To determine whether changes in BoP influenced by changes in PI, a mediation analysis was conducted assessing the relationship between the treatments (x), change in PI (ΔPI) (m, mediator), and change in BoP (ΔBoP) from baseline to T1 (y, primary outcome) (Fig. 2G). The total effect (c) of the treatment group on ΔBoP was 10.3 ($p < 0.001$), suggesting a strong impact. The direct effect (c') of the treatment group on ΔBoP, removing the effect of the PI, was 10.4 ($p < 0.001$), indicating a significant direct influence of treatment on BoP. The indirect effect (ab) of the treatment group on ΔBoP through the PI was −0.12. The confidence interval for the indirect effect included zero, indicating that this effect was not statistically significant. These results strongly indicate that the effect of the *L. brevis* CD2 treatment on changes in BoP was primarily direct, rather than mediated through changes in PI.

Effects of *L. brevis* CD2-containing lozenges on salivation rate, pH, and buffering capacity of saliva

The four-week *L. brevis* CD2 treatment had a remarkably significant ($p < 0.01$) impact on salivation rates compared to baseline, and the values remained significantly higher at washout ($p < 0.05$) (Fig. 3A). Analysis of data by smoking status showed that the probiotic increased salivation rates in both non-smokers and smokers. However, the effect was statistically significant for smokers only at both T1 and T2 (Supplementary file Fig. S2, panel A). In contrast, the placebo group showed no significant changes in salivation rate either at T1 or T2 compared to T0. Regarding the differences between the groups, the intake of *L. brevis* CD2 substantially enhanced the salivation rate both at post-treatment and washout compared to placebo, with a high level of statistical significance ($p < 0.01$) (Fig. 3D).

The four-week treatment with *L. brevis* CD2 did not significantly alter the salivary pH compared to baseline, and the values did not change even after the two weeks of washout (Fig. 3B). Even stratifying the data by smoking status, there were no significant differences in salivary pH values (Supplementary file Fig. S2, panel B). In subjects treated with the placebo, no significant changes in salivary pH values were detected either at T1 or T2 compared to baseline. Regarding the differences between the groups, an increase in pH was recorded in the probiotic group at T1 compared to placebo, although it was not significant (Fig. 3E). Even though the analysis did

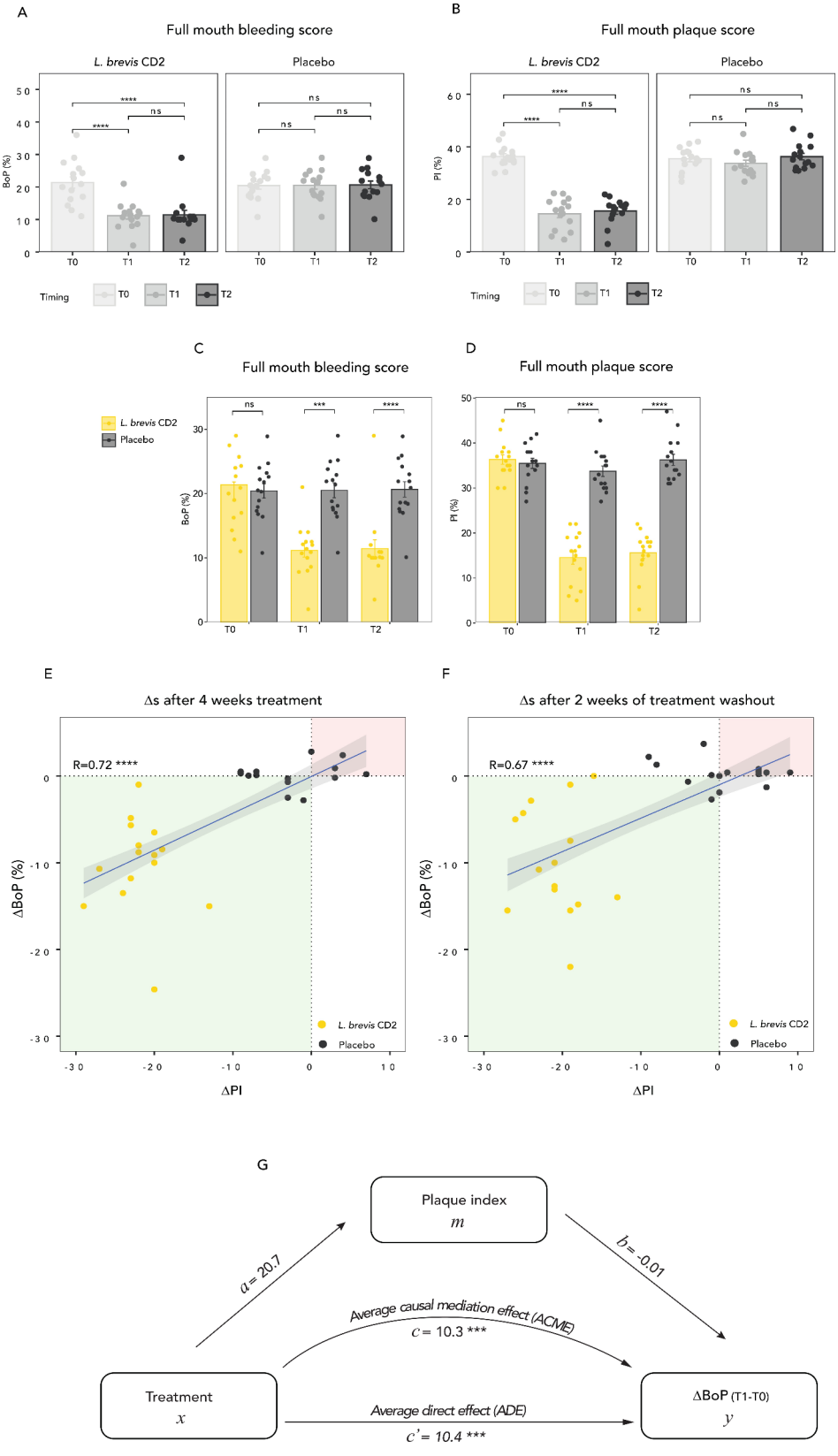

Fig. 2 Evaluation of clinical parameters (BoP and PI) in *L. brevis* CD2 and placebo groups at T0, T1, and T2. BoP (**A** and **C**) and PI scores (**B** and **D**) expressed as mean ± SE. Quadrant Analyses depicting changes (Δ) in BoP and PI scores at T1 (**E**) and T2 (**F**). Each dot represents an individual. Dots in the green and red areas represent, respectively, an improvement or a worsening in the scores. Correlation coefficient (R) and *p*-value of the relationship between the changes in BoP and PI scores. Mediation Analysis is also shown (**G**). ns = non-significant, ***$p < 0.001$; ****$p < 0.0001$

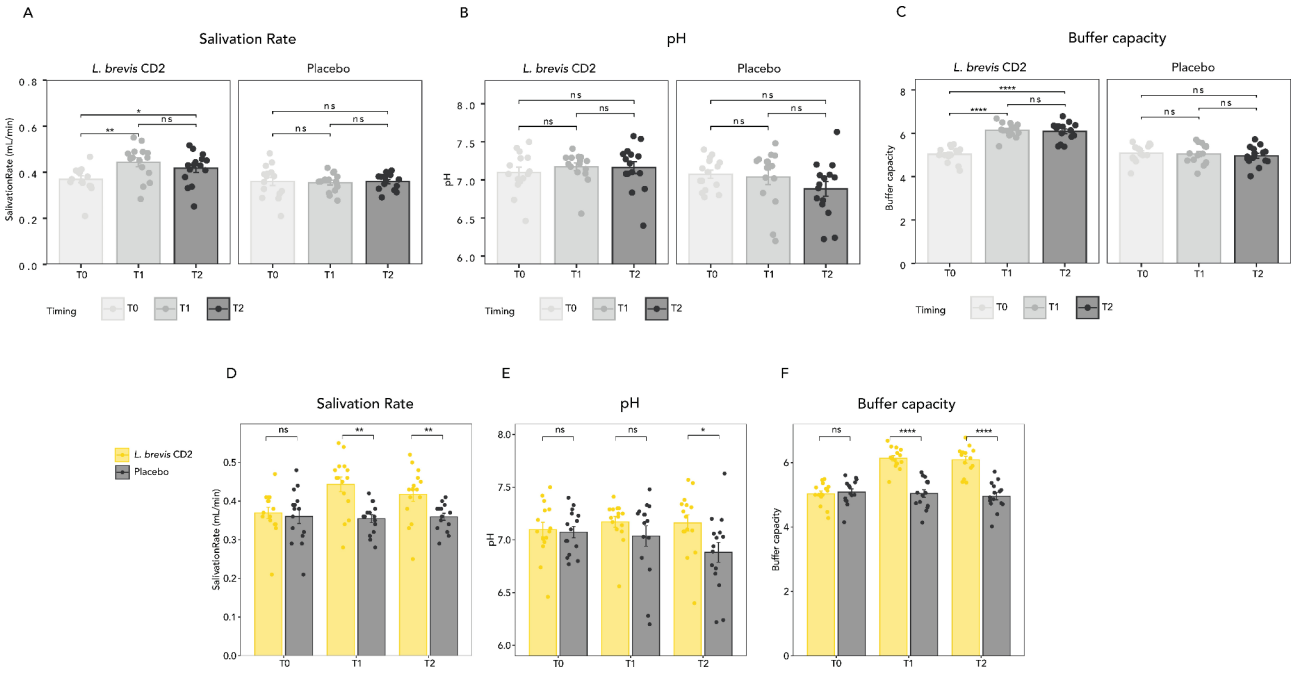

Fig. 3 Evaluation of salivary parameters (salivation rate, pH, and buffer capacity) in *L. brevis* CD2 and placebo groups at T0, T1, and T2. Salivation rate (**A** and **D**), pH (**B** and **E**), and buffer capacity (**C** and **F**) expressed as mean ± SE. ns = non-significant, *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$

not show any differences between salivary pH at T0 and T1, a statistical difference was observed at T2 ($p < 0.05$). After four weeks of *L. brevis* CD2 treatment, the buffering capacity significantly increased compared to baseline ($p < 0.0001$) (Fig. 3C), and the values remained substantially higher also at washout ($p < 0.0001$), demonstrating the prolonged effect of the probiotic. Additionally, the analysis of the data based on smoking status revealed that the impact of *L. brevis* on buffering capacity was not influenced by the smoking habits (Supplementary file Fig. S2, panel C).

The placebo group showed no significant changes in the buffer capacity either at T1 or at T2 with respect to T0. When comparing the groups, the *L. brevis* CD2 group exhibited the buffering capacity values considerably higher both at post-treatment and washout compared to placebo ($p < 0.0001$) (Fig. 3F).

Effects of *L. brevis* CD2-containing lozenges on salivary biomarkers

After four weeks of *L. brevis* CD2 treatment, a decrease in salivary glucose levels was depicted compared to baseline, even though the effect reached a statistical significance after the two-week washout (Fig. 4A) ($p < 0.05$). Conversely, no significant changes in salivary glucose levels were found in the placebo group at different time points. When comparing the groups, the *L. brevis* CD2 group displayed salivary glucose levels significantly lower than the placebo at post-treatment ($p < 0.05$) and even more so at washout ($p < 0.01$) (Fig. 4C). Additionally, the four-week intake with the probiotic significantly reduced salivary D-lactate levels with respect to T0 ($p < 0.01$), and the values remained significantly reduced even at T2 ($p < 0.01$). When analyzing the data based on smoking status, the probiotic was found to effectively lower D-lactate levels in both smokers and non-smokers at T1. Of note, in non-smokers the effect of *L. brevis* assumption remained statistically significant even at T2 (Supplementary file Fig. S3, panel B). The placebo group showed no significant changes in salivary D-lactate values at the various time points. Regarding the differences between the groups, the probiotic group exhibited D-lactate levels considerably lower both after four weeks of treatment and at follow-up compared to placebo ($p < 0.01$) (Fig. 4D).

The intake of *L. brevis* CD2 resulted in a highly significant increase ($p < 0.01$) in salivary sIgA levels at T1 compared to T0 (Fig. 5A), and the effect was supported even at T2, thus maintaining significantly higher values than T0 ($p < 0.01$). Notably, this effect was statistically significant only for the non-smoker group (Supplementary file Fig. S3, panel C). No significant changes were registered in the placebo group at post-treatment and washout compared to baseline ($p > 0.05$). When comparing the groups, the probiotic intake increased sIgA levels compared to the placebo, with the differences being statistically significant at T2 ($p < 0.05$) (Fig. 5B).

The heatmaps presented in Fig. 6 illustrate the changes (T1 vs. baseline) in clinical and salivary parameters of all subjects included in the trial (placebo and *L. brevis* CD2 groups). Darker shades indicate higher values, while

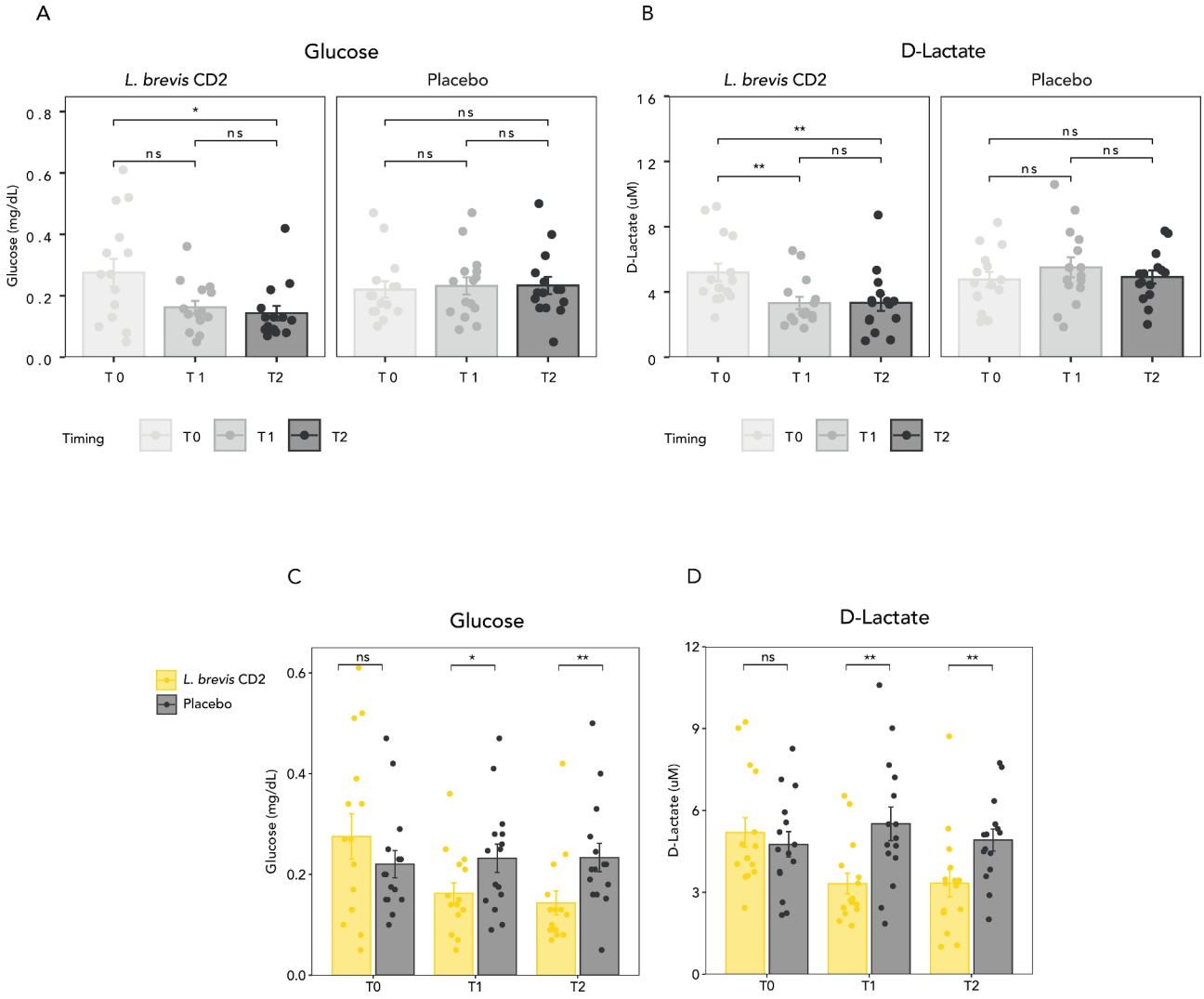

Fig. 4 Evaluation of salivary biomarkers (glucose and D-lactate) in the *L. brevis* CD2 and placebo groups at T0, T1, and T2. Salivary glucose (**A** and **C**) and salivary D-lactate (**B** and **D**) expressed as mean ± SE. ns = non-significant, $*p < 0.05$, and $**p < 0.01$

lighter shades represent lower values. After probiotic treatment at the individual level, the Δ scores (Δs) highlight a relevant improvement in all the evaluated clinical and salivary parameters compared to the placebo group. Additionally, a heatmap comparing smokers and non-smokers confirms that smoking status did not significantly impact the beneficial effects of the probiotic on the examined parameters (Supplementary file Fig. S4).

Discussion

The present double-blind, randomized, placebo-controlled clinical study aimed to evaluate the effects of a one-month treatment with the oral probiotic *Levilactobacillus brevis* CD2 (CNCM I-5566 on various oral health parameters in adult subjects. We specifically examined changes in full-mouth bleeding on probing (BoP) and plaque index (PI) scores. The results showed that the intake of *L. brevis* CD2 lozenges positively influenced these clinical parameters compared to both the baseline measurements and the placebo group. Of note, we found a strong and highly significant correlation between changes in BoP and PI scores after four weeks of treatment and after two weeks of washout, both within the *L. brevis* CD2 group and placebo. All subjects treated with the probiotic exhibited an improvement in both clinical parameter scores. Additionally, we also conducted a mediation analysis to verify whether the effect of the probiotic treatment on BoP was influenced by changes in PI. Our findings demonstrated that the *L. brevis* CD2 treatment's impact on BoP changes was primarily direct, rather than mediated through changes in PI.

Our study also uncovered an improvement in salivation rate among subjects treated with *L. brevis* CD2. To the best of our knowledge, our results represent the first

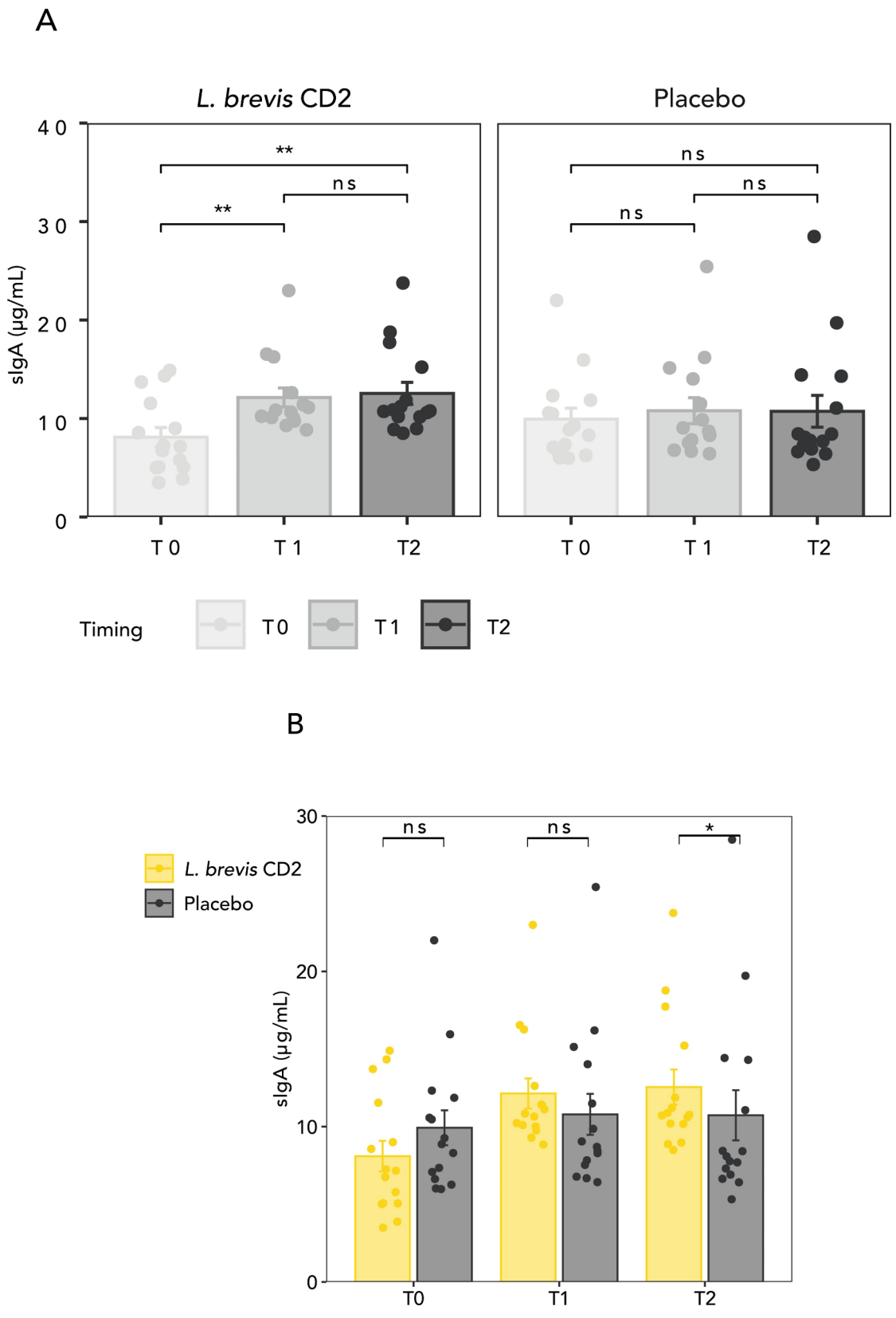

Fig. 5 Evaluation of sIgA levels in the *L. brevis* CD2 and placebo groups at T0, T1, and T2. sIgA values (**A** and **B**) expressed as mean ± SE. ns = non-significant, **p* < 0.05; ***p* < 0.01

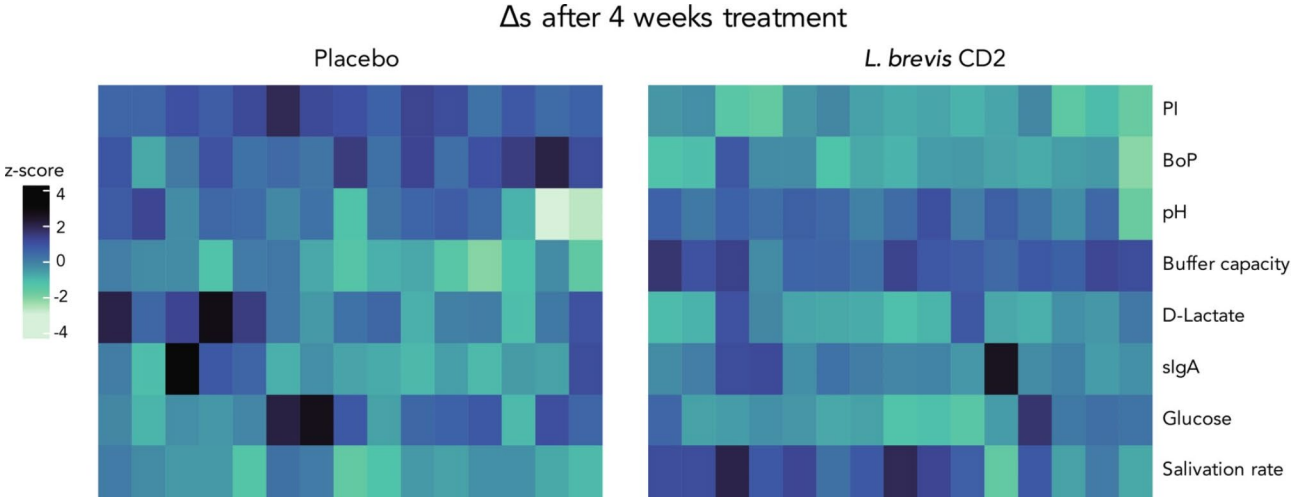

Fig. 6 Heatmap of changes (Δs at T1 vs. baseline) in both clinical and salivary parameters of all subjects included in the trial. Darker shades indicate higher values, while lighter shades indicate lower values. The left heatmap represents the placebo group, and the right represents the *L. brevis* CD2 group

evidence of the positive impact of *L. brevis* CD2 on this crucial parameter for maintaining both oral and systemic health [61, 62]. Previous research has shown that salivary flow rate is reduced in patients with increased caries activity and various associated pathological systemic conditions, such as Sjogren's syndrome and diabetes. Additionally, certain treatments, like drug intake or therapeutic radiation for head and neck cancers, can damage salivary glands [63–65]. Conversely, a higher salivary flow rate is associated with enhanced buffering capacity, improved remineralization/demineralization ratio, increased antimicrobial action, and better immune function. These factors collectively create a less favorable environment for the development of cariogenic biofilms [66], which is a crucial piece of knowledge for oral health professionals. Another key objective of this study was to investigate the ability of probiotic *L. brevis* CD2 lozenges not to alter salivary pH, a pivotal element in preserving the health of the oral cavity and a significant indicator of the carious process [67]. Low salivary pH fosters the growth of aciduric bacteria, facilitating the proliferation of acidogenic bacteria, thereby creating an unfavorable environment for protective oral bacteria. Our findings emphasize the distinct capability of the probiotic to not alter the pH value in the treated subjects. This effect can be attributed to the arginolytic nature of the *L. brevis* CD2 strain in the lozenges, leading to the generation of ammonia ions that can counteract and maintain the physiological salivary pH value > 7 [32, 42–44]. This result is particularly intriguing given that some probiotics tested in vitro have instead resulted in a decrease in salivary pH [68–70]. Saliva's buffering capacity is crucial, as it helps maintain pH levels, neutralizes the effects of acid exposure, and prevents enamel demineralization and dental caries [71]. Factors that positively influence salivary buffering capacity include bicarbonate ions (HCO_3^-), proteins, and phosphates, and a high salivary flow rate [72]. Our study found a relevant and statistically significant improvement in buffering capacity values in all subjects treated with *L. brevis* CD2 compared to baseline and placebo.

Regarding the impact of the probiotic treatment on salivary glucose levels, although the results did not reach statistical significance, a downward trend was observed following the intake of *L. brevis* CD2 compared to baseline. Considering the critical role of salivary glucose as a biomarker for dental caries and its association with diabetes, these findings could be highly relevant in preventing dental caries and systemic diseases, including diabetes [73–75]. D-lactate, another essential salivary component in oral health, resulted in a substantial reduction in the *L. brevis* CD2 group *versus* the baseline or placebo group, and the effect remained significant and stable even at follow-up. Also, this effect of *L. brevis* CD2 represents the first evidence of the ability of a probiotic to influence basic salivary D-lactate levels. This result could also be related to the ability of *L. brevis* CD2 to reduce salivary glucose levels, being D-lactate one of the two stereoisomers of lactic acid produced during the bacterial metabolism of carbohydrates, mainly glucose [76]. It has been reported that inflammatory bowel disease and metabolic disorders, which are closely associated with dental caries [77, 78], can affect D-lactate metabolism [79, 80]. Thus, *L. brevis* CD2 could prevent dental caries by decreasing salivary glucose and D-lactate levels. Probiotics may influence saliva's composition, including the concentrations of mucins and salivary immunoglobulins, which can affect the quantity and nature of saliva secreted. Among the various components of saliva, sIgA exemplify local immunity and host defense. sIgA play a

defensive role by opsonizing and agglutinating bacteria and preventing their adhesion to the epithelium of the gastrointestinal tract mucosa, including the oral cavity [26–28]. Notably, the four-week *L. brevis* CD2 treatment resulted in a significant and sustained increase in sIgA levels compared to the baseline. This effect was maintained even after the washout, providing a reassuring indication of the treatment's lasting impact. Furthermore, while the mean sIgA levels were higher in the *L. brevis* CD2 group compared to the placebo, statistical significance was only reached at follow-up. The promising results of probiotics in preventing major oral diseases like periodontitis and dental caries are encouraging and motivate further research and development in this area. Our findings show that *L. brevis* CD2 could have a remarkable prophylactic and preventive effect on the maintenance of oral diseases, significantly improving clinical parameters and salivary biomarkers. The clinical relevance of consuming dairy products containing this probiotic suggests that it could be a supporting approach to preventing dental caries and other oral diseases. The results of the current trial support the growing evidence that certain strains of *Lactobacillus* can enhance or maintain oral cavity health [81]. In general, the oral benefits of *Lactobacillus* species, the most widely studied as bacterial probiotics, involve several mechanisms. These include the production of organic acids, hydrogen peroxide, antibacterial compounds, bacteriocins, and adhesion inhibitors [82].

Some *Lactobacillus* strains release factors that have immunomodulatory and anti-inflammatory properties [81, 82]. These factors promote a balanced microbial community by engaging in competitive and antibacterial activities against pathogenic bacteria. Evidence has shown that consuming specific *Lactobacillus* strains can prevent periodontal disease by inhibiting the formation of dental plaque in the oral cavity [83]. This protective effect is linked to lipase enzymes produced by selected Lactobacillus strains, which can degrade pathogen biofilms. Of note, the beneficial impact of specific *Lactobacillus* on periodontal tissues has been attributed to their ability to enhance the proteolytic resistance of connexins, such as E-cadherin and β-catenin, in epithelial cells against pathogens like *P. gingivalis* [84, 85]. Moreover, *Lactobacillus* strains have been found to release metabolic products, such as exopolysaccharides, which exhibit strong antioxidant properties [86, 87]. Some species of *Lactobacillus* also show antioxidative functions by increasing the expression of antioxidant enzymes, such as heme oxygenase 1, in gingival epithelial cells [85].

Additionally, various biochemical and molecular mechanisms have been identified to explain how certain *Lactobacillus* strains inhibit immune-inflammatory responses [81, 82]. This includes the suppression of NOS2, matrix metalloproteinases (MMPs), and pro-inflammatory cytokines [32]. Notably, high levels of ADI present in *L. brevis* may competitively inhibit NOS, thereby helping to suppress inflammatory responses associated with periodontitis [32].

We readily recognize the limitations of our study. The relatively small number of participants requires planning further research with a larger sample size based on the results obtained in the present trial, with the promise of providing more robust and generalizable results. Moreover, even if similar time points were used in previous studies, a four-week probiotic intake and a two-week follow-up period could be considered relatively short to establish the long-term effect of *L. brevis* CD2. Some probiotics for oral care claim to enhance the body's natural defenses in the oral cavity. This claim emphasizes their ability to strengthen the immune response and promote oral health. Another limitation of our study is the lack of oral microbiome analysis. Notably, effective colonization of the oral cavity by *L. brevis* has been reported in individuals with healthy gums or mild gingivitis, even after a short period of probiotic administration [81, 88]. Although the participants in this trial were healthy, it would be crucial to investigate whether the intake of *L. brevis* can help maintain the balance of the oral microbiome, similar to findings reported for the gut microbiome [89–91].

Finally, although we evaluated the influence of *L. brevis* CD2 on some salivary markers (salivation rate, pH, buffering capacity, glucose, D-lactate, sIgA), to gain a complete picture of this probiotic's impact, it will be noteworthy to evaluate its effect on other salivary parameters equally indicative of oral health.

Abbreviations

BoP	Bleeding on probing
PI	Plaque index
sIgA	Secretory immunoglobulins A
L. brevis	*Levilactobacillus brevis*
NOS2	Nitric oxide synthase 2
NO	Nitric oxide
ADI	Arginine deiminase
MMPs	Matrix metalloproteinases
RCT	Randomized controlled trial
DMFT	Decayed, missing, and filled teeth

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-024-06000-1.

Additional file 1: Complete R Markdown file including the code and detailed steps of the analysis

Supplementary Material 2

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Author contributions
Conceptualization, S.A., F.L., and D.P.; methodology, S.A., F.L., F.R.A., B.C., and D.P.; formal analysis, S.A., F.L. and D.P.; investigation, S.A., F.L., F.R.A., and A.B.; data curation, S.A., F.L., F.R.A., A.B., and D.P.; original draft preparation, S.A. and F.L.; review and editing, M.G., B.C., and D.P.; supervision, M.G., B.C., and D.P.; project administration, B.D., and D.P. All authors have read and agreed to the published version of the manuscript.

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Data availability
The datasets underlying this article will be shared on reasonable request to the corresponding author.

Declarations

Ethics approval and consent to participate
The study was approved by the Internal Review Board (Protocol n. 48/2022, 22.11.2022) of the University of L'Aquila (Italy). Informed consent was obtained from all subjects.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Life, Health & Environmental Sciences, University of L'Aquila, Building Rita Levi Montalcini, Coppito, L'Aquila 67100, Italy
[2]PhD School in Medicine and Public Health, University of L'Aquila, L'Aquila, Italy
[3]Center of Oral Diseases, Prevention and Translational Research - Dental Clinic, L'Aquila, Italy

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